BRNO UNIVERSITY OF TECHNOLOGY

Faculty of Chemistry

DOCTORAL THESIS

Brno, 2021 Ing. Martin Szotkowski

BRNO UNIVERSITY OF TECHNOLOGY
VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF CHEMISTRY
FAKULTA CHEMICKÁ

INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY
ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

CONTROLLED PRODUCTION OF LIPIDS AND LIPIDIC SUBSTANCES BY SELECTED YEASTS AND MICROALGAE

ŘÍZENÁ PRODUKCE LIPIDŮ A DALŠÍCH LIPIDICKÝCH LÁTEK POMOCÍ VYBRANÝCH DRUHŮ KVASINEK A MIKROŘAS.

DOCTORAL THESIS
DIZERTAČNÍ PRÁCE

AUTHOR
AUTOR PRÁCE

Ing. Martin Szotkowski

SUPERVISOR
ŠKOLITEL

prof. RNDr. Ivana Márová, CSc.

BRNO 2021

BRNO UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMISTRY

Specification Doctoral Thesis

Department:	Institute of Food Science and Biotechnology	Academic year:	2020/21
Student:	**Ing. Martin Szotkowski**		
Study programme:	Chemistry and Technology of Foodstuffs		
Study branch:	Food Science		
Head of thesis:	**prof. RNDr. Ivana Márová, CSc.**		

Title of Doctoral Thesis:

Controlled production of lipids and lipidic substances by selected yeasts and microalgae

Doctoral Thesis:

The aim of this dissertation was the study of production properties of carotenogenic yeasts, microalgae and cyanobacteria with a focus on the production of lipids, lipidic metabolites (e.g. carotenoids, chlorophylls, ubiquinone, ergosterol) in connection with the utilization of waste substrates originating from agriculture and food industry. The work was divided into several units according to the tested types of microorganisms.

1) Processing of waste substrates and media formulation
2) Metabolic and production activity of carotenogenic yeasts
2) Cultivation and production properties of microalgae and cyanobacteria in laboratory and pilot scale
3) Co–cultivation of selected yeasts and microalgae
4) Comparison of production effectivness in tested strains

Deadline for Doctoral Thesis delivery: 13.2.2021:

Ing. Martin Szotkowski Student	prof. RNDr. Ivana Márová, CSc. Head of thesis	prof. RNDr. Ivana Márová, CSc. Head of department

In Brno dated 1.9.2020

prof. Ing. Martin Weiter, Ph.D.
Dean

ABSTRACT

Carotenoids are natural pigments found in microorganisms such as algae, yeast and cyanobacteria. They represent the most widespread group of antioxidants with a significant biological effect. Currently, there is a growing interest in carotenoids due to their beneficial effects on human health. Chlorophylls are green photosynthetic pigments that are used in the food industry as intensive green dyes. Coenzyme Q is known for its positive effect on the proper functioning of a number of organs in the human body. Ergosterol is an integral part of the membranes of yeasts and fungi. It is a provitamin D_2, which is an important part of the immune system. Microbial lipids, or ‚Single cell oils', are characterised by a high content of healthy unsaturated fatty acids that can be used in pharmacy or cosmetics. Microbial lipids are further studied as an alternative for the production of biofuels.

The dissertation focused on the study and possibilities of optimising the production of lipids and lipid substances by selected strains of carotenogenic yeasts, microalgae and cyanobacteria. The tested yeasts of the genera *Rhodotorula*, *Rhodosporidium*, *Cystofilobasidium* and *Sporidiobolus* were cultivated on a series of different media with different C/N ratios in the range of 13 to 100, containing processed waste substrates of the food industry. Selected strains were then cultivated in bioreactors with medium containing a combination of waste substrates.

Cultures of microalgae of the genera *Desmodesmus*, *Scenedesmus*, *Chlorella*, *Coccomyxa*, *Chlamydomonas*, *Botryococcus* dealt with the optimisation of individual components of the medium and the application of various stresses in order to increase the production of the studied metabolites. Pilot large-volume cultures in open tanks were performed as part of experiments with the extremophilic microalgae *Cocomyxa*. In the final part, pilot screenings and large-volume bioreactor experiments focused on the possibilities of co-cultivation of carotenogenic yeasts and microalgae were performed.

The tested yeast strains were able to utilise media containing hydrolysed waste substrates with varying success. The best strain was *Sporidiobolus pararoseus*, which achieved the highest biomass production and studied metabolites in the media. The tested waste substrates were a combination of waste frying oil and coffee grounds hydrolysate. Successful optimisation of the composition of the mineral medium's main components led to increased production of the studied metabolites. The most important effect exhibited particular P/N ratio and the application of oxidative stress. *Desmodesmus* and *Scenedesmus* microalgae achieved the best results. Large-scale cultures of *Coccomyxa onubensis* confirmed the resistance of the culture to contamination by external influences and the ability to grow at high temperatures and light intensities. Co-cultivation experiments confirmed the ability of symbiotic growth of yeasts and microalgae. The best results were obtained in all tested yeasts co-cultivated with microalgae of the genus *Desmodesmus* and *Scenedesmus* and, to a lesser extent, with the genus *Coccomyxa*.

KEYWORDS

Yeasts, microalgae, cyanobacteria, waste substrates, co-cultivation, lipids, fatty acids, carotenoids, chlorophylls, ergosterol, ubiquinone

ABSTRAKT

Karotenoidy jsou přírodní pigmenty vyskytující se v mikroorganismech jako jsou řasy, kvasinky a sinice. Představují nejrozšířenější skupinu antioxidantů s významným biologickým účinkem. V současnosti vzrůstá zájem o karotenoidy vzhledem k jejich příznivým vlivům na lidské zdraví. Chlorofyly jsou zelená fotosyntetická barviva, která nacházejí uplatnění v potravinářství jako intenzivní zelená barviva. Koenzym Q je znám svým pozitivním vlivem pro správnou funkci řady orgánů v lidském těle. Ergosterol je nedílnou součástí membrán kvasinek a hub. Je to provitamin D_2, který je důležitou součástí imunitního systému. Mikrobiální lipidy, nebo také „Single cell oils" jsou charakteristické vysokým obsahem zdraví prospěšných nenasycených mastných kyselin, které lze využít ve farmacii či kosmetice. Mikrobiální lipidy jsou dále studovány jako alternativa pro výrobu biopaliv.

Dizertační práce byla zaměřena na studium a možnosti optimalizace produkce lipidů a lipidických látek vybranými kmeny karotenogenních kvasinek, mikrořas a sinic. V rámci práce byly testované kvasinky rodu *Rhodotorula, Rhodosporidium, Cystofilobasidium* a *Sporidiobolus* podrobené kultivacím na sérií médií s různými C/N poměry v rozsahu 13 až 100, obsahujících upravené odpadní substráty z potravinářského průmyslu. Vybrané kmeny byly poté kultivovány v bioreaktorech v médiu obsahujícím kombinaci odpadních substrátů.

Kultivace mikrořas rodu *Desmodesmus*, *Scenedesmus*, *Chlorella*, *Coccomyxa, Chlamydomonas, Botryococcus* se zabývaly optimalizací jednotlivých komponent média a aplikací různých stresů s cílem navýšení produkce studovaných metabolitů. V rámci experimentů s extrémofilní mikrořasou *Coccomyxa* byly provedeny pilotní velkoobjemové kultivace v otevřených nádržích. V závěrečné části byl provedeny pilotní screeningové a velkoobjemové bioreaktorové experimenty zaměřené na možnosti kokultivace karotenogenních kvasinek a mikrořas.

Testované kmeny kvasinek byly s rozdílnou úspěšností schopny utilizovat média obsahující hydrolyzované odpadní substráty. Nejlepším kmenem byl *Sporidiobolus pararoseus*, který na médiích dosahoval nejvyšších produkcí biomasy i sledovaných metabolitů. Z testovaných odpadních substrátů byla nejlepší kombinace odpadního fritovacího oleje a hydrolyzátu kávové sedliny. Úspěšná optimalizace složení hlavních komponent minerálního média vedla k zvýšené produkci studovaných metabolitů. Největší vliv měl optimální poměr P/N a aplikace oxidačního stresu. Nejlepších výsledků dosáhly mikrořasy rodu *Desmodesmus* a *Scenedesmus*. Velkoobjemové kultivace *Coccomyxy onubensis* potvrdily rezistenci kultury proti kontaminaci vnějšími vlivy a schopnost růstu za vysoké teploty a intenzity světelného záření. Kokultivační experimenty potvrdily schopnost symbiotického růstu kvasinek a mikrořas. Nejlepších výsledků dosahovaly všechny testované kvasinky s mikrořasami rodu *Demsodesmus a Scenedesmus* a v menší míře i rodu *Coccomyxa.*

KLÍČOVÁ SLOVA

Kvasinky, mikrořasy, sinice, kokultivace, lipidy, mastné kyseliny, karotenoidy, chlorofyly, ergosterol, ubichinon

SZOTKOWSKI, Martin. *Řízená produkce lipidů a dalších lipidických látek pomocí vybraných druhů kvasinek a mikrořas. .* Brno, 2021. Dostupné také z: https://www.vutbr.cz/studenti/zav-prace/detail/134986. Dizertační práce. Vysoké učení technické v Brně, Fakulta chemická, Ústav chemie potravin a biotechnologií. Vedoucí práce Ivana Márová.

DECLARATION

I declare that the doctoral thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the doctoral thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

…..……..

Students signature

Acknowledgment:

At this point I would like to thank mainly the supervisor of the dissertation, prof. RNDr. Ivana Márová, CSc., for professional guidance and valuable advice during the entire doctoral study.

My thanks go to prof. Carlos Vilchéz for his willingness, help and valuable advice during the internship and training in the game Padel. I want to thank the whole BITAL group from the University of Huelva in Spain and Zaida Montero and Kevin Gordon for wonderful moments of fun and memories not only in the laboratories and for their friendship.

I would like to thank my colleagues and students from the biotechnology group for a great time in the laboratories and beyond. Namely Andrea Hároniková, Jiří Holub, Nikola Chrástová, Veronika Mariničová, Samuel Šimanský, Robert Majdloch, Pavlína Sikorová, Pavlína Sniegoňová, Dagmar Hladká, Marek Rapta, Přemysl Menčík and Dana Byrtus.

Last but not least, my greatest thanks go to my family and closest friends for their continued support and patience throughout the study.

TABLE OF CONTENTS

ABSTRACT **3**

KEYWORDS **3**

ABSTRAKT **4**

KLÍČOVÁ SLOVA **4**

1 INTRODUCTION **10**

2 THEORETICAL PART **12**

2.1 STUDIED METABOLITES **12**

2.1.1 Lipids *12*

2.1.1.1 Structure, properties and functions of lipids 12

2.1.2 Microbial lipids as a biofuel feedstock *13*

2.1.3 Lipid biosynthesis: phospholipids and triacylglycerols *14*

2.1.4 Fatty acids: properties and synthesis *15*

2.1.4.1 Fatty acid synthesis 16

2.1.5 Carotenoids *17*

2.1.5.1 Carotenoids commercial applications 18

2.1.5.2 Carotenoid properties 19

2.1.5.3 Carotenoid structure 21

2.1.5.4 Carotenoid biosynthesis 22

2.1.5.4.1 ISOPRENOID 22

2.1.5.5 Carotenoid biosynthesis in cells 25

2.1.6 Chlorophylls *27*

2.1.6.1 Chlorophyll biosynthesis 28

2.1.6.2 Photosynthesis 28

2.1.7 MIcrobial sterols *30*

2.1.7.1 Function and use of sterols 30

2.1.7.2 Sterol biosynthesis 30

2.1.8 Ubiquinone *31*

2.1.8.1 Functions and utilisation of ubiquinone 31

2.1.8.2 Microbial biosynthesis of ubiquinone. 33

2.1.9 Microbial extracelular lipases *33*

2.1.9.1 Structure and properties of Yeast lipase 34

2.1.9.2 Yeast lipase activity 34

2.1.10 Biosurfactants *35*

2.1.10.1 Biosurfactant application 35

2.2 MICROORGANISMS **36**

2.2.1 Yeasts *36*

2.2.1.1 Structure and shape of a yeast cell 36

2.2.1.2 Yeast reproduction 37

2.2.1.3 Microorganism Growth curve 38

2.2.1.4 Carotenogenic yeasts 38

2.2.2 Algae *40*

2.2.2.1 Structure and shape of microalgae cells 41

2.2.2.2 Microalgae cultivation 42

2.2.2.3 Microalgae reproduction 46

2.2.2.4 Microalgae genera description 46

2.2.3 Cyanobacteria *51*
2.2.3.1 Cyanobacterial cell structure and their occurrence 52
2.2.3.2 Cyanobacteria genera description 53
2.2.4 Microalgae and cyanobacteria industrial application *54*
2.3 CO-CULTIVATION **55**
2.3.1 Co-cultivation possibilities *56*
2.3.2 Biological stress *56*
2.3.2.1 Co-cultivation heterotroph - phototroph 57
2.4 WASTE MATERIALS FROM THE FOOD INDUSTRY AND THEIR POTENTIAL USAGE **58**
2.4.1 Waste animal fat *58*
2.4.1.1 High-grade fat 58
2.4.2 Whey *60*
2.4.2.1 Whey composition: 60
2.4.2.2 Whey processing techniques 61
2.4.3 Spent coffee grounds *62*
2.4.3.1 Biotechnological application of SCG 62
2.5 METHODS FOR MICROORGANISM CELLS AND BIOMASS ANALYSIS **63**
2.5.1 Gravimetric determinations and microscopic observations of microorganisms *63*
2.5.1.1 Optical and fluorescence microscopy 63
2.5.1.2 Cell biomass determination 64
2.5.2 Extraction *64*
2.5.3 Transesterification *64*
2.5.4 Chromatography *65*
2.5.5 HPLC/PDA *65*
2.5.6 GC-FID *66*

3 THE AIMS OF THE STUDY **67**

4 MATERIALS AND METHODS **68**

4.1 MICROORGANISM STRAINS **68**
4.1.1 Yeast strains *68*
4.1.2 Microalgae and cyanobacteria strains *68*
4.2 CHEMICALS **69**
4.2.1 Chemicals used for cultivation of carotenogenic yeasts, microalgae and cyanobacteria *69*
4.2.2 Chemicals used for waste substrates hydrolysis *69*
4.2.3 Chemicals used for biosurfactant production and lipolytic activity assay *69*
4.2.4 Chemicals used to process and extract biomass *70*
4.3 EQUIPMENT **70**
4.4 CULTIVATION TECHNIQUES AND MEDIA COMPOSITION **71**
4.4.1 Cultivation of carotenogenic yeasts and experimental design *71*
4.4.1.1 Inoculation 71
4.4.1.2 Production media 71
4.4.1.3 Bioreactor cultivation 72
4.4.2 Cultivation of microalgae and cyanobacteria *73*
4.4.2.1 Microalgae cultivation in Brno 73
4.4.3 Microalgae cultivation in Huelva *75*
4.4.3.1 Coccomyxa onubensis cultivation 75
4.4.3.2 Botryococcus braunii cultivation 78

4.4.4 Co-cultivation of carotenogenic yeasts and microalgae 79
4.4.4.1 The first phase – yeasts cultivation on mineral BBM media 79
4.4.4.2 The second phase – microalgae cultivation on BBM media with an organic carbon source 80
4.4.4.3 Third phase – co-cultivation of yeasts and microalgae in small scale 81
4.4.4.4 The fourth phase – co-cultivation in aerated 1l Pyrex flasks. 82
4.4.4.5 Fifth phase – large scale bioreactor co-cultivation 82
4.5 WASTE SUBSTRATES HYDROLYSIS 83
4.5.1 Animal fat hydrolysis 83
4.5.1.1 Animal fat enzymatic hydrolysis 83
4.5.1.2 Animal fat base hydrolysis 84
4.5.2 Whey hydrolysis 84
4.5.3 Spent coffe grounds hydrolysis 85
4.5.4 Spectrophotometric protein determination 86
4.5.5 Botryococcus braunii botryoterpenes cyclic extractions 86
4.6 BIOMASS AND METABOLITE ANALYSIS 86
4.6.1 Biomass production determination 86
4.6.2 Photosystem II quantum yield 86
4.6.3 Optical density 87
4.6.4 Spectrophotometric determination of carotenoids and chlorophylls 87
4.6.5 HPLC/DAD analysis of carotenoids, chlorophylls, sterols and ubiquinone 87
4.6.6 HPLC/RI analysis saccharides and alcohols: 88
4.6.7 Fatty acid profile and total lipid analysis: 88
4.6.8 Lipase activity 89
4.6.9 Biosurfactants production 89
4.6.9.1 Oil spreading assay 89
4.6.9.2 Solubilization of crystalline anthracene 89
5 RESULTS AND DISCUSSION 90
5.1 YEAST CULTIVATION RESULTS AND DISCUSSION 90
5.1.1 Processing of waste substrates 91
5.1.1.1 Waste animal fat hydrolysis 92
5.1.1.2 Whey hydrolysis 93
5.1.1.3 Spent coffee grounds hydrolysis 94
5.1.2 Erlenmeyer flask cultivation of yeast on waste substrates 94
5.1.2.1 Cultivation on media with waste animal fat 94
5.1.2.2 Cultivation on whey hydrolysate 99
5.1.2.3 Cultivation on spent coffee grounds hydrolysate 99
5.1.3 Bioreactor large volume cultivation on media with a combination of waste substrates 100
5.1.3.1 Cultivation on media containing whey hydrolysate and crude fat 101
5.1.3.2 Cultivation on media containing SCG hydrolysate and crude fat 101
5.1.3.3 Cultivation on media containing SCG hydrolysate and coffee oil 101
5.1.3.4 Cultivation on media containing SCG hydrolysate and waste frying oil 109
5.1.4 Partial conclusions - Yeast cultivation 117
5.2 MICROALGAE CULTIVATION RESULTS 119
5.2.1 Microalgae cultivation in Brno 119
5.2.1.1 Microalgae cultivation on different media types 120
5.2.1.2 Multicultivator cultivation with different nitrogen sources 121
5.2.1.3 Cultivation on media with different phosphorus concentration 127

5.2.1.4 Cultivation of microalgae with the application of stress factors 127
5.2.1.5 Conclusions: microalgae cultivation in Brno 129
5.2.2 Microalgae cultivation in Huelva.....129
5.2.2.1 Coccomyxa onubensis salt adaptation experiments 129
5.2.2.2 Raceway pond cultivations of Coccomyxa onubensis 146
5.2.2.3 Botryococcus braunii v. Showa-Bielefeld cultivation 158
5.2.2.3.5Botryococcus braunii botryoterpenes cyclic extraction.....174
5.3 CO-CULTIVATION OF MICROALGAE AND CAROTENOGENIC YEASTS RESULTS 177
5.3.1 The first phase – yeast cultivation on BBM and standard media 177
5.3.1.1 Carotenoid, sterol and ubiquinone production 179
5.3.1.2 Fatty acid profile and lipid production 182
5.3.2 The second phase – microalgae cultivation on BBM media 184
5.3.3 Third phase – small scale co-cultivation.....184
5.3.3.1 Third phase: Carotenoid, chlorophyll, sterol and ubiquinone production.185
5.3.3.2 Third phase: Fatty acid profile and lipid production.....188
5.3.3.3 GC analysis co-cultivation of the yeast R. toruloides 189
5.3.3.4 GC analysis co-cultivation of the yeast P. rhodozyma 190
5.3.4 Fourth phase – co-cultivation in aerated flasks 191
5.3.4.1 Fourth phase: Carotenoid, chlorophyll, sterols and ubiquinone production
……..192
5.3.5 Summary of the results of the 1st to 4th phase.....197
5.3.6 Fifth phase – bioreactor cultivation 198
5.3.6.1 Bioreactor co-cultivations of Rhodosporidium toruloides 199
5.3.6.2 Bioreactor co-cultivations of Rhodosporidium toruloides 209
5.3.7 Co-cultivation connclusions 210
6 DISSERTATION CONCLUSIONS.....212
7 LIST OF ABBREVIATIONS.....215
8 REFERENCES.....216
9 ŽIVOTOPIS – CURICILUM VITAE 232
10 BIBLIOGRAPHY.....233

1 INTRODUCTION

The development of biotechnology, biochemistry, and genetics has enabled a huge leap in our knowledge of all types of microorganisms in recent decades. Biotechnology has accompanied humanity since the development of ancient civilisations. Archaeological finds describe the use of microorganisms in many branches of society at the time. Whether it is food production (bread, cheese, sauerkraut), beer, wine or other alcoholic beverages. We still use these microorganisms and processes, and we cannot function practically without them. With the development of science in the early 20th century and our knowledge of microorganisms, biotechnology began to be used more in industries and agriculture. For example, the automotive industry used acetone produced by fermentation to process paints. An important milestone for biotechnology was DNA discovery, which opened the way for deeper research into microorganisms, modification, and DNA transfer. Nowadays, biotechnologies are used for food production, but more and more are used to produce drugs, hormones, vitamins, industrial chemicals and many others.

Yeast was one of the first biotechnologically used microorganisms. From the production of fermented beverages and food, yeast's biotechnological use has further expanded, for example, to the production of enzymes, polysaccharides, and proteins. Carotenogenic yeasts are a group of yeasts that differ from other yeasts in the presence of an enzymatic apparatus capable of synthesising carotenoids. They belong to oleogenic microorganisms because they are able to accumulate high amounts of lipids in cells. Like their relatives, carotenogenic yeasts have the ability to process various carbon sources, such as waste from the food and agricultural industries. Their rapid growth characterises yeasts, and in combination with waste substrates, it is possible to produce a large amount of biomass with a high content of desired substances in a short time.

Microalgae and cyanobacteria are autotrophic microorganisms capable of producing the same variety of metabolites as yeast, as well as many other valuable substances. With few exceptions, they are usually unable to process organic carbon and waste substrates. On the other hand, they process carbon dioxide and have lower demands on the salt content in the medium, which reduces the cost of cultivation. As a result, they become attractive for potential use as producers of substances with higher added value (carotenoids, chlorophylls). Like yeast, microalgae are oleogenic microorganisms and are able to accumulate more than 50% of lipids in biomass under suitable conditions. On the other hand, one of the disadvantages is their slow growth and low biomass production. To increase the growth rate, cultivation in specialised reactors with constant illumination is needed, which unfortunately increases the overall cost of the process.

The demand for carotenoids worldwide is growing every year. For many years, they were used in the food industry as pigments. Their strong antioxidant activity and positive effects on human health led to the intensive development of their use in pharmacy, food supplements and the cosmetics industry. Carotenoids have beneficial effects against ageing, chronic diseases and cardiovascular diseases. Another metabolite of microorganisms are microbial lipids, so-called single cell oils. These lipids are characterised by a high content of unsaturated fatty acids, which, like carotenoids, positively affect human health. High lipid production is also attractive for the production of 3rd generation biofuels, both in the form of untreated and fatty acid methyl esters. These microorganisms also produce sterols, which are a provitamin of vitamin D, which is an important part of the immune system. Coenzyme Q, produced by microorganisms, is a powerful antioxidant whose decline in the human body is associated with ageing. It is widely used mainly in the cosmetic and pharmaceutical industries. The microorganism cell is a separate production unit that can be suitably influenced to produce the metabolites we require.

As part of this dissertation, cultivations were performed with selected strains of carotenogenic yeasts, microalgae, and cyanobacteria to optimise the production of microbial biomass enriched with studied substances with added value: carotenoids, single-cell oils, ubiquinone, ergosterol, chlorophylls. The optimisation was focused on the economics of the process and finding the cheapest variant. In work, microorganisms were exposed to various stress factors, whether biological, physicochemical or nutritional. The experimental work is divided into several main parts according to microorganisms studied: i) yeasts, ii) algae and iii) their co-cultivation.

The aim of this dissertation thesis was to study the production properties of carotenogenic yeasts, microalgae, and cyanobacteria with a focus on the production of lipidic metabolites (carotenoids, chlorophylls, ubiquinone, ergosterol) and lipids, and other metabolites. Transfer the data and experience from laboratory small-scale Erlenmeyer flasks to large scale bioreactor cultivation with the aim to find compromise between biomass and metabolites production and process costs and its reduction. To reduce costs of yeast cultivation, several food industry waste materials were tested as a possible carbon source. To enhance production of studied metabolites, microorganisms were tested under various physico-chemical, nutritional and biological stress factors. The data obtained in this work could serve as a basis for potential pilot scale experiments and practical applications.

2 THEORETICAL PART

2.1 Studied metabolites

2.1.1 Lipids

Lipids are substances of biological origin. Together with saccharides, proteins, and nucleic acids, they create all living organisms' basic building blocks. It is a large group of substances with a different structure and function combined by their nonpolar character and solubility in organic solvents such as chloroform and hexane. Hence, they are easily separated from other groups of biological materials by extraction and partition between polar and nonpolar solvents, and from a chemical point of view, they are esters of alcohols and higher fatty acids. They are products of cells primary metabolism. Depending on their structure, lipids play a number of essential roles in microorganisms' cells and higher organisms. Lipids are divided into three basic groups [1][2]:

a) Simple lipids - further subdivided into acylglycerols (oils, fats) and waxes, which are esters of higher fatty acids and higher alcohols
b) Complex lipids - structurally they are acylglycerols, where one of the ester bonds is esterified with another acid, e.g. phosphoric acid. They also contain other bound components such as carbohydrates, alcohols and others. Due to the content of these substances, complex lipids acquire an amphipathic character. This group include phospholipids, glycolipids, sphingosine.
c) The last group are derived from lipids and precursors: Here we include sterols, steroid hormones, carotenoids, ubiquinone, fat-soluble vitamins, etc.

2.1.1.1 *Structure, properties and functions of lipids*

Lipids form a large group of heterogeneous organic molecules of predominantly nonpolar or amphipathic character. Nonpolar lipids are insoluble in water and other polar solvents, whereas lipids of an amphipathic nature form micelles whose surface and structure depend on the environment's polarity. In the cell, lipids perform many essential functions [1][3][4]:

- Structural functions: In the form of phospholipids (sphingomyelins and phosphatidylglycerols) are the basic building blocks of membranes of all cells and organelles

- Protective function: Higher organisms uses lipids as a thermal insulator and protection against external influences.

- Energy storage function: Energy is stored in long hydrocarbon chains of fatty acids, and it is released via β-oxidation [1].

- Metabolic and solvent function: Lipids in cells create a nonpolar phase, in which hormones, vitamins (A, D, E, K), carotenoids are stored [4].

Majority of lipids is made up of phospholipids and triacylglycerols. This group has the highest market and financial potential. Microorganisms known for high lipid production are referred to as oleogenic microorganisms [5][7][8]. Oleogenic microorganisms produce large quantities of triacylglycerols as a storage compound which are often called “single-cell oils” with stearyl esters to a lesser extent. Finding a suitable strain for large scale production of single-cell oils (SCO) is of great interest because the SCO produced by microorganisms have different fatty acid composition than humans. SCO contains a high content of healthy and dietary essential polyunsaturated fatty acids (arachidonic acid, linoleic and linolenic acid). Thus these lipids can serve as a dietary food supplement or as a part of pharmaceutical products. Last option is the transformation into biofuels [2][3].

2.1.2 Microbial lipids as a biofuel feedstock

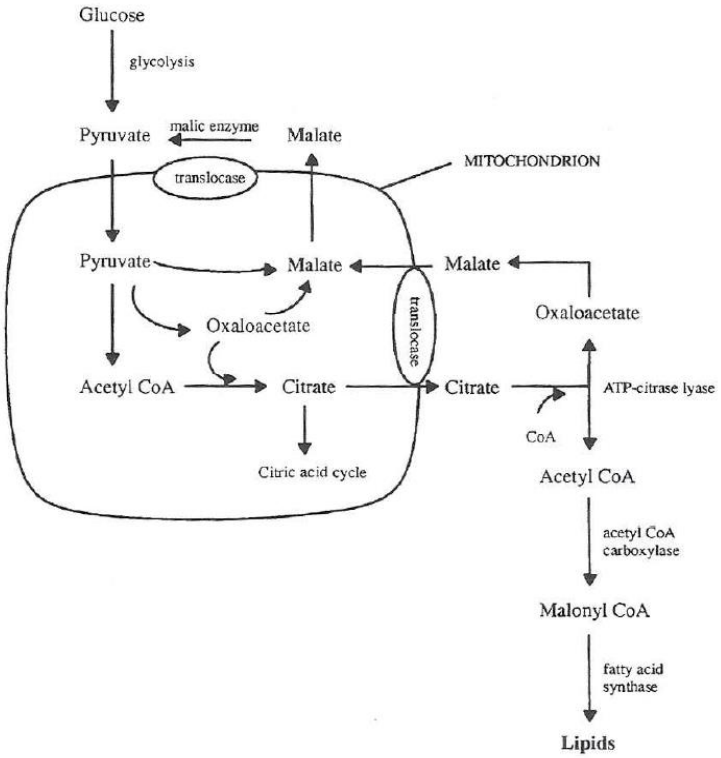
In recent years, the biotechnology industry has increasingly focused on microbial lipid production, which can be used in the food, pharmaceutical, and petrochemical industry. First-generation biofuels have pioneered this new area. First-generation biofuels were agricultural products, such as maise, sugar beet and cane, oilseed rape and others, transformed into bioethanol and biodiesel by chemical reactions [10].

Due to the new regulations concerning the content of the bio-component in fuels in individual countries of the world (e.g. the European Union), an accompanying phenomenon of biodiesel's growing consumption was dramatically rising price of lipid-containing vegetable, its raw materials and their associated food prices. This phenomenon then led to a change of strategy, in which other sources were sought that would not directly compete with the food industry. This gave birth to the idea of second-generation biofuels, where waste materials from agriculture and other sectors such as hay, straw and wood processing residues served as feedstock for conventional chemical reactions, like pyrolysis. The resulting product is called a syn-gas [10], which can then be transferred to various products, such as methanol, mixed alcohols, and others. On one side, the idea of second-generation biofuels eliminated the problem of using food and feed resources and saving precious farmlands. However, chemical methods and processes used in this production, do not provide the same quality and not even the high yields as first-generation biofuels. The problem lies in the overall complexity and structure of the waste materials, low content of desired transformable molecules and low theoretical yields [10].

With the development of environmental awareness in developed countries combined with the disadvantage mentioned above of first and second-generation biofuels, a platform has been created for a new generation of biofuels using microorganisms' enormous potential. Biofuels produced by microorganisms are referred to as third-generation biofuels. Microorganisms can utilise a wide range of waste substrates, be it cellulose wastes, fats, etc., and convert them into substances with higher added value. This leads to the creation of a zero-waste economy, where different microorganisms process the wastes of different industries to produce biomass with added value, for example, biomass with high lipid content [10][11][12].

Yeast, like all heterotrophic eukaryotes, needs an organic carbon source to grow and produce lipids. Yeasts are able to utilise a large variety of different carbon sources. Therefore, the main economic requirements are to reduce the costs associated with the substrate and medium costs as much as possible. The use of waste agricultural raw materials appears to be the ideal solution. Cultivation, which would allow the valorisation of this waste, thus reducing the burden on the environment and at the same time enabling the production of valuable microbial oils, could be of great interest. The most common and studied oleaginous yeasts include the genera *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Yarrowia*, *Candida* and *Lipomyces*. Some of these strains are able to accumulate up to 70-75% lipids in dry biomass. Another advantage of yeasts is the cultivation without the need of constant illumination, which allows continuous cultivation and reduces the potential costs of artificial lighting [12][13][15].

In contrast, algae are photoautotrophic microorganisms that require only solar energy and atmospheric CO_2 for growth. This makes them a very attractive source for biodiesel production. Common microalgae strains that achieve high lipid yields in dry biomass (from 20 to 75%) included, for example, the genus *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Botryococcus* or *Neochloropsis*. Another group, cyanobacteria are able to convert up to 10% of solar energy into biomass. Microalgae and cyanobacteria light-harvesting effectivity significantly exceeds conventional crops that possess less than 5% efficiency [16]-[18].

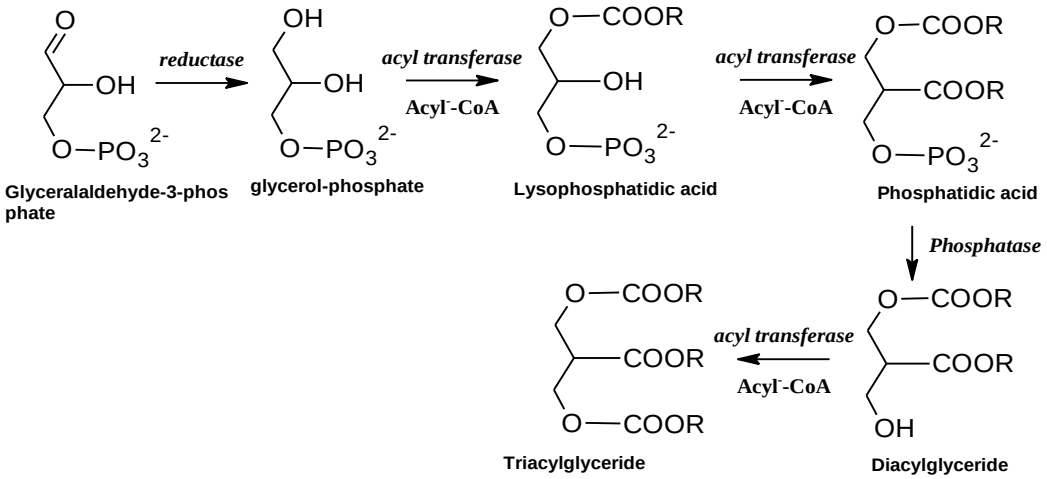


Scheme 1. *Biosynthesis of lipids in carotenogenic yeast cells [1][2].*

2.1.3 Lipid biosynthesis: phospholipids and triacylglycerols

The metabolic pathway of lipid synthesis is, in principle, conservative and the individual steps and chemical reactions are practically identical between all cell types and microorganisms [1]. The difference lies in the enzymatic composition, enzyme structure and the sequence of reactions. To form a new triacylglycerol or phospholipid, a microbial cell has to undergo a rather energy-demanding process. The whole process consists of several key steps. Starting with key precursor molecules, fatty acids and active glycerol. Activated forms of glycerol (glycerol-3-phosphate, dihydroxyacetone phosphate) are obtained mainly from glycolysis of carbohydrates. Active glycerol can also be synthesised via phosphorylation reaction by enzyme glycerol kinase, which forms glycerol-3- phosphate. Dihydroxyacetone phosphate can be incorporated into the pathway by reductive conversion to glycerol-3-phosphate, or the first acyl group is first added by a specific acyltransferase to its molecule, followed by reduction of the keto group to give 1-acylglycerol-3-phosphate [2][3] .

In the second step, two fatty acid chains are sequentially attached to active glycerol to form phospholipids. When phospholipid production requirements are met, and the cell has enough energy, the phosphate group on the third glycerol carbon atom is replaced with a third fatty acid molecule to form a triacylglycerol energy storage compound. The finished lipid structure can then be attached to other functional groups, such as monosaccharides and proteins, thus gaining new properties, function, and localisation in the cell. Biotechnologically important is especially the overproduction of lipids, which occurs under the lack of nutrients such as nitrogen, phosphorus, sulphur and at the same time an excess of carbon source. Lack of nutrients leads to the cessation of energy-demanding processes associated with cell division and proliferation. Lipid biosynthesis in eukaryotic cells occurs in the cytosol and is separated from its catabolic counterpart, located on the mitochondrial membrane's inner side [1][18][19][20].



Scheme 2. *Phospholipid and triglyceride synthesis [1]*

Lipid overproduction starts with metabolism shift regulated by several key enzymes. The first enzyme involved is the adenosine monophosphate deaminase. Its upregulation leads to cleaving the amino group attached to 6th carbon of AMP, forming inosine monophosphate (IMP) and ammonium ion. Lowered AMP concentration in the cell cytoplasm leads to the reduction of isocitrate dehydrogenase (ICDH) activity, thus increasing isocitrate concentration in mitochondria, which is transported into the cell's cytoplasm via citrate/malate translocase. Citrate lyase cleaves isocitrate, forming oxalacetate and Acetyl-CoA. Oxalacetate returns to the mitochondria in the form of malate by citrate/malate translocase (Scheme 1). Acetyl-CoA enters the process of fatty acid synthesis. In total, eight molecules of Acetyl-CoA and 16 molecules of NADPH are required to synthesise one palmitic acid molecule [8][21][22].

Microalgae are also capable of lipid synthesis inside chloroplasts. However, the production of lipids in chloroplasts mainly serves to cover the needs of the chloroplast itself, and only a small part is exported outside the membrane to the cytosol, where it is used to form storage substances. There are three different sources of fatty acids: external supply, endogenous lipid turnover, and the de novo synthesis and elongation. Cells tend to incorporate mono- and polyunsaturated fatty acids from external sources into their own membranes. The absorption and incorporation of PUFA and MUFA are given by the given cell's insufficient enzymatic apparatus (heterotrophic organisms) [8][15][23].

2.1.4 Fatty acids: properties and synthesis

Fatty acids play an essential role in cells of all living organisms. They provide a variety of important roles. Firstly, their long hydrophobic chain is able to create a force strong enough, to form a membrane bilayer structures. This structure serves as a basis for subcellular compartmentalisation. Second, fatty acids serve as precursors for biologically active compounds and thus bear the signalling function (arachidonic acid). Third, fatty acids are perfect for energy storage. Multiple C-C bonds contain large amounts of energy, that can be released via β-oxidation. This process generates molecules od Acetyl-CoA, which can be used as a building block for anabolic pathways, such as the isoprenoid pathway. Acetyl-CoA can be further catabolised in the Krebs cycle to provide more energy. Fatty acid synthesis is a rather a remarkable achievement of evolution because C-C bonds' formation is an extremely energy-demanding process [18][22][24]. Furthermore, the formation of hydrophobic fatty acid chains in the cell's hydrophilic environment places high demands on the enzymatic apparatus involved.

Fatty acids, on the other hand, pose a threat to the cell itself. At higher concentrations, amphipathic chains of fatty acids acts as detergents and disrupt the structure of membranes and proteins. For this reason, they are stored in cells in lipid droplets, most often bound in the form of esters (triacylglycerols) [1][2][12][23].

Fatty acid synthesis is a chemically conserved pathway that is virtually unchanged for all known organisms. Fatty acids are synthesised using a complex of multiple enzymes called fatty acid synthase (FAS), which has evolved into two forms during evolution [28]-[31]:

- **FAS I** – utilises a large multifunctional polypeptide, which is common to both fungi and animals. Type I was also found in the CMN group of bacteria (*Corynebacteria, Mycobacteria* and *Nocardia*), where it cooperates with FAS II, resulting in various products [26][28].
- **FAS II** – this complex utilises a group of discrete monofunctional enzymes. It is found in plants, algae, archaea and bacteria [28].

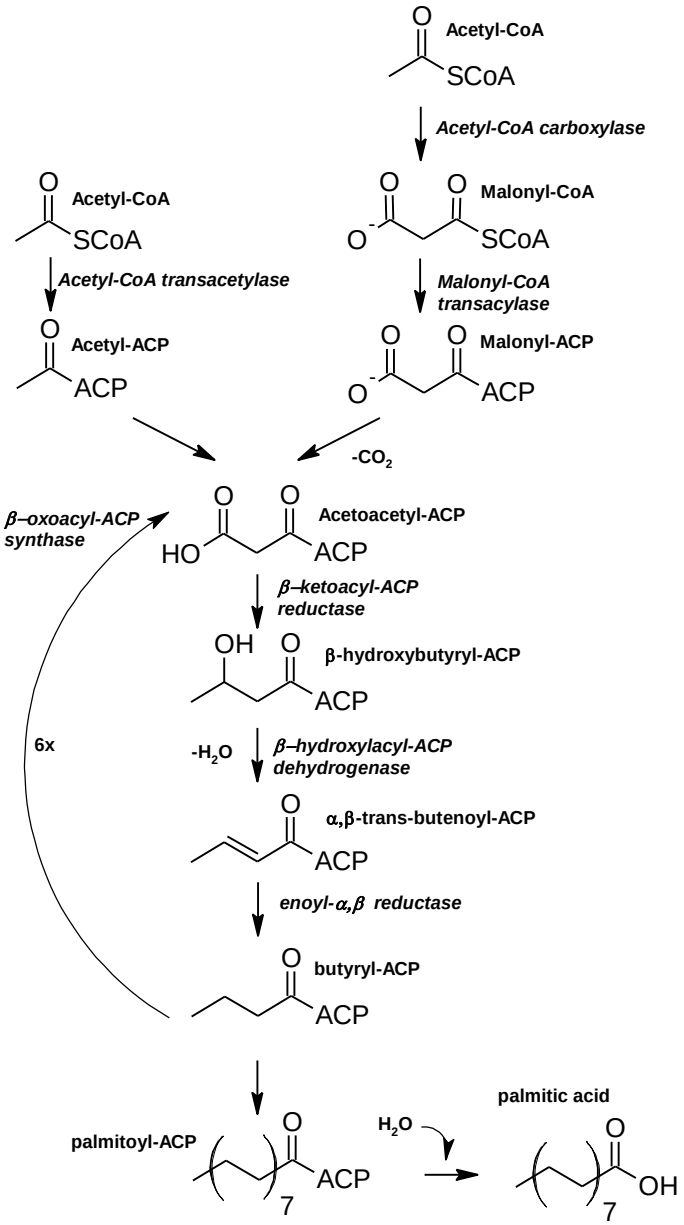
In yeast cells, FAS I complex resembles the shape of a rigid barrel with six active chambers. Every active site works independently, and thus the FAS complex is able to process six molecules at one time. Acyl carrier protein (ACP) serves as the only mobile domain. FAS I complex is anchored on the inner side of the cytoplasmic membrane.

2.1.4.1 *Fatty acid synthesis*

Fatty acid synthesis begins with transforming the Acetyl-CoA molecule to the acyl carrier protein by the enzyme acetyl-CoA transacetylase and subsequent transfer to the cysteine residue of the FAS I complex. Acetyl-CoA carboxylase converts the second molecule of acetyl-CoA to malonyl-CoA and then transfers it to ACP (Scheme 3). This step is the crucial step of the whole process. The energy stored in the carboxyl group is then used to condense both molecules and form a C-C bond. Simultaneously, the formation of malonyl-CoA is an irreversible process and is the main regulatory point of the entire pathway. Subsequent condensation done by β-oxoacyl-ACP-synthase forms a C_4 backbone. Reaction energy is provided by associated decarboxylation. The formation of beta-oxoacyl-ACP is the end of the first phase, which forms the C-C bond and extends the chain [1][31][34][39].

In the second phase, C_4 backbone is further processed by the FAS complex by a series of enzymatic reactions concentrated on a third carbon atom. Oxo group on third carbon is reduced by β-ketoacyl-ACP-reductase. Subsequent dehydration catalysed by β-hydroxyacyl dehydrogenase produces α, β-trans-butenoyl-ACP (Scheme 3). The last step is the second reduction with enoyl-α,β-reductase to give the final butyryl-ACP. This mechanism is repeated six times to form the C_{16} chain of palmitic acid (palmitoyl-ACP), which is hydrated by palmitoyl esterase and cleaved from the FAS complex. This pathway is only able to produce palmitic acid. All other fatty acids are produced by subsequent enzymatic reactions taking place mainly on the endoplasmic reticulum. The fatty acid profile is specific for each microorganism and depends on its genome, external conditions, and other factors [31][34][39].

Microalgae, as mentioned above, have the possibility to synthesise fatty acids inside chloroplasts. Thanks to the genome inherited from cyanobacteria. However, during evolution, genes encoding different types of fatty acids in chloroplasts have been lost, and today, most representatives of microalgae are able to produce palmitic, stearic, and oleic acids in chloroplasts. This phenomenon is probably due to redundancy. The microalgae cell does not need two synthetic sites to synthesise the required amount (small amounts) of polyunsaturated fatty acids. If needed, precursors are transferred to the endoplasmic reticulum and processed into the desired product. After transformation, the newly formed fatty acids are transported back to the chloroplast [40]-[44].



Scheme 3. *Scheme of fatty acid synthesis*

2.1.5 Carotenoids

Carotenoids are a fascinating group of natural pigments found throughout the planet and performing many essential functions. They are found in lower and higher organisms, such as cyanobacteria, bacteria, yeasts, algae and plants. Carotenoids are derived from terpenes, consisting of 8 isoprene (C5) units [2][3][45]. The concept of carotene was first introduced in 1831 by Wackenroder, who for the first time isolated and described carrot pigment, now known as β-carotene. One of the scientists that followed his research was Berzelius in 1837 with successful isolation of yellow pigments extracted from old leaves [46]. These group of pigments with related properties and structure as carotenes were later named and introduced by the term xanthophylls.

In the early 20th century, Mikhail Semyonovich Tsvet joined these two groups of chemically related pigments containing both carbohydrate carotenes, characterised by red-orange colour and their yellow-coloured xanthophyll oxygen derivatives in the group called Carotenoids [30][46][47]. In this work, he also laid the foundations of an analytical technique called chromatography, which is still used as the dominant method for the separation and analysis of carotenoids and many other molecules [46].

2.1.5.1 *Carotenoids commercial applications*

Overall production and demand for carotenoids have grown exponentially worldwide in recent decades. The global carotenoid market was estimated to be ~ 1,24 billion USD in 2016 and is projected to increase to ~ 1,53 billion USD in 2021 [50]-[52]. This trend is due to many factors. Rising awareness about a healthy lifestyle in the average population led to high demand for quality and nutrient-rich food and supplements with health benefits (such as strong antioxidants, immunity boosters and supplements rich in vitamins and proteins). Carotenoids have various medicinal properties, which allow them to be used as preventives against civilisation diseases, such as cancer, sight problems, diabetes, or reducing cardiovascular diseases [51][54]-[56]. Carotenoids have been traditionally used for decades in a wide range of food and agricultural industries, due to their colour and nutritional properties. They are considered safe natural pigment and are added to various food and products to enhance their colour (yoghurts, juices) [51]. Nowadays, they are widely used in cosmetics, pharmaceuticals and food supplements for athletes and the active population. Therefore, carotenoids' world market can be expected to continue to grow steadily in the coming years [1][51]-[53].

2.1.5.1.1 Agriculture industry

The largest consumer of carotenoids was the agricultural sector, more precisely the feed sector with a market share of 47% in 2014 with 562 million USD. This segment is expected to grow by about 3.5-4% per year [52]. In aquaculture, the fastest-growing segment of feed industry worldwide, carotenoids are supplemented via dried cells of astaxanthin producing algae *Haematococcus pluvialis*, to give the desirable red colour of crustaceans, salmonids and other farmed fish meat [57]. For example, in the poultry industry, the carotenoid supplemented diet promotes orange-yellow colour of egg yolk, oxidation stability of lipids and increases eggs longevity. Furthermore, hens supplemented with this diet produced more eggs and were in overall better health than the control group without carotenoid supplementation [57]. In addition to providing nutrition and colour, carotenoids are strong antioxidants. They are used as strong antioxidants to slow the oxidation process, and therefore, reducing the speed of food degradation and development of off-flavours [57][59][60].

2.1.5.1.2 Cosmetic industry

Similarly to agriculture/feed industry, carotenoids are used in cosmetics for their potent antioxidant properties and nutritional value. They serve as active ingredients with biological activity in lotions and creams. Rising demand from cosmetic companies for organic/bio and herbal products creates a great potential for carotenoid-containing products derived from microalgae and yeasts. Carotenoids are used as a topical treatment to serve as protection from UV-light. Interestingly, carotenoid colour properties are exploited in tan lotions. It was found out that diet rich in carotenoids can enhance skin protection capabilities against UV-light [61][62].

2.1.5.1.3 Nutraceutical and health market

The nutraceutical market is currently the fastest-growing segment of the carotenoid market with ~ 440 million USD per year. As mentioned above, carotenoids are on very high demand as dietary supplements. Oxidative stress, free radicals, pollution, and cellular redox imbalances are well known to trigger the development of many human diseases, including neurodegenerative diseases [64]. Carotenoids as antioxidants, neutralise free radicals, prevent or slow down chronic diseases and cellular damage. It has been reported that carotenoids can slow down inflammation, cancer, heart disease [65], neurodegenerative diseases (Alzheimer's disease, Parkinson's [63]), type 2 diabetes [66]), chronic eye diseases [67] and obesity [68]. With increasing environmental pollution and increasing life expectancy, the risk of a number of civilisational and genetic diseases increases. A diet rich in carotenoids or other antioxidants can reduce the risk of these diseases [71].

2.1.5.1.4 Carotenoid production

Currently, most carotenoids, that are sold on the market are prepared by chemical synthesis. Only a small portion is obtained from natural sources such as plants and algae. Carotenoids can be synthesised rapidly with low costs and inexpensive chemicals, thus negating living production organisms and subsequent extraction costs. On the other hand, the main drawback of this is that synthetic carotenoids do not have the same health-promoting properties as naturally synthesised relatives. Hence, they are less desirable and valuable product [50][51].

The percentage of naturally extracted carotenoids on the market is gradually increasing. Unfortunately, it is mostly made up of plant extracts. In this carotenoid extraction process, large agricultural areas are used as raw materials sources that could otherwise be used to grow crops primarily intended for food. In addition, this process is strongly dependent on weather, rainfall, pests and other factors that are very difficult to influence. Microorganisms, such as microalgae and carotenogenic yeasts offer a wide range of carotenoid products (and many others), the possibility of year-round cultivation, the use of cheap substrates, a controlled cultivation environment that is entirely under control [50][51].

The combination of several factors, such as the growing ecological awareness of the population, changes in opinion about synthetically prepared additives and colourants, the growing population of our planet and the effort to recycle the waste we produce, leads to an effort to use microbial producers, as a future source of carotenoids [51]. Therefore, carotenoids are currently a frequent topic of publications focusing on different microorganisms that can produce carotenoids and other valuable products and thus allow diversion from traditional sources of carotenoids such as fruits and vegetables synthetic chemistry [50][69].

2.1.5.2 *Carotenoid properties*

Carotenoids are lipophilic substances practically insoluble in water and other very polar solvents. They dissolve well in nonpolar solvents. Their properties are based on the molecule's structure, especially the conjugated chain of double bonds, which gives them many exciting properties. Carotenoids are relatively resistant to pH changes and reducing agents. However, they are susceptible to the effects of intense light radiation and oxidising agents, resulting in their oxidation and subsequent decomposition. A large number of multiple bonds in carotenoid molecules readily react with oxidising agents such as various types of oxygen radicals, peroxides, etc. The willingness to react strongly with oxidising agents in biological systems is used as protection against radicals, where this reactivity is referred to as antioxidant activity. Carotenoids are known to have one of the highest antioxidant activities in biological systems [1][3][46][48].

Probably the best-known property of carotenoids is their intense colour, which is based on the structure of the molecule itself. The ability to absorb light rays by carotenoid molecules is determined by the central chain structure containing a conjugated double bonds system that allows carotenoids to absorb light in the visible light region. The colour spectrum of carotenoids varies from yellow to deep red. The maximum absorption band ranges from 400 to 500 nm, whereby the structure of the side chains, in particular, the presence of conjugated bonds and oxygen atoms shifts the absorption spectrum to lower or higher wavelengths. Shift to higher wavelengths (red colour) is observed in carotenoids with a longer chain of conjugated double bonds. Presence of polar oxygen groups on the carotenoid structure shifts the maxima to lover wavelengths (yellow colour) [1][3][46][48].

Carotenoids serve many functions in the metabolism of microorganisms, plants and animals. The primary function is given by their potent antioxidant activity mentioned above. Cell carotenoids serve as one of the dominant free radical scavengers and other reactive oxidative agents that arise spontaneously in different metabolic pathways or by external agents. These radicals act on biologically essential compounds such as proteins and nucleic acids in the body. Their interaction with radicals causes a change in function, cell structure, more extensive tissue damage or tumour growth. Due to their function, carotenoids are found mainly in the area of individual organelles' membranes, where they also prevent the peroxidation of lipid membranes and thus cell death. In the case of animals, carotenoids serve as vitamin A precursor, which are essential for the sight's proper functioning [1][3][46][48].

One of the essential functions of carotenoids is associated with phototrophs and photosynthesis (). In the photosynthetic apparatus of all phototrophs, carotenoids serve as antenna pigments. They are concentrated in thylakoids of prokaryotes and eukaryotic organisms. This function is again based on a conjugated chain of unsaturated bonds. The carotenoid molecule absorbs incident light and enters the excited state. In this state, the excited electron serves as an energy carrier and is transferred through the molecule chain to other antenna pigments until it finally arrives at the photosynthetic reaction centre containing the chlorophyll molecule, where its energy is used to produce reduced NADPH cofactor and hydrogen proton gradient [1][3][46][48].

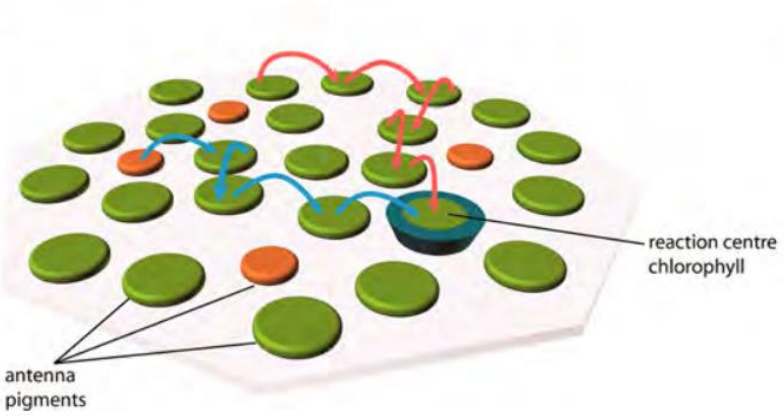


Figure 1. Schematic of light absorption system in the photosynthetic apparatus. Carotenoids (orange) and chlorophyll B serve as antenna pigments, absorbing light energy and transferring to the reaction centre [70].

2.1.5.3 *Carotenoid structure*

From a biochemical point of view, carotenoids belong to the group of nonpolar molecules called terpenoids, precisely the tetra-terpenes. They are the product of a secondary metabolism pathway originating from the isoprenoid pathway. Chemically, carotenoids are a group of polyunsaturated aliphatic and cyclic hydrocarbons and their respective oxygen derivatives. The general structure of all carotenoids consists of three parts. The first part is the central linear hydrocarbon chain consisting of 22 carbons, which is the same for all representatives of this group (Figure 2). The chain is formed by a system of conjugated double bonds with four substituted methyl groups along the chain. This part is relatively conservative, and there are only a few variations that differ in the spatial arrangement of the methyl groups to the main chain [1][72][74].

Most carotenoids prefer trans configuration of unsaturated bonds in the central region over cis configuration because of the whole molecule's spatial and energetic stability. The other two parts marked as R^1 and R^2 on the picture below are linked to both sides of the central chain. These two chains are then responsible for the wide variety of carotenoids. These parts R^1, R^2 can be formed both by linear, branched or cyclic chains of 9 carbons. Xanthophylls differentiate from carotenes by the presence of oxygen atoms in the form of hydroxyl, carboxyl or carbonyl groups [1][72][74].

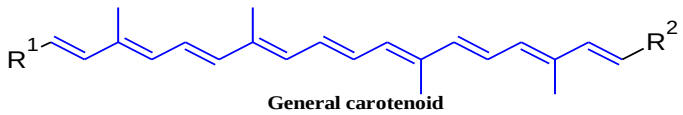


Figure 2. The general structure of carotenoids.

Generally, carotenoids are divided by two simple parameters. The first is based on the shape of the side-chains mentioned above, according to which we divide carotenoids into three groups [1][72][74]:

a) Acyclic - a linear chain terminates both side chains. (δ-carotene, lycopene, neurosporene)

b) Monocyclic - One side chain is cyclised to form a five or six-membered cycle while the other has a linear structure (torulene, torularhodin, γ-carotene)

c) Bicyclic - Both side chains are terminated by a cycle (β-carotene, astaxanthin, lutein)

The second distinguishing parameter is the presence of oxygen atoms on the side chains of the molecule, which divides carotenoids into [1][72][74]:

a) Anoxygenic hydrocarbons: Carotenes

b) Oxygen derivatives: Xanthophylls

Xanthophylls form the largest group of carotenoids. This includes biosynthetic representatives as well as various derivatives formed by oxidation by non-enzymatic reactions [1][72][74]. As mentioned above, carotenoids are located in various compartments in microorganisms' cells, especially in biomembranes, where they serve as antioxidant protection. For this purpose, carotenoids are further enzymatically treated to form fatty acid esters. The esterified form of carotenoids provides greater stability to oxidising agents. Furthermore, it allows the cell to accurately distribute and anchor carotenoids in organelle membranes where they are needed. The second part of carotenoids is concentrated in lipid bodies inside cells and organelles, where it serves as an immediate backup if needed. Most carotenoid-producing microorganisms, in adverse conditions, accumulate larger amounts in these lipid droplets [1][72][74].

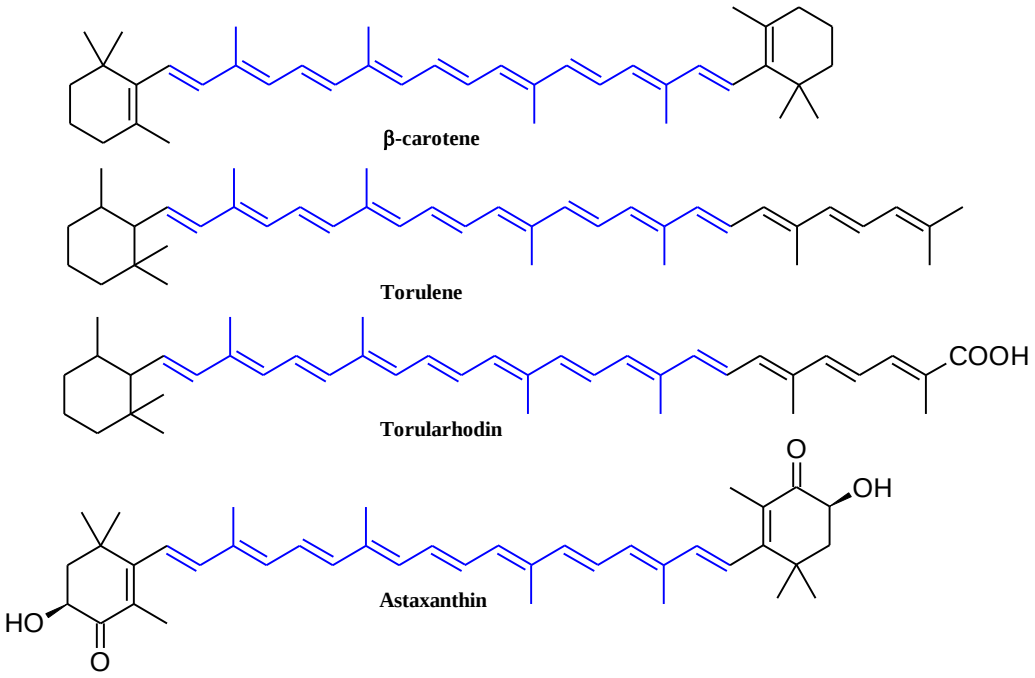


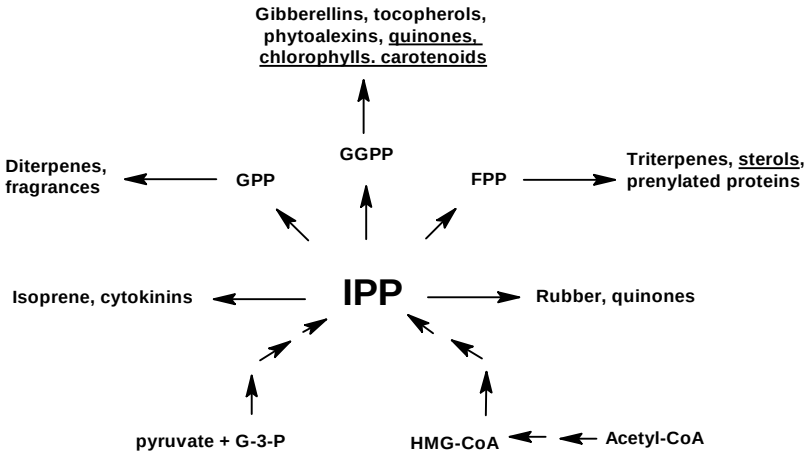
Figure 3. Carotenoid pigments produced by carotenogenic yeasts [1].

2.1.5.4 *Carotenoid biosynthesis*

As mentioned in the previous chapters, carotenoids are products of the isoprenoid pathway that is part of both primary and secondary metabolism. Carotenoid biosynthesis is a two-step process. The first step is the synthesis of the active isoprene in the form of isopentenyl pyrophosphate (IPP) or dimethylallyl pyrophosphate (DMAPP). This part of secondary metabolism is essential for all organisms because, in addition to carotenoids, the active form of isoprene is used to synthesise various other essential molecules (sterols, steroid hormones or electron transporters such as ubiquinone). The synthesis of IPP is followed by a series of condensation reactions in the second phase. These activated units can condense in three ways: "head - heel", "head - head" or "heel - heel" [1][3][74][75].

2.1.5.4.1 Isoprenoids

Isoprenoids are one of the oldest and most extensive groups of substances on earth. This large group of substances contains over 30,000 representatives with very different functions and structures, such as essential oils, poisons, growth factors, fragrances, sex attractants, and polymers found in all living organisms (Scheme 4). Large unsaturated isoprene chains are found in the structure of ubiquinone, known as coenzyme Q_{10}, which plays an indispensable role as an electron transporter. Isoprenoids include sterols, which are essential components of biomembranes providing fluidity and serve as precursors to steroid hormones and bile acids. Last but not least, isoprenoids include the aforementioned important group of carotenoids [79]-[82]. Isoprenoids are derived from the five-carbon skeleton of isoprene. The enormous diversity of structures and the variety of features and functions of individual representatives are determined by the number of condensed isoprene units, their cyclisation, cleavage, oxidation, and other reactions. Generally, the terpenes are divided into groups by the number of condensed isoprene units [79]-[82].



Scheme 4. *Scheme of products of the isoprenoid pathway [1][2]*

2.1.5.4.1.1 Isoprene and synthesis of IPP and DMAPP

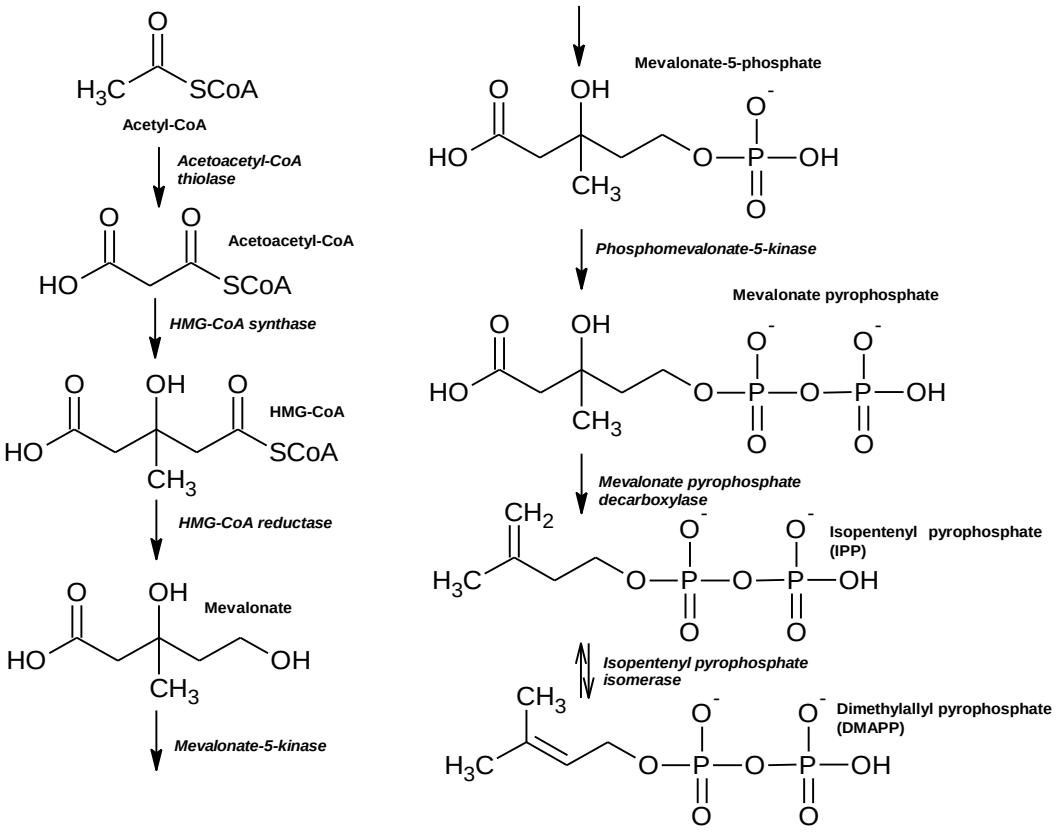
Isoprene is an unsaturated hydrocarbon of a nonpolar nature, derived from 1,3-butadiene, from which it is distinguished by the methyl group attached to the second carbon. It occurs in nature in fossil fuels and living organisms in two forms: isoprene itself or its active forms isopentenyl pyrophosphate and dimethylallyl pyrophosphate (DMAPP). Isoprene itself occurs mainly in plants that synthesise it from dimethylallyl pyrophosphate using isoprene synthase enzyme. Increased isoprene production occurs at slightly elevated temperature, where it serves as plant protection from thermal stress. Active forms of isoprene undergo condensation reactions to produce isoprenoids [79]-[82].

There are two distinct IPP biosynthesis pathways in nature: HMG-CoA reductase pathway (known as the mevalonate pathway) and the later discovered pathway, called deoxy-D-xylulose-5-phosphate pathway (DXP pathway), based on studies of the historical evolution of the enzymes present in these two pathways and phylogenetic distributions in microorganisms. According to these studies, the mevalonate pathway developed in *Archaebacteria* and deoxy-D-xylulose-5-phosphate pathway in *Eubacteria*. In eukaryotic cells, genes for isoprene synthesis were inherited from prokaryotes and mevalonate pathway mostly predominates. The only exception is phototrophic eukaryotes, which can synthesise active isoprene in photosynthetically active plastids (chloroplasts) in addition to the mevalonate pathway. This fact confirms the endosymbiotic theory of the formation of phototrophic eukaryotic cells, where the prokaryotic cyanobacterial cell was probably assimilated, from which chloroplast gradually developed as we know it today. The results also suggest that gene transfer between *Eubacteria* representatives also played an essential role in the development of plastids and thus, the transfer of genes responsible for DXP pathway development [79]-[82].

2.1.5.4.1.1.1 The Mevalonate pathway

The mevalonate pathway is an essential metabolic pathway for many bacteria and all higher Eukaryotes. This pathway's products are isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which are then used for isoprenoid synthesis, protein prenylation, N-glycosylation, or hormone synthesis. Acetyl-CoA is the input molecule for this pathway and at the same time the sole carbon source. Mevalonate pathway is a metabolic pathway located at the interface of smooth endoplasmic reticulum and cell cytoplasm. In prokaryotes, enzymes are dispersed in the cytoplasm. The mevalonate pathway can be divided into two parts based on all representatives' common features, the so-called upper and lower parts [79]-[82].

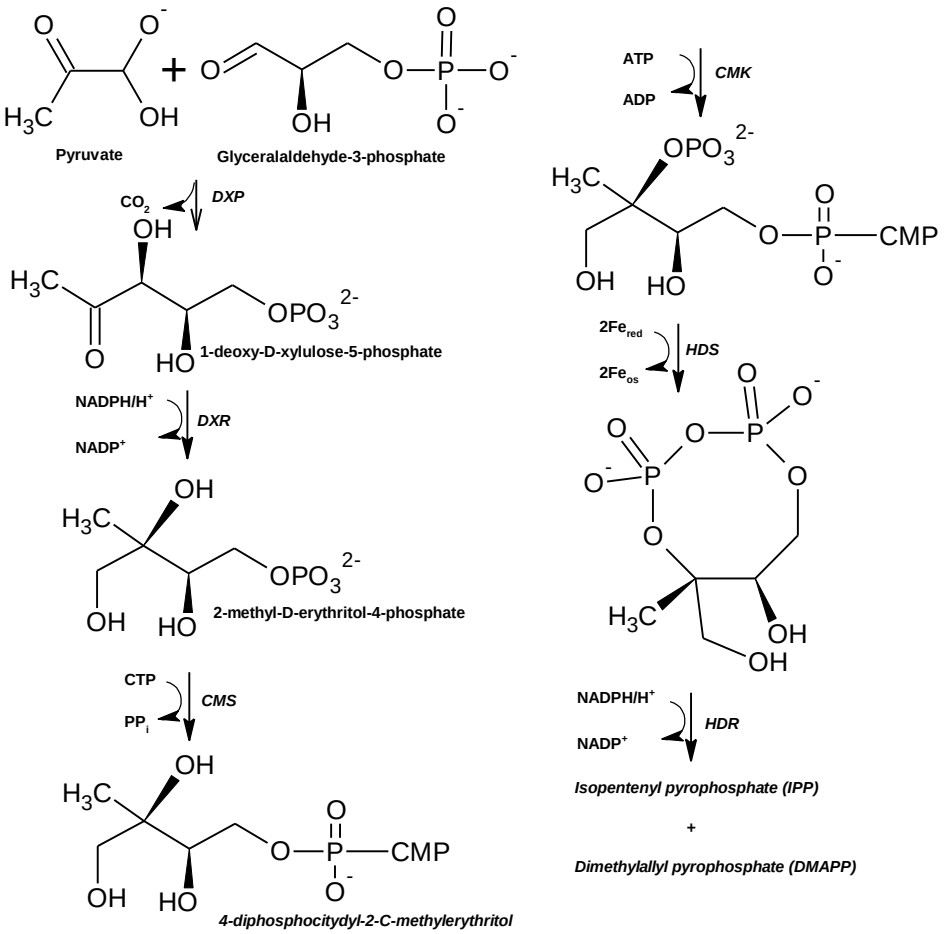
The upper part is conservative for all representatives and begins by condensing two Acetyl-CoA molecules to form Acetoacetyl-CoA, which provides β-hydroxymethylglutaryl-CoA (HMG-CoA) by condensation with the third Acetyl-CoA molecule. This phase's last step is controlled by the enzyme HMG-CoA reductase, which transforms HMG-CoA to mevalonic acid. This enzyme is crucial for the entire metabolic pathway, functioning as its central regulatory point (Scheme 5). The second part of the mevalonate pathway is different within each organism group. In the case of Eukaryotes, double phosphorylation of mevalonic acid occurs to form mevalonate pyrophosphate, followed by final decarboxylation to form IPP. For most *Prokaryota* and *Archaea*, mevalonic acid is decarboxylated and phosphorylated in one step to form isopentenyl phosphate. The latter is then phosphorylated a second time to form the resulting IPP: Isomerisation enzyme converts IPP to DMAPP if necessary [79]-[82].



Scheme 5. *IPP and DMAPP synthesis in the mevalonate pathway [79]-[82]*

2.1.5.4.1.1.2 Deoxy-D-xylulose-5-phosphate pathway

Unlike the mevalonate pathway, plants, *Protozoa* and *Eubacteria*, developed an alternative pathway to produce IPP and DMAPP, using pyruvate and D-glyceraldehyde-3-phosphate molecules. This metabolic pathway is located in the cytoplasm of prokaryotic cells. Phototrophic eukaryotes synthesise it predominantly in chloroplasts. Biosynthesis begins with the condensation of pyruvate and D-glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose-5-phosphate (DXP). IPP and DMAPP products are then generated by a series of enzymatic reactions, starting with the conversion of DXP to 2C-methyl-D-erythritol-4-phosphate, followed by binding of the cytidyl moiety and phosphate group to form 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (Scheme 6). This molecule then undergoes a cyclisation reaction associated with the phosphate rearrangement to form 4-hydroxy-3-methylbut-2-enyl pyrophosphate. The last reduction step produces isopentenyl pyrophosphate and dimethylallyl pyrophosphate [84]-[86].



Enzymes:

DXP - 1-deoxy-D-xylulose-5-phosphate synthase ***DXR - 1-deoxy-D-xylulose-5-phosphate reductase***

CMS - 2-C-methyl-D-erythritol-4-phosphate citydyltransferase ***CMK - 4-diphosphocitidyl-2-C-methyl-D-erythritol kinase***

MCS - 2-C-mehyl-D-erythritol 2,4.cyclodiphosphatesynthase ***HDS - HMB-PP synthase***

HDR - HMB-PP reductase

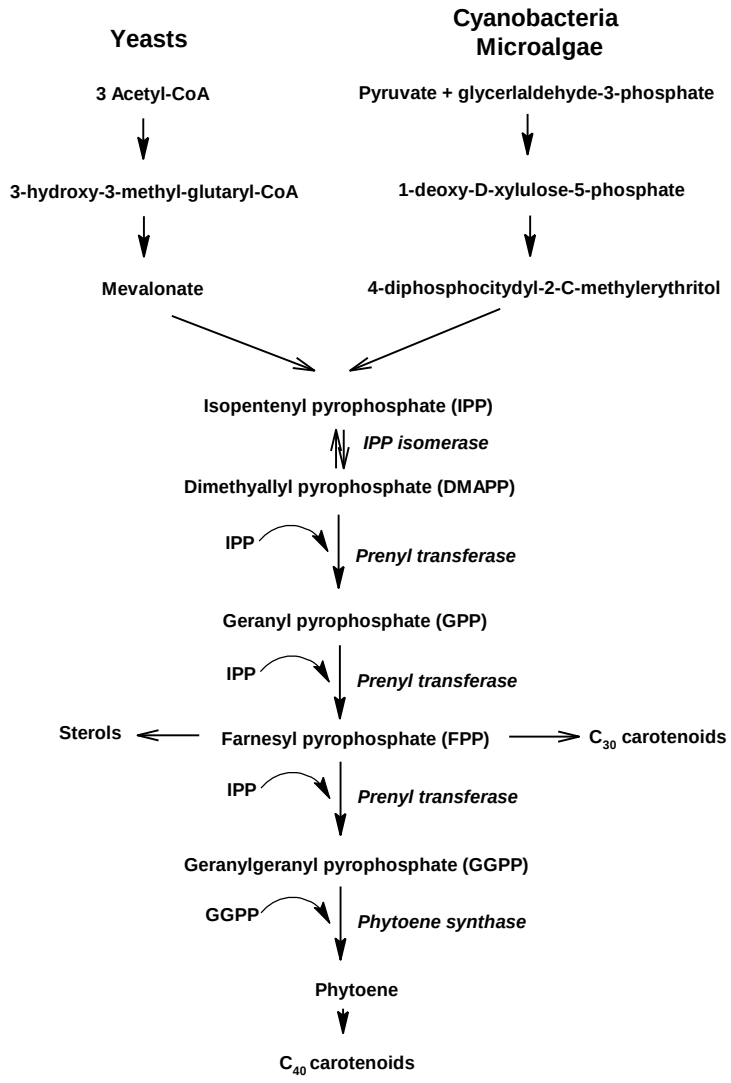
Scheme 6. *Scheme of the Deoxy-D-xylulose-5-phosphate pathway [84]-[86]*

2.1.5.5 *Carotenoid biosynthesis in cells*

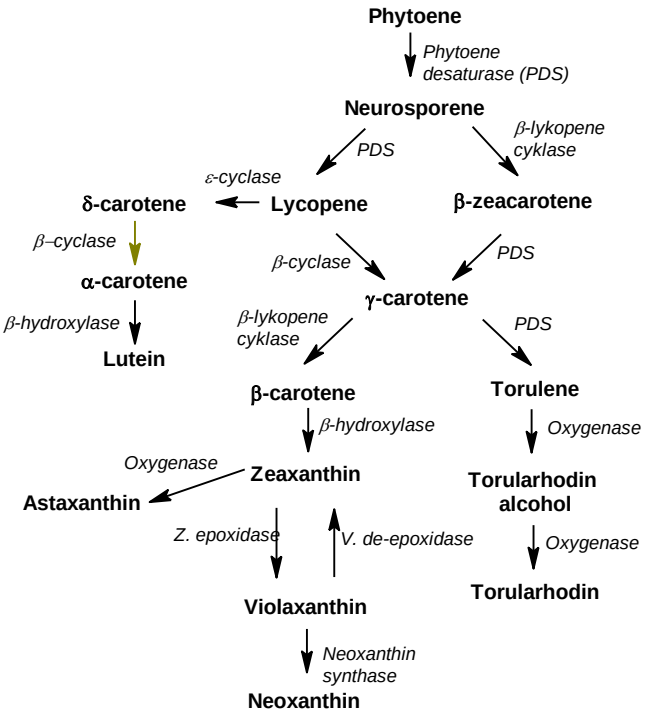
Biosynthesis of carotenoids in heterotrophic eukaryotes, similarly to the synthesis of isoprene, occurs with the participation of endoplasmic reticulum enzymes and in the case of phototrophic eukaryotic organisms also occurs in chloroplasts. This metabolic pathway is relatively conservative in its origins and is virtually identical in all production organisms. As mentioned above, active isoprene molecules are the starting molecule entering carotenoid biosynthesis. The sequence of reactions begins with the condensation of two activated isoprene units "head to tail" by geranyl pyrophosphate synthase to form geranyl pyrophosphate (GPP). The subsequent reaction is a condensation of two GPP units to form geranylgeranyl pyrophosphate [1][74]-[78][83].

Phytoene synthase enzyme condensates two geranylgeranyl pyrophosphate molecules "head to head" to produce carotenoid precursors phytoene. The resulting phytoene then undergoes a series of 4 consecutive desaturation reactions to form lycopene containing 11 unsaturated bonds. The energy required for these energy-demanding condensation reactions is obtained by hydrolysis of pyrophosphate groups [1][74]-[78][83]. The formation of lycopene ends the most important and conservative part of the metabolic pathway. Lycopene is then cyclised to β-carotene by two β-cyclizations or to α-carotene by one β- and one ε-cyclization (Scheme 7).

These two types of circles are formed by different enzymatic mechanisms by lycopene β-cyclase and lycopene ε-cyclase. The formation of ε-rings and the synthesis of β,ε-carotenoids leading to the synthesis of α-carotene and its derivatives are two key differences distinguishing carotenoid biosynthesis in plants and biosynthesis in cyanobacteria, fungi and bacteria. Depending on the individual organisms' enzymatic equipment, the lycopene molecule undergoes a series of desaturation, cyclisation and oxidation reactions to form various representatives of carotenoids (Scheme 8) [1][74]-[78][83].



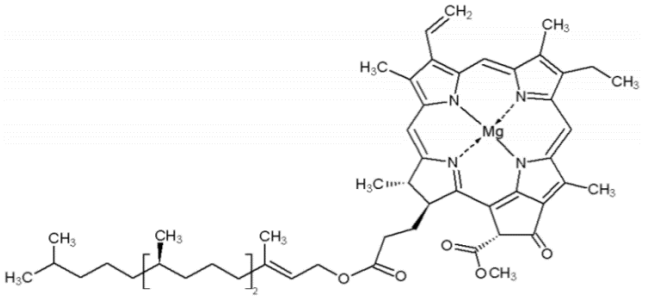
Scheme 7. *The carotenoid biosynthetic pathway [1][74]*



Scheme 8. *Carotenoid biosynthetic pathway detail [1][74][78]*

2.1.6 Chlorophylls

Chlorophylls are pigment molecules found in plants, algae and cyanobacteria. They play a crucial role in the photosynthesis process. They are responsible for plants' green colour and occur in the thylakoid membranes of Eukaryotes chloroplasts bound to hydrophobic proteins. Organisms capable of photosynthesis capture sunlight using chlorophylls and other necessary molecules and transform it into chemical energy. Their green colour is determined by the absorption of red and blue spectra of sunlight. Their general structure consists of a porphyrin ring with a bound chelate Mg^{2+} cation in the molecule's centre. Along hydrocarbon chain (phytol) is attached to the outer carbon of the porphyrin ring. In nature, we find several forms of the chlorophyll molecule, which are marked alphabetically. The differences between chlorophylls A and B are due to the methyl group's exchange for the aldehyde group on the C-7 carbon. This chemical structure difference causes a yellowish colouration of chlorophyll B (Scheme 9). Chlorophylls A and B are the most represented and almost ubiquitous for all autotrophs. Other types of chlorophylls are present in minor amounts in specific types of organisms, such as brown and red algae. Each group of photoautotrophic organisms has its ratio of chlorophylls, which is determined by evolution, areas of occurrence and conditions in the medium. In the case of microorganisms, the ratio of chlorophylls in the cell often changes as part of the stress response [3][88]-[91].



Scheme 9. *Chlorophyll A structure [88]*

2.1.6.1 *Chlorophyll biosynthesis*

Chlorophyll biosynthesis occurs inside the chloroplasts of eukaryotic cells and cytoplasm of prokaryotes. The biosynthetic pathway is conservative for these types of microorganisms and begins with the synthesis of precursor 5-aminolevulinic acid (ALA) via Beale pathway. The pathway starts with a glutamate molecule condensation with a tRNA to form glutamyl-tRNA by enzyme glutamyl-tRNA synthase. Glutamyl-tRNA reductase transforms newly formed molecule into glutamate-1-semialdehyde. Subsequent transamination produces 5-aminolevulinic acid (ALA), creating the necessary precursor for porphyrin synthesis. Chlorophyll synthesis continues with condensation of two ALA precursors by porphobilinogen synthase enzyme to porphobilinogen. Four porphobilinogen molecules with pyrrole rings condense together forming hydroxymethyl bilane (HMB). Hydrolysis of HMB by uroporphyrinogen synthase gives the structure of a cyclic tetrapyrrole uroporphyrinogen III. This enzyme catalyses several further transformations leading to coproporphyrinogen III and then protoporphyrin IX. Mg-chelatase then catalyses the incorporation of Mg^{2+} to give Mg-protoporphyrin IX. Subsequent cyclisation reaction produces divinyl protochlorophyllide. This is followed by a reduction reaction where chlorophyllide is formed. Chlorophyll synthase catalyses the esterification of propionic acid side-chain with phytyl diphosphate and thus creating chlorophyll molecule [3][88]-[91].

2.1.6.2 *Photosynthesis*

Photosynthesis is a two-step process in which phototrophic organisms capture solar energy and transform it inside the cell as chemical energy. Several photosynthesis types have evolved during evolution, the common element of which is the capture of sunlight. They differed in the source of the reducing agent. The original photosynthetically active microorganisms probably used hydrogen, sulfane or other similar compounds. Over time, microorganisms have adapted to water as a reducing agent, creating oxygen photosynthesis as we know it today. The first organisms to use oxygen photosynthesis were cyanobacteria, whose assimilation transferred this biochemicall process to algae and plants. The process of oxygen photosynthesis is divided into two parts [92][93]:

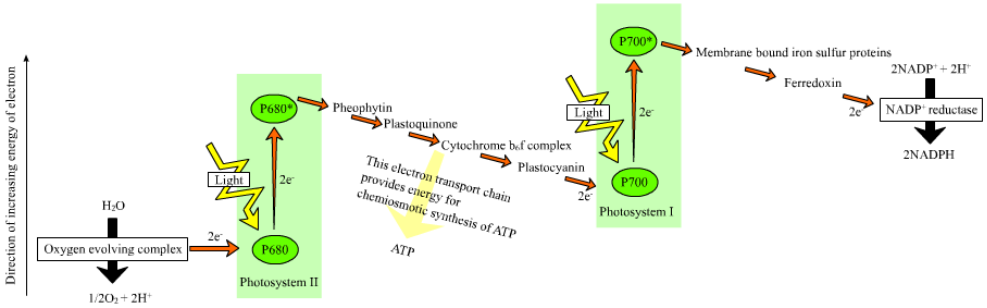
- Light phase - energy transformation, synthesis of NADPH and ATP
- Dark phase - CO_2 incorporation, glucose synthesis

2.1.6.2.1 Light-dependent phase

In the first phase of photosynthesis, the cells of phototrophic microorganisms absorb solar radiation in the range of 400 – 700 nm, i.e. in the visible light band. In cyanobacterial cells, photosynthesis is located on the membrane in a specialised region of the cytosol interior. Light is absorbed in specialised organelles chloroplasts in eukaryotic cells, which contain all the necessary components for photosynthesis. These organelles have an internal membrane system called thylakoids. There are two photosystems (PS I and PS II) on the surface of the thylakoid membrane.

Photosystems contain photochemical reaction centres that are made up of chlorophyll molecules and antenna pigments. Antenna pigments serve as energy carriers, and include carotenoids, chlorophylls and others [92][93].The process of photosynthesis can be described according to the so-called Z-scheme [94]. Photosynthesis begins with the absorption of light radiation by the antenna pigments of Photosystem II. An electron is excited and then gradually transferred to the photosystem II's reaction centre, which contains chlorophyll pigment. Subsequently, the electron is converted to a pheophytin molecule formed by modifying the chlorophyll molecule. Pheophytin transports the electron further to the plastoquinone and further in the electron transport system to the photosystem I and replenishes the missing electrons in this system (Scheme 10).

Excited electrons from photosystem I are converted to NADP-reductase by ferroprotein system. The reductase-cytochrome b6F complex reduces $NADP^+$ to NADPH. During electron transport, a gradient of electrochemical potential is created, which is used to create a gradient of hydrogen protons in the chloroplast's intermembrane space. This gradient then passes through the ATP synthase transmembrane protein complex channel and is used for ATP synthesis. The electrons provided by PS II are replenished in the Hill reaction, which is the photolytic decomposition of water. The resulting hydrogen protons serve to form a proton gradient, and oxygen is released out as waste. This phase's final products are ATP molecules and reduced cofactor NADPH, which are further used in the dark phase [92][93].



Scheme 10. *Light-dependent photosynthesis - Z-scheme [94]*

2.1.6.2.2 Dark phase – Calvin Benson cycle

In the Calvin Benson cycle, ATP and NADPH are used to fix carbon dioxide and reduce it to form a hydrocarbon skeleton. This cycle involves a series of reactions that ultimately yield a glucose molecule. The most crucial enzyme here is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco enzyme), which is the only known enzyme capable of fixing carbon dioxide. The Calvin cycle is divided into three phases: carboxylation, reduction and regeneration. In the first phase, the Rubisco enzyme carboxylates the ribulose-1,5-bisphosphate molecule to form a 6-carbon precursor, which is immediately cleaved into two 3-phosphoglycerate units. In the subsequent reaction, 3-phosphoglycerate is phosphorylated using ATP to 1,3-bisphosphoglycerate. The reduction phase of the cycle ends with the reduction of 1,3-bisphosphoglycerate to glycerate-3-phosphate. The regeneration phase of the cycle is similar to the pentose cycle, and in a series of reactions, the carbon units are joined together to form ribulose-5-phosphate, which is phosphorylated by ATP and enters the next cycle. Thus, three cycle revolutions are required to produce one molecule of 3-phosphoglycerate. 3-phosphoglycerate is then in gluconeogenesis pathway transformed into glucose. There are a total of 13 reactions in the Calvin cycle and 11 enzymes involved. Most of them are identical to other metabolic pathways (pentose cycle, gluconeogenesis). Only Rubisco enzyme and ribulose-1,5-bisphosphate regeneration are specific for this cycle [92][93].

2.1.7 MIcrobial sterols

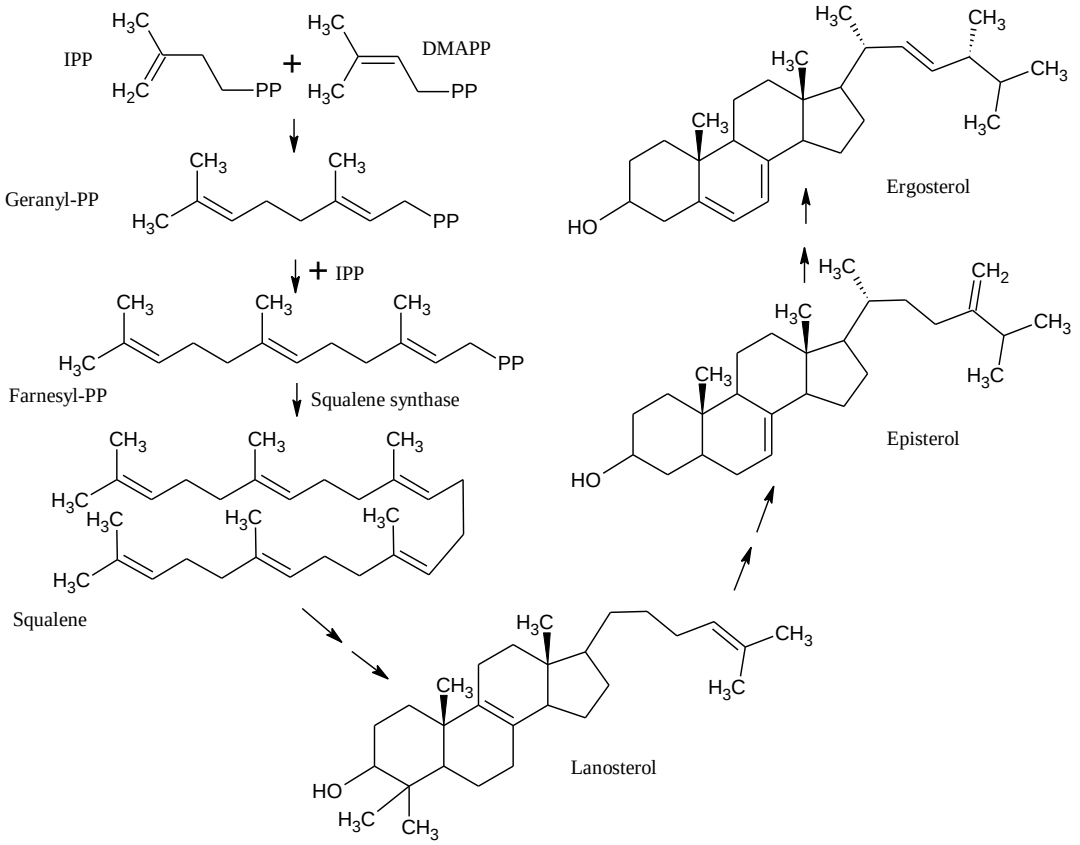
Microbial sterols are a group of bioactive molecules derived from the sterane structure containing at least one hydroxyl group. This vast group of molecules contains yeast ergosterol, microalgae clionasterol, fuconasterol and steroid hormones like testosterone aldosterone and others. Ergosterol was the first isolated and described member of this group, isolated from the ergot sclerotia growing on a rye [1][95]-[97].

2.1.7.1 *Function and use of sterols*

Sterols are an essential component in all cell membranes. They provide the necessary fluidity of the membrane, which would otherwise be very rigid due to its structure. Therefore, it prevents tearing or other damage to the membranes and thus the viability of each cell. Ergosterol is also a vitamin D_2 provitamin known as ergocalciferol. Ultraviolet exposure initiates a photochemical reaction whereby ergosterol is transformed into ergocalciferol. For biotechnological production, mushrooms are irradiated with UV light after harvesting to increase vitamin D concentration artificially. After extraction, the mixture is then most often used as food supplements. The specific occurrence of different types of sterols in the cells of microorganisms is due to the long-term evolution in different environmental conditions, which resulted in the development of a relatively broad group of phytosterols, especially in phototrophic microorganisms. In the case of eukaryotic yeast and protozoa, predominantly ergosterol is produced in various forms, which differ in the 3D structure and minor changes in the number of atoms contained. Essentiality of ergosterol for the life of fungi, protozoa and protist made it an ideal target for antifungal and other medicines against diseases caused by these organisms. Combined with its absence in animal and human cells, it has enabled the development of many safe antimicrobial drugs that interfere with or bind to the ergosterol structure and alter its physicochemical properties. Thus, the properties of the entire membrane. Another option is drugs that block or inhibit the enzyme activity of one of the ergosterol biosynthetic pathway [1][95]-[97].

2.1.7.2 *Sterol biosynthesis*

Sterol biosynthesis is based on the isoprenoid pathway and is mostly conservative for all organisms. Synthesis in eukaryotic cells occurs at the cytoplasm and endoplasmic reticulum interface and in semiautonomous organelles (chloroplasts, mitochondria). The input molecule is an activated form of isoprene. Biosynthesis begins with gradual „head to tail“ condensation of two IPP units and one unit of DMAPP to form the C15 chain of farnesyl pyrophosphate . Then two molecules of farnesyl pyrophosphate are coupled together „head to head“ by squalene synthase enzyme to produce the C30 chain of squalene. The squalene precursor synthesis is crucial in the sterol biosynthesis since the squalene molecule formed can only be used for subsequent sterol reactions. In contrast, the prior intermediates such as farnesyl-PP and geranyl-PP can be used for carotenoid synthesis. The squalene molecule then undergoes a two-stage cyclisation to form lanosterol. Lanosterol is then converted by a series of reactions to the appropriate microbial sterol. The scheme below (Scheme 11) shows yeast ergosterol synthesis [97][98].



Scheme 11. *Diagram of ergosterol biosynthesis [1][95]]*

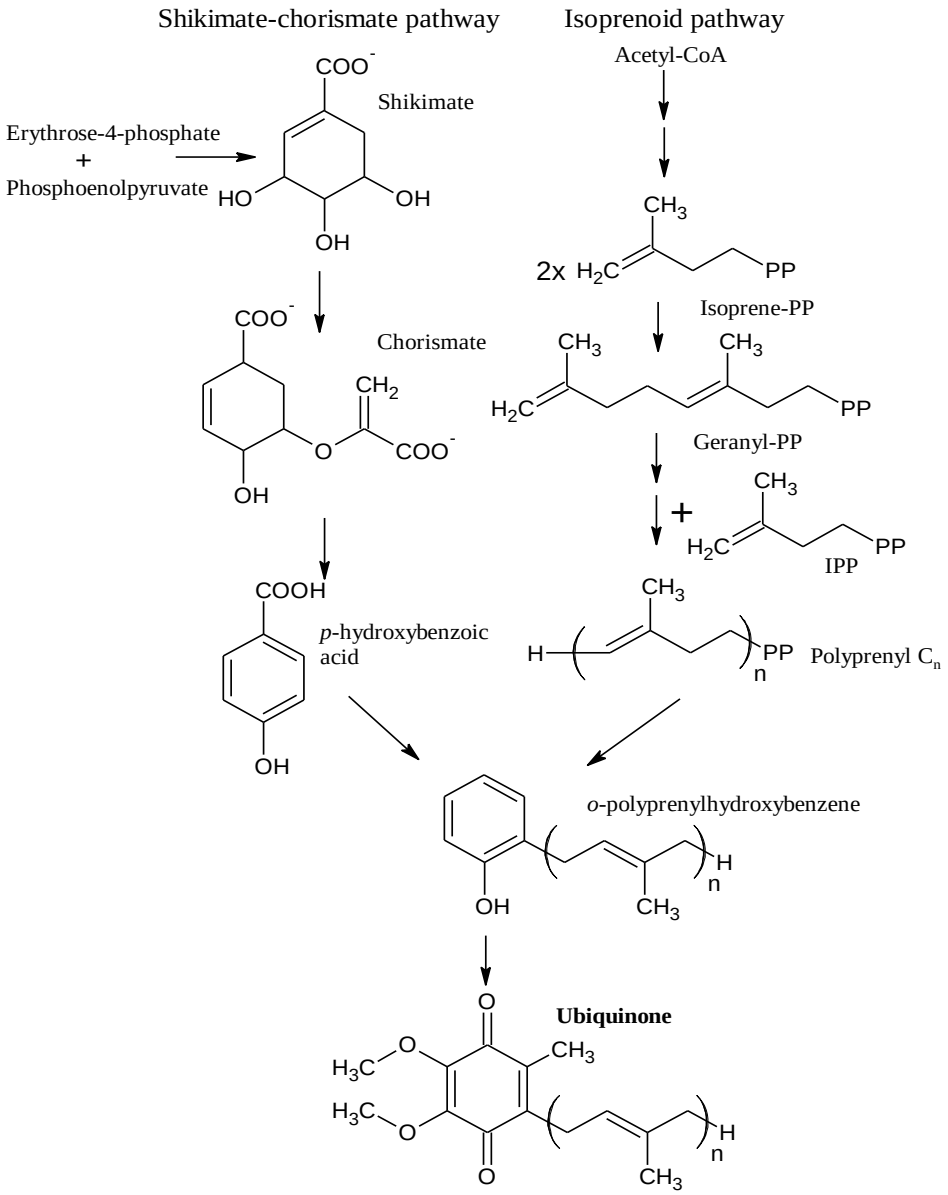
2.1.8 Ubiquinone

Called also as Coenzyme Q, it belongs to the group of molecules that shares the basic structure of the quinone nucleus. The second part of the molecule consists of the polyprenyl chain formed by the condensation of isoprene units. The number of isoprene units and the chain length is expressed by a number in the name, for example, coenzyme Q_{10}. The Ubi- prefix is derived from ubiquitous (found in all living organisms). Individual ubiquinone representatives are distributed according to the length of the isoprenoid unit side chain. Vitamin K also has a similar structure to ubiquinones. The best-known representative of this group is coenzyme Q_{10}. Coenzyme Q_{10} is present in most eukaryotic cells, primarily in mitochondria [100]-[102].

2.1.8.1 *Functions and utilisation of ubiquinone*

The primary function of ubiquinone lies in the respiratory chain. Here, ubiquinone serves as a part of the electron transport chain, in which the electrons collected during the reoxidation of the $FADH_2$ and $NADH+H^+$ cofactors are transported through the inner membrane space employing different electron potential, which includes the ubiquinone mentioned above. This function is enabled by the ubiquinone structure, which has two parts. The first part is the quinone nucleus, which is responsible for ubiquinone's ability to receive electrons and reduce itself in the process. The second part is the polyprenyl chain, which increases the molecule's nonpolar nature and allows ubiquinone to exist in the nonpolar inner part of the biomembrane. Ubiquinone also acts as a potent antioxidant and protects lipid membranes from peroxidation and oxidative destruction [100]-[103].

Ubiquinone use is mainly associated with the cosmetic and pharmaceutical industries. For many years, ubiquinone has been used in cosmetic products associated with the skin ageing process. The ageing process of skin and organism is also associated with decreasing levels of ubiquinone in cells. In its absence, larger amounts of free radicals are released, mitochondrial DNA is mutated, and energy production in the cell is disrupted, leading to cancerous growth. High concentration in the heart muscle is essential, where a small decrease in the concentration of coenzyme Q leads to impaired heart function. Long-term studies have confirmed the assumption that the long-term use of ubiquinone leads to an overall improvement in the state of the organism and a reduction in cardiovascular, inflammatory and tumour diseases [100]-[103].



Scheme 12. *Ubiquinone biosynthesis scheme [1][100]*

2.1.8.2 *Microbial biosynthesis of ubiquinone.*

Ubiquinone biosynthesis is accomplished by a combination of two metabolic pathways, the shikimate-chorismate pathway and the isoprenoid pathway (Scheme 12). First, a quinone core (p-hydroxybenzoic acid precursor) is synthesised in the shikimate-chorismate pathway located in the cell's cytoplasm. This pathway uses erythrose-4-phosphate and phosphoenolpyruvate as input molecules. Their condensation and cyclisation results in a shikimate which is converted to chorismate by condensation with another phosphoenolpyruvate molecule. The pyruvate is then cleaved from the chorismate molecule to form *p*-hydroxybenzoic acid. P-hydroxybenzoic acid then condenses with polyprenyl pyrophosphate created in the isoprenoic pathway to form o-polyprenylhydroxybenzene. o-polyprenylhydroxybenzene then undergoes a series of oxidation reactions and is double methoxylated to form ubiquinone [1][100]-[102].

2.1.9 Microbial extracelular lipases

Enzymes are biocatalysts that play an important role in the metabolic and biochemical processes of cells. The primary source of industrial enzymes are microorganisms. Biotechnological production of microbial enzymes has many advantages. Enzyme production can be induced by choosing an appropriate strain with high production capabilities. Then modulating the productivity by optimisation of cultivation parameters, carbon substrate, nitrogen substrate, C/N ration. Temperature, cultivation time and many more. Enzyme production can be increased using genetic techniques, either by modifying and increasing production in a given strain or by transferring the genes responsible for enzyme synthesis to more suitable microorganisms (mainly in bacteria) with faster growth. In overall bacteria and yeasts are best enzyme producers, due to their fast growth in large quantities [111][112].

The most biotechnologically produced enzymes are hydrolases. Hydrolases are a class of enzymes exhibiting broad substrate specificity. The mechanism of their action lies in the catalytic hydrolysis of bonds formed by condensation. Thus, peptide, amide, ester, glycosidic and the like bonds. Lipolytic enzymes (abbreviated lipases) are a group of hydrolases focused on ester bonds' cleavage and formation. We divide lipases into two groups [111][112]:

- Carboxyl ester hydrolases, or "true esterases" (EC 3.1.1.1.), which preferentially hydrolyse simple esters and triacylglycerols with short-chain fatty acids than C6.
- Lipases (EC 3.1.1.3.) are particularly effective against water-insoluble substances such as long-chain fatty acid triacylglycerols.

Most lipases that cleave triacylglycerols act primarily at the sn1 and sn3 positions. Triacylglycerols are a natural substrate for lipases. Triacylglycerols are large molecules and cannot pass across the cell wall. In order to be transported, they must first be broken down into partial glycerides and free fatty acids. This process is provided by lipases located in the extracellular environment. Although carbon metabolism in microorganisms is based primarily on carbohydrate sources, lipases' presence allows the processing of unconventional carbon substrates, such as fats. Many lipase-producing microorganisms used in biotechnological production of lipases have been isolated from lipid-rich environments (fat and oil industries, dairy products) [111]-[113].

2.1.9.1 *Structure and properties of Yeast lipase*

The size of the yeast lipase is in the range of 20-60 kDa. It has a three-dimensional structure which is characterised by α/β fold. The lipase contains a cover segment that protects the lipase active site. If a hydrophobic substrate appears near the enzyme, the lid will open immediately, and the enzyme activity will increase. The enzyme's central region is formed by a parallel β-sheet in which the active site is located. The active site contains a catalytic triad comprising a nucleophilic residue (serine), a catalytic acid residue (glutamate/aspartate) and a histidine residue. According to the nucleophilic residue in the active site, lipases are classified as serine hydrolases. The lipase active sites' geometry varies considerably and has an impact on the biochemical properties of the enzyme. In general, it is a deep hydrophobic pocket into which the fatty acid of the substrate fits exactly. According to the binding centre shape, we distinguish three types of active sites: tunnel (*Candida rugosa*, *Geotrichum candidum*), funnel-shaped (pancreatic lipase, lipase B *Candida Antarctica*, *Pseudomonas*) and slit-shaped (*Rhizomucor*, *Rhizopus*). A common characteristic of lipases' primary structure is the repeating consensus sequence Gly-X-Ser-X-Gly (X= amino acid) [111]-[113].

Both intracellular and extracellular lipases are stereospecific and regiospecific, i.e. they act on a certain bond in the triacylglycerol molecule. Due to the opposite polarity of the enzyme (hydrophilic) and the substrate (hydrophobic), the lipase reaction occurs at the oil-water interface. Yeast extracellular lipase is located on the protrusion of the outer trance of the cell wall. These cell-associated lipases can be inducible or constitutive in nature. Over time, the lipase is released freely into the medium. In the presence of detergent in the medium, lipase is bound from the cell wall. Both types of extracellular lipases show the highest activity at different times of the cultivation. Lipase activity located on the cell membrane is very closely related to the growth of biomass. For example, in the yeast *Yarowia lipolytica*, cell-associated lipase activity peaks at about 20 hours. The lipolytic activity of extracellular free lipase increases significantly after 80 hours of culture. At 50 hours there is a significant drop in the activities of both enzymes. The free enzyme activity is positively stimulated by triacylglycerol fats, liquid fatty acids, and myristic acid. In contrast, in cultivations using carbohydrates as a carbon source, lipase activity is significantly suppressed or does not reach significant values. However, in the yeast *Rhodotorula glutinis*, lipase activity may also be induced by the carbohydrate present [112]-[114].

2.1.9.2 *Yeast lipase activity*

Most yeast lipases are active in the temperature range between 40-50 °C. Unlike bacterial, yeast lipases are not thermotolerant. Their denaturation already occurs at 60 °C. The temperature stability of lipases varies depending on the location. The temperature stability of cell-bound *Rhodotorula glutinis* lipase is much higher than that of lipase secreted into the environment. Adjusting the optimal pH of the environment is very important to maintain lipase activity. The optimal pH of lipases may vary depending on the microorganism. The lipolytic activity of the yeast *Rhodotorula glutinis* is highest in a pH 7.5 environment at 35 °C. In *Rhodotorula mucilaginosa*, on the other hand, the enzyme is most active around pH 5 and 30 °C. As already mentioned, lipase reactions take place at the oil-water interface. Unlike esterases, lipases do not follow the kinetics of Michaelis-Menten. Only a minimal amount of substrate is required for high enzyme activity [112]-[114].

2.1.10 Biosurfactants

Surfactants are compounds with an amphipathic character used to stabilize emulsions (water/oil, oil/water, water/air, etc.).Their structure consists of hydrophilic and hydrophobic parts, which in solution are oriented to their counterpart with the same polarity. Their accumulation on the two immiscible phases' interface helps to reduce the surface tension and thus increase solubility hydrophobic and non-polar organic molecules' solubility in an aqueous medium. Surface tension in water solution decreases with increasing surfactant concentration until a certain point, in which surface reduction tension causes micelles' formation. This phenomenon is called critical micellar concentration (CMC) [115][116]. After reaching this concentration, the surface tension no longer decreases, and micelle concentration increases. Surfactants are used daily in various applications. The majority of surfactants used are synthetically prepared substances such as TWEEN 100. However, naturally occurring surfactants with identical or even better properties are found in nature. We denote these surfactants by the prefix bio. These biosurfactants are substances produced by plants or microorganisms. In addition to efficiency, their main advantage is their biodegradability, thanks to which these products do not burden the environment. Thus, biosurfactants are an important biotechnological product. The molecular weight of biosurfactants is in the range of 500–1500 Da. Most of the biosurfactants produced are anionic or ionic. Only nitrogen-containing biosurfactants can take on a cationic character [115][116].

Microbial biosurfactants can be classified according to their origin and chemical structure. The hydrophilic part is made mostly of amino acids, peptides, carboxylic acids, phosphate groups or sugar alcohols. Long hydrocarbon chains of saturated and unsaturated fatty acids are attached to this structure, forming the hydrophobic part. Most microbial biosurfactants are in the form of glycolipids, lipopeptides, lipoproteins, phospholipids and polymers. The best-known producers of biosurfactants are primarily bacteria, as well as a number of filamentous fungi and yeasts. In particular, those microorganisms which exhibit lipase activity and are capable of processing non-polar substrates such as oils and fats in the polar environment must produce biosurfactants in some form. The type of biosurfactant produced depends on the cultivated strain, cultivation conditions, type of substrate, nitrogen source, phosphorus [115]-[117].

2.1.10.1 *Biosurfactant application*

One of the most important biosurfactants' uses is bioremediation, including removing heavy metals and their salts/organic forms from polluted waters and soils. Biological surfactants are also used in the regeneration of petroleum oils industry. In the pharmaceutical industry, they could serve in the field of genes transmission or pharmaceutical preparations with antimicrobial, antiviral and anticancer activity, etc. Biosurfactants can further be used in biotechnology to increase non-polar substrates' solubility and increase the availability of substrates for strains with insufficient or no biosurfactant production [118][119].

2.2 Microorganisms

2.2.1 Yeasts

Yeasts are heterotrophic unicellular eukaryotic organisms belonging to the Fungi kingdom. They live all over the planet and so far about 1,500 species of yeast have been described, estimated to be about 1% of the entire yeast population on the earth. Yeast can live singly or in colonies, where they form so-called pseudomycelia or false mycelia. The size and shape of a yeast cell depend on the species, the method of vegetative propagation, the environment and the nutritional conditions of the environment in which they live. In general, however, the yeast size is in the range of 3-4 μm. The shape of the cell is ellipsoidal, ovoid to spherical. In individual cases, there are also other shapes, such as cylindrical in the genus *Schizosaccharomyces* or triangular in the genus *Trigonopsis* [120][122]-[125].

2.2.1.1 *Structure and shape of a yeast cell*

The yeast cell consists of a strong cell wall, with a cytoplasmatic membrane underneath protecting the cytoplasm. In the cytoplasm, there is a nucleus separated by a double membrane froma cytoplasm, as well as other organelles typical for eukaryotic cells, such as the endoplasmic reticulum, Golgi apparatus, mitochondria, plastids and vacuoles. Last but not least, ribosomes [120][122]-[125].

Cell wall: The cell wall's primary goal is to protect the cell from external influences (mechanical, osmotic shock) and separate it from the environment. At the same time, however, it must also ensure the transport of nutrients further into the cell's inner parts. It also allows communication between individual cells in the colony. The cell wall of yeasts, especially carotenogenic yeasts, has a very strong and resistant cell wall, consisting of a chitin layer on which a layer of β-glucans sits. Its composed of monosaccharide units, whose chain is made of 1,3-glycosidic bonds and branching is provided by carbohydrates attached by 1,6-glycosidic bonds. Proteins and phosphate layer forms up the final layer also containing short chains of mannose [122]-[125].

The cytoplasmic membrane of yeast is composed of a phospholipid bilayer, in which proteins, sterols, antioxidants, signalling molecules and other essential parts are further dissolved and anchored. The cytoplasmic membrane encloses the protoplast of the cell. The membrane is semipermeable and forms an interface between the environment and the cell. It allows small molecules and gases to pass through on its own. Larger molecules must pass into the cell on both sides via transport channels [122]-[125].

The cytoplasm is a transparent mass in which all cell organelles, enzymes and other storage substances are located. There is an endoplasmic reticulum (ER) formed by a system of membranes, responsible for the synthesis and modification of proteins in the area of the so-called rough ER. Many other transformations and modifications of lipids, polysaccharides, carotenoid synthesis, and other enzymatic reactions occur on the smooth ER surface. Furthermore, there are mitochondria, which serve as cellular power plants, are home to respiratory chain enzymes and oxidative phosphorylation enzymes. Mitochondria are among the semi-autonomous organelles similarly to chloroplasts, which means that they themselves have the ability to replicate, transcribe and translate nucleic acids [120][122]-[125].

The vacuole is a component in the cytoplasm, forming mostly spherical shapes surrounded by a monolayer membrane. The vacuole serves as a storage reservoir for all necessary substances and enzymes. In budding cells, the vacuoles are small and found in small numbers. As the cell ages, the number of vacuoles decreases and size increases. Another function of the vacuole is the hydrolysis reactions of molecules with a shorter half-life. The Golgi apparatus is an intramembrane formation that has a form of vesicles. The primary functions are post-translational modifications of proteins, their transport to designated sites and synthesis of polysaccharides [122]-[125].

Another important organelle of the cell is the nucleus, which is separated from the cytoplasm by a nuclear membrane with pores. It is the bearer of genetic information. A nucleolus in cooperation with the pole body forms microtubules and ensures the necessary processes in cell division [120][122]-[125].

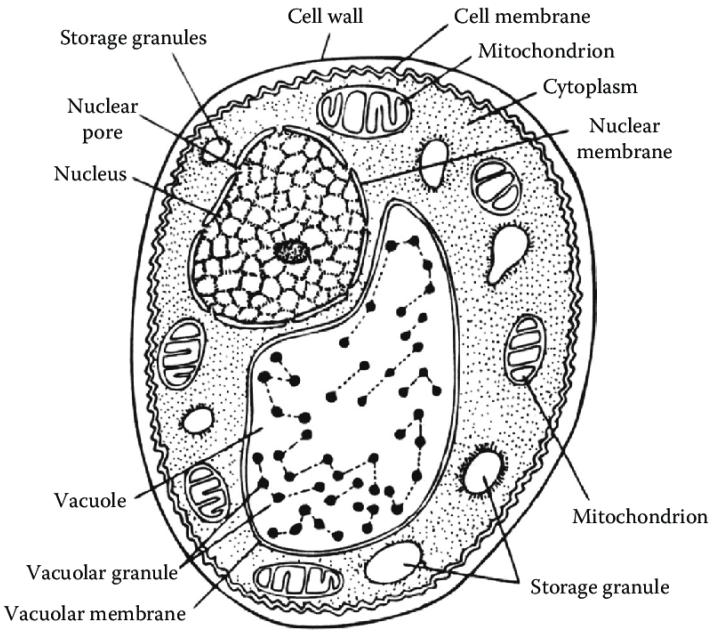


Figure 4. Diagrammatic view of electron micrograph of a yeast cell [153].

2.2.1.2 *Yeast reproduction*

There are two types of reproduction in yeasts, sexual and vegetative (asexual), which regularly alternate in most members of this species. Asexual vegetative reproduction is further divided into two types: budding and division. In the case of sexual reproduction, they create two types of spores, namely ascospores and basidiospores. Based on this difference, the yeast is divided into *Basidiomycetes* and *Ascomycetes*. Carotenogenic yeasts prefer mainly asexual reproduction. Only under optimal conditions, sexual reproduction occurs through the formation of basidiomycetes, in which karyogamy occurs [122]-[125].

2.2.1.2.1 Vegetative propagation

The vast majority of yeast representatives prefer budding as a type of vegetative propagation. In the budding process, a small daughter cell is formed on the surface of the mother cell. It is connected to the mother cell by a canal. At the beginning of budding, the ER membranes fuse together, then divide. The process is repeated to create the necessary amount. Furthermore, the shape of the mitochondria changes to long elongated ones. This process is followed by the process of nucleus mitotic division and at the same time, its migration together with the prepared organelles (mitochondria, vacuoles, etc.) towards the newly emerging bud. Together with other components in the cytoplasm, they migrate into space of the newly formed bud. Finally, the channel between the mother and daughter cell is closed. The endoplasmatic reticulum's metabolic activity is increased, a new cell wall is formed, and small vacuoles join into one. This ends the budding process, which leaves a characteristic scar on the surface of the mother cell [122]-[125].

2.2.1.3 *Microorganism Growth curve*

The growth curve describes the growth of the cell culture over time. After inoculation into the cultivation medium, the culture goes through a series of stages during its adaptation and growth in the medium. The first stage following inoculation is called the lag phase. At this stage, the culture adapts to new conditions. It triggers enormous enzymatic pathways to process nutrient sources; the cells gradually enlarge and prepare for the division. The length of this phase depends on the microorganism cells' physiological state, the composition of the medium and its size. The lag phase is followed by a phase of accelerated growth, in which the cells divide intensively and accelerate. This phase then passes into an exponential growth phase, in which the cells multiply geometrically and produce a large number of primary metabolites. The exponential phase is characterized by the shortest generation time, which does not change during the entire stage. With the gradual depletion of nutrients in the medium, cell division rate decreases, which is referred to as the phase of slow growth. This phase is followed by a stationary phase when there is practically no increase in the number of cells because the rate of cell proliferation is balanced with the rate of cell death. At this stage, there is an increased production of secondary metabolites in response to stress caused by nutrient limitation, oxygen, light and other factors. The last phase is cell death when a lack of nutrients stops cell division, and the culture slowly dies [122]-[125].

2.2.1.4 *Carotenogenic yeasts*

They are a group of yeasts that contain an enzymatic apparatus capable of producing carotenoid pigments. Due to carotenoids' production, these species are characterized by their colour ranging from pale yellow to red, caused by the accumulation of carotenoid pigments in the cell. They are therefore also called "red yeasts". The main produced carotenoids of these yeasts include β-carotene, torulene and torularhodine. The composition of carotenoids in individual cells differs mainly based on the type of yeast, the nutrient medium and environmental conditions such as the light spectrum, its intensity, and oxidants' presence. Carotenogenic yeasts belong to the class *Basidiomycetes*. This class includes studied yeast genera known for the overproduction of carotenoids, such as *Cystofilobasidium*, *Rhodotorula* and *Sporobolomyces*. Under suitable conditions, these genera are further characterized by high lipid production. The composition of fatty acids in these lipids is characterized by a high content of polyunsaturated and monounsaturated fatty acids, which can be widely used in the food, pharmaceutical and agricultural industries. Within the work, the studied genera are described below [122]-[125].

2.2.1.4.1 Genus Cystofilobasidium

Yeasts of the genus *Cystofilobasidium* multiply vegetatively and sexually. They are capable of fermenting monosaccharides. Asexual reproduction occurs through the polar budding of haploid or diploid cells. It forms colonies of orange, salmon or cinnamon colour with a glossy surface. The cells have the shape of an elongated oval, and some species of this genus are able to form pseudohyphae. Sexual reproduction is done through the formation of holobasidium inside which karyogamy and formation of spores occur. Torulen serves as a major carotenoid pigment, and under stress conditions, his oxidized analogues are produced. The genus belongs to the family *Rhodotorulacea* and is related to the genus *Rhodotorula*. It differs by the mentioned formation of unicellular basidia of round to elongated shape (so-called holobasidium). The best-known representatives are *Cystofilobasidium bisporidii, Cystofilobasidium macerans, Cystofilobasidium capitatum* and *Cystofilobasidium infimominiatum* [125][132]-[134].

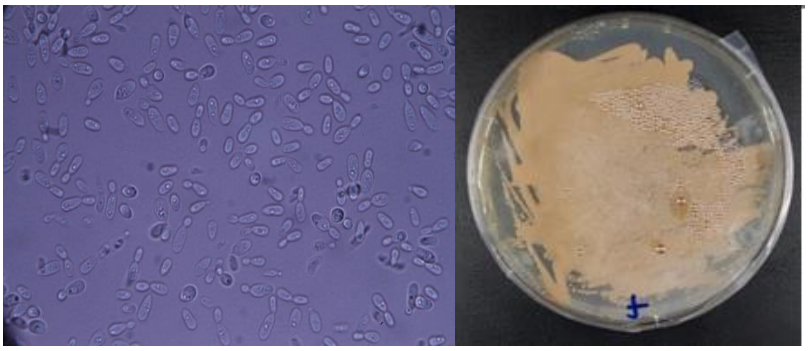


Figure 5. Cystofilobasidium macerans colony a) under a microscope and b) on a petri dish

2.2.1.4.2 Genus Rhodotorula

The genus is characterized by ellipsoidal to oval cells with a size around 5 μm. Yeasts of the genus *Rhodotorula* cannot ferment sugars and have a very well developed pentose cycle. They are mostly ubiquitous mesophilic microorganisms found in the air, soil and phyllosphere around the planet. Compared to other representatives of carotenoid yeasts, the genus *Rhodotorula* is the least demanding on cultivation and nutrient sources in the medium, especially on the amount of nitrogen. It is therefore often found in soils with a lower content of this element. During the exponential growth phase, they are able to produce glycogen. *Rhodotorula* accumulates lipids and mixture of carotenoid in the stationary phase, consisting mainly of β-carotene, torulene, and torularhodine. Due to carotenoid pigments' higher content, the colonies acquire a creamy pink, red to yellow colour. The cells are round to elongated oval in shape. They reproduce by multilateral or polar budding, produce pseudomycelium and are capable of sexual reproduction by teliospores [125][135]-[140].

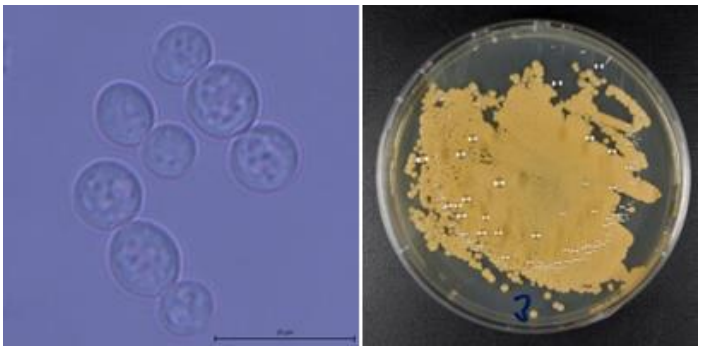


Figure 6. Rhodotorula kratochvilovae colony a) under a microscope and b) on a petri dish

2.2.1.4.3 Genus Rhodosporidium

Due to higher concentrations of carotenoids, colonies of *Rhodosporidium* cells turn red, orange colour. Strains can occur in both mycelial and yeast form. Like other yeast, they form ovoid cells that multiply by budding and can form a pseudomycelium. Sexual reproduction is both homothallic and heterothallic. Best-known strain *Rhodosporidium toruloides* was isolated from the wood pulp of conifers. Other members of the genus were isolated from air, sewage water and soil. It is able to process diverse carbon sources, for instance, hexoses and pentoses, oligosaccharides (maltose, cellobiose, trehalose), alcohols (glycerol, mannitol, sorbitol) and organic acids (acetate, lactate). Also, increased production of Coenzymes Q-9 and Q10 was observed in the cells. The genus *Rhodosporidium* is a perfect form of the genus *Rhodotolula*. As with the genus *Rhodotorula*, the genus *Rhodosporidium* is strictly aerobic, so it cannot ferment sugars, nor is it able to process lactose. Representatives of the genus include *Rhodosporidium toruloides* and *Rhodosporidium babjevae* [125][141]-[143].

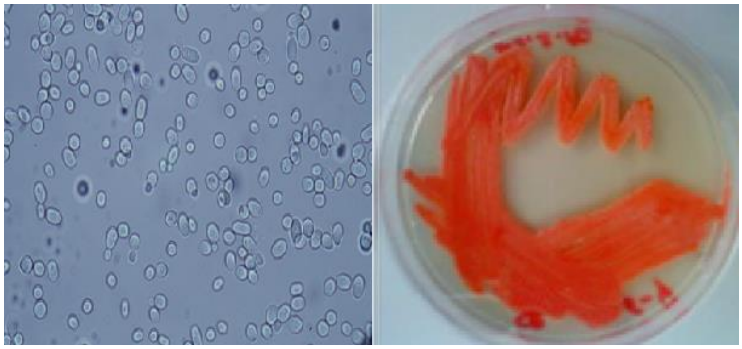


Figure 7. Rhodosporidium toruloides colony a) under a microscope and b) on a petri dish

2.2.1.4.4 Genus Sporidiobolus

The genus *Sporidiobolus* is the perfect form of the genus *Sporobolomyces*. The cells' shape is oval, ellipsoidal to cylindrical, and the colonies are flat and smooth. The genus *Sporidiobolus* is characterized by carotenogenesis with higher production of β-carotene, torulene and torularhodine, so its colonies take shades of pink-red colour. Furthermore, the genus is characterized by the formation of teliospores. Some species are able to form the true mycelium. Sporidiobolus cells can take the form of pseudohyphae and hyphae on which blastoconidia form. Representatives of this genus are not able to ferment sugars. These include, for example, *Sporidiobolus pararoseus* or *Sporidiobolus salmonicolo*r [125][144]-[148].

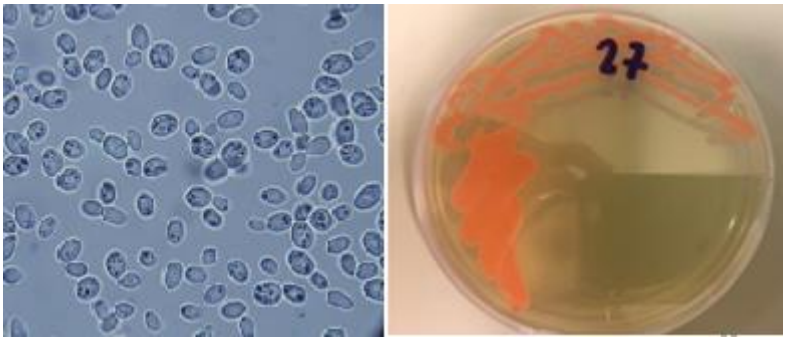


Figure 8. Sporidiobolus pararoseus colony a) under a microscope and b) on a petri dish

2.2.2 Algae

Algae is a group of oxygenic phototrophic multicellular and unicellular eukaryotic organisms, classified among kingdom *Plantae*. It is a diverse polyphyletic group that has no common ancestor, including species form very distinct clades. Organisms in this group range from large multicellular organisms such as *Macrocystis pyrifera*, which are believed to be the ancestors to higher plant, and small unicellular algae *Chlorella* and *Chlamydomonas*. In simple terms, algae can be divided into two main groups, namely multicellular and unicellular algae. Multicellular algae are a higher developmental stage, and they are already differentiated into structures that perform different functions.

Algae can be found throughout the planet and need, to some extent, the presence of water for their growth. With more complex multicellular algae, the water demand is much higher, and the presence of water is strictly required. These members are found in fresh waters and saltwater. Multicellular algae are currently used as a source of food, or chemicals useful in the food (agar), pharmaceutical, and medical industries. Generally, there is no accepted definition of algae. For example, the whole group shares some features, such as green pigment chlorophyll, which serves as the primary photosynthetic pigment. Furthermore, the overall structure and morphology of chloroplasts suggest the same origin from cyanobacteria. However, the process of endosymbiotic acquisition went differently. Phylum algae are divided into several divisions [149]-[152]:

(a) *Rhodophyta* (Red algae)
(b) *Dinophyta*
(c) *Cryptophyta*
(d) *Chromophyta* (Brown algae)
(e) *Euglenophyta*
(f) *Chlorophyta* (Green algae)

Unicellular representatives of algae are referred to as microalgae. They are a lower developmental form of algae. Their origin came from endosymbiosis of heterotrophic eukaryotic cells with cyanobacteria. Microalgae can be found everywhere on earth, even in arctic conditions. Evolutionarily, they have evolved and adapted to almost all possible conditions, whether they are the driest places on earth (*Chroococcosidiopsis* sp.) or one of the most acidic places on earth (*Coccomyxa sp*.). They serve as an essential part of the food chain and nutrient cycle in nature. Most of them contain chlorophyll pigment, which allows photosynthesis to take place, and together with cyanobacteria, they are responsible for up to 50% of the photosynthetically produced O_2 on Earth. The total number of microalgae species is estimated at 200 to 800 thousand, but only about 40.000 to 50.000 have been described in the literature so far. Microalgae serve as an integral part of the food chain and nutrient cycling in nature [149]-[152].

Biotechnology seeks to exploit the vast potential of microalgae from both the autotrophic and mixotrophic way of life. Microalgae have the ability to efficiently decompose, purify and utilize wastewater full of various forms of nitrogen fertilizers, phosphates, to produce enriched biomass. Furthermore, microalgae produce polysaccharides, lipids, and carotenoids under stress conditions and are sources of molecules that can serve as antibiotics, immunostimulants, anti-inflammatory drugs, and many others [149]-[152].

2.2.2.1 *Structure and shape of microalgae cells*

The microalgae cell shares the essential characteristics and structure of eukaryotic cells described in the previous chapter. The main difference is the presence of chloroplasts, which originated from endosymbiosis with cyanobacterial cell. This theory was confirmed by studying the chloroplast genes as a semi-autonomous organelle, and it was found that genetic information is in many parameters and metabolic pathways identical to cyanobacteria. Chloroplasts are semi-autonomous organelles, capable of replication, transcription and translation of genes. There are many metabolic pathways (isoprenoid pathway, fatty acid synthesis, glycolysis and others). However, evolution over a million years has led to the loss of functionality and alteration of some genes. Therefore, chloroplasts' genetic information is not the same as that of cyanobacteria [149]-[152].

Chloroplasts are formed by an outer biomembrane, which encloses the inner matrix of the chloroplast. An inner membrane system forms disc-like formations of thylakoids, which are stacked on top of each other and form a granule. Photosynthesis process takes place inside thylakoids. In the light-dependent phase, a molecule of chlorophyll inside photosystem II (PSII) absorbs a photon. It passes electron via an electron transport chain leading to the ultimate reduction of $NADP^+$ to NADPH. In addition, the electrochemical potential gradient of a passing electron is used to create a proton gradient on both sides of the thylakoid membrane that uses the enzyme ATP synthase to form ATP. PSI supplements the missing electron in PSII. The decomposition of water, known as the Hill reaction, serves to compensate for PSI's lack of electrons. By-product of this reaction is the formation of O_2. Same as with the cyanobacteria, water serves as electron and proton source [149]-[152].

The algae cell wall's primary building material is cellulose, which is oriented in several layers forming a thin protective layer. Other groups of molecules that appear, depending on the species variation, are lignins, hemicelluloses, alginate, fucin and fucoidin. Extremophilic strains have a much thicker cell wall, and its composition is dependent on the conditions in which the extremophile lives. One of these algae's common strategies is the formation of colonies that are encapsulated by a "capsule" consisting of derivatives of polysaccharides, terpenes, or lipids. Another strategy is forming a shell of a similar structure to the above-mentioned single-cell colonies. However, cell wall thickness cannot be too large. Its thickness its compromise between the protective function and the ability to receive and transmit as much light as possible inside the cell [149]-[152].

2.2.2.2 *Microalgae cultivation*

The importance of microalgae lies in the wide range of uses and a wide range of products. As mentioned above, microalgae can serve as a producer of lipids, carotenoids, enzymes, biopolymers, pigments, polysaccharides, and many others. Whole microalgae biomass is used as nutritional supplements, or its complex extracts serve as additive of cosmetics, pharmaceuticals. Microalgae are used in wastewater treatment as an in situ source of oxygen for bacteria and scavenger of phosphorus, nitrogen from agricultural fertilizers. Microalgae are also studied as a possible source of biofuels or “green energy”. Their use lies primarily in exceptional properties, which make them more attractive than other energy plants. These properties are [151][152]:

- Possibility of continuous cultivation, independent on seasons
- Biomass can be used as complete (dried cells) or extracted to gain different products
- Ability to tolerate a wide range of temperature, pH and salinity
- Reduction of industrially produced and atmospheric CO_2
- Sustainability and renewability of their production
- High yields of target products
- Possibility of cultivation on marginal soils, non-agriculture lands

Microalgae cultivation requires specific regulation of cultivation conditions, such as temperature, pH, salinity, gas transmission, nutrient supply, and light intensity. Optimization of process parameters such as aeration, mixing, hydrodynamic stress and harvesting frequency is necessary for feasible microalgal growth [1][2][151]-[153].

2.2.2.2.1 Temperature

The optimal algal growth temperature varies depending on the species and their desired response. Every strain has its temperature limits. The most interesting is the optimal growth temperature, where the highest possible growth rate is achieved. Most species of microalgae grow best in the temperature range of 20 to 30 °C. Deviation from the optimal temperature leads to changes in culture. At a higher temperature growth rate of microalgae decreases due to a negative effect on photosynthesis. With higher temperatures, the activity of Rubisco enzyme and generally other enzymes decreases and CO_2 solubility decreases. At some point, the medium's temperature is so high that growth is inhibited, and the culture reproduction stops. On the other hand, higher ambient temperature is intertwined with higher accumulation of storage compounds, such as lipids and polysaccharides. Like yeast, algae change the level of saturation of their fatty acids as a function of temperature. High temperature is responsible for accumulating saturated lipids and higher production of antioxidants, such as β-carotene due to higher free radicals production. In contrast, a low temperature is responsible for a higher accumulation of unsaturated lipids. Low temperature has also been shown to be favourable for carotenoid production [1][2][151]-[153].

2.2.2.2.2 pH

The pH value of the medium directly affects the metabolism of microalgae by regulating the enzyme activity and the supply of ions by influencing phosphorus availability and inorganic carbon. Most microalgae strains are quite sensitive to pH changes, where each has its own pH optimum. In open cultivation systems, measuring and controlling the pH can be quite tricky and time-consuming, due to the size of the cultivation area and the medium's imperfect mixing. In contrast, in closed systems, pH regulation is significantly faster and more efficient. For these reasons, the culture media contain buffers that help maintain a stable pH and reduce significant pH fluctuations. These buffers are incorporated into a medium such as Bolds Basal Medium, where the buffer is a mixture of KH_2PO_4 and K_2HPO_4 [1][2][151]-[153].

2.2.2.2.3 Nutrients

For proper microalgae growth, cultivation medium must contain a sufficient amount of all necessary macronutrients, micronutrients and vitamins in bioavailable forms [1][2][151]-[153].

Macronutrients include:

• Carbon: is the most abundant element in microalgal biomass making up to 50% of dry weight; the primary carbon source is inorganic CO_2, which is bound in the Calvin cycle of photosynthesis by the enzyme Rubisco. Mixotrophic microalgae are also able to assimilate organic carbon sources, such as acetate, glucose, glycerol.

• Nitrogen: is the second most abundant macroelement in microalgal biomass, where it serves as an essential component of key molecules. Microalgae obtain nitrogen from both inorganic sources (nitrate, nitrite, ammonia) and organic sources (urea, amino acids, proteins). However, the most abundant source of nitrogen (N_2), in both the atmosphere and water, is only used by some strains of cyanobacteria and bacteria that are able to fix N_2. In general, microalgae cannot use this source, and if so, it is only in symbiosis with the above-mentioned bacteria and cyanobacteria.

• Phosphorus: is essential for DNA, RNA, membrane phospholipids function and also ATP. Microalgae assimilate phosphorus mainly in the form of phosphates by intra and extracellular enzymes attached to the plasma membrane.

• Sulfur: is an integral part of some amino acids, vitamins and lipids; sulfur is primarily bound from sulphates (SO_4^{2-}) because sulfites are toxic for microalgae.

Another integral part for microalgae cultivation media are micronutrients, which play a crucial role in enzyme and vitamin activity [1][2][151]-[153]:

• Magnesium: in the form of Mg^{2+} serves as an activator of several enzymes and part of the photosynthetic apparatus (chlorophyll); its usual sources are $MgSO_4$ and $MgCl_2$

• Calcium: is often added to the medium in the form of $CaCl_2$; Ca^{2+} ions affect cell division and morphogenesis.

• Iron: is involved in essential biochemical processes such as oxygen metabolism, chlorophyll synthesis, electron transfer or nitrogen assimilation.

• And other trace metals such as Mn, B, Mo, K, Co and Zn

Nutritional stress caused by a lack of nitrogen, phosphorus, sulfur, and chlorine can stimulate excessive β-carotene production by affecting the photosynthetic pathway by altering photoprotective pigments' ratio to chlorophyll [1][2][151]-[153].

2.2.2.2.4 Mixing

Mixing the medium is important to prevent algae sedimentation and maintain homogeneity and even algae transfer between the more and less illuminated parts of the bioreactor. Without sufficient agitation, a situation could easily arise where algae on the surface would be exposed to too much light intensity and thus photoinhibition, while algae deeper in the medium would suffer from a lack of light. It is also important to emphasize that many microalgae species do not have a good tolerance for intensive mixing and the resulting large shear forces [151]-[153].

2.2.2.2.5 Light intensity

Chlorophylls, carotenoids and other pigment molecules absorb the energy of solar radiation and through electron transport chain convert it into chemical energy in the form of short-term energy molecules (ATP) through the process of photosynthesis. Thus, it is clear that there is a direct relationship between the intensity of light radiation and the growth rate of culture. However, if the light intensity exceeds the limit value, photoinhibition and photooxidation phenomena begin to occur, which have a negative effect on cell growth. However, such a stressful situation has a remarkable effect on carotenoids' production because carotenogenesis is one of the natural physiological strategies for defense against excess light radiation. Therefore the optimal light intensity must always be determined experimentally for each strain [1][2][151]-[153].

2.2.2.2.6 Cultivation systems

Various cultivation techniques are used for large-scale production of microalgal biomass. In principle, we can divide them into two groups, namely open systems (ponds and reservoirs) and closed systems (photobioreactors). Examples of culture systems are shown in Figure 9 and Figure 10. Open systems usually consist of a simple water tank made of concrete, dug into the ground. The source of light is sunlight, and the source of CO_2 is usually atmospheric air. A paddlewheel usually ensures the mixing of cells and nutrients. Open systems are the most used due to their low start-up costs. More than 80% of the globally produced microalgae biomass comes from open cultivation systems. The advantages, disadvantages and comparison with closed culture systems are summarized in Table 1.

A photobioreactor is a closed cultivation system in which the culture medium circulates between a series of transparent tubes or plates and between a central reservoir. This cultivation mode represents higher operating costs in the form of artificial lighting, CO_2 and culture medium, but offers better options for regulating cultivation conditions and protection against contamination. The advantages, disadvantages and comparison with open culture systems are summarized in Table 21. [154][155].

Figure 9. a) Open pond cultivation strain Dunaliella bardawil NTB company, Eilat, Israel. b) Nannochloropsis open pond cultivation, Texas, USA.

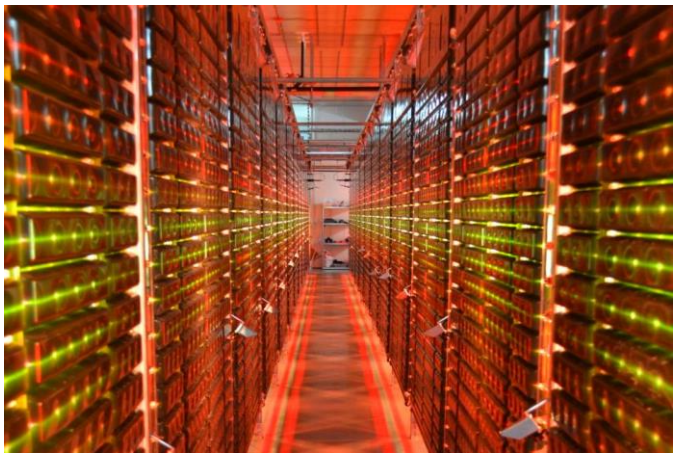


Figure 10. Haematococcus pluvialis cultivation in indoor photobioreactors Algamo company, Czech republic.

Table 1. Comparison of microalgae cultivation in enclosed photobioreactors and open systems [192][193].

Parameter	Photobioreactor	Open pond cultivation
Space required	Small	Large
Contamination risks	Small	high
Energy requirements	Large	Small
Strain selection	Almost all strains	Limited
Water evaporation	Almost none	Very high
CO_2 loss	Almost none	High
Process reproducibility	Possible under certain conditions	Almost impossible
Process flexibility	Relatively easy changes, in strains, products	Changes are quite complicated
Process management	Simple, effective	Very challenging
Weather effect	Insignificant	Significant
Biomass production	High (4-10 g/L)	Low (0,2-1,0 g/L)*
Process effectivity	Larger biomass concentration, faster growth, higher effectivity	Low effectivity, low biomass concentration, slow growth

** Production in open raceway ponds*

2.2.2.3 *Microalgae reproduction*

Most microalgae strains are capable of sexual and asexual reproduction. In nutrient-rich conditions, especially nitrogen, microalgae prefer the asexual mitotic reproduction. One of the most studied life cycles is that of *Chlamydomonas reinhardtii. T*hus the basic principles of microalgae reproduction will be described on this alga. The life cycle of *Chlamydomonas reinhardtii* can be divided into two main cycles: the sexual and vegetative cycle. Vegetative cell division occurs in nutrient-rich conditions. It starts with cells becoming immobile after the loss of flagella. Flagella basal bodies migrate towards cell nucleus, followed up by its division. The mitotic process continues with chloroplast division and migration of other organelles towards each cell's end, followed up by cytokinesis. Daughter cells then elongate, their protoplasts rotate and chloroplast reforms back to the original shape. This cell division mechanism can be repeated several times to form up to 32 daughter cells within one mother cell. Finally, new cell wall forms around each daughter cell and flagella are reformed. Daughter cell is then released using lysin like proteolytic enzyme called sporangin, which breaks down the mother cell wall [149]-[190].

When nitrogen concentration falls below a certain threshold, sexual reproduction begins. *Chlamydomonas reinhardtii* cells have two mating types mt^- and mt^+. Vegetative cell with locus mt^+ differentiate into gametes mt^+ and similarly mt^- cells into mt^- gametes. Within a short amount of time, gametes agglutinate to form binucleate quadriflagellated cells (QFCs) Two nuclei fuse together to form a zygote which subsequently becomes dormant, gaining resistance to freezing and desiccation. When conditions improve, the dormant cell initiates meiosis and form four recombinant haploid products that resume vegetative growth [149]-[152].

2.2.2.4 *Microalgae genera description*

2.2.2.4.1 Genus Scenedesmus

Scenedesmus is a genus of non-motile colonial green algae growing as a single cell or in colonies forming cenobia of 4, 8 or 16 individuals arranged in a row. It is one of the most common freshwater microalgae. There are currently 74 taxonomic species of *Scenedesmus* accepted, with a quite large diversity in morphology, making it very hard to identify and classify. Thus there are several approaches to the division of representatives into the individual genus. According to Hegewald, representatives of genus *Scenedesmus* can be distinguished into three sub-genera *Acutodesmus*, *Desmodesmus* and *Scenedesmus*, which differ in cell poles. *Acutodesmus* is characterized by sharp/acute cell poles, whereas *Desmodesmus* and *Scenedesmus* have obtuse/truncated cell poles. Strain *Scenedesmus* grows independently in optimal light and nutritional conditions. Cenobia is formed in the presence of a predator, unbalanced nutritional conditions or absolute deficiency. To counter the presence of predator *Scenedesmus* produces strong cell wall with a slimy envelope, that makes them indigestible or produces selective toxic substances [150][159].

Members of this genus have been discovered among the first strains of microalgae and have been studied since the first half of the 18th century. Nowadays, they are used as basic microorganisms for the study of photosynthesis and environmental pollution. For example, in cooperation with bacteria, they are used in sewage treatment processes, where they serve as a source of oxygen for bacteria capable of breaking down organic waste. This genus includes *Scenedesmus obliquus* and *Scenedesmus acuminatus* [150][159].

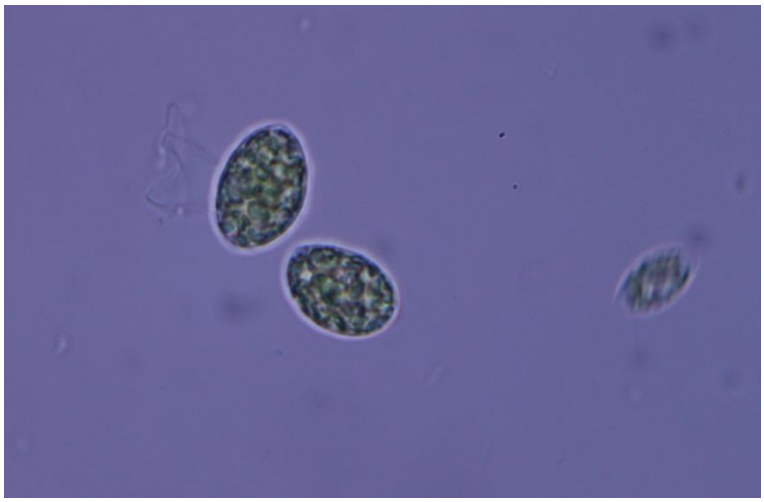


Figure 11. Scenedesmus obliquus CCALA 455

2.2.2.4.2 Sub-genus Desmodesmus

Desmodesmus differs from the previous two subgenera mainly by forming a 4-layer envelope on the outside of the cell wall, compared to 3 layers in the subgenus *Acutodesmus* and *Scenedesmus*. This fourth layer appears as granules or ribs under a light microscope. Short or long spines characterize most representatives of *Desmodesmus* strains. This genus includes *Desmodesmus dimorphus*, *Desmodesmus quadricauda* and *Desmodesmus abundans* [150][158].

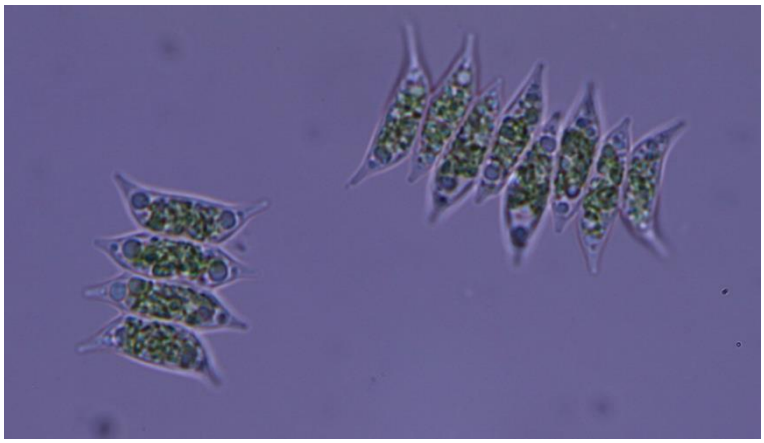


Figure 12. Desmodesmus dimorphus CCALA 443

2.2.2.4.3 Genus Chlorella

The genus *Chlorella* is one of the most famous commercially used algae in the world. It was the first algae, that was isolated in culture by Beijerinck. Morphologically, *Chlorella* is unicellular and immobile species of green freshwater algae, that can be found in nutrient-rich waters and soils. There are also some seawater species identified. This genus is characterized by a spherical or ellipsoidal shape of cells with a size of 2-15 μm. It is nonmotile and prefers unicellular growth. Only a small number of representatives prefer to grow in colonies. All representatives of this genus lack flagella. In ideal conditions and sufficient nutrients, genus *Chlorella* is known for its fast growth rate, compared to other microalgae species. The genus served as a model organism for the study of photosynthesis and for the study of the process of assimilation of carbon dioxide into cells (citace). This genus includes *Chlorella sorokiniana*, *Chlorella lobophora*, *Chlorella kessleri* and *Chlorella vulgaris* [150][160][161].

Genus was intensively studied in the 1940s and the early 1950s due to fear of overwhelming population boom. Many people saw *Chlorella* as a new and promising food source that would solve the problem and provide high-quality food source (about 45 % protein, 20% lipids, 20% saccharides, 5% fibre and 10% minerals and vitamins in dry mass) with relatively low-cost production. Intensive research was practically stopped with advances in agriculture and better crop efficiency. Furthermore, laboratory results proved to be rather difficult to transfer to open field conditions. To reach full potential, microalgae would have to be grown in highly modified artificial conditions. To this date, production costs remained high, and *Chlorella* is mostly sold as food supplement, animal feed, or cosmetics [150][160][161].

Chlorella is characterized by a photosynthetic apparatus that can efficiently convert light radiation into chemical energy. Photosynthesis efficiency can reach up to 8 %, which exceeds high-efficiency crops like sugar cane. Remarkably, it can perform photosynthesis at a relatively low light intensity. The value of light intensity for the photosynthetic apparatus's maximum efficiency is 80 to 400 $\mu E \cdot m^{-2} \cdot s^{-1}$. A maximum light intensity that *Chlorella* cells can withstand is above 2500 $\mu E \cdot m^{-2} \cdot s^{-1}$. Unused energy is then converted to heat. *Chlorella* is able to thrive on media with scarce nutrients. Under certain stress conditions (nitrogen deficiency), reduced protein production occurs, and conversely increased lipid production within cells is observed. The concentration of nitrogen in the medium is crucial for regulating cell growth and metabolism in all microorganisms. The growing interest in microalgae *Chlorella* is due to abovementioned growth properties and mainly due to content of biologically beneficial substances for human nutrition and food supplements. High lipid content could be exploited for the production of biofuels.

Over the last 20 years, results from biochemical and molecular studies led to a major reassessment of genus *Chlorella,* with many species being transferred to other genera. Currently, there are 44 species of *Chlorella* recognized. However, this number is not final and will most likely change in the coming years [150][160][161].

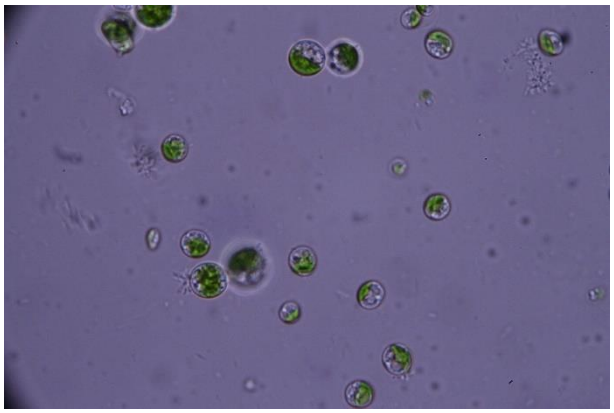


Figure 13. Chlorella sorokiniana CCALA 259

2.2.2.4.4 Genus Chlamydomonas

Chlamydomonas is a genus of single-celled green algae, consisting of around 300 species. Genus inhabits stagnant waters, seawater, damp soil and even snowy landscapes. *Chlamydomonas* cells have a glycoprotein wall with two equal flagella. A substantial part of the inner cell space is filled with a cup-shaped chloroplast with a single large pyrenoid, which encloses the nucleus inside. Two contractile vacuoles connected to flagella are located above the chloroplast. Chloroplast contains thylakoid bands, which are not organized in grain-like structures. This is one of many differences that distinguish the genus *Chlamydomonas* from other genera of microalgae. *Chlamydomonas* cells also contain rhodopsin ion channels that act as sunlight sensors that control cell phototaxy.

These channels further control the electrically polarizable cell membranes, the cell's internal pH, and the flow of calcium ions into the cell. Genus feeds mostly autotrophically, but some members are semi-autotrophic, capable of utilizing organic carbon sources, such as acetate. In recent years, *Chlamydomonas* strains were proposed as a possible starch producer for food and non-food applications. C*hlamydomonas reinhardtii* (Dangeard, 1899) serves as a model organism in molecular biology. Especially for the study of chloroplast dynamics, photosynthesis, flagellar motility, its genetics and biogenesis. To this day, *Chlamydomonas* is one of the most studied microalgae representatives and serves as a model organism [150][162].

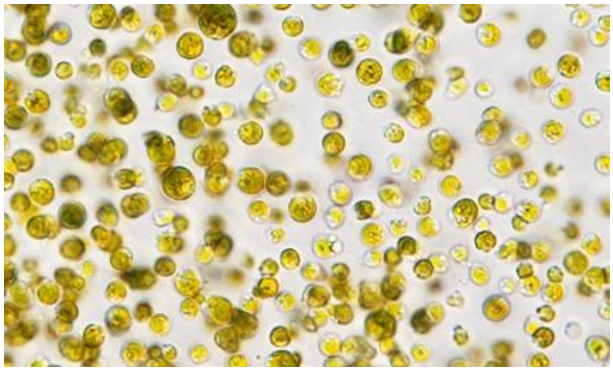


Figure 14. Chlamydomonas reinhardtii CCALA 973

2.2.2.4.5 Genus Coccomyxa

Coccomyxa belongs to the family *Coccomyxaceae* and its defined by its relatively small size, compared to other microalgae. Cells have a spherical or elliptical shape and have a simple chloroplast. Microalgae *Coccomyxa* can be found all around the world, inhabiting both marine and freshwater environments. *Coccomyxa* displays a wide variety of different ways of life. It can be found living in mild conditions or very harsh conditions with high temperature, large salt concentrations and very low pH. Members of this genus live in terrestrial biofilms, as a part of soil ecosystems, associated with mosses, associated with fungi and higher plants, or even living as a parasite in mussels. Genus plays a significant role in ecosystems. Based on the formation of brown akinetes, two subgenera can be distinguished, namely *Coccomyxa* and *Pseudococcomyxa* [163]-[165]. To this day, 40 members of genus *Coccomyxa* were identified according to the information described above. *Coccomyxa* often serves as a model organism, and its genome is being completely sequenced. This genus is considered as an attractive producer of next-generation biofuels [163]-[165].

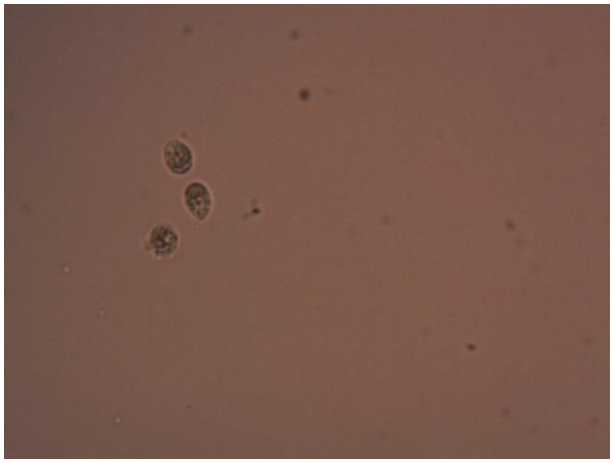


Figure 15. Coccomyxa sp. CCALA 912

2.2.2.4.6 Genus Botryococcus

The genus *Botryococcus* belongs to the group of algae with great potential for the use of energy production based on microorganisms. The chemical composition of this microalga could be used for biofuels such as bioethanol or biodiesel. However, the main disadvantage of *Botryococcus* algae compared to other microalgae is mainly the slow growth. The main representatives include the species *Botryococcus braunii*, which can be found in colonies growing in star-shaped clusters and occurs mainly in temperate or tropical lakes, reservoirs or brackish ponds. It is almost ubiquitous, except in the areas around Antarctica [166]-[168].

Botryococcus strains usually grow in colonies, which vary in size; however, we can observe almost exclusive single cells in some cultures. *Botryococcus* has been widely studied as a promising biofuel producer since the discovery that this alga accumulates substantial amounts of long-chain hydrocarbons. These hydrocarbons share the similar properties of crude oil , and thus can be used as a fuel analogue. Currently, there are 17 species recognized, which are classified into races, depending on the type of hydrocarbons produced. Most promising is the Race B, which has the potential to accumulate up to 86% of dry weight in hydrocarbons called botryococcenes (botryoterpenes) with secondary products methylsqualenes. Both groups can be quite easily converted to biofuels [166]-[168].

Botryococcus cells have a fibrous cell wall consisting of β-1,3 and β-1,4 glucans. Extracellular matrix (ECM) of the colony comprises long-chain polyacetal hydrocarbons that are cross-linked with hydrocarbons specific to each *Botryococcus* race. Matrix is filled with liquid hydrocarbons, and the whole colony is protected by an outer wall consisting of arabinose-galactose polysaccharides. Mechanism of releasing hydrocarbons into ECM and its accumulation is still not known. Each cell inside the colony contributes to the synthesis of the protective layer and liquid hydrocarbons. The cross-linked network between cells helps in vegetative reproduction and daughter colonies' formation [166]-[168].

Accumulation of hydrocarbons in ECM of *Botryococcus* cultures led to the proposal of non-destructive extraction referred to as “milking”. Instead of harvesting the cells and its destruction followed by extraction of hydrocarbons, cultivated cells are repeatedly gently extracted with appropriate biocompatible solvents, such as hexane and n-heptane, without killing them. The whole process's advantage is that the cells do not need to be harvested, inoculated again, etc. Thanks to that, the energy costs of this process are lowered [166]-[168].

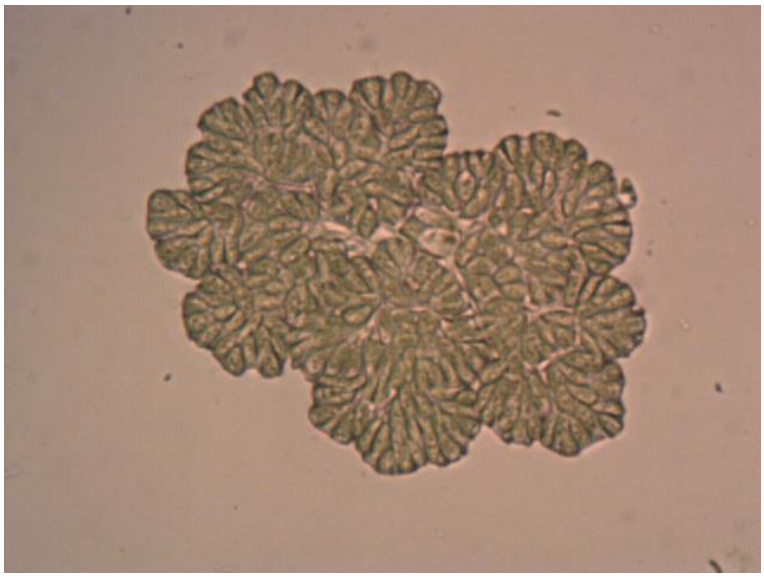


Figure 16. Botryococcus Braunii v. Showa-Bielefeld – exponential phase of growth

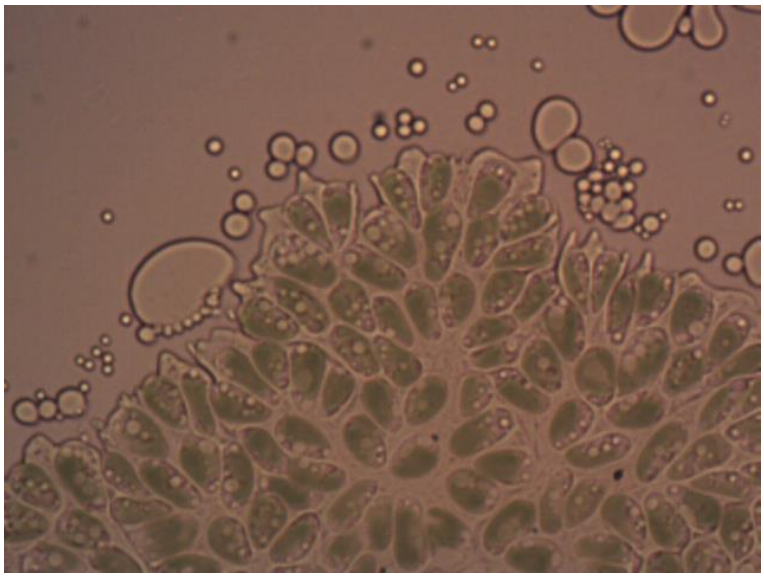


Figure 17. Botryococcus Braunii v. Showa-Bielefeld – late stationary phase. Formation of lipid droplets

2.2.3 Cyanobacteria

Cyanobacteria are a group of organisms that enabled the development of life on earth as we know it today — also referred to as blue-green bacteria that developed in the late part of the so-called Prekambria (the period since the creation of the earth - 542 million years). They are the first photosynthetically active microorganisms that use water as an electron source and produce oxygen as a waste product. They are therefore referred to as oxygen phototrophs, preceded by photosynthetic anaerobic bacteria using inorganic, mainly sulfur compounds. Phylogenetically, cyanobacteria are classified based on the structure of ribosomal subunits and cell wall structure into the *Bacteria* domain, more specifically, gram-negative bacteria. The primary characteristic defining cyanobacteria from other bacterial species is photosynthetic PSI and PSII systems and their phototrophic growth pattern. Some species also developed a partial chemoheterotrophic growth pattern during evolution. Cyanobacteria are a blind developmental branch in the field of bacterial phylogeny, but on the other hand, their endosymbiosis with eukaryotic cells has developed the development of algae [169]-[171].

Cyanobacteria are found in unicellular spherical cells, filamentous cells, or colonies of various shapes and sizes depending on each type of cyanobacteria. The forms mentioned above are the so-called vegetative forms of cells. They reproduce asexually by cell division and fibre fragmentation. Cyanobacteria have also developed unique structures such as heterocysts and akinetes during their development. Akinetes are a protective form of cyanobacteria cells created in unfavourable conditions and survive in a viable state for several decades. This survival structure is formed by enveloping cell wall with several additional layers. Inside the cell, various storage materials are accumulated. This akinete can withstand desiccation and cold. However, they are susceptible to heat [169]-[171].

Heterocysts are cells capable of binding air nitrogen in the form of ammonium ions. Specialized nitrogen-fixing cells are formed during nitrogen starvation by filamentous cyanobacteria, such as *Nostoc punctiforme* or *Anabaena sphaerica*. N_2 is fixed by nitrogenase enzyme inside heterocyst into ammonia ions, which are then transformed into organic nitrogen forms. This process supplies cells with enough processable nitrogen to overcome this stressful state and continue with necessary biosynthesis. Nitrogenase is inactivated with oxygen, so the formed heterocyst must undergo several key steps to create anaerobic state inside it. Heterocyst, therefore, have a unique structure and physiology. The whole process starts with the transformation of a vegetative cell. Here are some of the most crucial steps [169]-[171].

- The cell wall is reinforced by three new layers, one of which is made up of glycolipids that form a non-polar oxygen barrier.
- Nitrogenase and other enzymes involved in N_2 fixation are produced in sufficient concentration.
- Photosystems inside cells are degraded, to stop oxygen production.
- Proteins that scavenge the last remains of oxygen are produced.
- Cyanophycin is produced, which slows down cell-to-cell diffusion.
- Glycolytic enzymes are up-regulated

2.2.3.1 *Cyanobacterial cell structure and their occurrence*

The structure of a cyanobacterial cell is based on the structure of a prokaryotic cell. As already mentioned, cyanobacteria are classified as gram-negative bacteria. This means that the cell wall's main components that form the upper protective layer of the cell are lipopolysaccharides, which are coated on the surface with a membrane of phospholipids. In case of unfavourable conditions, the cell wall can be transformed and incorporated into protective shells, which are very common (e.g. glycocalyx). Below the cell wall, a cytoplasmic membrane is formed of phospholipids supplemented with the necessary sterols ensuring fluidity and transport, regulatory and signalling proteins or their complexes [3][169]-[171].

In many ways, cyanobacteria have typical features of prokaryotic organisms. The cytoplasm contains a bacterial type of ribosome and a circular DNA molecule. The prokaryotic cell in the cytoplasm does not contain any organelles and membrane bodies except for primitive membrane structures called thylakoids, which in cyanobacteria serve as the centre of the photosynthetic apparatus. Unlike bacteria of the genus *Chlorobium*, which uses bacteriochlorophyll, cyanobacterial thylakoids contain chlorophyll A as the main photosynthetic pigment. The auxiliary photosynthetic pigment is mainly β-carotene and to a lesser extent other carotenes and xanthophylls. Phycobilisomes formed by phycobiliproteins are anchored on the surface of thylakoids. These contain phycoerythrin, phycocyanin and allophycocyanin, which form antenna structures and serve as a light collection component. Core is made of allophycocyanin, from which rods of stacked phycocyanin or phycoerythrin discs are oriented outwards. The so-called chromatic adaptation characterizes cyanobacteria. It is an evolutionary mechanism that changes phycobiliproteins' composition, allowing optimal use of the red and green parts of the solar radiation spectrum for photosynthesis [3][169]-[171].

Some cyanobacteria are able to bind airborne nitrogen due to the presence of the enzyme nitrogenase, which can reduce airborne nitrogen to form ammonium ions. Since the anaerobic environment determines nitrogenase activity, nitrogen fixation takes place mainly in non-green heterocysts in which photosynthesis does not occur. Vegetative green cells can only fix air nitrogen in the dark part of the day when the otherwise strongly photosynthetic apparatus is suppressed [3][169]-[171].

Cyanobacteria inhabit the sea and freshwater waters of the entire planet, where they move freely in the water like plankton, but also on the bottom adjacent to the ground. No less important environment is the soil environment where cyanobacteria are an irreplaceable factor affecting the circulation of nutrients in the ecosystem. As one of the few microorganisms, they can survive in the hostile environments of volcanic dust, hot springs, rocks and many more. Cyanobacteria produce many biologically active substances that can be used as pharmaceuticals, biological pesticides and other industrially important substances. We divide Cyanobacteria into four subdivisions [3][169]-[171].

(a) *Chroococcales*
(b) *Pleurocapsales*
(c) *Oscillatoriales*
(d) *Nostocales*

2.2.3.2 *Cyanobacteria genera description*

2.2.3.2.1 Genus Synechococcus

Synechococcus is a genus of widespread marine cyanobacteria with size varying between 0,8 to 1,5 μm. Cells have cylindrical-ovoid shape and reproduce by traverse fission. Genus is one of the vital parts of autotrophic plankton in tropical and subtropical oceans. They prefer well-lit shallow waters, where it can reach high growth rates and become abundant. Genus was first described in 1979 according to cell shape and reproduction. However, this description of the genus *Synechococcus* was very broad, and so individuals with relatively different genetic makeup were included in this genus. therefore, the whole group was divided into several subgroups, for example, according to phycoerythrin pigment. Cells of the genus are gram-negative, they have a structured cell wall, which contains phosphate inclusions, glycogen or PHA granules in the cell's interior [172]-[174].

One of the most interesting characteristics is the presence of carboxysomes. According to the latest theories, they have evolved as a result of increasing concentrations of oxygen on ancient earth atmosphere, which is a competing substrate for the enzyme Rubisco. The enzyme is responsible for the ability of cells to bind CO_2. With high oxygen concentration, the assimilation of CO_2 in the Calvin cycle is inhibited and energy-intensive photorespiration occurs. Instead of carboxylation of ribulose 1,5-bisphosphate, its oxidation takes place in photorespiration, and the energy obtained in the light phase of photosynthesis is consumed. Thus, this oxidation reaction reduces the overall efficiency of photosynthesis. Carboxysomes suppress this negative effect by concentrating carbon dioxide, which gradually diffuses inwards in the form of HCO_3^- ions. They are an example of microcompartments that evolved inside bacteria cells [172]-[174].

Synechococcus cells lack the flagella but are still motile, using gliding method, which is still not fully understood and characterized. All *Synechococcus* strains are obligate autotrophs, and marine strains do not have the nitrogenase activity. The best-known representatives include *S. elongatus*, *S. nidulans* and *S. marinus* [172]-[174].

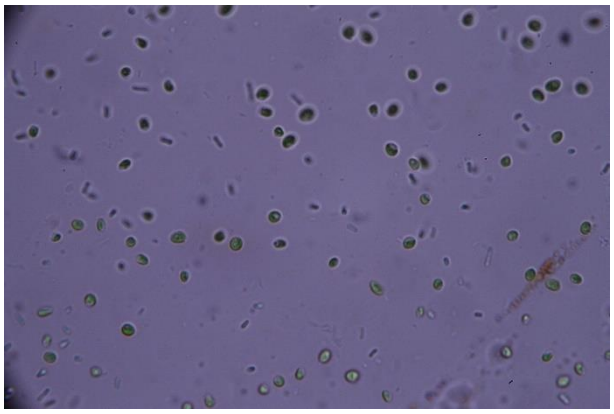


Figure 18. Synechococcus nidulans CCALA 188

2.2.3.2.2 Genus Arthrospira

Arthrospira is a genus of filamentous cyanobacteria, that was firstly isolated from brackish and saline tropical waters. *Arthrospira* prefers alkaline waters with high concentrations of carbonate and bicarbonate. Long filamentous cells are twisted in a left-hand helix. Shape, radius, and other coil parameters differ between each species and even between the same species' cells. Thus, in media with different conditions, we can observe tight coil or even straight filament. Filaments are always solitary and reproduce by binary fission. Cell size varies between 2-12 μm. The genus *Arthrospira* is often confused with the genus *Spirulina*, which has certain identical properties, such as the fibrous shape of the cell. The main distinguishing difference is the formation of sept in the case of the genus *Arthrospira* [175]-[177].

Arthrospira biomass is used as food and a dietary supplement called “Spirulina”. It has a very high protein content (53-68 %), including all essential amino acids. Furthermore, biomass contains high amounts of polyunsaturated fatty acids, like γ-Linolenic acid, photosynthetic pigments with antioxidant activity, vitamins and minerals. Majority of this biomass is produced in open pond outdoor cultivations. Arthrospira could not serve just as a food supplement; according to the latest research, promising results were found in treating certain types of cancer, hepatitis, allergies and vascular diseases. Furthermore, *Arthrospira* can serve as animal feed or in a technical application, such as silver nanoparticle biosynthesis [175]-[177].

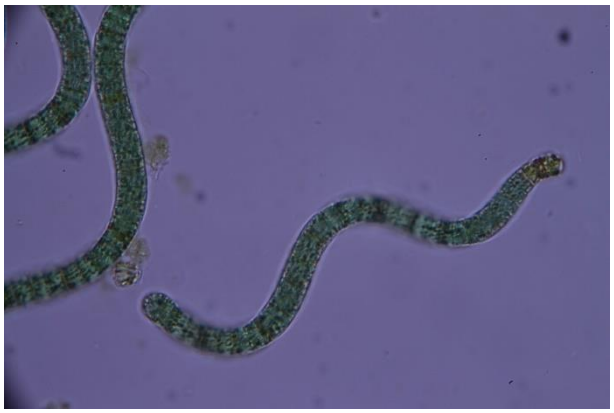


Figure 19. Arthrospira maxima CCALA 027

2.2.4 Microalgae and cyanobacteria industrial application

Industrial use or potential is based on the algal and cyanobacterial cell composition itself, it's way of life, and the wide range of products they can produce. It copies industrial potential and metabolites produced by yeasts. From the beginning, microalgae were considered as a promising food and feed ingredient, thanks to their potential. Their nutritional features vary between classes, even members of the same family. Based on cultivation conditions (light, nutrients, temperature), scientists can, to some extent, modulate production strategy in microalgae strains and thus produce different types of high-value products. Microalgae biomass contains many biologically active compounds, such as oligosaccharides, vitamins, provitamins, pigments, and polyunsaturated fatty acids [179]-[182].

Microalgae have already found a wide variety of industrial applications. Examples of this success include fertilizers, food and feed supplements, health products, cosmetics. They are used for wastewater treatment and are still studied for biofuel production. A large part of the research and some technological results and findings have failed to reach a commercial level due to obstacles and limitations including high production costs, small market size and strong competition with chemically synthesized or alternative products isolated from other microorganisms (bacteria, fungi). Another limitation is connected with strict regulations in terms of quality specifications, environmental impact, safety etc. On the other hand, microalgae and cyanobacteria have a huge biotechnological potential, which is limited mainly by our own limited abilities and their knowledge. And in the future, this potential will be used more and more [179]-[182].

2.3 Co-cultivation

The idea of co-cultivation arose as one of the possibilities of solving major challenges in heterotrophic and photoautotrophic microorganism's biotechnology regarding the production of biofuels, carotenoids, etc. Variety of different oleaginous microbes were studied as potential production strains, capable of accumulating more than 20%of lipids in cell dry mass. From the beginning, the research dealt with the use of two groups of microorganisms, heterotrophic (yeast, bacteria) and phototrophic (cyanobacteria, algae) with different production capabilities. Laboratory and small-volume experiments have provided interesting results, but under conditions that are difficult to achieve in commercial high-volume production. Each group of microorganisms has its own set of pros and cons [183]-[187].

Heterotrophic microorganisms grow very fast and produce large amounts of biomass, that can be used as whole or extracted to get the desired group of biomolecules such as lipids, carotenoids, and polysaccharides. They tend to sediment quite fast in the latter stages of growth, which can be exploited during harvesting. This approach's downside is the high costs of inputs such as carbon source (glucose, glycerol), salts, and nitrogen source. Furthermore, biomass production and growth of these microorganisms are directly proportional to the amount of dissolved oxygen in the medium, which requires very intense aeration in the entire volume of the culture vessel, suitably oriented inlet nozzles and vigorous stirring. All these inputs increase the costs of the whole biotechnology process. As discussed in the previous chapter, the replacement of artificial carbon and nitrogen sources with waste materials from the food and agriculture industry reduces initial costs. However, majority microorganisms do not grow as well as on artificial sources [122]-[125].

Biotechnological use of autotrophic microorganisms, on the other hand, offers the possibility of relatively low input costs. The production medium is practically composed only of salts, and atmospheric air or waste carbon dioxide serves as a carbon source. However, the main negatives are prolonged growth compared to heterotrophs, susceptibility to contamination, low biomass production and the need for virtually constant lighting. Another complication is harvesting a large volume of low-density cultures. Adding to the bill is also the extraction and purification process. Due to the information mentioned above and other parameters such as the still low price of petroleum fuels, the production of biofuels by microorganisms is economically not feasible on a comparable scale [151][183]-[187].

2.3.1 Co-cultivation possibilities

Co-cultivation of different microorganisms offers the possibility to overcome the problems and obstacles of classical monoculture cultivation. In this cultivation, a single culture microorganism can only be stressed out by using nutrient deficiency, salt stress, and heat stress. These physical and chemical stress conditions lead to higher productions of desirable products (lipids, polysaccharides, carotenoids). However, they have limitations, mainly in lowered biomass growth and thus no significant increase in productivity. The last type of stress that has long been neglected is biological stress. For many years, scientists have been involved in the isolation, purification, and identification of individual types of microorganisms, which have been deposited in extensive collections in the form of axenic cultures. Follow-up work was based on identifying produced metabolites, optimising the medium and culture conditions to achieve the highest possible productivity. Many of the microorganisms thus selected were then transferred to industry and are in use today. After using the maximum natural potential, the increase in production was made possible by genetic modification of these microorganisms or suitable substitutes. This procedure is effective and suitable for many heterotrophic microorganisms [183]-[187].

2.3.2 Biological stress

For millions of years, microorganisms have evolved, competed, and interacted with each other. Evolution has led to several basic approaches that can be observed throughout the living world. The first path is the path of predation, and microorganisms that specialize in this path have gained various weapons (antibiotics) and advantages (faster proliferation) that have allowed them to gain a competitive advantage over other consumers of the same sources. The second way linked to the previous one was an adaptation to another source of carbon, nitrogen etc. The last-mentioned path is the path of cooperation and symbiosis when two or more different microorganisms form a more resilient symbiotic consortioum that takes advantage of the original organisms. A typical case is the evolutionary formation of eukaryotic algae. As mentioned in the previous chapter, originally predatory assimilation of a cyanobacterial cell by a eukaryotic cell led to microalgae development. Another case is a consortium of different microorganisms living separately, but mutually supportive [183]-[187].

Therefore, we consider a foreign organism's presence to be biological stress, which affects the strain we cultivate at a certain level. This effect can be negative (predation - culture contamination) where a foreign strain outgrows our studied strain. Or a positive symbiotic effect. Thus, co-cultivation is combined cultivation of two or more microorganisms in one culture medium with the aim of symbiotic growth. Several types of co-cultivation can be distinguished:

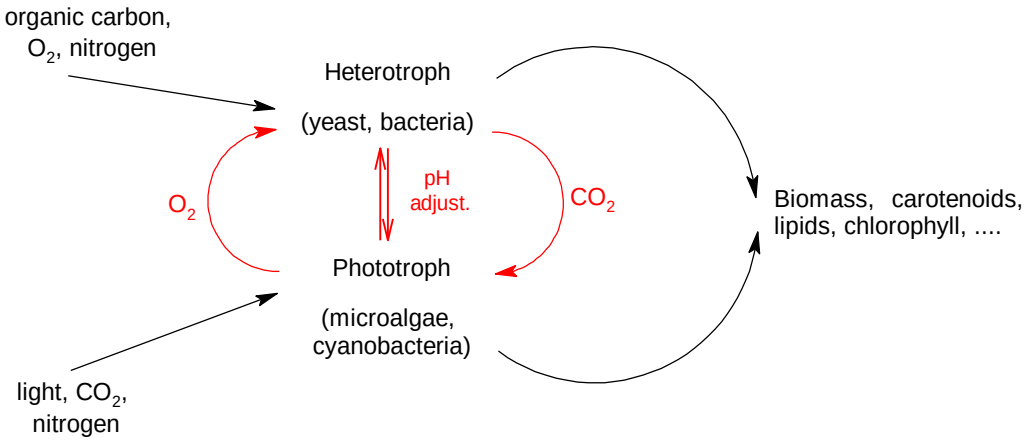
1. Cultivation autotroph – autotroph
2. Cultivation heterotroph – heterotroph
3. Cultivation phototroph – heterotroph

By cultivation two types of similar microorganisms, it is possible to increase the desired metabolites' production, increase their diversity and ensure the utilization of the maximum amount of embedded resources. If the microorganisms share a source of nutrients, there may occur be unnecessary competition, reduced biomass production, and no significant improvement. This approach may be appropriate when using combined nutrient sources (e.g. waste substrates) where one microorganism consumes only a portion of the nutrients and does not compete with the other microorganism. However, finding a suitable combination can be more difficult if representatives from the same species (yeast-yeast) are selected because their enzymatic equipment and nutrient sources preferences are very similar [183]-[187].

Co-cultivation of two phototrophic organisms could provide some advantages. For example, vide variety of products, higher productivity, and more biomass. The best option here could be the mixed cultivation of cyanobacteria and algae, where both cultures simultaneously produce carotenoids, chlorophylls, lipids. Nevertheless, also producing specific products of their species, for example, phycobiliproteins from cyanobacteria. However, there is a problem of competing for a carbon dioxide as carbon source, that can be solved by adding more. However, some cyanobacteria and algae strains could not coexist together because of different media composition demands, pH level, and many more. Also, one microorganism could expel molecules that inhibit the growth of the second one [183]-[187].

2.3.2.1 *Co-cultivation heterotroph - phototroph*

The third type of mixed cultivation offers a possibility of an almost ideal combination of the high variability of combinations of individual microorganisms with an extensive range of produced and extractable substances and finally, no competition for a carbon source. An ideal situation is depicted in the picture below (Scheme 13). A heterotrophic microorganism (carotenogenic yeast) consumes a source of organic carbon and dissolved oxygen through aerobic metabolism and produces carbon dioxide into the medium. The CO_2 thus produced increases the natural concentration of dissolved CO_2 in the medium. CO_2 is processed by a phototroph (microalgae) and grows faster thanks to its increased concentration. On the other hand, the phototroph's oxygen induces a faster growth of the heterotrophic partner [183]-[187].



Scheme 13. *Schema of co-cultivation heterotroph and phototroph*

Unfortunately, this ideal situation can never be achieved, and many obstacles need to be addressed and appropriate compromises found. When cultivating, it is necessary to consider, the difference in the medium's composition, the pH optimum for the cultivated strains, their mutual compatibility and many others. For example, carotenogenic yeasts have a pH optimum between 5-6.8, whereas most microalgae strains prefer pH higher than 7. As mentioned above, each type of microorganism has its issues and demands regarding the cultivation process conditions (carbon source, oxygen). The process of optimizing the composition of the media and selecting suitable pairs of strains will be lengthy and demanding [183]-[187].

On the other hand, this process's benefits are very high. They can bring exciting innovations in processes and understanding of microorganisms' symbiotic relationships, despite the economic benefits of this product combining the benefits of both yeast and microalgae. Based on these results and innovations, it is possible to simplify and streamline both types of microorganisms' cultivation processes. We encounter various co-cultivation examples within the literature, such as co-cultivation of oleogenic yeasts or bacteria with microalgae in aquaculture, chemical production, and waste treatment. This co-cultivation of carotenogenic yeast and microalgae can be carried out to induce production of lipids, carotenoids, polysaccharides or other valuable compounds. This consortium yeast-microalgae can have different physical properties, be easily harvested, grown in wastewater, process waste from the food industry, and many more [183]-[187].

2.4 Waste materials from the food industry and their potential usage

The food industry worldwide produces large amounts of solid and liquid waste material, which was mostly unused in previous decades. This material mostly ended up in waste incinerator or waste treatment plant. The growing population of the planet and the associated increased consumption are continually increasing food production demands, increasing waste production and more intensively depleting the planet's resources and capabilities. Researchers are currently looking for ways to use this material for further use in any area of life. Waste from the food and agricultural industries still contains a number of nutrients and substances that can be further used to increase the efficiency of processing these materials. With the development of biotechnology and genetic techniques, our knowledge of microorganisms, the ability to work with microorganisms, and direct them to metabolites production, which finds application in many sectors of human life, is developing [188][191].

2.4.1 Waste animal fat

Waste animal fat is the product of the meat industry. With rising population each year, the amounts of processed meat and the associated production of waste by-products are growing steadily. More than 16 million tons of by-products of animal waste fat are processed in the EU every year. According to the regulations (1069/2009, 142/2011), animal fat is divided into three categories depending on its origin and constitution. The total amount of waste fat is expected to grow continuously, due to consumers rising focus on low fat (mainly low saturated fat) diets [191][197][198].

1. Lowest grade waste fat – this fat can be used only as an energetic source
2. Medium-grade waste fat – derived from condemned slaughterhouse animals and dead farm stock, can be used as an energetic source and for some technical applications
3. High-grade fat – fat derived from approved animals in slaughterhouses, highest quality fat, named “edible fat”.

2.4.1.1 *High-grade fat*

Although called “edible fat”, it is not used as food or supplement for the human population. It serves mainly as a cattle feed. However, the composition of waste fat is not ideal for animal feed purposes because it contains a high percentage of saturated fatty acids (Figure 20). There are two ways to solve the problem. The first way is to supply MUFA and PUFA acids from an external source artificially [191]. E.g. from another type of oil (rapeseed, sunflower). However, the problem is the high cost of this process and the need to use a large amount of oil. Another disadvantage is that this process can practically only adjust the FA composition parameter. The second way is the use of biotechnological transformation using selected strains of microorganisms. Microorganisms are able to utilize this fat in raw or modified form and produce enriched biomass, which contains in addition to lipids with a high content of MUFA and PUFA, also other added substances proteins, carbohydrates, vitamins, etc [191].

Studied strains of microorganisms such as carotenogenic yeast and biomass also contain many antioxidants - carotenoids. Thus, the enriched biomass can be added to feed, or isolated and processed in the form of supplements and preparations for humans. The chemical composition of fat consists of triacylglycerols, free fatty acids and minor admixtures of sterols, phospholipids and other substances of a lipophilic nature (vitamins A, D, E, K and antioxidants). In this work, waste animal fat provided by the Norwegian company Norilia was used. The melting point of the animal fat ranged from 40-45 ° C. The nonpolar nature of fat causes its insolubility in aqueous media, i.e. also in culture media [191].

From a biotechnological perspective, waste fat can be classified as a simple waste substrate that does not require complex pre-treatment. Most species of heterotrophic microorganisms produce lipases in two forms. The first is localized freely in solution and the second is non-covalently anchored on the outer surface of the cell wall (it can be released, for example, by the ultrasound). Structurally, both enzymes are practically identical. However, the activity of microbial lipases is insufficient for the biotechnological production of microorganisms on waste fat. To speed up the whole process and make it more efficient, the waste fat is therefore treated before cultivation itself. The following three methods can be used for fat pre-treatment [191][197][198]:

1. Acid hydrolysis:

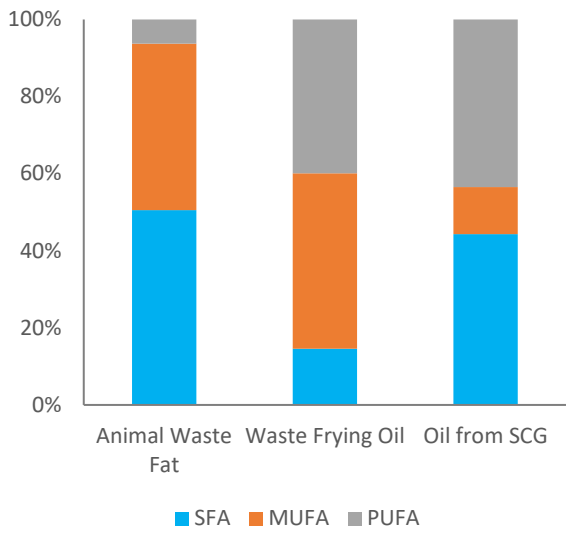
Acid hydrolysis produces glycerol and free fatty acids. This reaction has the disadvantage of establishing an equilibrium of the reaction. Equilibrium is established because the hydrolysis reverses the esterification process, and part of the product is thus transformed back into the starting materials. As a result, complete hydrolysis of the material cannot be achieved without continuously removing some of the products. The advantage of this reaction is that the acid is only a catalyst and is not consumed [191][197][198].

2. Base hydrolysis

During base hydrolysis, a strong base's action irreversibly cleaves the ester bond to form glycerol and fatty acid salts. This reaction is fast and complete hydrolysis of the fat can be achieved. The disadvantage of the reaction is that the hydroxide is consumed during the reaction and must be replenished. If the hydrolysis were to take place directly in the culture medium, complete hydrolysis would result in a medium with a high content of salts of fatty acids and salinity, which negatively inhibit most microorganisms' growth [191][197][198].

3. Enzymatic hydrolysis

This process is based on the use of enzymatic reactions for fat hydrolysis. This reaction takes place selectively at the polar: nonpolar environment interface. The enzymatic reaction rate can be influenced by setting the optimal conditions (temperature, pH, amount of enzyme) and emulsifying the fat before starting the reaction. As in base hydrolysis, the reaction products are free fatty acids and glycerol. The disadvantage of this reaction may be the different pH optimum of the enzymes and the optimum for the cultivation of microorganisms [191].



Graph 1. *Fatty acid profile of used waste animal fat, waste frying oil and coffee oil [191]*

2.4.2 Whey

Milk and the products produced from it have long been considered an integral part of human civilization. The dairy industry is one of the central and indispensable parts of the food industry, playing a significant role in the global economy. More than 811 million tons of milk are produced annually (2017), and this production is continuously increasing. This industry generates an enormous amount of waste, which can be divided into solid and liquid waste. Waste products discharged at the end of the process have different properties, depending on the type of product that has been produced, the climate, the operating conditions and the cleaning procedures. In milk processing and the production of dairy products, 80-90% of the total volume of milk is discarded as a by-product called whey [188][192][193].

Whey can be characterized as a milk serum free of fats and casein, in which the remaining parts are homogeneously dispersed. World whey production is around 200 million tonnes and is growing every year. The leading producers with more than 70% share are the United States and the European Union. Virtually the amount of whey produced approaches equal the amounts of produced milk. Simple disposing of millions of tons of whey is forbidden nowadays, due to its toxicity to the environment. This main problem stems from its potential to cause significant damage to the environment. Whey has a huge biochemical oxygen demand (BOD) between 40,000-60,000 mg/L and at the same time, a high chemical oxygen demand (COD) of 50,000-80,000 mg/L. For comparison, the same oxygen consumption corresponds to 150-175 times the amount of domestic wastewater. The main component causing this problem is a high concentration of lactose. Spilling whey into the environment, thus leads to virtually immediate consumption of all oxygen [192][193][195][196].

2.4.2.1 *Whey composition:*

Whey is a rich source of macronutrients and micronutrients. The content of individual components in whey is very variable. It depends on many factors, such as the origin of the milk used (cow, sheep, goat, etc.), the health of the animals, how the milk is processed, the type of product produced, etc. One of the main parameters is the method of milk protein precipitation. When milk protein is precipitated with mineral acids, so-called acid whey is created, characterized by lower pH, lower carbohydrate content, and higher mineral content. This process is characteristic for the production of Cottage cheese. Enzymatic precipitation of milk, e.g. with chymosin or other proteolytic enzymes, produces so-called sweet whey. This procedure is applied, for example, in the production of Cheddar cheese. The basic composition and parameters of whey are given in the table below (Table 2) [192][193][196].

Table 2. Sweet and acid whey composition.

	Whey type	
Component	Sweet whey	Acid whey
Water	93-94 %	
Dry matter	6-7 %	
pH	6-7	<5
Lactose	56-52	44-46
Protein	6-10	6-8
Calcium	0.4-0.6	1.2-1.6
Phosphate	1-3	2-4.5
Lactate	2	6.4
Chloride	1.1	1.1
Fats and oils	<1.5	<1.5

** Concentrations expressed in g/L, except for pH*

2.4.2.2 *Whey processing techniques.*

There are three options for processing waste whey. The first is based on the maximum use of individual components of whey. Using various physical-chemical processes (flocculation, precipitation) and filtration techniques, individual components (lactose, proteins, etc.) are isolated from whey. In this area, it is worth mentioning the high market development for products and food supplements for athletes and active people. The best-known products are various protein preparations, initially produced by simply drying whey, which is no longer used today. Simply drying the whey produced a material high in carbohydrates and other undesirable substances. Therefore, the use of ion-exchange chromatography in protein separation has been and is being introduced. The disadvantage of this process is the disruption of some substances' biological activity by the inorganic salts and acids. Today's most widely used technique is cross-flow microfiltration, in which protein and other components are obtained without disturbing biological activity [192][193][195][196].

The second possibility of whey processing is its biological degradation as a source of nutrients for plants. This process offers several advantages, e.g. high content of nitrogen sources, minerals, and organic compounds can be used as growth factors. The main obstacle in this process is the high salt content of whey, which most agriculturally used plants, except halotolerants, cannot tolerate. Whey must undergo a process of base precipitation with calcium. Then the resulting sludge is used in combination with water. From the results of research on the tomato tree, it was found that the application of such a fertilizer leads to the formation of sweet fruits with a higher content of proteins, potassium and antioxidants. Whey is also used as part of cattle feed [192][193][195][196].

2.4.2.2.1 Biotechnological transformation of whey

The third possibility is a biotechnological transformation of whey using microorganisms. For decades, whey has been considered a surplus and burdensome waste, an ecologically incompatible liquid without practical use. In recent decades, with the development of biotechnology, the view of whey has changed significantly. The research began to deal with the possibility of using whey and its transformation into a valuable substrate. Whey is one of the complex waste substrates, containing macro and micronutrients sources for the growth of microorganisms. The carbon source here is primarily the disaccharide lactose and to a lesser extent the amino acids of milk protein.

The nitrogen source is a milk protein and to a lesser extent, inorganic mineral nitrogen. Globally, considerable efforts are being made worldwide to find ways to transform excess whey into higher value-added materials. Lactose as the main carbon source in the medium is a relatively simple substrate for processing by microorganisms. Many microorganisms naturally have lactase enzymes and can thus be produced in untreated whey, which improves the economic balance of the process. Within the literature and industry, we find a wide range of applications of this material. We can also mention the production of yeast extract, antibiotics, bioethanol, bioplastics - polyhydroxyalkanoates and lactic acid [192][193][195][196].

2.4.3 Spent coffee grounds

Coffee as a drink has come a long way since its discovery due to the colonisation of the American continent in the 16th and 17th centuries and the subsequent spread throughout the world over the years. Today, more than 2 billion coffee drinks are drunk daily. According to data published by the International Coffee Organization, more than 171 million coffee bags (1 bag = 60 kg) were produced and exported last year. Instant coffee alone produces more than 6 million tonnes of spent coffee grounds (SCGs) per year [199][200]. This waste material is the source of a variety of useful compounds. SCG can be divided into several fractions, namely oil fraction, lignocellulose fraction, alkaloids and proteins. The oil fraction is a triglyceride source with a higher mono- and polyunsaturated fatty acid content, phenolic compounds, and others [200].

Spent coffee grounds can be used as fuel for industrial heaters, so they are most often used in the coffee processing industry. Coffee grounds are difficult to process in composts in large quantities due to residual concentrations of caffeine, antimicrobials and polyphenols, which are toxic to the soil, and can ignite spontaneously due to high oxygen requirements. Coffee grounds contain 10 to 20% of coffee oil, which can be used after extraction with non-polar solvents to produce biofuels, in particular biodiesel, which can reach a higher cetane number than diesel produced from petroleum. The lignocellulosic fraction comprises hemicelluloses and celluloses, which, as with other comparable types of lignocellulosic waste substrates, can be utilised and transformed into fermentable carbohydrates, which serve as a cheap carbon source [203][205]. They make up 50 % of SCG dry weight and are rich in glucose, arabinose, mannose and galactose. For lignocellulose to be used for yeast cultivation, this material must be hydrolysed and long polysaccharide chains therefore cleaved. Acid hydrolysis is most often method used for this. Wang et al. optimised the acid hydrolysis process of SCG and obtained about 521.6 mg of fermentable carbohydrates from 1 g of SCG. The hydrolysis of SCG mainly uses a dilute solution of sulfuric or hydrochloric acid in a 1-5% concentration. The hydrolysis time is given by the temperature used. In general, the higher the hydrolysis temperature, the less time it takes [201]. Another suitable choice is enzymatic hydrolysis with commercial cellulases and hemicellulases enzymes.

2.4.3.1 *Biotechnological application of SCG*

SCG hydrolysates can be used to produce a wide range of valuable products [203], such as polyhydroxyalkanoates [205], lactic acid [207], bioethanol [203] and carotenoids [205]. The level of hydrolysis depends on the selected microorganism and its enzymatic equipment. In the case of carotenogenic yeasts, SCG needs to be hydrolysed entirely [205], since these microorganisms do not in themselves form the required cellulases. On the other hand, fungi and bacteria are known for their production of cellulases and hemicellulases, and therefore SCG does not need to be so strongly hydrolysed [207]. The disadvantage or a problem of SCG hydrolysates is the presence of other accompanying substances, which can be called co-contaminants or stress inducers.

Three groups of substances can act toxic towards microorganisms. The first group are extractives (terpenes, alcohols and tannins), substances that are extracted in the process. The second group is represented by molecules formed as breakdown/cleavage products of cellulose and hemicellulose matrix (hydroxymethylfurfural, furfural and acetic acid). The last group are substances releasing from lignin decomposition (phenolic substances, lignin degradation products). For successful biotechnological use of SCG, it is necessary to study and verify the effect of these antimicrobial compounds on microorganisms' growth and productivity. In the literature, we find extensive research dealing with this area.

Furfural and hydroxymethylfurfural have been found to inhibit the respiratory mechanism in microorganism cells [208]-[210] and cell proliferation [211]. Phenolic substances are generally known for their antimicrobial activity [210][212]. High concentrations of carboxyl acids in cells affect cell morphology and overall pH of the medium [212]. The problem is that these experiments are focused on the effects of individual groups of antimicrobials. It can be said with certainty that the combined antimicrobial effect of all groups of substances will be much higher [209][212].

2.5 Methods for microorganism cells and biomass analysis

2.5.1 Gravimetric determinations and microscopic observations of microorganisms

Microscopic techniques and cytological techniques give us information about the size and anatomy of cells. It is the so-called morphological description of individual cell components. We can observe differences between individual species of microorganisms (if it is a mixed culture), differences between individual representatives of the same species, responses to stress environment, utilising isotopes to follow the path of individual molecules and atoms by cell and mutation of cells. We can further study intercellular communication, intra-cell communication, the interconnection of metabolic pathways, etc. All these processes directly impact the production of important metabolites, and therefore the gain of this information is crucial for the efficient use of the microorganism in both laboratory and industrial biotechnology.

2.5.1.1 *Optical and fluorescence microscopy*

The basic optical microscopy techniques provide information on the shape, size and morphology of cells, cell multiplication, localisation of specific organelles in the cell, and other parameters. Using confocal and optical microscopes, we can find relatively limited information. Using different types of contrasting pigments, we can target individual organelles, metabolic pathways. At 1000-fold magnification, vacuoles and large cell organelles can be observed. Fluorescence microscopy allows direct observation of fluorescence-capable molecules (autofluorescence). There are different types of molecules capable of autofluorescence in the cell. They contain aromatic cycles or conjugated multiple bonds (vitamins, hormones, cofactors, pigments). By adding fluorescent pigments, substances or organelles that cannot be capable of autofluorescence can be observed and studied to virtually locate most of the metabolites and organelles in the cell [214][217].

2.5.1.2 *Cell biomass determination*

The determination of the number of cells or their concentration in the medium is crucial in microbiological and subsequently biotechnological experiments. Many direct and indirect methods are used to determine the amount of biomass, which is based on calculation or absolute weight determination. Traditional methods include microscopic counting or counting by counting chambers. With the advancement of technology, devices have taken on the counting task, which is more accurate, efficient, and often provide and record more parameters at once than just the number of cells—for example, flow cytometry and automatic cell counters. Indirectly, cell count can also be determined turbidimetrically [214][217].

Another traditional technique is to determine the cell biomass gravimetrically, where the culture sample is centrifuged or filtered, then washed several times and dried to constant weight. The second option is then lyophilization, which is much more gentle, especially considering thermolabile substances.

2.5.2 Extraction

The purpose of the extraction is primarily to isolate the analyte from the matrix or substances causing the determination's interfering effects. The extraction process is a separation method that uses the selective transfer of a particular component between two immiscible liquids or between a liquid and a solid phase. The particles/molecules are transferred from the solid phase to the solution or, in the case of two liquid phases, from one liquid phase to another. Interactions between solvent molecules and between analyte molecules apply here. The choice of a suitable solvent depends on the type of interactions that apply to the system. In liquid-liquid extraction, the main condition for the extraction is to establish an equilibrium between the analyte and the solvent. In this case, the metabolites determined are predominantly non-polar in nature and occur in the cytoplasmic membrane and other cellular organelles. In the case of carotenoid yeasts, a strong cell wall protects the cell's interior and the organelles in which the desired metabolites are found. Thus, before or during the extraction, it is necessary to disrupt the cell wall and make the solvent's cytosol volume available. Algae and cyanobacteria also require cell disruption, but the extraction is more efficient due to the thinner cell wall. Mechanical, chemical and enzymatic methods can be used to disrupt the cell wall [215][216].

2.5.3 Transesterification

Transesterification is a reaction in which carboxylic acids (fatty acids) are esterified with alcohols in the presence of a suitable acid catalyst. This reaction is used in biotechnology for the analysis of lipids and fatty acid profile produced by microorganisms. The products of this reaction are carboxylic acid esters with a reduced boiling point compared to carboxylic acids and thus allow determination by gas chromatography. One of the main disadvantages of transesterification is its reversibility. The reaction proceeds as long as the equilibrium between the products and the reaction's starting materials is established. To increase the efficiency and yield of transesterification, the reaction a large excess of alcohol is used. The equilibrium shifts towards ester products [222].

2.5.4 Chromatography

Chromatographic methods are a group of separation methods úsed in analytical chemistry. The sample analytes are separated according to different solubility, adsorption or distribution coefficient between two physically and chemically different immiscible phase. The first phase is stationary and the second mobile. The separation process itself is formed by the repeated creation of equilibrium states of analytes based on physico-chemical interactions between the analyte and the mobile phase, the analyte and the stationary phase, as well as the mobile and stationary phases. The separation mechanisms used in chromatographic separations are dissolution, precipitation, chemisorption, adsorption, sieve effect, complex formation, ion exchange, etc. Chromatographic separation is characterized by the fact that it employs several separation mechanisms at once. The total time that a given analyte spends in the chromatographic column is then given by the resulting affinity for the two phases individually. Thus, those substances with the highest affinity for the stationary phase are the system's most retained molecules. This separates the individual components and less retained substances first reach the column's end [219]-[222].

2.5.5 HPLC/PDA

High-performance liquid chromatography techniques were developed from column chromatography techniques, where a column with average lengths of about 50 cm and a diameter of 2-3 cm was packed with sorbent particles of relatively large diameter. A sample was loaded onto the top of the column, and then the mobile phase was poured, through which the individual components of the mixture were gradually eluted. Elution was driven only by gravity, and the separation was lengthy and inefficient. The newly formed liquid chromatography differed from the column chromatography in several basic points. The mobile phase flow was ensured by pumps that supplied a constant flow of the mobile phase at the same pressure. The stationary phase particles have been significantly reduced, thus increasing the active surface of the stationary phase and thus increasing the separation efficiency. However, as the stationary phase's particle size decreased, the resistance of the stationary phase to the flow of the mobile phase also increased proportionally. Thus, new assemblies have been developed with pumps capable of operating at extremely high pressures. Thus, through low-pressure liquid chromatography has developed high-performance liquid chromatography (HPLC) [219]-[222].

High-performance liquid chromatography is today a ubiquitous technique for separating complex mixtures of analytes. In this method, the mobile phase is pumped under high pressure by pumps to the column where the separation itself occurs. For lipid metabolites (carotenoids, sterols, ubiquinone, chlorophylls) analysis, an arrangement with the non-polar stationary and polar mobile phases is preferred. The diode array detector (PDA) is one of the preferred detectors to analyse studied metabolites. The main part of the detector is a series of photodiodes that sense individual wavelengths. Thus, the entire absorption spectrum of a given substance can be scanned in real-time, and the given substance can be identified on this basis [219]-[222].

2.5.6 GC-FID

Gas chromatography is used to separate substances with a relatively low boiling point. In gas chromatography, the mobile phase is a carrier gas that does not interact with the sample in any way. Hydrogen, nitrogen and helium are used as carrier gases. In gas chromatography, the separation is based only on the interaction of analytes with the stationary phase. Packed and capillary columns are used for gas chromatography. The packed columns are structurally and functionally identical to HPLC columns. The capillary columns are formed by a hollow quartz capillary coated with polyamide. The inner walls of the capillary are coated with a thin layer of stationary phase. Due to the large ratio between the column's length ranging from 10 to 100 meters and the inner diameter (hundreds of micrometres) of the column forms a huge separation area, the capillary columns achieve the highest separation efficiencies. The disadvantage is the possibility of analyte analysis with a boiling point up to about 240 °C. Molecules that cannot normally be separated by gas chromatography due to their high melting point are transformed into lower boiling derivatives (e.g. the conversion of fatty acids to their corresponding methyl esters) analysis. Flame ionization detector is a universal detector for almost all organic molecules'. It has a linear response over a wide range of concentrations. [219]-[222].

3 THE AIMS OF THE STUDY

The aim of this dissertation was the study of production properties of carotenogenic yeasts, microalgae and cyanobacteria with a focus on the production of lipids, lipidic metabolites (carotenoids, chlorophylls, ubiquinone, ergosterol) and possibly other metabolites in connection with the utilization of waste substrates originating from agriculture and food industry. The work was divided into several units according to the tested types of microorganisms.

The experimental part of the dissertation solves the following goals:

1) Cultivation of carotenogenic yeasts
    - Screening of yeast strains cultivated on untreated waste substrates of the waste industry
    - Optimization of pretreatment of waste substrates for cultivation of carrotenogenic yeasts.
    - Study of the production of extracellular lipases and biosurfactants on waste animal fat
    - Screening cultivations on waste substrates in media with different C/N ratios
    - Large-volume bioreactor cultivations of yeast on media with a combination of two waste substrates
2) Cultivation of microalgae and cyanobacteria
    - Screening cultivation of microalgae and cyanobacteria on different types of media
    - Optimization of medium composition (N, P, S) and culture conditions
    - Cultivation under stress conditions (salt stress, oxidative stress).
    - Large-scale cultivation of extremophilic microalgae *Coccomyxa onubensis*
3) Co-cultivation of yeasts and microalgae
    - Screening cultivation of yeast on BBM medium
    - Cultivation of microalgae on media with an organic carbon source
    - Laboratory tests of co-cultivation of yeasts and microalgae
    - Large-scale bioreactor co-cultivation

4 MATERIALS AND METHODS

4.1 Microorganism strains

4.1.1 Yeast strains

All studied carotenogenic yeast strains were obtained from Culture Collection of Yeasts (CCY), Slovak Academy of Sciences, Bratislava, Slovakia. Carotenogenic yeasts strains were kept at -80°C in a cold storage box. Storage media consisted of 1:1 mixture of yeast culture in YPD media and 50% of glycerol as cryoprotectant.

Table 3. Carotenogenic yeasts used in this dissertation.

Microorganism name	Culture collection code
Rhodotorula kratochvilovae	CCY 20-2-26
Rhodotorula mucilaginosa	CCY 19-4-6
Rhodosporidium toruloides	CCY 062-0002-001
Sporidiobolus pararoseus	CCY 19-9-6
Cystofilobasidium macerans	CCY 10-1-2
Phaffia Rhodozyma	CCY 77-1

4.1.2 Microalgae and cyanobacteria strains

All studied microalgae, and cyanobacteria strains except *Coccomyxa onubensis* and *Botryococcus Braunii* v. Showa-Bielefeld were bought from Culture Collection of Autotrophic Organisms (CCALA), Czech Academy of Sciences, Třeboň. Abovementioned microalgae *Coccomyxa onubensis* and *Botryococcus braunii* were obtained from Algal Biotechnology group, University of Huelva, Huelva, Spain. Microalgae were stored in tubes with slant agar media with appropriate medium.

Table 4. Microalgae and cyanobacteria used in this dissertation.

Microorganism name	Culture collection code
Athrospira maxima	CCALA 030
Chlorella vulgaris	Beijerinck CCALA 897
Chlorella sorokiniana	Shihira et Krauss CCALA 259
Chlamydomonas reinhardtii	P. A. Dangeard CCALA 973
Scenedesmus cf. acutus	(Krueger) Migula CCALA 258
Scenedesmus cf. obliquus	Meyen CCALA 438
Scenedesmus dimorphus	(Turpin) Kuetzing CCALA 443
Synechococcus nidulans	(Pringsheim) Komarek CCALA 188
Desmodesmus quadricauda	CCALA 464
Coccomyxa sp.	CCALA 912
Coccomyxa onubensis	Isolate form Rio Tinto
Botryococcus braunii	v. Showa-Bielefeld

4.2 Chemicals

4.2.1 Chemicals used for cultivation of carotenogenic yeasts, microalgae and cyanobacteria

Yeast autolysate (Himedia, India)
Bacteriological pepton (Himedia, India)
Agar-agar (Himedia, India)
Polysorbate 80, (Tween 80) (Lach-Ner Ltd., Czech Republic)
Triton X 100, (Lach-Ner Ltd., Czech Republic)
Material animal fat mixed, (Norilia, Norway)
Spent coffee grounds, Department of Food Science and Biotechnology coffee machine
Whey, Dairy Kromik a.s., (Czech Republic)
Na_2-EDTA.$2H_2O$ p.a., (Sigma-Aldrich, Germany)
$(NH_4)_5Fe$ $(C_6H_4O_7)_2$ p.a., (Sigma-Aldrich, Germany)
Manganese chloride tetrahydrate p.a., (Sigma-Aldrich, Germany)
Sodium molybdate dihydrate p.a., (Sigma-Aldrich, Germany)
Cobalt nitrate hexahydrate p.a., (Sigma-Aldrich, Germany)
Zinc chloride p.a., (Lach-Ner Ltd., Czech Republic)
Cobalt Chloride Hexahydrate p.a., (Lach-Ner Ltd., Czech Republic)
Potassium Chloride p.a., (Lach-Ner Ltd., Czech Republic)
Ampicillin sodium salt, (Sigma-Aldrich, Germany)
Clotrimazole, (Sigma-Aldrich, Germany)

4.2.2 Chemicals used for waste substrates hydrolysis

B-Galactosidase from *Escherichia coli*, (Sigma-Aldrich, Germany)
Hemicellulase *Aspergillus niger*, (Sigma-Aldrich, Germany)
Cellulase from *Trichoderma resei*, (Sigma-Aldrich, Germany)
Cellulase from *Aspergillus niger*, (Sigma-Aldrich, Germany)
Cellulase enzyme blend, (Sigma-Aldrich, Germany)
Lipase from *Aspergillus niger*, (Sigma-Aldrich, Germany)
Lipase from *B Candida antarctica*, (Sigma-Aldrich, Germany)
Lipase from *Candida rugosa*, (Sigma-Aldrich, Germany)
Lipase from *Mucor miehei*, (Sigma-Aldrich, Germany)
Lipase from *Pseudomonas cepacia*, (Sigma-Aldrich, Germany)
Lipase from *Pseudomonas fluorescens*, (Sigma-Aldrich, Germany)
Lipase from *Rhizopus oryzea*, (Sigma-Aldrich, Germany)
Lipase from *Rhizopus niveus*, (Sigma-Aldrich, Germany)
Lipase from *porcine pancreas*, (Sigma-Aldrich, Germany)

4.2.3 Chemicals used for biosurfactant production and lipolytic activity assay

p-nitrophenol (Sigma-Aldrich, Germany)
p-nitrophenol palmitate (Sigma-Aldrich, Germany)
Natrium dodecylsulphate (Sigma-Aldrich, Germany)
Anthracene p.a., (Sigma-Aldrich, Germany)
Rapeseed oil commercial, Tesco stores (CZ)
Vizualization agent, (Sigma-Aldrich, Germany)

4.2.4 Chemicals used to process and extract biomass

Acetone p.a., (Lach-Ner Ltd., Czech Republic)
Chloroform p.a., (Lach-Ner Ltd., Czech Republic)
Methanol p.a., (Lach-Ner Ltd., Czech Republic)
Hexane p.a. (Lach-Ner Ltd., Czech Republic)
Sulfuric acid 96%, (Lach-Ner Ltd., Czech Republic)
Ethyl acetate HPLC grade (Lach-Ner Ltd., Czech Republic)
Acetonitrile HPLC grade (Lach-Ner Ltd., Czech Republic)
Methanol HPLC grade (Lach-Ner Ltd., Czech Republic)
Hexane HPLC grade (Lach-Ner Ltd., Czech Republic)
Heptadecanoic acid analytical standart (Sigma-Aldrich, Germany)
Carotenoids mix analytical standard (Sigma-Aldrich, Germany)
Ubiquinone analytical standard (Sigma-Aldrich, Germany)
Ergosterol analytical standard (Sigma-Aldrich, Germany)
Chlorophyll A analytical standard (Sigma-Aldrich, Germany)
Chlorophyll B analytical standard (Sigma-Aldrich, Germany)
Glycerol analytical standard (Sigma-Aldrich, Germany)
Saccharides kit analytical standard (Sigma-Aldrich, Germany)

4.3 Equipment

HPLC Thermo Fischer Scientific Dionex UltiMate 3000 series (USA):

- Pump with degasser
- Autosampler with column oven
- Vanquish series Diode Array Detector
- Chromeleon 7.2 chromatography software
- Column Kinetex Core Shell C18 150x5.0x4.6 + C18 precolumn Phenomenex

HPLC/MS Thermo Finnigan Surveyor LCQ series (USA)

- MS pump with degasser
- Column Oven ECOM (CZ)
- PDA Plus Detector
- Xcalibur software
- Column Kinetex Core Shell C18 EVO 150x5.0x2.6 + C18 precolumn Phenomenex

GC Thermo Fischer Scientific Gas Chromatograph TRACETM 1300

- Column oven
- Autosampler AI 1310
- Flame ionization detector
- Chromeleon 7.2 software
- Zorbax ZB-FAME 30 m x 0.25 mm x 0.2 μm

Vortex Genie, Scientific Industries, Inc. (USA)
Freeze-dryer Labconco FreeZone 4.5 Freeze Dryer (USA)
Thermoblock VWR, (CZ)
Syringe filters 0.4 μm PTFE Chromservis, (CZ)
Syringe filters 0.4 μm Nylon Chromservis, (CZ)
Box Aura mini iBiotech (CZ)
Systech Autoclave (USA)
Orbital shaker WiseSHake SHO (USA)
TLC plates, (Sigma-Aldrich, Germany)
Analytical balances Boeco (Germany)
Multicultivator MC 1000 OD Photon System Instruments (CZ)
OD-View Software Photon System Instruments (CZ)

AquaPen-C AP-C 100 Photon System Instruments (CZ)
RALF Bioengineering bioreactor, Bioengineering (Switzerland)

- 3.0L vessel with heating jacket and cooling finger
- pO_2 sensor
- pH sensor
- Temperature sensor

Centrifuge Z 366 – Hermle, Hermle (Germany)
Safemate ECO 1.8 m sterile box, BioAir (USA).

4.4 Cultivation techniques and media composition

4.4.1 Cultivation of carotenogenic yeasts and experimental design

4.4.1.1 *Inoculation*

All yeast strains were cultivated using double inoculation method. For fresh stock culture preparation, YPD media with 2% agar was sterilized in high-pressure pots and poured into sterile Petri dishes. After cooling down in the sterile conditions in flow-box under UV light, Petri dishes were closed by parafilm and kept in the fridge for later use. For inoculation, several loops from cryotube stock cultures were then inoculated onto Petri dish with YPD agar. Petri dishes were kept in laboratory temperature under constant illumination. After optimal cell proliferation (3-4 days), cultures on agar plates were prepared for inoculation into liquid media. YPD media was prepared and sterilized for 30 minutes at 121 °C in Erlenmeyer flasks in high-pressure pots with open valve (preventing Maillard reaction). Culture from the agar plate was transferred into flasks by a sterile loop. Inoculation was performed with ratio one loop of culture per 10 mL of inoculation media (unless otherwise mentioned). Inoculum I was cultivated at room temperature under constant illumination on a reciprocal shaker. After 24 hours, Inoculum I was transferred to Inoculum II with ration 1:5 (V/V) yeast culture: fresh media. The composition of the YPD inoculation media is shown in the table below.

Table 5. YPD media composition

Media type		Components	Amount
YPD solid media	YPD liquid media	Water	1000 mL
		Pepton bacteriological	20 g
		Yeast autolysate	10 g
		Glucose	20 g
		Agar bacteriological	20 g

4.4.1.2 *Production media*

Production media was prepared according to the procedure above. Sterilized and cooled Erlenmeyer/Pyrex flask with production media was inoculated in a sterile flow-box with same 1:5 (V/V) ratio as mentioned above (unless otherwise mentioned in some experiments). Carotenogenic yeasts were cultivated in a variety of production media. Composition of basic production media, nitrogen sources, carbon sources, and others is listed in tables below (Table 6-Table 8). The flask was cooled down after sterilisation and inoculated with inoculum II with ratio 1:5 inoculum: production media. All experiments in Erlenmeyer flasks were cultivated for 96 hours under constant irradiation at room temperature on a reciprocal shaker.

Table 6. Yeast production media composition

Components	Amount
Tap water	1000 mL
KH_2PO_4	4 g
$MgSO_4 \cdot 7H_2O$	0.696 g
$(NH_4)_2SO_4$	4 g
Glucose	23.63 g*

*C/N ratio 13

Table 7. Nitrogen sources for yeast production media

Nitrogen source	Amount
Urea	1.818 g
$(NH_4)_2SO_4$	4 g
Coffee hydrolysate	*
Whey hydrolysate	*

*The amount depends on the composition of the waste material used and the hydrolysate prepared

Table 8. Carbon sources for yeast production media

	CN 13	CN 25	CN 50	CN 100
Glucose [g]	23.63	45.44	90.89	181.77
Glycerol [g]	24.16	46.46	92.92	185.84
Animal fat [g]	9.10	17.50	35.00	70.00
Coffe oil [g]	9.10	17.50	35.00	70.00
Waste frying oil [g]	9.10	17.50	35.00	70.00

4.4.1.3 *Bioreactor cultivation*

All yeast cultivations were carried out in a small scale 3L laboratory bioreactor filled with 1.5 L of production media. Prior to cultivation, pH electrode was calibrated using standard pH calibration solutions (pH=4.01; 7.00 and 9.0). Bioreactor bottle was washed and cleaned properly and filled with production media. Prepared bioreactor bottle with the necessary accessories was sterilized in an autoclave at 121 °C for 15 minutes and then carefully cooled down to 22 °C, without aggressive stirring and shaking to maintain a low concentration of gases in the medium. pH adjustment was made using 10% sodium hydroxide and sulfuric acid solution to 5.8. With stabilized temperature and pH, dissolved oxygen electrode was calibrated firstly to 0% and then after vigorous aerating with compressed air and stirring to 100%. During the cultivation, the bioreactor's internal conditions were controlled by the operating system to achieve pO_2 conditions in the range of 25-30%, a pH=5.8 and a 22 °C temperature. To avoid cell destruction due to shear forces, the maximum agitation limit was set at 1000 rpm. All substances and accessories entering the medium were pre-sterilized (air filters, silicon tubes, sodium hydroxide and sulphuric acid) by heat sterilization. Compressed air was used as an oxygen source for the cultivation.

The preparation of production media started with the preparation of hydrolysate. Sample of finished hydrolysate was measured, using HPLC/RI saccharide analysis, and the amount of dissolved sugars was calculated. Waste fats and oils were measured by GC-FID. According to that results, the exact amount of hydrolysate was mixed with tap water to prepare 1,5 L of media. The medium was poured into bioreactor flask and supplemented with salts and waste fat. Total carbon content was divided between sugar hydrolysate and waste fat by ratio 1:3. If needed, potassium hydroxide was added to the medium in tenths to the weight of the fat to hydrolyse the fat during sterilization partially. The final medium was inoculated with 300 mL of yeast culture grown on YPD media for 24 hours.

4.4.2 Cultivation of microalgae and cyanobacteria

In this work, microalgae and cyanobacteria were cultivated at two different workplaces under different conditions, and in the following subchapters, therefore, the experimental procedures in both workplaces are described separately.

4.4.2.1 *Microalgae cultivation in Brno*

Microalgae strains were stored on several types of agar plates and slant agar tubes under room temperature for long-term storage. Two sets of agar plates containing the appropriate medium for the strain and second set also contained antibiotics. For microalgae culture preservation, algae mineral media was prepared with 2% agar and sterilized. While hot, a portion of media was poured into sterile polystyrene Petri dishes. Another part was mixed with a sterile solution of antibiotics and poured into other sets of Petri dishes. Petri dishes with cultures were regularly checked for the presence of contamination and overall healthiness. For stock culture preparation, 30 mL of appropriate mineral media was poured into 100 mL Erlenmeyer flask and sterilized in an autoclave or high-pressure pot at 121°C for 30 minutes. Flask was cooled down in a sterile box under UV light. Using the inoculation loop, a small portion of microalgae culture was transferred from Petri dishes to liquid media. To keep axenic culture or limit the growth of unnecessary contamination, a small amount of Ampicillin and Clotrimazole solution was introduced to media via sterile 0,45 μm filter (*Cyanobacteria* stock cultures were mixed only with Clotrimazole). After sufficient growth, stock culture was transferred to 250mL Erlenmeyer flask with 120 mL of pure media. These stock cultures were then used for inoculation of production media. Stock cultures were renewed every two months and periodically checked for contamination. All stock cultures were cultivated under constant illumination on orbital/reciprocal shakers.

4.4.2.1.1 Production media

Microalgae strains were cultivated in Erlenmeyer flasks and Pyrex flasks. Production media was prepared and sterilized according to the procedure mentioned above. After sterilization, the flask with medium was cooled down to room temperature. Before inoculation, a sample of stock culture was taken under sterile conditions, checked for contamination and its absorbance at 680 nm measured.

In cultivations aimed at studying the effect of the concentration of various media components (N, P, S), a stock solution was prepared and sterilized separately. The stock culture was then added in a given amount to a flask with the sterile medium before inoculating the microalgae culture. The same procedure was applied when testing the effect of oxidative stress (Fe, Cu). In salt stress tests, the appropriate amount of salt was weighed into a culture flask. All microalgal cultivations were performed on the media listed in the table below (Table 9 and Table 10). In the case of experiments monitoring the effect of different temperatures, the shaker with the test Erlenmeyer flasks was placed in a room with a controlled temperature. To test the possible mixotrophy mode of growth, the culture flasks were wrapped entirely in aluminium foil and thus cultivated in the dark.

4.4.2.1.2 Multicultivator MC1000 cultivation

A specialized bioreactor, called a multicultivator, was also used to cultivate microalgae. This device is designed for simultaneous cultivation of 8 samples in 80mL tubes. The multicultivator contains a tempered bath where the tubes are placed, allowing the temperature to be regulated during cultivation. Each of the tubes is illuminated by its own source, and a different illumination intensity can be set for each tube. During cultivation, the instrument sensors measure the optical density at A_{680} and A_{720} of each tube and measure the microalgal growth curve under given conditions. The preparation of the cultivation experiment was performed according to the following procedure. The bath was cleaned and filled to the mark with distilled water. The instrument was turned on and warmed to the desired cultivation temperature.

The optical density sensor was then zeroed and calibrated using non-sterile vials with the medium. Cleaned tubes with stopper and tubing connected were sterilized in a pressure cooker. In a sterile box, 700 mL of medium was mixed with a microalgal stock culture so that the resulting optical density was A_{680} = 0.150. 80 mL of the prepared culture was dispensed into each tube using a graduated cylinder and then supplemented with the necessary sterile solutions of various nutrient sources depending on the experiment. The prepared tubes were placed in a multicultivator; the lighting was set and connected to an air source. The cultivation was started, and the optical density was read at regular intervals by an OD sensor. After cultivation, the culture was centrifuged and lyophilized for further analysis. The data measured by the instrument were converted into a growth curve.

Figure 20. Multicultivator MC 1000, Photon systems instruments, Drásov – cultivation chamber detail

Table 9. Composition of BBM and Spirulina algae media

Media type				
BBM			**Spirulina medium**	
Component	Concentration [g/L]		Component	Concentration [g/L]
$NaNO_3$	0.2500		$NaNO_3$	2.50
$MgSO_4.7H_2O$	0.0750		$MgSO_4.7H_2O$	0.20
$CaCl_2.2H_2O$	0.0250		$CaCl_2.2H_2O$	0.04
Citric acid	0.0060		$FeSO_4.7H_2O$	0.71
K_2HPO_4	0.0750		K_2HPO_4	0.50
KH_2PO_4	0.1750		NaCl	1.00
NaCl	0.0250		EDTA	0.58
EDTA	0.0500		$NaHCO_3$	13.61
$FeSO_4$	0.00498		Na_2CO_3	4.03
KOH	0.0310		K_2SO_4	1.00
$ZnSO_4.7H_2O$	0.0088		$ZnSO_4.7H_2O$	0.001
$MnCl_2.4H_2O$	0.0014		$MnSO_4.7H_2O$	0.002
MoO_3	0.0007		H_3BO_3	1.00
$CuSO_4.5H_2O$	0.00157		$Co(NO_3)_2.6H_2O$	0.001
$Co(NO_3)_2.6H_2O$	0.0005		$CuSO_4.5H_2O$	$0.005 \cdot 10^{-3}$
			$Na_2MoO_4.2H_2O$	0.001

Table 10. Composition of NPK media, K9 media and Chu media

Media type				
Spirulina medium			**K9 media**	
Component	Concentration [mM]		Component	Concentration [g/L]
NO_3^-	0.250		KNO_3	2.290
NH_4^+	0.450		K_2HPO_4	0.500
P_2O_4	0.400		K_2SO_4	3.950
K_2O	0.640		CaCl	0.010
Micronutrient solution*	0.4mL		$MgCl_2$	0.410
			KCl	0.100
			Hutners solution	5 mL
Chu medium				
Component	Concentration [g/L]			
$CaCl_2 \cdot 2H_2O$	0.367			
$MgSO_4 \cdot 7H_2O$	0.369			
K_2HPO_4	0.087			
$NaNO_3$	0.850			
$Na_2SiO_3 \cdot 9H_2O$	0.284			
Citric acid	0.335			
Ferric citrate	0.335			
Chu micro. sol.	10 mL			

*Micronutrient solution (Microfer Complex, Fercampo, Málaga, Spain).

4.4.3 Microalgae cultivation in Huelva

As part of the Erasmus+ internship, cultivations were performed with the microalga *Botryococcus braunii* v. Showa-Bielefeld and *Coccomyxa onubensis*. The experiments were performed at the research institute of the BITAL group, which is part of the University of Huelva. This group deals with laboratory and large-scale cultivations of various types of microalgae.

4.4.3.1 *Coccomyxa onubensis cultivation*

Coccomyxa onubensis belongs to the extensive genus *Coccomyxa*, including versatile green representatives that can be found worldwide. This group's representatives can be found in aquatic and terrestrial ecosystems, soils and ponds with very high metal concentrations or low pH. Furthermore, representatives of this group can be found in symbiotic associations with different microorganisms. This alga is one of the extremophilic representatives because it is able to live in an environment with a very low pH and a high concentration of metals. All cultivations performed with this extremophilic microalgae can be divided into two large experiments.

4.4.3.1.1 Salt adaptation experiments

The first experimental part dealt with the adaptation of the culture to salt stress and the subsequent optimization of the components of the used K9 medium, focusing mainly on the content of N, P and S. The optimization began with a search for the pH optimum, followed by the selection of a suitable nitrogen source. Three standard nitrogen sources were tested here, namely ammonium sulphate, sodium nitrate and urea. Tests of the effect of its concentration on the microalgae were performed with a best nitrogen source.

Furthermore, various phosphorus concentrations were then tested to find optimal phosphorus to nitrogen ratio (P/N ration). In the last part, the effect of increased concentration of iron atoms on the production of studied metabolites was tested. All cultivations here were carried out in 500mL Erlenmeyer flasks containing 200 mL of a media at the beginning of the cultivation.The microalgae stock culture was maintained on agar Petri dishes, 10 mL vials and slant agar glass tubes. K9 medium with 2% agar was used as the stock medium. To prepare a liquid stock culture, 100mL Erlenmeyer flask was fitted with a polystyrene foam stopper with a hole in the centre through which a glass tube passed. All openings were closed with aluminium foil and sterilized in an autoclave. 100 mL of sterile K9 medium was prepared in a side bottle with a cap. A portion of the culture was transferred to the liquid medium in an Erlenmeyer flask under sterile conditions with a loop in a sterile box. After inoculation, the stock culture bottle was placed in a temperature-controlled cultivation box with lighting. Using a glass rod, the culture was connected to 2% CO_2-enriched air distribution.

The culture was thus gently bubbled under constant illumination using fluorescent lamps emitting warm white light with a total intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photons. During culture growth, culture samples were taken regularly to determine culture growth, medium pH, and quantum fluorescence yield. After sufficient growth of the culture, the NaCl concentration was gradually increased into the medium at 7-day intervals up to a final value of 0.5 $mol \cdot l^{-1}$. The adapted culture was then inoculated into a 1000mL Erlenmeyer flask with 400 mL of K9 medium with 0.5 M NaCl, which served as a stock culture for all experiments. The stock culture was regularly checked and replenished with fresh medium. Experimental flasks were prepared via the same procedure. After sterilization, the chosen amount of medium was put into all flasks with a graduated cylinder. As part of experiments with different nutrient contents, the required amount of sterile stock solutions was added by pipette. The finished medium's pH was then measured, and any deviations were adjusted with 20% sulfuric acid and sodium hydroxide solutions.

Subsequently, the media was inoculated with the stock culture so that the absorbance was A_{680}=0.150. The flasks were connected to a source of enriched air, and the degree of aeration in all flasks was adjusted using hose clamps. Cultivation was performed for three weeks in a cultivation box under the same conditions as in the case of stock culture. All air supply components and microbial filters were carefully sterilized before each cultivation. An example of cultivation is shown in the figure below (Figure 21). Within this work, experiments were performed according to the following scheme. All cultivations were performed in duplicate, and the results shown are the average of these duplicates.

1. Cultivation at different pH - 2.0–7.0
2. Testing of nitrogen sources - ammonium sulphate, urea, sodium nitrate
3. Nitrogen source concentration - concentration 20-150%
4. P/N ratio - interval 0.05-0.30
5. Sulphur source concentration
6. Oxidative stress – Fe^{3+} concentration - interval 100-300mM Fe^{3+}

Figure 21. Example of cultivation in aerated Erlenmeyer flasks

4.4.3.1.2 Raceway ponds cultivation

The second direction of experimental work was to test the growth of *Coccomyxa onubensis* in outdoor open ponds. In this experiment, the microalgae were cultivated in NPK fertilizer media under non-sterile conditions in open raceway tanks with a total volume of 300 L of media. At the beginning of the experiment, a stock culture was prepared in the same procedure as in the paragraph above. The stock culture was grown in a 3000mL Erlenmeyer flask in standard K9 medium with pH=2.5. After sufficient growth, the culture was inoculated into an intermediate stage, which consisted of plastic bags with a medium volume of 25 L. For preparation, a piece of sufficient length and volume was cut from the supply roll. It was then water tightly closed on both sides and hung on a metal structure using a rope. In the upper 4/5 of the length, the bag was slightly cut, and the medium was pumped through the created hole. The medium was prepared under non-sterile conditions with the tap water and common commercial fertilizers. Each bag was filled with 25 L of NPK medium, the composition of which is given in the table above (Table 10). *Coccomyxa onubensis* from stock culture was inoculated into the prepared medium under non-sterile conditions. Subsequently, a tube supplying 2% CO_2-enriched air was introduced into the medium. This cultivation took place under constant illumination of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$. In total, five large bags were prepared by this procedure. After 14 days of cultivation, the stock cultures were ready for inoculation of raceway ponds.

Before inoculation, the tanks were cleaned of impurities, and the mixing system was tested, and then ponds were filled with tap water and nutrient solution to create 300 L of NPK medium. After a short time, where the medium was mixed properly, a microalgae stock culture was inoculated, and the experiment began. The final volume was 300 L of medium + 20 litres of culture. In this experiment, a total of three raceway ponds were inoculated with different conditions, which are shown in the scheme below (Table 11). The first tank contained twice the amount of nutrients and was aerated. The second tank contained NPK medium with a standard concentration of nutrients and was also aerated. The last tank was no longer aerated. The culture was mixed with a paddle wheel at 20 rpm.

The main goal of this cultivation was to test whether the microalga *Coccomyxa onubensis* is able to grow under these conditions and at what rate. Furthermore, its resistance to contamination by other microorganisms was studied. Last but not least, the production of selected metabolites was also studied. Samples from each raceway pond were taken every other day (Monday – Wednesday – Friday) to measure these parameters: Quantum Yield, temperature, absorbance 680 nm, media pH, and biomass production. Biomass was then analysed for carotenoid, chlorophyll, and lipid production. Culture samples were periodically checked for microbial contamination.

Table 11. Raceway pond outdoor cultivation conditions

	Media	Aeration
Raceway pond 1	2xNPK	Aerated (2 L/minute)
Raceway pond 2	NPK	Aerated (2 L/minute)
Raceway pond 3	NPK	Without aeration

4.4.3.2 *Botryococcus braunii cultivation*

Laboratory cultivation of *Botryococcus braunii* v. Showa-Bielefeld microalgae were performed under the same conditions as in the case of microalgae *Coccomyxa o*. Chu medium was chosen as the cultivation medium for this microalgae. The culture was stored in vials with a liquid and solid medium. To prepare a stock culture, 500 mL of Chu medium was prepared in a 2000mL Erlenmeyer flask. After sterilization and cooling, a sterile nitrogen source solution was added to the prepared medium, and the medium was inoculated with a stock culture. The prepared flask was then transferred to a cultivation box and connected to an aeration system. Samples of the stock medium were periodically checked for the presence of contamination and the overall condition of the culture. Once the microalgae reached sufficient optical density, a portion of the culture was inoculated into the prepared test media. The stock culture was periodically replenished with fresh sterile medium.

In experimental cultivations of microalgae *Botryococcus braunii*, the medium was prepared, and 200 mL of medium was put into individual flasks. After sterilization and cooling of the flasks, the necessary nutrients from sterile stock solutions were added depending on the experiment type. The prepared flasks were inoculated with the stock culture so that the resulting absorbance was A_{680}=0.150. All experimental cultivations were performed in a cultivation box with a stable temperature and illumination of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photons. Cultures were bubbled with air enriched to 2% carbon dioxide. Cultivations were run for 21 days and in the case of the last experiment with diluted media, cultivated for 28 days in Roux cultivation flasks with a total medium volume of 800 mL. Periodically taken culture samples were analyzed to determine the following parameters: optical absorbance, pH of the medium, quantum fluorescence yield, and biomass production. Biomass samples were analysed using various chromatographic techniques to determine lipid production, fatty acid profile, carotenoid a chlorophyll content. All cultivations were performed in duplicate, and the results shown are the average of these duplicates.

The last experiment with microalgae *B. braunii* was cyclic extraction of botryoterpenes. As part of this experiment, a 1000mL Erlenmeyer flask with 400 mL of Chu medium was prepared. Once a sufficient increase in culture absorbance was achieved (A_{680}=2.0), a microalgae culture was added to a sterile flask with 50 and 100 mL of hexane, and then the mixture was gently stirred for 20 minutes. Subsequently, the hexane extract was separated from the culture under sterile conditions. The culture was returned to the flask for further cultivation. The hexane extract was evaporated and weighed. The extraction process was periodically performed on Monday, Wednesday and Friday.

4.4.4 Co-cultivation of carotenogenic yeasts and microalgae

The whole co-cultivation experiment was divided into several phases. Each one of them was addressing specific issue or condition of possible co-cultivation of carotenogenic yeast and microalgae. Namely:

- Yeast growth curve on mineral BBM medium with different carbon and nitrogen sources in short 4-day experiments.
- The ability of the mixotrophic way of life of selected strains of microalgae with a focus on the utilization of glucose or glycerol in BBM media.
- Small scale co-cultivation experiments in Erlenmeyer flasks to determine the compatibility of yeasts and microalgae.
- Co-cultivation in aerated round flasks under illumination) to confirm small scale experiments.
- Co-cultivation under controlled conditions in the 3L bioreactor

4.4.4.1 *The first phase – yeasts cultivation on mineral BBM media*

Co-cultivation experiments began with the selection of a suitable medium. The algal medium was chosen because it contains all the necessary minerals for yeast and microalgae growth, unlike the mineral yeast medium mentioned in 4.4.1.2. The first medium tested was Bolds Basal Medium (BBM), suitable for all chosen microalgal strains. In these pilot experiments, the concentrations of individual micro and macro biogenic elements were not adjusted in any way. It was therefore based on the basic BBM medium with the addition of a carbon source. Glucose and glycerol were used as carbon sources. Other types of carbon sources will be tested in follow-up works. The nitrogen sources tested were urea, sodium nitrate and sodium sulphate. The amount was always adjusted in different nitrogen sources to match the sodium nitrate amount in the basic BBM medium recipe. The composition of the medium used is given in table 14. The mineral yeast medium was chosen as the control medium, the composition of which is shown in table 13.

As mentioned above, this phase was crucial to determine the yeast ability to grow on mineral BBM media and the production capabilities of carotenogenic yeast compared to standard mineral yeast media. Carotenogenic yeasts were in the first phase of the experiment also cultivated on a mineral medium with different carbon and nitrogen sources, to compare the growth properties and production of the monitored metabolites on selected sources. The cultivation scheme of the first phase is shown in the table below (Table 12). Yeasts were cultivated in Erlenmeyer flasks containing 40 mL of production medium. The same procedure as in chapter 2 was chosen for the preparation of the yeast inoculum. The inoculation, therefore, took place in two stages, always cultivated for 24 hours. Inoculation of the production medium was performed in the same ratio of 1 to 5. Thus, the total volume of production medium was 48 mL. Yeasts were cultivated on mineral BBM medium for 4 and 10 days.

A longer cultivation time was chosen due to future co-cultivation with microalgae, which have a much lower growth rate than carotenogenic yeasts. Cultivation was performed at room temperature with constant lighting and shaking on a reciprocating shaker. Ammonium sulphate + glucose medium also served as a control medium.

Table 12. Co-cultivation: first phase experimental scheme

Yeast mineral cultivation media						
	Flask 1.	Flask 2.	Flask 3.	Flask 4.	Flask 5.	Flask 6.
Carbon source	Glucose	Glucose	Glucose	Glycerol	Glycerol	Glycerol
Nitrogen source	Urea	$(NH_4)_2SO_4$	Yeast autolyzate	Urea	$(NH_4)_2SO_4$	Yeast autolyzate
BBM mineral media						
Carbon source	Glucose	Glucose	Glucose	Glycerol	Glycerol	Glycerol
Nitrogen source	Urea	$(NH_4)_2SO_4$	Yeast autolyzate	Urea	$(NH_4)_2SO_4$	Yeast autolyzate

Table 13. Yeast mineral media composition

Yeast mineral media	
Component	Concentration [g/L]
Glucose**	30.0
Glycerol**	29.97
$(NH_4)_2SO_4$*	4.0
Urea*	1.91
Yeast autolyzate*	7.85
$MgSO_4.7H_2O$	0.696
KH_2PO_4	4.0

Only one *N and **C source was used for cultivation at the same time

4.4.4.2 *The second phase – microalgae cultivation on BBM media with an organic carbon source*

After completion of the first phase experiments, microalgae were tested under the same conditions. The second phase of the experiments' main goal was to test the selected strains of microalgae and cyanobacteria on a medium containing glycerol and glucose. The main parameters here were whether the selected strains are capable of a mixotrophic growth, i.e. assimilating organic carbon. Alternatively, whether they are capable of phototrophic growth in the presence of an organic carbon source, i.e. whether they can tolerate its presence. The experimental scheme is shown in table 15. In this experiment, the yeast autolysate was replaced with sodium nitrate. Previous tests have shown that selected strains of microalgae cannot assimilate yeast autolysate, and its presence has led to the death of the culture. The medium containing only sodium nitrate, without organic carbon, served as a control medium. These cultivations were performed in Erlenmeyer flasks with 40 mL of culture at room temperature and constant reciprocal shaking. Cultivation were also performed in the dark, to confirm or disprove the mixotrophy in selected microalgae and cyanobacteria strains, by covering the Erlenmeyer flasks with aluminium foil. A stock culture of microalgae in standard BBM medium was used for inoculation. The absorbance of the culture at 680 nm was measured before inoculation. Subsequently, the production medium was inoculated with an amount of stock culture so that the initial A_{680} was approximately 0.150.

Table 14. Media composition used for co-cultivation experiments

BBM media	
Component	Concentration [g/L]
*$NaNO_3$	0.2500
*Urea	0.0883
*$(NH_4)_2SO_4$	0.1943
**Glucose	30.0000
**Glycerol	30.6700
$MgSO_4.7H_2O$	0.0750
$CaCl_2.2H_2O$	0.0250
Citric acid	0.0060
K_2HPO_4	0.0750
KH_2PO_4	0.1750
NaCl	0.0250
EDTA	0.0500
$FeSO_4$	0.0049
KOH	0.0310
$ZnSO_4.7H_2O$	0.0088
$MnCl_2.4H_2O$	0.0014
MoO_3	0.0007
$CuSO_4.5H_2O$	0.0016
$Co(NO_3)_2.6H_2O$	0.0005

Only one *N and **C source were used for cultivation at the same time

Table 15. Co-cultivation: second phase experimental scheme

BBM mineral media							
	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5	Flask 6	Flask 7
Carbon source	Glucose	Glucose	Glucose	Glycerol	Glycerol	Glycerol	-
Nitrogen source	Urea	$(NH_4)_2SO_4$	$NaNO_3$	Urea	$(NH_4)_2SO_4$	$NaNO_3$	$NaNO_3$

4.4.4.3 *Third phase – co-cultivation of yeasts and microalgae in small scale*

After evaluating the results from the first and second phases of the experiment, a pilot co-cultivation experiment was performed. The experiments were again performed on a small scale in Erlenmeyer flasks. The obtained data found that the best source of nitrogen for selected strains of microorganisms is urea. Therefore, a series of cultivations with urea as a nitrogen source were performed. The carbon source remained glucose and glycerol. The organic carbon source was selected based on previous results from purely yeast experiments. The experimental scheme involved cultivation the yeast alone on BBM medium containing organic carbon, microalgae on standard BBM medium, and then yeast with a selected strain of algae on BBM medium containing organic carbon. Microalgae were inoculated into the production medium, and then yeast was added after 24 hours. Cultures were inoculated so that the absorbance of the microalgae culture was A_{680} = 0.200 and the yeast was inoculated as standard at a ratio of 1 to 5. (in the future referred to as 2:1 ratio microalgae/yeast). The procedure for inoculating yeast and microalgae was the same as in the previous stages of the experiment. Cultivation lasted for ten days after inoculation of the yeast culture.

4.4.4.4 *The fourth phase – co-cultivation in aerated 1L Pyrex flasks.*

Based on the evaluated data from the third phase of the experiment, experiments were designed on a larger scale. Compatible yeast and algal strains were cultivated in an aerated medium in 1L Pyrex flasks. The experimental scheme of the experiment is given in table 16. In this experiment, yeast was grown on BBM medium with the best carbon source, then a series of yeast and algae in different inoculation ratios of 1: 1, 1: 2, 1: 4, where the ratios are absorbances of cultures when inoculated into the medium (A_{680} microalgae and A_{530} yeast). Next, pure microalgae bottles were cultivated in a standard BBM and the other BBM with an organic carbon source. In these bottles, the microalgae were inoculated to an absorbance of A_{680} = 0.100. Cultivation was again performed for ten days under constant illumination and aeration with sterile air at 2 L/min. The total volume of the medium was 400 mL. As in the previous case, the microalgae was inoculated 24 hours earlier than the yeast.

Table 16. Fourth phase – experimental scheme

Co-cultivation scheme: aerated Pyrex flasks					
Flask 1	Flask 2	Flask 3	Flask 4	Flask 5	Flask 6
Yeast	Yeast: Algae 1:1	Yeast: Algae 1:2	Yeast: Algae 1:4	Algae	Algae
BBM+ C	BBM+ C	BBM+ C	BBM+ C	BBM+ C	BBM

4.4.4.5 *Fifth phase – large scale bioreactor co-cultivation*

The experiment's final phase consisted of a series of controlled cultivations in a bioreactor with a volume of 3.0L. Based on previous data, suitable yeast-microalgae pairs were selected for a large volume cultivation. Due to the generally faster growth of cultures in the bioreactor than conventional flask cultures, the culture ratio C/N was raised from the original C/N = 13 to a ratio C/N 25 (while maintaining the same amount of nitrogen in the medium). The bioreactor's preparation consisted of preparing BBM medium, which was poured into a clean reactor vessel. Then the vessel was closed, a probe measuring dissolved oxygen was inserted. Before mounting the pH probe, it was calibrated using a series of pH buffers (4.10; 7.00 and 9.01). All outlets were closed and wrapped in aluminium foil.

The air supply was equipped with a sterilizable filter with a pore size of 0.2 μm and closed with a clamp before sterilisation. The vessel was placed in an autoclave and sterilized at 121 °C for 15 minutes. After sterilization, the vessel was allowed to cool until the next day. Solutions of 5% KOH and 5% H2SO4 were sterilized together with the vessel. There was also a solution of 80% glycerol, which served as a carbon source, supplemented with a small antifoam addition. After sterilization, the medium's pH was adjusted to 6.5 and temperature to 22 °C with gentle stirring at 50 rpm. Under these conditions, the calibration of the oxygen probe began. The zero-oxygen content was calibrated immediately after reaching the optimum temperature and pH. The medium was then aerated for 15 minutes with vigorous aeration and stirring. Subsequently, the calibration was set to 100% dissolved oxygen. Microalgae were inoculated into the bioreactor, followed by a yeast inoculum. Cultivation was performed by the fed-batch technique, where the carbon source was gradually added to the medium in the first three days of cultivation according to the following procedure: 0 hour - 50%, 24 hours 25% and 48 hours 25%. Cultivations always lasted six days, i.e. 144 hours. Bioreactor process parameters during cultivation are listed in the following table (Table 17).

Table 17. Bioreactor process values during co-cultivation

Media volume	2,0 L
Stirring*	300-1000 rpm
pH	6,5
pO_2	30%
Temperature	22 °C
Aeration	2 L per minute
Ilumination	200 $\mu mol \cdot m^{-2} \cdot s^{-1}$

*Stirring was regulated by oxygen consumption.

During the experiment, biomass production, growth rate, and lipid substances (carotenoids, sterols, coenzyme Q), lipids and fatty acid profile were monitored. In the case of microalgae cultivations and co-cultivations, chlorophyll production was also monitored. The results' graphs included the total production of carotenoids, major carotenoid pigments, chlorophylls, sterols, ubiquinone, and biomass. Graphs showing the results of GC lipid analysis were divided into two parts. Results from the first to the third phases are listed in a table describing the percentage composition of each type of fatty acid and the percentage of lipids and biomass. In the last part of the experiment, the results of the GC analysis are shown in graphs. The first graph describes the % composition of fatty acid groups (SFA, MUFA, PUFA). The second graph shows the percentage of lipids in biomass and biomass production.

4.5 Waste substrates hydrolysis

4.5.1 Animal fat hydrolysis

4.5.1.1 *Animal fat enzymatic hydrolysis*

To test the efficiency of animal fat hydrolysis by various enzyme preparations, 10 g of fat was weighed into 250mL Erlenmeyer flasks and 100 mL of buffer was added. The flasks were placed on a tempered shaker and heated to 37 °C. After heating to the hydrolysis temperature, 25 mg of the enzyme preparation was weighed into each flask. To compare commercial preparations with yeast-produced lipase, 5 mL of medium containing lipase produced by yeast *Rhodotorula kratochvilovae* was pipetted into another Erlenmeyer flask. The reaction was run for 30 hours on a tempered shaker at 300 rpm. At regular intervals, 1 mL of sample was taken into an Eppendorf vial. For quantitative and qualitative analysis of hydrolysis efficiency, samples were analyzed by TLC chromatography and liquid chromatography with a refractometric detector.

A silica gel plate of appropriate dimensions was prepared for TLC analysis of the hydrolytic reaction. 1 cm from the bottom, the beginning was marked, on which samples of enzymatic hydrolysates were applied with a micropipette. After drying the solvent, the TLC plate was immersed in a hexane: diethyl ether: acetic acid 70: 30: in a chamber. After reaching the end point, the plate was dried from the solvents and sprayed with the visualization mixture (10% H_3PO_4 and 10% $CuSO_4$). The plate was dried at 110 °C for 10 minutes. Individual groups (glycerol, fatty acids, monoacylglycerols, diacylglycerols and triacylglycerols) were identified on the dried plate. The retention factor R_f was calculated from the ratio of the distance of the analyte from the onset and the total distance. For quantitative analysis of hydrolysis efficiency, 0.5 ml of p.a. was added to 1 mL of the reaction mixture solution in an Eppendorf tube. hexane and after vigorous shaking, the mixture was centrifuged at 5000 rpm for 3 minutes. The lower aqueous phase containing glycerol was collected for HPLC/RI analysis as described in the section below (4.6.6).

The enzymatic hydrolyzate for the preparation of production media was prepared in a similar manner. The required amount of fat was weighed into a Pyrex bottle and supplemented with a buffer solution, the volume of which depended on the final concentration of the substrate for production media. The solution was placed on a heated magnetic stirrer and warmed to 37 °C. After reaching the temperature, the commercial enzyme from *Candida* ugosa was added and the reaction started. The concentration of glycerol released was determined by regular sampling and the reaction was terminated when the desired value was reached. The solution containing the released glycerol was then used as a basis for preparing the media.

4.5.1.2 *Animal fat base hydrolysis*

The base hydrolysis tests were performed according to the following procedure. 10 g of fat was weighed into 250mL Erlenmeyer flasks and made up to 100 mL with sodium or potassium hydroxide solution in various concentrations. After heating to the selected temperature on a heated magnetic stirrer, samples were taken at regular intervals to determine the glycerol content. The quantification procedure is the same as in the previous chapter (4.6.6), before the analysis, the base hydrolyzate sample was neutralized with a sulfuric acid solution to neutral pH. After a series of tests, the most suitable hydrolysis method (temperature, fat/hydroxide ratio) was selected, which was then used to prepare a fat hydrolyzate. To prepare a larger hydrolyzate amount, the required amount of fat was weighed into a bottle, mixed up with a hydroxide solution and heated to 70 °C. The hydrolysis was carried out for 4 hours with constant stirring.

The reaction was quenched by the addition of sulfuric acid solution and neutralization of the hydroxide used. The resulting mixture was analyzed by HPLC/RI and stored at 4 ° C after sterilization. To prepare the medium, the hydrolyzate was suitably diluted and supplemented with other components. A similar procedure was used for acid hydrolysis assays.

4.5.2 Whey hydrolysis

Due to the presence of protein in whey, acid hydrolysis is very inefficient and requires precipitation and removal of the protein, which complicates and increases the process. Therefore, enzymatic hydrolysis using commercially purchased lactase was used to hydrolyze whey. The hydrolysis procedure was as follows. Dairy whey was sterilized for 20 minutes at 121 °C to eliminate lactic acid bacteria. After sterilization, the whey was cooled to a lactase temperature optimum of 37 ° C. With constant stirring, the enzyme was added to the whey, and the reaction began. Samples were taken at regular intervals and subsequently determined by HPLC/RI. A whey sample was taken in an Eppendorf tube and centrifuged at 14,000 rpm for 5 minutes. The obtained supernatant was filtered through a 0.45 µm Nylon filter and suitably diluted. The analysis of the sample was performed according to the procedure in chapter 4.6.6. After complete hydrolysis of the lactose present in the medium, the hydrolyzed whey was cooled and stored at 4 °C. To prepare the medium, the required amount was diluted with water and supplemented with other medium components.

4.5.3 Spent coffe grounds hydrolysis

A combined process of acid and enzymatic hydrolysis was chosen for the preparation of coffee grounds hydrolyzate. The fresh coffee grounds were dried at 80 °C for 24 hours and ground to smaller particles using a grinder. The dried ground coffee grounds contained coffee oil, which inhibits hydrolysis of cellulose and hemicellulose. To remove coffee oil, a series of solvent mixtures with different ratios (isopropanol: hexane, ethanol: hexane) were tested. 20 g of coffee grounds were weighed into a cellulose cartridge and placed in a Soxhlet apparatus. The precipitate was extracted for 90 minutes, with 200 mL of extraction mixture (approximately 30 cycles). The extracted coffee grounds were dried at 80 °C for 24 hours and weighed on analytical balances. The extract was dried on a vacuum evaporator. The most suitable solvent pair was selected from the product weight comparison. 10 g of defatted coffee grounds were mixed with a series of 100 mL sulfuric acid solutions and hydrolyzed for 24 hours at temperatures between 80-130 °C. During the hydrolysis, samples were taken for the determination of monosaccharides by HPLC/RI. The same experiment was performed using commercial hemicellulases and cellulases. Based on the obtained data, the final procedure for the preparation of SCG hydrolyzate was determined.

High-volume SCG hydrolysates' preparation was as follows: Fresh coffee grounds were dried and ground to smaller particles. Subsequently, 100 g of sediment was extracted with 400 mL of a 50:50 mixture of isopropanol: hexane for 90 minutes in a Soxhlet extractor. 200 g of defatted coffee grounds were mixed with 1000 mL of water and autolyzed at 130 °C for 90 minutes. The autolysate was cooled to 40 °C and further hydrolyzed with a mixture of commercial cellulase and hemicellulase (0.5 mL Cellulase enzyme blend, Sigma Aldrich) with constant stirring for 6 hours. The pH of the mixture was maintained at an optimum of 5.0. After completion of the reaction, the hydrolyzate was vacuum filtered via filtration paper (fast filtration speed) and stored at 4 °C. The carbohydrate content of the hydrolyzate was regularly measured by HPLC/RI (4.6.6). The coffee hydrolyzate was warmed to room temperature to dissolve any precipitated carbohydrates and then suitably diluted with water and supplemented with other medium components to prepare the production medium. To prepare media with a high C/N ratio, the hydrolyzate was frozen at -36 ° C and lyophilized to the required carbohydrate concentration.

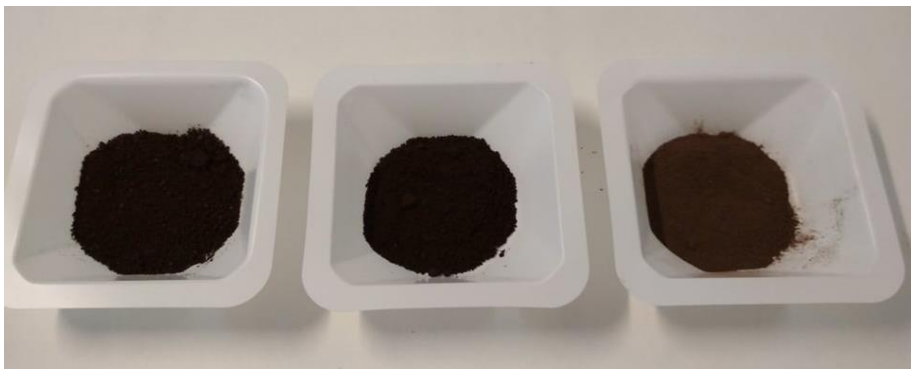


Figure 22. Process of spent coffee ground hydrolysis: a) Fresh dried spent coffee grounds b) milled spent coffee grounds c) defatted spent coffee grounds

4.5.4 Spectrophotometric protein determination

For the determination of proteins in hydrolysates, the Lowry method was chosen, in which copper ions interact with proteins. It is a colourimetric determination based on a two-component reagent. The first component is a Biuret reagent and the second a Folin-Cicalteu reagent (phosphomolybdic acid and phosphotungstinic acid). Tyrosine, tryptophan residues of the protein reduce acids to produce a blue colour. 1 mL of a suitably diluted hydrolyzate sample was mixed with 0.9 mL of Biuret Reagent A. The sample was incubated at 50 °C for 10 minutes. Subsequently, the sample tubes were cooled to room temperature and supplemented with 0.1 mL of Biuret reagent B. The tubes were re-incubated at 50 °C for 10 minutes. After cooling, 3 mL of Folin-Cicalteu reagent was rapidly added. After repeated incubation, the absorbance at 650 nm was measured. Bovine albumin was used to prepare the calibration curve. The samples were measured in triplicate and the resulting value was the average of these values [218].

4.5.5 Botryococcus braunii botryoterpenes cyclic extractions

The possibilities of cyclic extraction of botryoterpenes produced by colonies of *Botryococcus braunii* v. Showa-Bielefeld microalgae cells were tested in the experiment. The extractions were performed according to the following procedure. A 400 mL microalgae culture was grown in standard Chu medium until the absorbance reached A680 = 2.0. Subsequently, a 1000mL Pyrex bottle was sterilized at 121 ° C for 20 minutes. Under sterile conditions, 400 mL of microalgae medium was poured into a bottle and supplemented with hexane. The bottle was fitted with a sterile magnetic stirrer and closed with a cap. The mixture was gently stirred on a magnetic stirrer for 10 minutes at 100 rpm. After extraction, the culture was separated from the hexane in a sterile separating funnel and returned to the culture flask. The hexane portion was filtered to remove residual culture and then slowly evaporated. The dried extract was then weighed on analytical balances.

4.6 Biomass and metabolite analysis

4.6.1 Biomass production determination

Determination of biomass production is an essential parameter of the biotechnological process. A culture sample of the selected volume (40 mL on average) was taken from the culture vessel and centrifuged at 7000 rpm for 4 minutes (the speed was increased if necessary), and the culture was washed with distilled water and centrifuged again. The washed biomass sample was frozen at -36 °C for 24 hours. Subsequently, the culture sample was placed on a freeze drier for 48 hours. The lyophilized biomass was then weighed on analytical balances, and the total production in g/L medium was determined.

4.6.2 Photosystem II quantum yield

Photosystem II (PSII) quantum yield (QY) measurements were done using a method described by Schreiber [224]. QY measurements are done using portable modulated pulse amplitude fluorimeter AquaPen-C AP-C 100 (Photon System Instruments, Drásov, Czech Republic). 1 mL of fresh culture sample was put into a plastic cuvette and stored at room temperature in the dark for 15 minutes. If necessary, the culture sample was diluted with distilled water. After dark-acclimation cuvette was immediately put inside chamber and QY was measured using 630 nm orange-red LED emitter. QY is calculated using the equation below, where F_v represents the minimum level of fluorescence after dark-acclimation and F_m is a maximum fluorescence measured after exposure to a short pulse of actinic light [224].

$$QY = \frac{F_v}{F_m}$$

4.6.3 Optical density

Culture optical density was measured at 680 nm and 720 nm using a spectrophotometer to determine different media growth rates. 1 mL of the fresh culture sample was put into a quartz cuvette, put inside spectrophotometer and absorbance was measured immediately. If the absorbance was too high, the sample was diluted with Mili Q water to reach absorbance values between A=0.2-0.8.

4.6.4 Spectrophotometric determination of carotenoids and chlorophylls

Spectrophotometric determination of microalgae pigments is a fast and effective method of gaining necessary information about pigment production, culture adaptation on the medium and overall state of the culture. Spectrophotometric determination of pigments was done by optimized Lichtenthaler method [218]. 5 mL of the culture sample were centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded, and biomass was washed with distilled water and centrifuged again. Biomass sample in glass tubes was mixed with 5 mL of methanol and put into a water bath at 60°C for 30 minutes. Every 10 minutes, samples were vortexed for 10 seconds.

After 30 minutes, samples were put in the dark in 4°C to cooldown. Cooled samples were then measured on spectrophotometer in glass cuvette. Pure methanol served as a blank. Samples were measured at three wavelengths: 470 nm, 652 nm and 665 nm. Samples with high absorbance were diluted to match interval between A=0.200-0.600. Data of total carotenoid, chlorophyll A and chlorophyll B were evaluated using the equations below [218]. All samples were measured three times and the results are reported as the average of their values.

$$Chl_a = \left(16.72 \cdot A_{665.2} - 9.16 \cdot A_{652.4)}\right) \cdot \text{dilution factor}$$

$$Chl_b = (34.09 \cdot A_{652.4} - 15{,}28 \cdot A_{665.2}) \cdot \text{dilution factor}$$

$$Chl_{tot} = Chl_a + Chl_b$$

$$Carot_{tot} = \frac{\text{dilution factor} \cdot 1000 \cdot A_{470} - 1.63 \cdot Chl_a - 104.96 \cdot Chl_b}{221}$$

4.6.5 HPLC/DAD analysis of carotenoids, chlorophylls, sterols and ubiquinone

Carotenoid, chlorophyll, ubiquinone and sterol content in biomass was determined using the HPLC/DAD method. 15-20 mg of freeze-dried biomass sample of yeast/microalgae culture was weighed into a 2mL threaded Eppendorf tube and rehydrated with 1 mL of Mili Q water for 30 minutes. Excess water was removed by centrifugation at 10,000 rpm for 3 minutes. 1 mL of p. a. quality methanol was added to the Eppendorf tube containing the sample biomass, together with approximately 0.6 mL of glass beads (0.2-0.5 mm diameter). Eppendorf tube was closed with a screw cap and put on the vortex for 20 minutes at 2800 rpm. Mixture with disintegrated cells was quantitatively transferred into 15mL falcon tube and washed with 2 mL of p. a. chloroform, and the mixture was further vortexed for 10 minutes. 1 mL of distilled water was added, and the tube was shaken for 1 minute to create two separate phases. The lower chloroform phase was quantitatively transferred to a clean tube and dried under an inert nitrogen atmosphere.

The dried sample was dissolved in 1 mL of chromatography grade 2: 1 EtAc: ACN and filtered through a 0.45 µm PTFE filter into a glass 1.8mL vial. The prepared samples were stored at -36 °C until analysis. Samples were measured on Dionex Ultimate series HPLC with Vanquish DAD detector (Thermo Fischer Scientific, USA) on Kinetex C18-EVO column 150 mm x 4.6 mm x 5 µm (Phenomenex, USA) using gradient separation with mobile phase (MP) A (ACN:MeOH: 100mM Tris HCl pH=8; 84:2:14) and mobile phase B (MeOH: EtAc; 60:40) at flowrate 1.2 mL/min and 25 °C.

Gradient programme is listed below table 18. Carotenoid pigments were detected at 445 nm and chlorophylls at 445 nm and 455 nm. Ergosterol and ubiquinone were detected at 285 nm. Chromatographic data were evaluated using Chromeleon 7.2 software. Chlorophyll and carotenoids were identified and evaluated using commercial standards (Sigma Aldrich) and external calibration. All samples were measured twice, and the results are reported as the average of their values.

Table 18. HPLC gradient analysis programme:

Time [min]	MP A	MP B
0.0	100 %	0 %
13.0	0 %	100 %
19.0	0 %	100 %
20.0	100 %	0 %
25.0	100 %	0 %

4.6.6 HPLC/RI analysis saccharides and alcohols:

The HPLC/RI method was used for the analysis of the waste substrates and especially their hydrolyzates. 1 mL of hydrolyzate was appropriately diluted and filtered through a 0.45 µm nylon filter into an Eppendorf tube. If necessary, the pH of the sample was adjusted to 7. The hydrolyzate sample was analyzed on an Dionex UltiMate 3000 Series with diode array detector and refractive index detector (Thermo Fischer Scientific, USA) on a Luna Omega sugar 250 mm x 4.6 mm x 3 µm column (Phenomenex, USA) using isocratic elution in ACN: H_2O 80: 20 and a flow rate of 1.5 mL/min. The separation temperature was 25 °C. Chromeleon 6.0 was used to evaluate the results. Commercial standards (Sigma Aldrich, Germany) were used for the qualitative and quantitative determination of analytes.

4.6.7 Fatty acid profile and total lipid analysis:

Biomass lipid content and fatty acid profile were determined using gas chromatography with a flame ionization detector. Approximately 10-15 mg of freeze-dried biomass sample was weighed into 2.0mL crimp neck vial. 1.8 mL solution of 15 % (v/v) H_2SO_4 in chromatography grade methanol was added, and the vial was capped with aluminium cap. Vials with samples were heated at 85 °C for 2 hours in block heater. After the transesterification process, vials were cooled down and caps removed. Sample mixture was transferred quantitatively into 5 mL vial and neutralized with 0.5 mL of 5mM NaOH solution. After neutralization 1 mL of chromatography grade n-hexane was added into a vial and shaken vigorously for 5 minutes. Fatty acid methyl esters (FAME) dissolved in non-polar n-hexane phase. The final sample was prepared by diluting 100 µL of the n-hexane phase was with 900 µL of n-hexane into a glass vial. The final dilution of the extract was performed to reduce the signal response and further increase the measurement's sensitivity.

GC analysis of FAMEs was carried out on a TRACETM 1300 Gas Chromatograph (Thermo Fischer Scientific, USA) equipped with a flame ionization detector and Al 1310 autosampler. 1 µL of the sample was injected into silica-based column Zebron ZB-FAME (30 m, 0.25 mm id, 0.20 µm film thickness) (Phenomenex, USA). Hydrogen was used as carrier gas at a constant flow rate of 1,0 $mL \cdot min^{-1}$ with a sample split ration 10: 1. Injector and detector internal temperature was set to 260 °C. The detector was fed with 30 $mL \cdot min^{-1}$ of hydrogen, 350 $mL \cdot min^{-1}$ of air and 40 $mL \cdot min^{-1}$ of nitrogen gas.

FAMEs were identified using commercial standard Supelco 37 Component FAME Mix (Sigma Aldrich, SRN). The internal standard method was used for quantification via the addition of heptadecanoic acid (Sigma Aldrich, SRN) into the transesterification mixture in concentration 0.5 mg/mL. Chromatography data were evaluated using Chromeleon software 7.2. Temperature gradient programme is listed below (Table 19). All samples were measured twice and the results are reported as the average of their values.

Table 19. GC analysis temperature program.

No	Retention time [min]	Rate [°C/min]	Target value [°C]	Hold time [min]
1	0.000	Run		
2	1.000	0.00	80.0	1.00
3	5.000	15.00	140.0	0.00
4	21.667	3.00	190.0	0.00
5	25.467	25.00	260.0	1.00
6	25.467	StopRun		

4.6.8 Lipase activity

The lipolytic enzymes' activity in the supernatant was determined by a colourimetric method using p-nitrophenyl palmitate, which is enzymatically cleaved to yellow p-nitrophenol. This product is then measured spectrophotometrically at a wavelength of 405 nm. The reaction medium was optimized to ensure maximum lipase activity. A series of buffers were used to optimize the determination of lipase activity: 0.1M Tris HCl buffer pH 7.2; 0.1M Tris HCl buffer pH = 8.4 and 0.1M phosphate buffer pH = 7.2. Enzyme activity was evaluated as the average of absorbances from two parallel cultures.

4.6.9 Biosurfactants production

The oil spreading assay method was chosen for selected strains to detect and prove the production of extracellular surfactants in experiments with waste animal fat. The oil stain spreading method was also used in cultivation with vegetable oil induction. In the experiments, the solubilization method of crystalline anthracene was performed for semi-quantitative determination of biosurfactant production.

4.6.9.1 *Oil spreading assay*

This method serves to prove the presence of biosurfactants in the medium qualitatively. 40 mL of Milli Q of water were poured into a 15 cm diameter Petri dish. In the middle of the Petri dish, 100 µl of oil was added to the water surface with the addition of visualizing agent. 10 µl of supernatant was then added to the oil drop. The degree of rupture of the oil drop showed the presence of a surfactant. Similarly, positive, and negative controls were tested.

4.6.9.2 *Solubilization of crystalline anthracene*

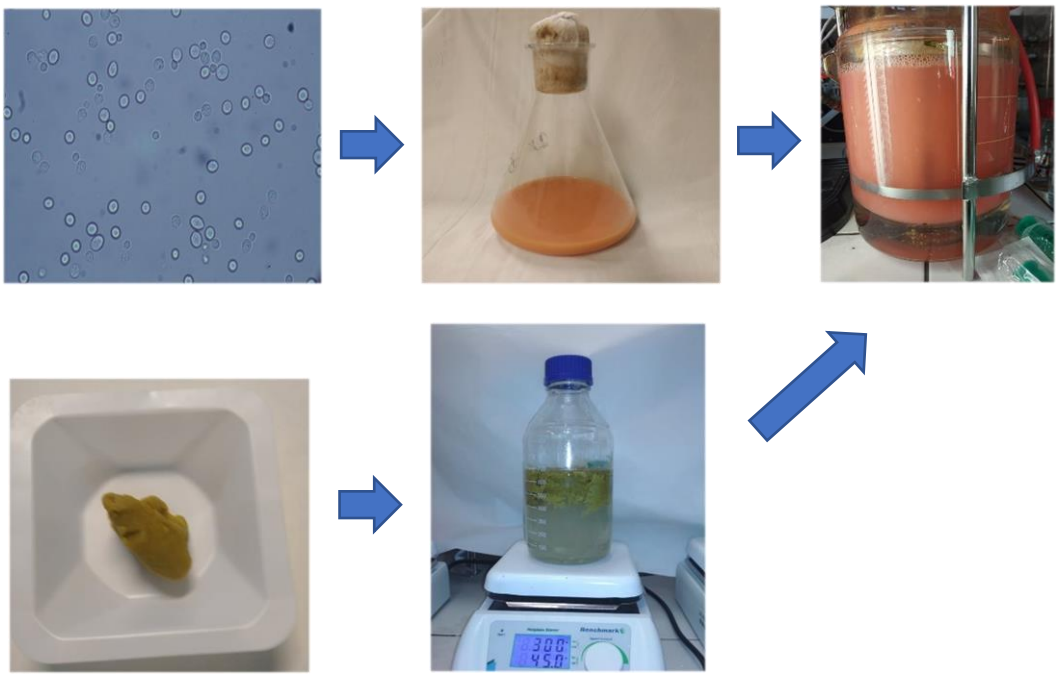
5 mL of supernatant was pipetted into a centrifuge tube, and a defined amount of hydrophobic anthracene was weighed. A positive and negative control were prepared similarly. The sample tubes were placed on a shaker and stirred vigorously at room temperature for 24 hours. Subsequently, the contents were centrifuged for 5 minutes at 6000 rpm. The prepared sample's supernatant was subjected to spectrophotometric measurement in quartz cuvettes at a wavelength of 354 nm. The measured absorbance corresponds to the concentration of anthracene dissolved in the surfactant present. Negative control was used as a blank. To exclude medium interference, absorbance was also measured in tubes containing only pure culture media.

5 RESULTS AND DISCUSSION

The following chapter presents selected results of experiments performed in this dissertation. The results are divided into three chapters depending on the strains of microorganisms used.

5.1 Yeast cultivation results and discussion

In this work, experiments with carotenogenic yeasts focused on optimizing the cultivation medium's composition, cultivation conditions, yeast biomass and metabolites (carotenoid pigments, lipids, coenzyme Q and sterols). The yeasts were cultivated on a basic mineral medium containing the necessary mineral nutrients (N, P, K, S, Mg) and an organic carbon source. The dissertation's main goal was to test the influence of various physicochemical stress factors on yeasts' growth and productivity and the use of various waste substrates from the food industry as a replacement for nitrogen and organic carbon source. Carotenogenic yeasts are strictly aerobic heterotrophic microorganisms capable of growing on various carbon sources (carbohydrates, fatty acids, alcohols, amino acids). Food and agriculture wastes, namely glycerol, whey, animal/vegetable fat, and spent coffee grounds were tested in this work. A part of the results was already published in the form of article [191], conference poster/abstract [190][227][228] or students' diploma works, which I have supervised. The summary of the main results is listed here.



Scheme 14. *Yeast cultivation process graphical abstract*

Cultivation of carotenogenic yeast was performed according to the following procedure:

1. Laboratory cultivation on untreated wastes
2. Waste hydrolysis experiments and hydrolysis process optimization
3. Laboratory cultivation on media with pre-treated waste substrates
4. Large-scale cultivation in 3.0L bioreactor on pre-treated waste substrate
5. Large-scale cultivation in 3.0L bioreactor on media with combined waste substrates

Firstly, cultivation of the yeast on the selected waste in the pure untreated form to determine the extent to which the yeast can utilize this material. The glycerol cultivation results showed a high ability to utilize this material, and yeasts cultivated on this carbon source were characterized by high biomass productivity, comparable to the control glucose medium. Furthermore, in many cases, yeasts cultivated on glycerol medium achieved even higher yields. From this point of view, glycerol is considered to be a practically ideal carbon source for yeast cultivation. A slight negative that was observed in some cultivations was the reduced content of valuable substances in biomass.

In cultivation on untreated waste animal fat, it was observed that the yeast produced some lipolytic enzymes that decomposed the fat in the medium into glycerol and free fatty acids. However, the rate of this hydrolysis was insufficient, and in the time frame of cultivation, the production of biomass together with the studied metabolites was low. Cultivation on waste whey from the dairy industry and waste spent coffee grounds produced the worst results. The results showed that carotenogenic yeasts do not have enzymes that would be able to hydrolyze spent coffee grounds (cellulases and hemicellulases), nor do they have enzymes that can break down lactose, which is the major source of carbon in whey. In whey cultivations, only a slight ability to process the whey protein was observed. Based on these results, it is clear that under these conditions, the cultivation and production of biomass by yeast would take too long or not at all. Therefore, it was decided that the waste substrates would be processed into a better usable form before cultivation [191].

5.1.1 Processing of waste substrates

As has been found in previous pilot cultivations on crude waste substrates, carotenogenic yeasts do not have robust enzymatic equipment capable of processing these materials in their native form. Three hydrolytic processes can be used to modify them, namely acidic, basic and enzymatic hydrolysis. Each of the wastes substrates has its own characteristic structure and properties, to which the given hydrolysis process must adapt. Various hydrolysis methods were tested on all complex waste substrates to find a compromise between time, price, hydrolysis efficiency, and compatibility with yeast. From an economic point of view, hydrolytic processes using an acid or a base appear to be the best, because they are cheap. The hydrolysis process is very fast under optimal conditions. On the other hand, their use significantly affects the medium's pH, and it is necessary to compensate for it, which requires additional costs. This neutralization increases the salt stress exerted on the yeast culture. High salt concentrations can have a lethal effect on a given microorganism culture. Simultaneously, it is often necessary to heat the material, and undesired side reactions can occur here due to heat or reagents used. Enzymatic hydrolysis is efficient, and the waste material can be specifically modified according to our requirements. At the same time, the specificity of the enzymes limits the amount of by-products formed. However, enzymatic hydrolysis also has its downsides. Enzymes are many times more expensive than acids and bases. They require a specific pH optimum for their function. Also, a mixture of enzymes needs to be used to process complex matrices [190][191][227][228].

5.1.1.1 *Waste animal fat hydrolysis*

Experiments with the use of waste animal fat were part of the international project Lipofungi, the aim of which was the microbial transformation of waste fat rich in saturated fatty acids into yeast biomass enriched with lipids with a high content of PUFA and other valuable substances. All types of hydrolysis were tested in the processing of waste animal fat. The basic hydrolysis parameters tested included: temperature, reaction time, reagent concentration and various types of reagents. Three types of acid (H_2SO_4, HCl, and HNO_3) were used for acid hydrolysis. Sodium hydroxide and potassium hydroxide were tested in the basic hydrolysis. Enzymatic hydrolysis was tested on a series of commercial enzymes and compared to the lipase from the tested yeast *Rhodotorula kratochvilovae*.

The hydrolyses were tested under the various conditions listed in the table below. Hydrolytic efficiency was determined by measuring the content of released glycerol. In the case of enzymatic preparations, the substrate specificity of the enzymes was monitored by TLC [191].

Table 20. Summary of the hydrolysis conditions

Reagent conc. [%]	Temperature [°C]	Time [hours]	Fat: reagent ratio [g/mL]
Acid hydrolysis			
1; 3; 5; 10; 15	40-120	0.3-24	10:100
Base hydrolysis			
1 – 20	20-100	0.1 -24	10:100
Enzymatic hydrolysis			
25 mg x 10 types	37	0-24	10:100

Table 21. List of enzymes used

Lipase	Lipase source
1	*Aspergillus niger*
2	*Candida antarctica, recom. from Aspergillus oryzae*
3	*Candida rugosa*
4	*Mucor miehei*
5	*Pseudomonas cepacia*
6	*Pseudomonas fluorescens*
7	*Rhizopus oryzea*
8	*Rhizopus niveus*
9	Porcine pancreas
10	*Rhodotorula kratochvilovae**

* lipase isolated from yeast (strain *R. k.*) cultivated on medium with crude animal fat at C/N ratio 50

The maximum measured efficiency of acid hydrolysis did not exceed 15%. Only in the experiments with the highest temperature and reaction time for more than 2 hours, the efficiency was close to 15%. Furthermore, the dependence of the hydrolysis efficiency on the reagent concentration was not found in the experiments. Of the tested inorganic acids, sulfuric acid achieved the highest efficiency. In general, acid hydrolysis is not a suitable method for processing waste fat and has not been used in further experiments. The results of enzymatic hydrolysis show consistent results. During hydrolysis, the reaction rate increases to the point where all active sites are saturated and then the rate of hydrolysis is constant.

Finally, the enzyme activity is inhibited by the reaction product and the reaction rate of hydrolysis decreases. We can see from the data that in the case of enzymatic hydrolysis, the hydrolysis efficiency can be modulated by the amount of enzyme and substrate used and the total reaction time. Of the enzymes tested, the most effective was the enzyme from *Candida rugosa*, which from the beginning outperformed other tested preparations. Furthermore, this enzyme was characterized by non-specific activity and from the very beginning, hydrolyzed TAG and intermediates formed by hydrolysis of TAG (diacylglycerols and monoacylglycerols). The lipase activity produced by the carotenogenic yeast *R. kratochvilovae* did not reach the activity of commercial enzymes. It should be added that the lipase-containing supernatant was used in the experiment in contrast to the refined form of the commercial preparations [191].

The last tested hydrolysis was base hydrolysis, also referred to as saponification. From an economic point of view, this method is the best choice for fat hydrolysis. It is characterized by high efficiency, speed and low-temperature requirements. In experiments, it was found that the optimal temperature for hydrolysis is in the range of 50-60 °C when the fat turns into a liquid phase. By controlling the amount of hydroxide used, the hydrolytic activity is then simply modulated. Thus, only saponification and enzymatic hydrolysis were used for the cultivation of carotenogenic yeasts [190][227][228].

5.1.1.2 *Whey hydrolysis*

Dairy whey is classified as a complex waste substrate, thanks to its composition. It can serve as a source of carbon and nitrogen. The main problem with the use of whey lies in the yeast's inability to hydrolyze lactose. All studied carotenogenic yeast strains do not show any lactase activity and cannot process this substrate without the necessary pre-treatment. Lactose is a disaccharide composed of a glucose and galactose molecule linked by a β-(1→4) glycosidic bond. Two units of monosaccharides are formed by cleaving it, which are a suitable substrate for yeast cultivation. This bond is practically inert to the action of bases, and therefore, only the possibilities of acidic and enzymatic hydrolysis could be tested. For acid hydrolysis tests, similar conditions were used to hydrolysis of animal fat using sulfuric acid and hydrochloric acid. The commercial enzyme lactase was purchased for enzymatic hydrolysis. Unfortunately, the results of the acid hydrolysis of native whey showed very low efficiency. The proteins contained in the whey reacted with the acid used and inhibited the whole hydrolysis process. To increase acid hydrolysis efficiency, it is first necessary to precipitate proteins and remove them from solution by filtration. The remaining amino acids are then removed, for example, with Carrez reagent. However, this procedure significantly increases the cost of the whole process and greatly complicates it. Therefore, in this case, even acid hydrolysis was not chosen as the optimal option. On the other hand, Enzymatic hydrolysis was very efficient in the hydrolysis of native whey. As with the previous case, the hydrolysis efficiency can be modulated by regulating the enzyme: substrate ratio and reaction time. Due to the yeast's inability to process lactose, complete hydrolysis of all lactose present was chosen as the best solution. Final enzymatic hydrolysis requires just gentle stirring at 40 °C for a short time.

5.1.1.3 *Spent coffee grounds hydrolysis*

Of all the materials used, coffee grounds were the most difficult to process. This solid material is composed of an oil fraction (7.9-26.4%), poly and oligosaccharides (47.7-52.1%), lignins (25-33.0%), phenolics (2.5%), caffeine (0.02%) and other substances [203]. This complex varies according to the type of coffee beans, growth conditions, fermentation etc. The complex composition of spent coffee grounds practically prevents direct hydrolysis and the use of basic hydrolysis methods because hydrolysis aims to break the bonds between monosaccharide units. To verify this assertion, a series of direct hydrolyses with various acids were performed at high temperatures. The efficiency of this hydrolyzation ranged from 4 to 10% of the released carbohydrates. Even lower yields were obtained by direct enzymatic hydrolysis. The main obstacle to successful hydrolysis is the presence of coffee oil and the inaccessible structure of the carbohydrate chain.

Thus, the proposed hydrolysis procedure started with treating dried SCG in a grinder to create smaller particles in order to increase the active surface for the following steps. The following description already includes the final optimized procedure. Ground dried SCGs were extracted with hexane: isopropanol 1: 1 for 90 minutes in a Soxhlet apparatus. The defatted SCGs were dried from residual solvents and prepared for the hydrolysis of complex carbohydrates. At this stage, standard experiments were performed with different acid concentrations as for other waste materials.

Furthermore, the possibility of autolysis was tested, where the acidic groups present in the substrate matrix serve as hydrolysis catalysts. The final procedure for the preparation of SCG hydrolyzate for yeast cultivation is as follows. The defatted SCGs are hydrolyzed autolytically in a 1: 5 SCG: water (w/v) ratio for 90 minutes at 130 °C. After cooling to 40 degrees, the enzymes cellulase and hemicellulase are added to the solution. After 3-4 hours of enzymatic hydrolysis, the mixture is filtered to obtain SCG hydrolyzate with an efficiency of 85-90% extracted sugars. All prepared hydrolysates were measured by HPLC or GC, before used. An equivalent amount corresponding to the amount of glucose in the standard medium was used to prepare the medium. The whole hydrolysis process was designed to achieve the lowest possible process costs.

5.1.2 Erlenmeyer flask cultivation of yeast on waste substrates

After optimizing the hydrolyzate preparation, a series of screening cultivations were performed in 250mL Erlenmeyer flasks with 60 mL of medium. Cultivation was performed for 96 hours at room temperature, constant illumination and constant shaking on a reciprocal shaker. Cultivations were performed at a total of 4 C/N ratios (13; 25; 50; 100) where the amount of carbon source was increased. The standard glucose medium served as a control medium. Cultivation at multiple C/N ratios was chosen to test under which conditions carotenogenic yeast strains produce the most carotenoids and lipids. In general, with increasing C/N ratio, the content of lipids in biomass increases and, conversely, the content of carotenoid pigments decreases.

5.1.2.1 *Cultivation on media with waste animal fat*

The cultivation of carotenogenic yeasts on waste animal fat was divided into two parts. The first part of the experiment dealt with the use of enzymatic fat hydrolyzate and the study of the adaptation of carotenogenic yeasts to media containing this substrate. The second part was focused on cultivation on basic fat hydrolyzate.

5.1.2.1.1 Cultivations with enzymatic fat hydrolysate

For each yeast culture at a given C/N ratio, five types of media were prepared:

1. Glucose medium - control
2. Glycerol medium - control, test for induction of lipase and biosurfactant production
3. Fat medium - medium with untreated crude fat
4. Fat medium with an emulsifier
5. Hydrolysed fat medium
6. Fat + enzyme medium - sterile enzyme solution added at the time of inoculation

In this experiment, the following standard parameters were determined: biomass production, lipid content, fatty acid profile, carotenoid production, ergosterol, ubiquinone. Furthermore, the lipase activity of yeast extracellular lipases and the production of biosurfactants were determined. This large-scale experiment was published in [191]. The following paragraphs briefly summarize the results of this work.

5.1.2.1.1.1 Lipase induction and activity

Lipase activity was measured by the decomposition method of *p*-nitrophenol. Samples for the determination of lipase activity were taken during the growth of the yeast culture. Lipase activity was measured in all carotenogenic yeast strains on all media types, indicating that the yeast produces a basal amount of lipase under all cultivation conditions. Induction of increased lipase production was observed on all fat-containing media, especially on pure, untreated fat media. The highest lipase activities (5-fold induction) on the fat medium were measured in the yeasts *R. mucilaginosa* and *C. macerans*. Induction of lipase activity was associated with a higher increase in these yeasts' biomass and was further reflected in increased lipid production. The induction of lipase activity was lesser on hydrolyzed fat compared to untreated fat. This effect is probably due to the degradation products of the lipids present in the medium. The addition of a commercial enzyme at the beginning of the cultivation did not have a negative effect on the production of yeast lipases, and the measured activity was identical to the medium on untreated fat. The presence of an emulsifier then had a strong inhibitory effect in all cases, and the production of lipases was practically zero [191]. Compared to commercial enzymes, lipases produced by carotenogenic yeast achieved 20-50 times less activity. The measured values of yeast lipase activity ranged from 0.01 to 0.025 U/mL. The low total lipase activity is also due to the large dilution in the medium's whole volume. Studied strains were able to produce lipase on all types of medium, with different success [191].

5.1.2.1.1.2 Biosurfactants

Biosurfactants are substances that, together with lipases, are generally involved in the processing of oil substrates. Biosurfactants are substances consisting of a polar and a non-polar part, which reduce the surface tension between the phases and allow lipases in the polar environment to process non-polar lipids, by lowering the surface tension, the solubility of non-polar components in the polar phase increases. Biosurfactants are widely used in various industries, and their production by microorganisms is an alternative to their synthetic counterparts. The relationship between lipases and biosurfactants produced by carotenogenic yeasts has not yet been fully described. The presence and amount of biosurfactants were tested using the oil spreading method and the anthracene method. Based on the quantitative method, the yeast cultivated on glucose, glycerol and untreated fat showed high productivity of biosurfactants. The oil layer in the petri dish was completely emulsified in these samples. The emulsifying activity of the individual yeasts was arranged as follows *C.m.* > *S.p.* > *R.m.* > *R. k*. By comparison with the commercial emulsifier TWEEN 100, it was found that yeast biosurfactants' activity corresponds to a TWEEN 100 concentration of 6.5 g/L [191].

The results of the quantitative determination by the anthracene method confirm the previous qualitative tests. The highest concentration of biosurfactants was reached by the *C. macerans* strain, followed by the *S. pararoseus* strain. The results show that the highest production was achieved on media with a C/N ratio 13, which was several times higher than in other tested C/N ratios. Simultaneously increased production of lipases and biosurfactants was then observed on fat-containing media, mainly in *C. macerans* and *S. pararoseus* strains [191].

5.1.2.1.1.3 Biomass production

Biomass production was specific to each strain depending on the type of medium. While yeast cultures on glycerol and glucose gave relatively identical results, larger inter-strain differences were observed in fat-containing media. Biomass production was about a 20% lower in the fat medium. The studied yeast strains produced slightly more biomass on all fat media than in glycerol media. An exception here is the *S. pararoseus* strain, which produced several times higher amounts of biomass. Within fat media, yeasts achieved the largest production on enzymatically hydrolyzed fat. This production is closely related to the medium's pH. Due to the presence of the buffer used for enzymatic hydrolysis, it maintained the pH optimum for a longer time, and the yeast produced more biomass. The growth curves recorded during 96 hours varied according to the strain and the composition of the medium. In general, all strains experienced a decrease in growth rate at the end of the second day of cultivation, which is related to the loss of carbon source and the end of the exponential growth phase. The *S. pararoseus* strain achieved the highest increase in biomass on fat media [191].

5.1.2.1.1.4 Carotenoid, ubiquinone and sterol production

Metabolite production was monitored at regular intervals during growth. Within the glucose medium, carotenoid production regularly increased with increasing cultivation time. Yields ranged from 2.0-5.8 mg/L of a medium, with *R. kratochvilovae* and *S. pararoseus* strains being the best producers. Cultivation on glycerol already showed fluctuations between strains in the stationary phase of growth, which confirmed the presumed osmotic stress role of glycerol. In the case of the genus *Rhodotorula*, the final yields on glucose, glycerol and fat media were similar. Furthermore, the medium with raw fat showed a much more stable production, and in the case of *S. pararoseus* and *C. macerans* strains the achieved production was 2-3 times higher than in the control medium. The *S. pararoseus* strain achieved the absolute highest production of 13.4 mg/L of medium [191].

Hydrolyzed fat medium also led to the induction of carotenoid pigment production. Ergosterol production was relatively stable during the stationary phase and comparable for all strains. A slight increase in ergosterol content was observed with the cultivation time. Stable ergosterol production is due to the need to keep functional membranes in the stationary phase of cultivation. Ubiquinone showed similarly stable production. Very high productions were observed in the *S. pararoseus* strain, whose production was 3-5 times higher than in other strains. Fatt medium induced increased ubiquinone production in all strains. This phenomenon is probably associated with a change in oxidation conditions and more efficient oxygen use in the respiratory cycle. With increasing C/N ratio, lower production of ubiquinone and ergosterols was observed in all strains. The assumption that higher carotenoid production is accumulated on media with a lower C/N ratio was confirmed. As the C/N ratio increased, carotenoid production decreased dramatically, except for the *R. mucilaginosa strain*, at a C/N ratio 50. In general, yeasts on the medium with hydrolyzed and emulsified fat achieved the highest production of metabolites [191].

5.1.2.1.1.5 Lipid production and fatty acid profile

Lipid production in all cultivations increased slowly with increasing time. It was observed that the accumulation of lipids on fat media is 1.3-2 times greater than in the control glucose or glycerol medium. Significantly higher amounts of lipids were produced in fat media in *R. mucilaginosa* and *S. pararoseus* strains. The accumulation of more lipids is probably associated with the induction of higher production of extracellular lipase and biosurfactant, which together form a complex metabolic response to the presence of fat as a carbon source. In all strains, lipid production increased up to a C/N ratio 50. After that, no significant effect of a higher C/N ratio was observed [191].

The *S. pararoseus* strain was the best strain on fat medium, accumulating 53.2% of lipids in biomass at a C/N ratio of 25 and 50. The same conditions were suitable for the *C. macerans* strain, which produced 47% of the lipids in the biomass. Representatives of the genus *Rhodotorula* achieved lower productions in the interval 24-28%. The fatty acid profile was also measured when determining the lipid content. We see from the results that the majority of saturated fatty acids of carotenogenic yeasts are stearic acid (C16:0) and palmitic acid (C18:0). Furthermore, monounsaturated oleic acid (C18:1n9c) and polyunsaturated linoleic acid (C18: 2n6c) accounted for the majority of all unsaturated fatty acids. In all samples, oleic acid was the most common, accounting for an average of 35-45% of the total fatty acid content. Linoleic acid formed only small amounts. We observed that the ratios of fatty acids are different within both the studied strains and within cultivation on various media [191].

Yeast cultivated on glucose medium produced equally the highest oleic acid content (63-81%). Changing the carbon source led to a change in the fatty acid ratio in the glycerol media, where the MUFA content decreased, and the PUFA content increased 3-4 times. The fat-containing media was strain-specific. A higher content of PUFA acids was found in samples of the yeast *C. macerans* (37%) and *R. kratochvilovae* (33%). The addition of an emulsifier had an effect on the production of PUFA acids by the *C. macerans* strain, where the PUFA content increased to 44%. Cultivation on hydrolyzed fat resulted in increased PUFA acid content in all strains. In terms of lipid production on the waste medium, the yeast C. macerans is the most suitable candidate [191].

5.1.2.1.2 Cultivation on base hydrolysed fat

The same experimental scheme was chosen for cultivations on base hydrolyzed fat. These experiments were also part of the Lipofungi project. Selected yeast strains were cultivated in Erlenmeyer flasks with 4 C/N ratios (13; 25; 50 and 100) on 3 media with different carbon sources: glucose, glycerol, base hydrolyzed fat. This chapter's main goal was to optimize the cultivation conditions in Erlenmeyer flasks and subsequent bioreactor cultivation in a laboratory bioreactor. As part of the comparison of production under conditions of different C/N ratios, a base hydrolyzate with the same state of hydrolysis 85-90%, was prepared in all flasks (the ratio of glycerol: unhydrolyzed fat is maintained in the medium). Laboratory cultivations were performed for 96 hours at room temperature, constant illumination and shaking on a reciprocal shaker.

The results of laboratory cultivations showed a negative effect of overly hydrolyzed fat. The emulsion formed by fat hydrolysis contained a high concentration of free fatty acids, which had a strong inhibitory effect on yeast cells' growth and viability. In media with a low C/N ratio, this emulsion was diluted sufficiently with water, and the inhibitory effect was not so strong. In terms of growth and biomass production, the most suitable media were with a C/N ratio of 13 and 25, where similar or slightly lower biomass production was achieved compared to the control glucose or glycerol medium. At higher ratios, growth was already strongly inhibited. In this experiment, the best strains were both members of the genus *Rhodotorula*, which achieved the highest biomass production and showed the greatest resistance to high concentrations of free fatty acids on media with C/N> 25. The other two tested strains practically did not grow at all [227][228].

In terms of carotenoid production, identical results were obtained in all strains in the range of 3.5-4.5 mg/g dry biomass and were comparable with control cultures. For all cultivations on fat hydrolyzate with C/N ratios of 13 and 25, stable high ergosterol production was measured in all yeasts, which was always higher than 4 mg/g dry biomass. The yeast *S. pararoseus* then reached the highest production of 6.50 mg/g on this medium. Ubiquinone production in these cultivations was marked by a low growth rate of the cultures and reached the range of 1.0-1.6 mg/g dry biomass. As in cultivations on an enzymatic hydrolyzate, the base hydrolyzate positively affected lipid production. All cultivated yeast strains produced more lipids on the fat hydrolyzate medium than on the control medium. Strains *R. mucilaginosa, R. kratochvilovae* and *C. macerans* produced on average 15-45% of lipids in biomass. As the C/N ratio increased, the amount of lipids in the biomass increased. The best results were obtained by the *S. pararoseus* strain, which achieved more than 50% lipids in the biomass on all cultivations. At a C/N 50 ratio, the accumulated lipid content reached 63.34%. In terms of fatty acid profile, an increased amount of polyunsaturated fatty acids was induced in the fat hydrolyzate media at the expense of the monounsaturated fatty acid content. Furthermore, it was observed that in these media, there was a jump in the content of saturated fatty acids, which in many cultures reached values of about 50% [227][228].

From the cultivation results on various fat hydrolysates, it was found that there is no ideal solution where the yeast would produce a sufficient amount of carotenoids and lipids. Yeast prefers lower C/N ratios for carotenoid and sterol production and higher ratios for lipid production. Increased lipid production was induced in all fat media, and in the case of enzymatic hydrolysates, the content of unsaturated fatty acids increases. Furthermore, the yeast was found to be sensitive to the concentration of fatty acids in the medium, and their high concentration leads to growth inhibition. Based on these data, a compromise medium was used in subsequent high-volume cultivations, where the carbon source is partially hydrolyzed fat.

5.1.2.2 *Cultivation on whey hydrolysate*

Whey is one of the most common waste substrates. It is a waste product of the dairy industry containing, in addition to minerals, two essential components. Lactose, which upon hydrolysis yields glucose and galactose, which are simple sources of carbon. The second component is whey protein, which is a complex source of nitrogen. In pilot experiments, cultivations were performed on pure, untreated whey. It was found that carotenogenic yeasts do not have enzymes for hydrolysis of lactose and can also partially process whey protein. In this case, it was mainly short-chain peptides or free amino acids. The rate of protein utilization under normal conditions was small, and therefore an enzymatic whey carbohydrate hydrolyzate was prepared in the hydrolysis optimization process.

The whey protein was removed by filtration. Soluble peptides were determined for nitrogen content and supplemented with urea to the required concentration for cultivation. Experiments dealing with whey protein hydrolysis have not been processed in this work and will be the subject of further studies. Laboratory cultivations with whey in Erlenmeyer flasks showed that all studied yeast strains were able to utilize the processed form of whey. In terms of biomass production, all yeasts on whey medium achieved at least similar productions and in many cases, 1.5-3.0 times higher biomass production, which further increased in all strains with increasing C/N ratio. Carotenoid production was not adversely affected by whey media. In contrast, in most samples, we see increased production compared to the control glucose medium. In the case of sterol production, no significant adverse effect of whey was observed, and the total production is comparable to the control glucose medium. Only in the case of ubiquinone is there a slight decrease in production in all strains.

In terms of lipid content, we do not see a clear trend as with previous metabolites. In general, whey had minimal effect. In most cases, production is comparable to control medium or slightly increased, especially for media with a higher C/N ratio. The fatty acid profile of yeasts cultivated in whey shows an increased monounsaturated fatty acid content, which is on average 5-10% higher than the control. In the case of MUFA, we also observe a slight increase in the monounsaturated fatty acids content. The majority of fatty acids consists of the standard quaternary palmitic, stearic, oleic and linoleic acids. From the point of view of lipid production, a separate whey hydrolyzate is not a suitable medium. We can see from the results that it is more suitable for the production of carotenoids, sterols and ubiquinone. Overall, the best strain for whey hydrolyzate utilization was *Rhodotorula mucilaginosa* and *Rhodotorula kratochvilovae* [227][228].

5.1.2.3 *Cultivation on spent coffee grounds hydrolysate*

The last waste material tested was coffee grounds hydrolyzate prepared according to the hydrolysis procedure mentioned in chapter 3.1.3. In addition to evaluating the production properties of carotenogenic yeasts, this experiment aimed to monitor the effect of antimicrobial substances present in the medium on yeast growth. Laboratory tests again included a series of media with different C/N ratios cultivated in Erlenmeyer flasks with 3 C/N ratios (13, 25, 50). In terms of biomass production, coffee hydrolyzate is an excellent source. Higher biomass production was observed in all tested ratios than in the control glucose medium. The best results were obtained by the *S. pararoseus* strain, which produced more than 10 g/L (C/N 13) and 12 g/L (C/N 50) at all tested C/N ratios. In the best sample, the yeast even produced 17 g/L, which is an unprecedented value for classical laboratory cultivations in flasks under given conditions. In the experiment, other related representatives of the genera *Sporobolomyces* and *Sporidiobolus* were tested, and even these yeasts achieved excellent results in the produced biomass. In terms of pigments, sterols, and ubiquinone production, we see a uniform trend where their production decreases with a gradually increasing C/N ratio. The highest production was achieved on media with C/N 13.

The production of carotenoids in the studied strains ranged from 3.5-5.0 mg/g of dry biomass, which is slightly higher than the production in control glucose media (5-10%). The production of ergosterol and ubiquinone is then practically comparable to the control medium. In terms of lipid accumulation, coffee hydrolyzate had a negative effect. Lipid production in biomass was on average 5-8% lower in media with a C/N ratio of 13 and 25. Only at a C/N ratio 50, did the lipid content equal the control medium. Cultivation on coffee hydrolyzate had an interesting effect on the profile of fatty acids produced. SFA production was significantly reduced to a minimum and replaced by an increased poly and monounsaturated fatty acid content. Like whey, this saccharide hydrolyzate is more suitable for the production of pigments and sterols.

5.1.3 Bioreactor large volume cultivation on media with a combination of waste substrates

The results of previous experiments show that the yeasts are able to utilize many different waste substrates. However, we encounter the problem that waste substrates used to cultivate yeasts must be treated and hydrolyzed to some degree. The main reason is the low activity of enzymes produced by yeast or their absence. As the substrate becomes more hydrolyzed, it becomes a more available source of nutrients for the yeast, leading to faster culture growth, but not always to higher metabolite production. The waste substrate in its untreated form acts as a complex substrate and thus exerts more stress on the yeast, leading to increased production of the desired metabolites. In the case of animal fat, a compromise can be reached by partial hydrolysis of the fat to release a sufficient amount of a simple carbon source glycerol. Ideally, a given amount of glycerol is consumed in the exponential growth phase.

Subsequently, the remaining unhydrolyzed part slows down yeast's growth, and their metabolism will lead to the formation of storage molecules (carotenoids, lipids). In experiments with partially hydrolyzed fat, a problem has arisen in the high concentration of free fatty acids, which have a strong inhibitory effect on yeast growth. In other substrates, such as SCG, the yeast does not have the necessary cellulase enzymes and must therefore be completely hydrolysed. The same problem occurs in the use of lactose from the whey.

Based on this idea and the obtained data, the possibility of using a combination of waste substrates was proposed. The first substrate is completely hydrolyzed and provides a fast, simple carbon source that provides sufficient yeast biomass growth. The second substrate is then minimally or not modified and is processed by the yeast in the second phase of yeast growth. The final stage of cultivation with yeasts was a series of cultivations in laboratory bioreactors, in which two waste materials were combined. The first simple source of saccharides was whey hydrolysates or coffee grounds. The second complex source was fat, either animal waste fat, used frying oil or coffee oil. The cultivation conditions were set so that the simple: complex substrate ratio was always 1: 3. The first experiments performed were cultivation the yeast in a bioreactor with a medium consisting of whey hydrolyzate and waste animal fat. The C/N ratio was chosen for these bioreactors cultivation based on the best results in laboratory cultivations. Cultivations lasted 96 hours and samples were taken at regular intervals to determine biomass and metabolite production. Here is a brief summary of the results achieved. For better comparison, productions are multiplied by total biomass and expressed as total productions in mg/L medium.

5.1.3.1 *Cultivation on media containing whey hydrolysate and crude fat*

All strains were able to grow on medium with a combination of whey hydrolyzate and fat. Different growth rates and biomass production were observed between the strains. A characteristic feature of all was the initial rapid increase due to whey hydrolyzate, after which the yeast metabolism adapted to waste fat differently and with varying success. The production of carotenoids, ergosterol and ubiquinone in the bioreactors increased linearly, except for the *C. macerans* strain. Carotenoids' production is less than 2.0 mg/g dry biomass and kept very low throughout the cultivation. Lipid production has steadily increased since the beginning of the cultivation, reaching a maximum between 40 and 48 hours, depending on the strain. The highest yield of ubiquinone 88.645 mg/L and carotenoids 147.751 mg/L of medium, was achieved in the culture of *R. kratochvilova*e at the 48th hour of cultivation. On the other hand, the most suitable strain for ergosterol production was *Sporidiobolus pararoseus with* 206.455 mg/L of a medium at 48 hours of cultivation. This strain was also the most suitable lipid producer with a production of approximately 5 g/L medium.

5.1.3.2 *Cultivation on media containing SCG hydrolysate and crude fat*

These experiments were followed by experiments in which whey hydrolyzate was replaced by coffee grounds hydrolyzate. Results of yeasts cultivated on a combination of coffee hydrolyzate and crude fat are significantly more strain different from experiments with whey hydrolyzate with crude fat. Yeast achieved on average worse results in biomass production than in the case of the combination of whey hydrolyzate + crude fat. One reason for this decline is the presence of antimicrobial phenolic agents, which are likely to inhibit growth partially. Another reason may be the simple incompatibility between coffee hydrolyzate and waste fat. The *Sporidiobolus pararoseus* strain achieves three times less biomass production on coffee hydrolyzate. On the other hand, the production of metabolites is significantly increased. We observe a similar trend in other strains. The slightly increased lipid content further compensates for the low increase in biomass.

Reduced biomass production affected the overall yield of metabolites in biomass. The highest carotenoid yield of 70.229 mg/L medium is obtained in the yeast *S. pararoseus* at the end of cultivation. At the same time, this strain also achieved the highest yield of ubiquinone 70.803 mg/L medium. The highest ergosterol production was shown by the *R. toruloides* strain, which reached a maximum of 119.191 mg/L of medium in the 30th hour of cultivation. The highest lipid content of 28% was measured in *C. macerans* strain at 28 hours of culture.

5.1.3.3 *Cultivation on media containing SCG hydrolysate and coffee oil*

Based on the yeast results on a medium with coffee hydrolyzate and fat, experiments were proposed using another similar substrate. Coffee hydrolyzate rich in glucose, mannose, arabinose, and xylulose served as a fast source of nutrients, which the yeast preferentially utilizes. The second carbon source here was coffee oil, which was obtained in the SCG hydrolysis process. Coffee oil is a very valuable source of unsaturated fatty acids in comparison with, for example, waste animal fat. Other components that the oil contains are phenolic substances, which are absorbed into the oil during the extraction process. This experiment aimed to compare yeast's growth rate and production properties compared to animal fat and waste frying oil. At the same time, this experiment made it possible to monitor the yeast culture's resistance or response to phenolic substances present in the medium, which are known for their antimicrobial activity. During the cultivation, 40 mL culture samples were taken, which were further processed and analyzed. The following tables and graphs summarize the biomass production and metabolite analysis of 5 carotenogenic yeast strains cultivated on this carbon source combination.

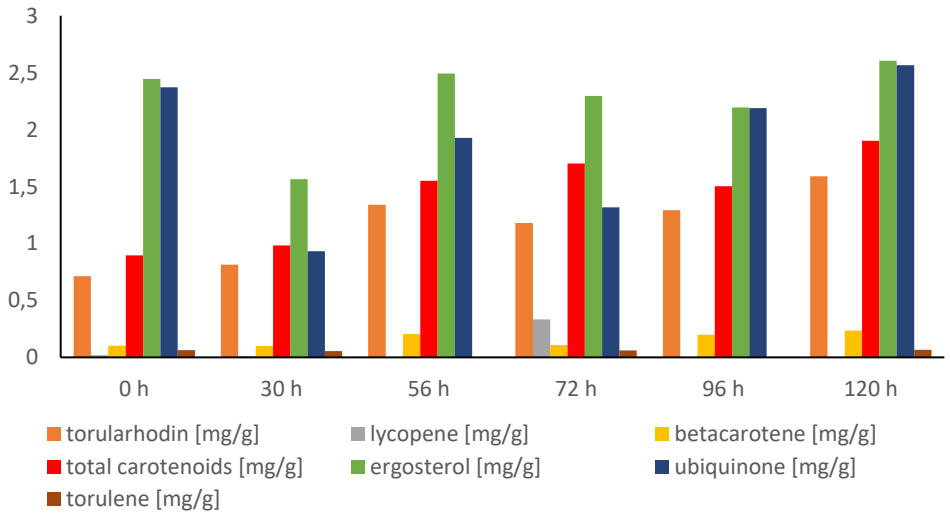
5.1.3.3.1 Rhodotorula kratochvilovae results:

During cultivation, this strain shows a very rapid increase in biomass, where 30 hours after the beginning of cultivation the production already reaches 8.11 g/L is achieved. Then growth rate decreases significantly and increases linearly to a final value of 10.40 g/L (Table 22). We see here that the yeast initially consumed all the sources of simple carbon very quickly. Then at the end of the second day of cultivation, the metabolism is reoriented to lipid processing. Unfortunately, we can see from the values of biomass growth in this period that this yeast could not utilize this carbon source well, and therefore the growth in this phase is very slow. In terms of carotenoid production, we see a linear increase in carotenoid production from the beginning to 72 hours, where we see a local maximum of 1.70 mg/g (Graph 2). After a slight decrease on the fourth day of cultivation, the maximum carotenoid production reaches 1.90 mg/g of dry biomass. The major pigment was torularhodin throughout the cultivation, which accounted for more than 70 % of the total carotenoid content. Ergosterol production is relatively balanced in the range of 2.20-2.60 mg/g. An exception is a sample at 30 hours with lower production of 1.56 mg/g. The ubiquinone content decreased at the beginning of the cultivation, but then its content increased to the final 2,57 mg/g of dry biomass.

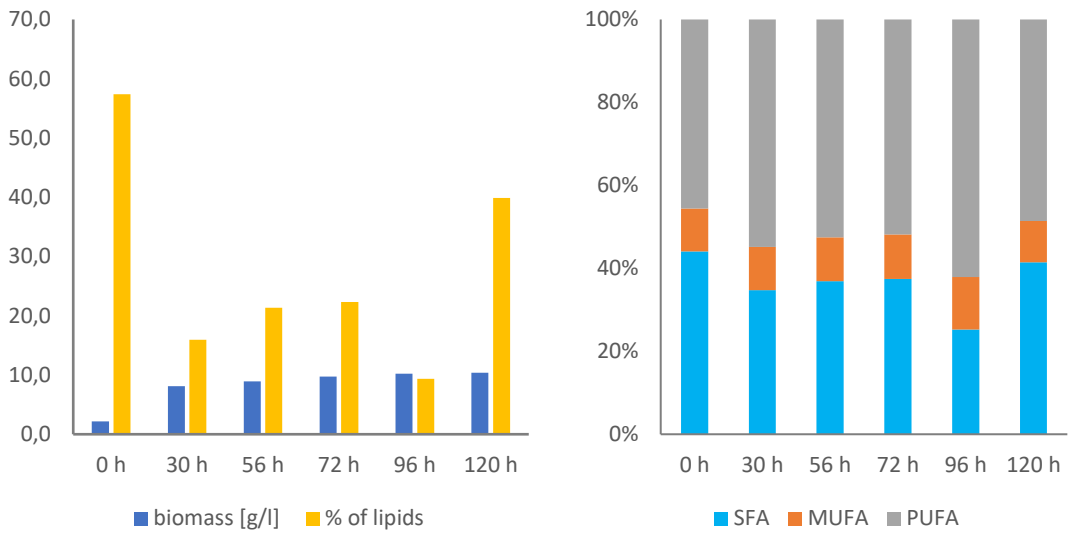
After the inoculation on the first day of culture, the initial high lipid content (57.4 %) decreases rapidly to approximately 16.0 % (Graph 3). Then, it grows practically linearly (except for the sample at 96 hours) to the final 39.9 % at 120 hours of cultivation. The fatty acid profile produced by the yeast *R. kratochvilovae* shows a stable high production of PUFA acids maintaining in the range of 52-55 %. The production of saturated fatty acids gradually decreases until the 96th hour of cultivation, where we subsequently observe a jump to the final 41.4 % on the last day. The least represented group is MUFA, whose content is in the range of 10-12 %. The sample at the final 120 hours is the best time for harvesting the culture. In general, we observe very low productions, so it can be said that this combination of substrates is not a suitable combination for this strain. The presence of phenolic substances also has a significant influence on this.

Table 22. R. kratochvilovae bioreactor cultivation SGC hydrolysate + coffee oil

Biomass [g/L]					
0 h	30 h	56 h	72 h	96 h	120 h
2.19	8.11	8.90	9.72	10.20	10.40



Graph 2. *R. kratochvilovae bioreactor cultivation on SGC hydrolysate and coffee oil: HPLC results*



Graph 3. *R. kratochvilovae bioreactor cultivation on SGC hydrolysate and coffee oil: lipid production and fatty acid profile*

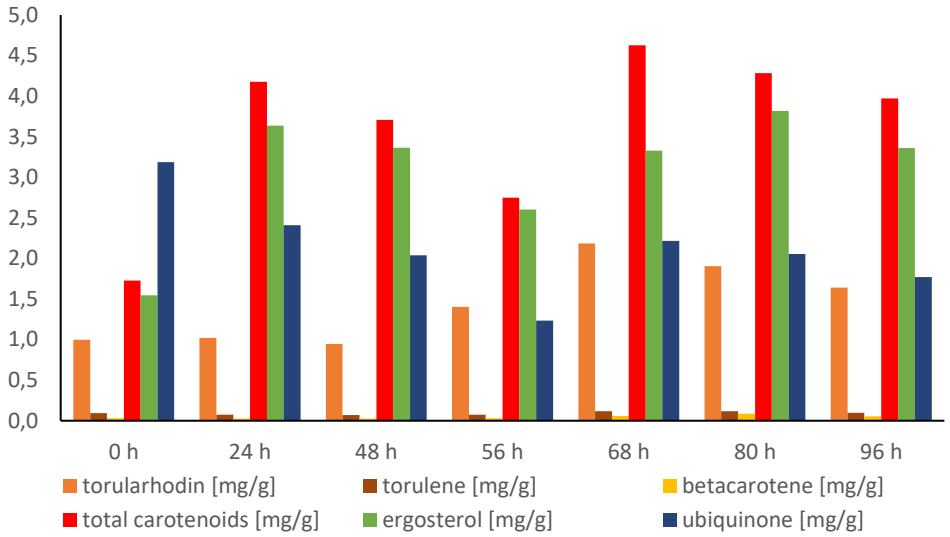
5.1.3.3.2 Rhodotorula mucilaginosa results:

The following data summarize the cultivation results of a second member of the genus *Rhododtorula*. Biomass production is generally lower by 1.3 g/L (Table 23). This phenomenon is observable at the beginning of cultivation where we see that *R. mucilaginosa* achieves a comparable increase with *R. kratochvilovae* only at a later stage of cultivation. Even in this case, we can say that the yeast could not utilize coffee oil well and after depletion of the hydrolyzate, the biomass increase is minimal. At first glance, we see that compared with previous results of the *R. kratochvilovae* strain, the *R. mucilaginosa* strain achieved significantly better results in the production of lipidic substances. The highest production of carotenoids is achieved in two samples. The first local maximum of 4.18 mg/g at 24 hours and the second at 68 hours of culture (Graph 4). After that, the carotenoid content decreases. Even in this cultivation, torularhodin was again the major pigment, the content of which during the cultivation ranged from 40-50%. In terms of ubiquinone production, the content gradually decreases during cultivation. Ergosterol production is fluctuating here, and the maximum is reached 3.82 mg/g of dry biomass.

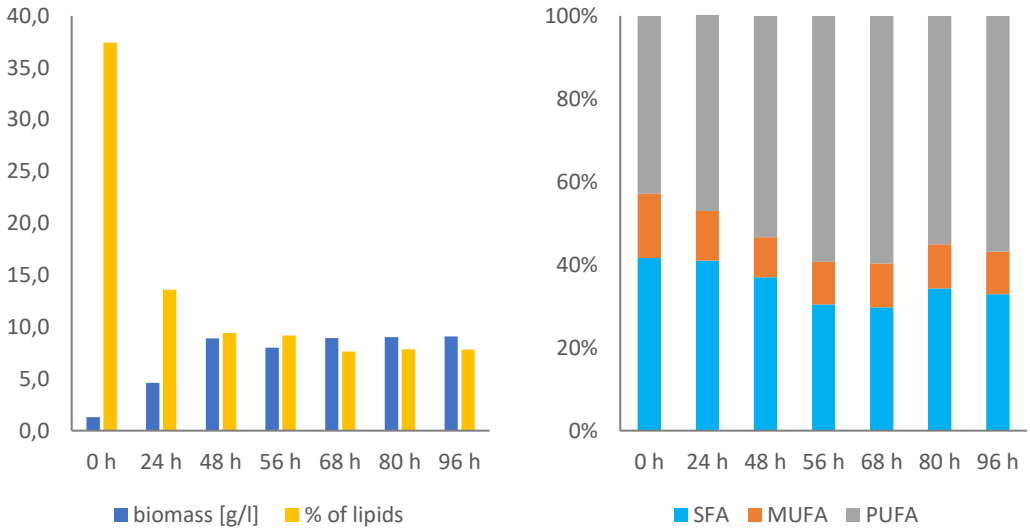
As in the previous case, the production of lipids by the yeast *R. mucilaginosa* shows the same parameters. The initial high lipid content is consumed on the first day of cultivation and drops to 13.58 % (Graph 5). In the following days of cultivation, the lipid content decreases to 7.82 % in the last 96 hours of cultivation. The fatty acid profile shows a stable tendency for the PUFA content, increasing to 60% by the 68th hour of cultivation, after which it stabilizes at an average of 55.56% in the final hours of cultivation. SFA production is gradually declining in favour of PUFA. In terms of MUFA, this yeast also maintains a stable production of 10-13% MUFA (Graph 5). Therefore, it can be stated that, within the genus *Rhodotorula*, this medium does not affect the production of monounsaturated fatty acids and the changes concern only the other two groups of fatty acids (PUFA and SFA). From the point of view of the overall yield of lipid production, this strain is completely unsuitable under these conditions. The best compromise for harvesting the culture is at 68 hours, where the culture produced the highest amounts of carotenoids and ubiquinone.

Table 23. R. mucilaginosa bioreactor cultivation SGC hydrolysate + coffee oil

Biomass [g/L]						
0 h	24 h	48 h	56 h	68 h	80 h	96 h
1.30	4.62	8.89	8.01	8.93	9.04	9.07



Graph 4. *R. mucilaginosa bioreactor cultivation on SGC hydrolysate and coffee oil: HPLC results*



Graph 5. *R. mucilaginosa bioreactor cultivation on SGC hydrolysate and coffee oil: lipid production and fatty acid profile*

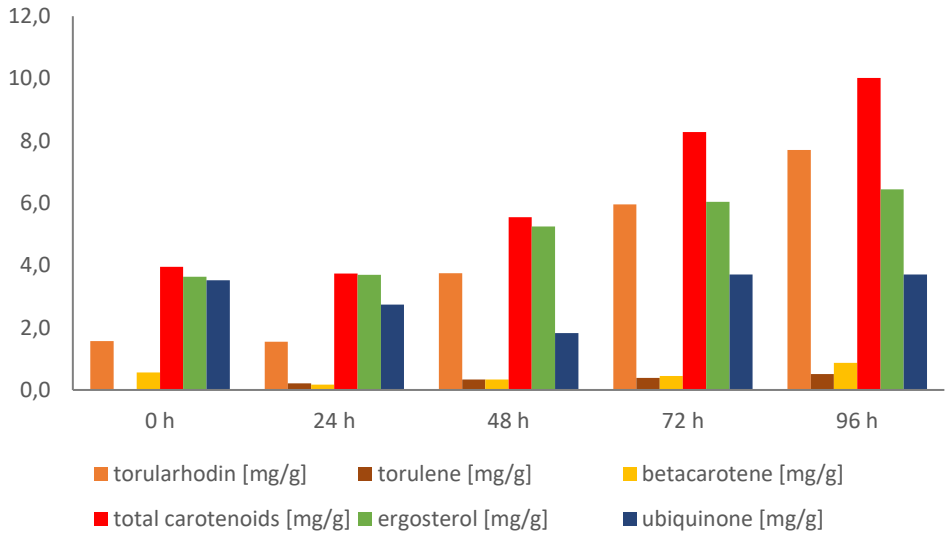
5.1.3.3.3 Rhodosporidium toruloides results:

The results show that this strain was able to better utilize the substrates in the medium. The culture's growth curve again shows a very fast exponential growth phase in the first two days of cultivation (Table 24). After depleting the carbohydrate source, growth is slowed down, and biomass growth reaches a maximum of 10.43 g/L at 96th hour of cultivation. The production of lipid substances has been steadily increasing from the outset. In the 96th hour of cultivation, carotenoids' production reaches 10.02 mg/g, ergosterol 6.44 mg/g and ubiquinone 3.71 mg/g dry biomass (Graph 6). From the overall trend of the obtained data, it can be assumed that by prolonging the cultivation in the case of this strain, an even higher production of all metabolites would be achieved. The profile of carotenoid pigments share the same features with previous cultures, and even here, torularhodin form the majority of the production of all carotenoids.

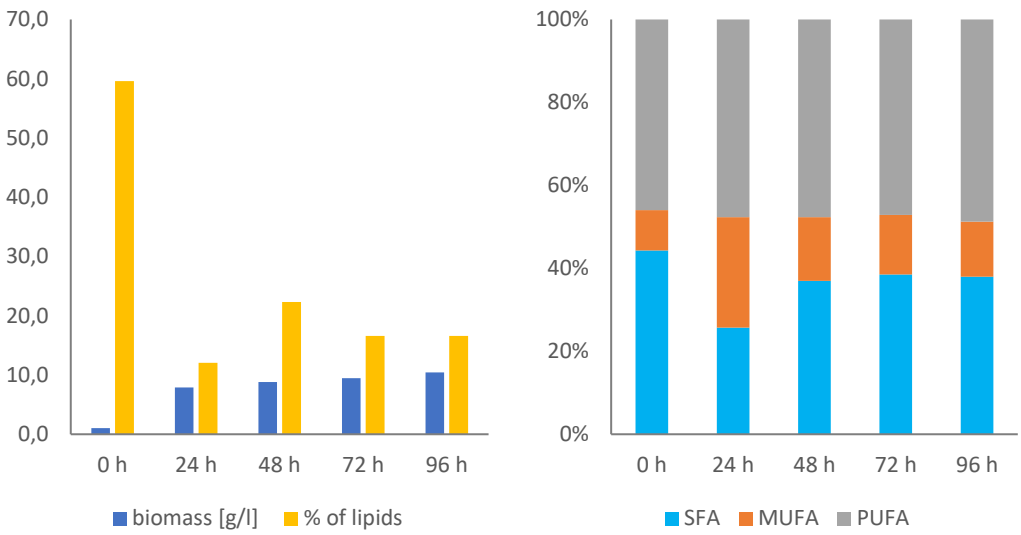
Lipid production of *R. toruloides* strains shows a declining character during the whole cultivation and reaches 16.58% at the end of the cultivation (Graph 7). In comparison with the related genus *Rhodotorula*, we see practically the same results in terms of lipid production with only slightly higher production, by 2%. On the other hand, we see significant differences in the profile of fatty acids. This yeast maintains a very stable fatty acid profile during growth, not counting sample at 24 hours. The PUFA content is 8-10% lower here than in the genus *Rhodotorula*. This decrease is slightly offset by an increase in the MUFA content by approximately 2-3%, but the greater part is in favour of SFA production. In terms of biotechnological use, the best time to harvest the culture is at the end of cultivation, where the highest production of carotenoids, ergosterol and ubiquinone was achieved.

Table 24. R. toruloides bioreactor cultivation SGC hydrolysate + coffee oil

Biomass [g/L]				
0 h	24 h	48 h	72 h	96 h
1.06	7.91	8.79	9.44	10.43



Graph 6. *R. toruloides bioreactor cultivation on SGC hydrolysate and coffee oil: HPLC results*



Graph 7. *R. toruloides bioreactor cultivation on SGC hydrolysate and coffee oil: lipid production and fatty acid profile*

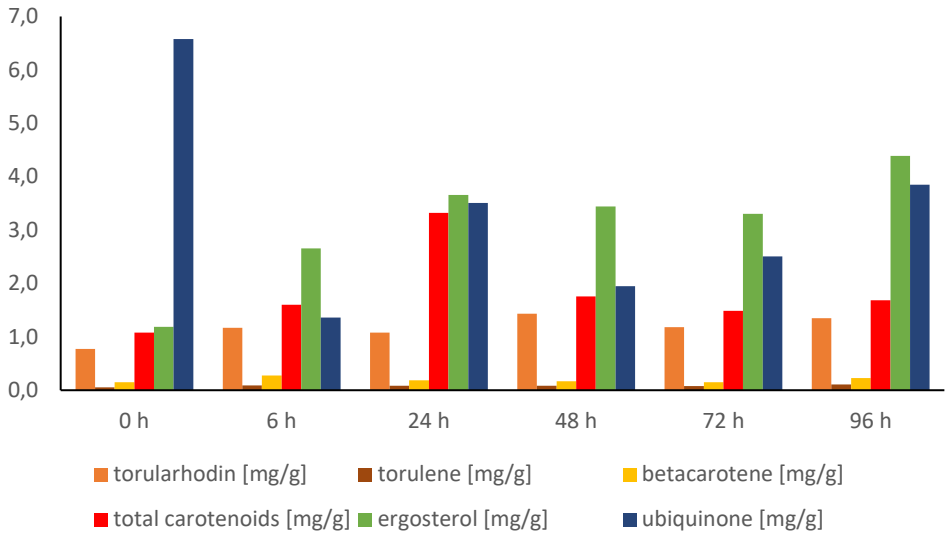
5.1.3.3.4 Cystofilobasidium macerans results

Compared to other strains, the *C. macerans* strain's cultivation results show the worst results in biomass production, reaching only 7.84 g/L. From the data, we see a growth stagnation between the 1st and 3rd day of cultivation. On the last day, the culture probably successfully reoriented its metabolism and started to process a complex oil substrate. In the case of pigment production, we see a comparable production with the *R. kratochvilovae* strain. The maximum output of carotenoids is 3.32 mg/g at 24 hours of cultivation (Graph 8), and then the production of carotenoids is practically halved. The maximum production of ergosterol (4.37 mg/g) and ubiquinone (3.85 mg/g) was on the last day of cultivation.

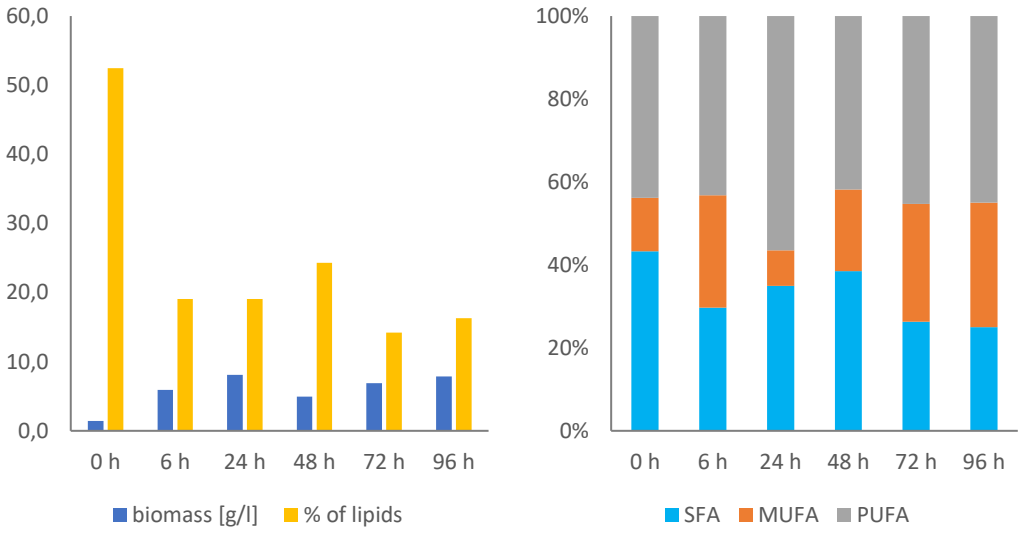
Data of lipid production show almost the same results as previous strains' cultivations (Graph 7 and Graph 5). The only exception is the first half of the cultivation, where the biomass's lipid content rises to 24.30% (Graph 9). However, it falls to 16.28% in the following days, which is a comparable production to previous strains. The fatty acid profile here shows a more significant fluctuation in the MUFA content. MUFA content drops to 8.53% on the first day, then increases significantly to 30.0% in the following days. The increased content of MUFA acids is at the SFA expense, whose production during cultivation continuously decreases to 25.01%. PUFA production is then stable in the 42-45% range for *C. macerans*. From an economic point of view, under the given cultivation conditions and results, the best time to harvest the culture would be the 24th hour, which is a compromise solution in terms of biomass production and all lipid substances.

Table 25. C. macerans bioreactor cultivation SGC hydrolysate + coffee oil

Biomass [g/L]					
0 h	6 h	24 h	48 h	72 h	96 h
1.44	5.92	7.09	6.94	6.90	7.84



Graph 8. *C. macerans bioreactor cultivation on SGC hydrolysate and coffee oil: HPLC results*



Graph 9. *C. macerans bioreactor cultivation on SGC hydrolysate and coffee oil: lipid production and fatty acid profile*

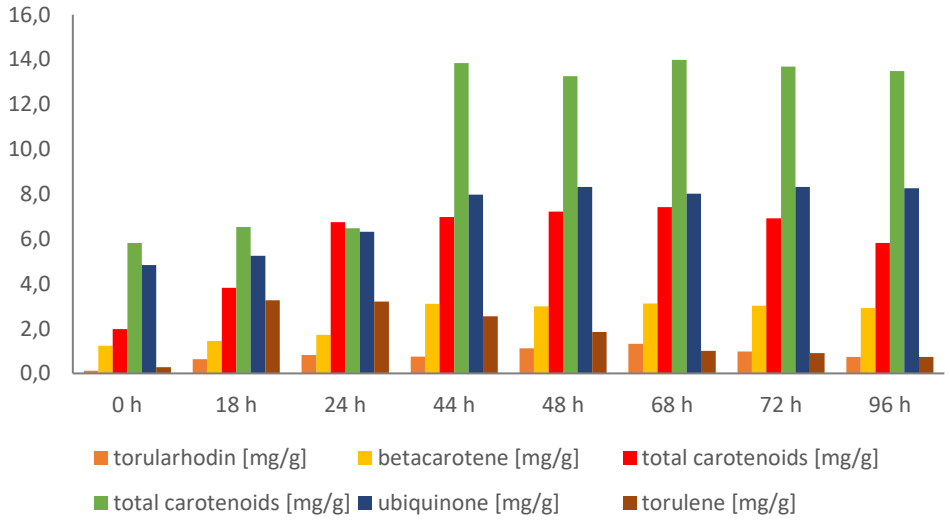
5.1.3.3.5 Sporidiobolus pararoseus results

The absolute best results of cultivation were achieved with the *S. pararoseus* strain. From the very beginning of cultivation, we observe a very rapid growth of the culture. From the results, we see that this yeast was able to adapt fast and best to coffee oil. The maximum biomass production is 23.40 g/L in the last sample at the end of cultivation (Table 26). Based on data growth, it can be assumed that if the cultivation is extended by a day or two, the increase in biomass would increase even further. From the data of the chromatographic analysis of lipids, we observe significantly high production of metabolites. From the beginning of the cultivation, carotenoids' content increases and reaches a peak of 7.22 mg/g in 48th hours of cultivation (Graph 10). In the following days, the production is more or less stable, and in the end, we observe a slight decrease in carotenoid content. The same trends follow ergosterol and ubiquinone production. Their production peaks at 44th hour (13.84 mg/g ergosterol). Moreover, at 48th hours yeast reaches a maximum ubiquinone production (8.32 mg/g). In the following days of cultivation, the content is stable and does not change.

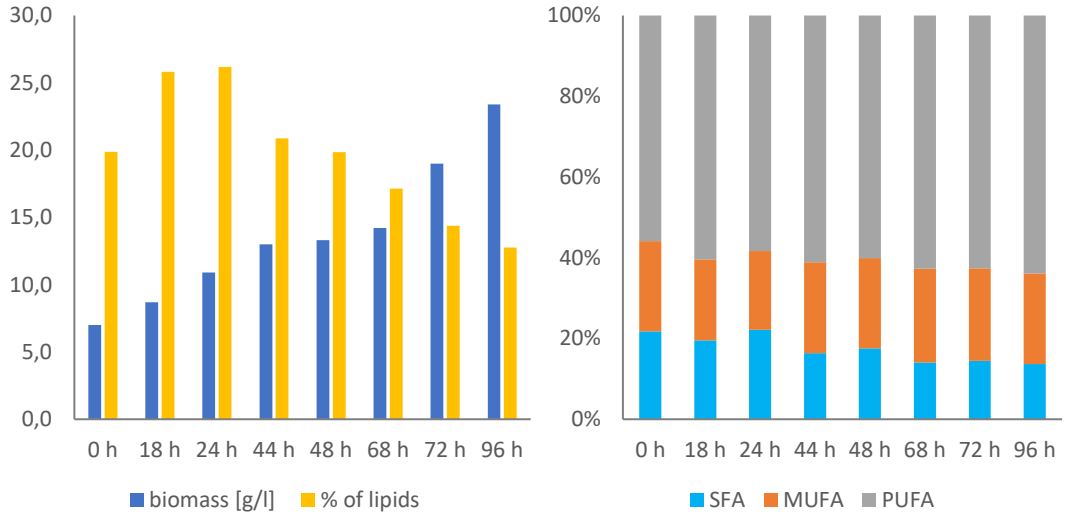
Lipid production data indicate differences from the previous four yeast strains. On the first day of cultivation we observe both the rapid growth of biomass and accumulation of lipids in the biomass with a maximum of 26.19% lipids at 24 hours (Graph 11). A linear decrease follows into 12.76% lipids in biomass, which is the lowest value of all tested strains. The fatty acid profile of the *S. pararoseus* strain is very consistent, and the PUFA content increases at the expense of SFA with increasing cultivation time. MUFA production is stable in the range of 22-24%. This strain showed the highest production of unsaturated fatty acids, which at the end of the cultivation make up 66.33% of lipids. From an economic point of view, the most appropriate time to harvest the culture is 96 hours. However, we see from the data that even in previous days, it would be possible to obtain high production, at the cost of losing a certain amount of biomass.

Table 26. Sporidiobolus pararoseus bioreactor cultivation SGC hydrolysate + coffee oil

Biomass [g/L]							
0 h	18 h	24 h	44 h	48 h	68 h	72 h	96 h
1.20	7.02	10.90	12.48	13.29	14.21	19.02	23.40



Graph 10. *S. pararoseus bioreactor cultivation on SGC hydrolysate and coffee oil: HPLC results*



Graph 11. *S. pararoseus bioreactor cultivation on SGC hydrolysate and coffee oil: lipid production and fatty acid profile*

From the overall point of view, this experiment's clear winner is the *S. pararoseus* strain, which surpassed the other tested strains in all monitored parameters. In other strains, the limitation was a lesser ability to adapt and utilize the complex substrate and lesser resistance to antimicrobials from coffee present in the medium. Due to the low production of lipids in all tested strains, this medium is not suitable for the production of lipids and is more suitable for production of lipid substances (carotenoids, sterols, ubiquinone).

5.1.3.4 *Cultivation on media containing SCG hydrolysate and waste frying oil*

As part of the testing of various complex lipid sources, a series of bioreactor cultivations were performed. The yeast was cultivated under the same conditions as in the previous experiment. In this experiment, however, coffee oil was replaced by waste frying oil. The aim was to test how much the presence of phenolic substances derived from coffee oil affects yeast's growth also to compare growth and production properties of liquid lipid waste with solid lipid waste (animal fat) The general composition of frying and coffee oil in terms of a fatty acid profile is relatively similar. Simultaneously, coffee oil is a valuable commodity in its composition, which could be better applied in its natural form in the cosmetic or food industry. The following graphs and tables summarize the results of cultivation five strains of carotenogenic yeasts. Cultivation was performed on a medium with the same C/N ratio and the same ratio of saccharide SCG hydrolyzate and waste frying oil.

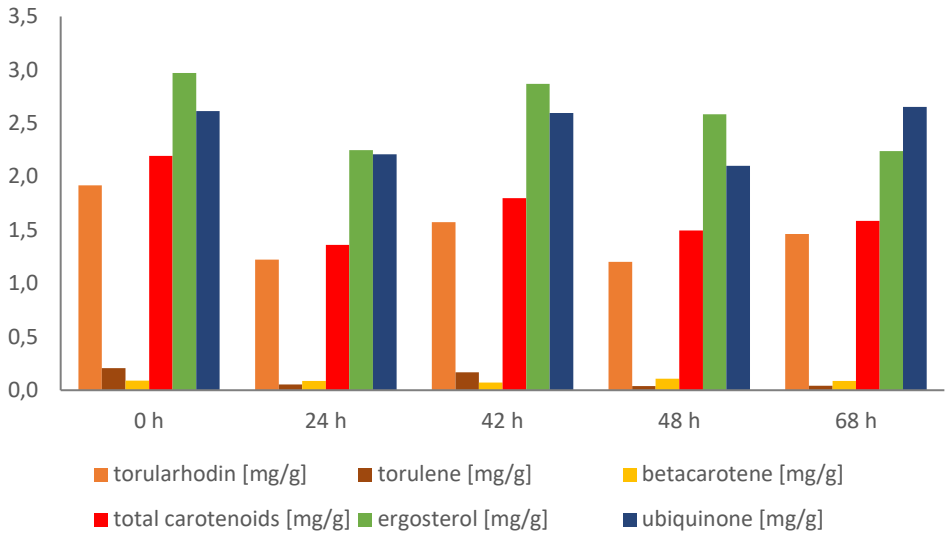
5.1.3.4.1 Rhodotorula kratochvilovae results

In the case of cultivation of the yeast *R. kratochvilovae*, a very low increase in biomass was observed from the beginning, which continued until the premature termination of the cultivation at 68 hours. Microscopic observations did not detect any external influence/contamination, and it is likely that the yeast culture was somehow inhibited and could not produce and grow naturally. The phenolic substances present in the medium may have partially contributed to this. As part of this cultivation, the samples taken were analyzed and provided partial information. The production of carotenoid pigments has a decreasing character during cultivation and decreases from the original 2.19 mg/g to 1.59 mg/g (Graph 12). As the vitality of the cells deteriorates, the content of ergosterol in the cells also decreases. Only ubiquinone production shows a relatively stable trend and at the end of the experiment is comparable to the initial concentration. As part of the experiment, it would be appropriate to repeat this cultivation.

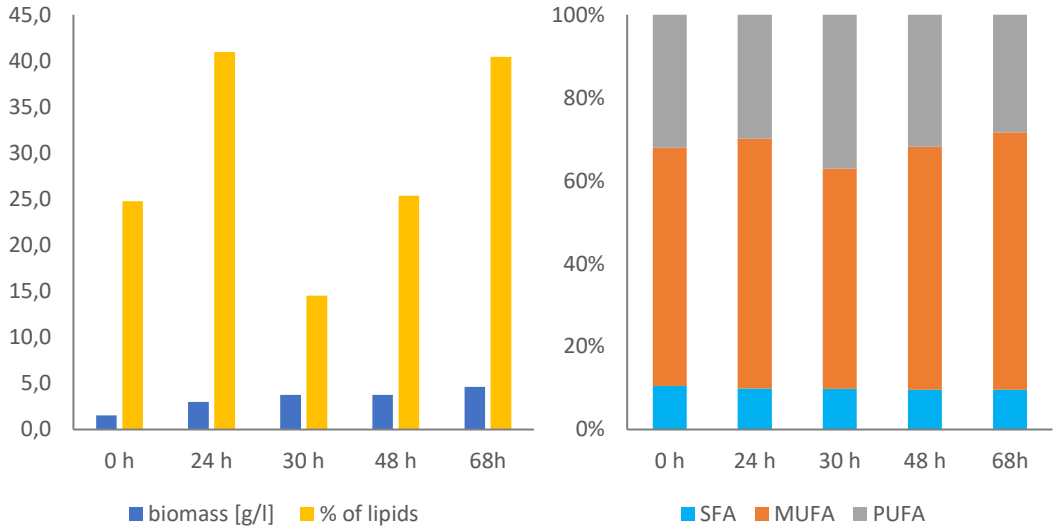
The GC analysis of lipids of the first cultivation on waste frying oil shows a high production of lipids. Although biomass production is reduced, on the other hand, this strain achieves a lipid production of 40.45% in the 68th hour of cultivation (Graph 13). It can be assumed that by prolonging the cultivation, lipid content would decrease slightly, but it will still be much higher than this strain was able to produce on a medium with coffee oil (Graph 3). The fatty acid profile is diametrically different in this culture. In this cultivation, monounsaturated fatty acids predominate, which make up 56-62% of all lipids' content. Furthermore, SFA production is significantly lower, and it is only on average 10%. The total fatty acid content thus consists of more than 90% of unsaturated fatty acids.

Table 27. R. kratochvilovae bioreactor cultivation SCG hydrolysate + frying oil

Biomass [g/L]				
0 h	24 h	42 h	48 h	68 h
1.55	3.00	3.58	3.75	4.64



Graph 12. *R. kratochvilovae bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 13. *R. kratochvilovae bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production and fatty acid profile*

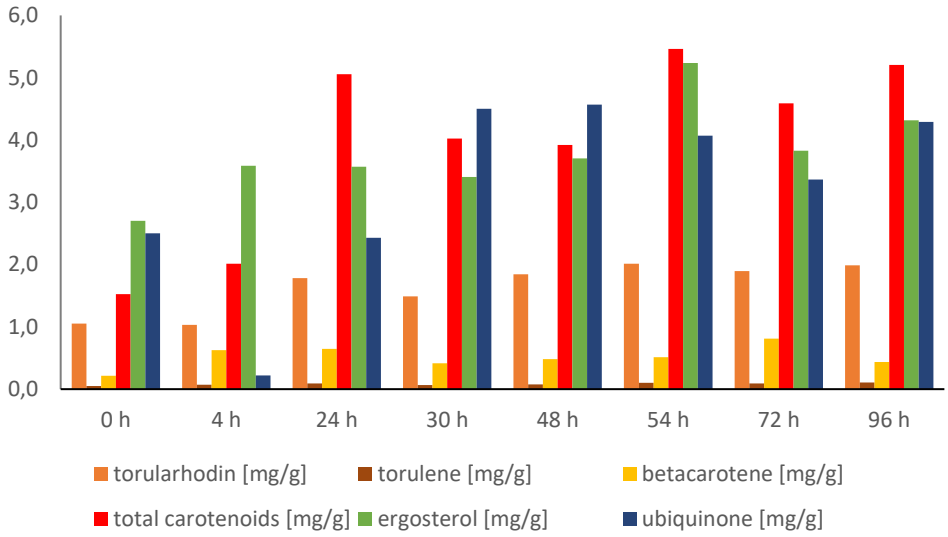
5.1.3.4.2 Rhodotorula mucilaginosa results

The results of cultivation with waste frying oil show much better conditions for cultivation of this yeast. Biomass production is more than 50% higher than that of coffee oil and reaches a maximum of 16.09 g/L at 96th hour of cultivation (Table 28). Here again, we see a rapid increase in biomass production in the exponential growth phase on the first day. With the depletion of carbohydrates in the medium, the culture switches to oil processing and the growth curve flattens out more. From the point of view of carotenoid production, the yeast was more successful in this medium. In general, the content of carotenoids during the whole cultivation is higher than on the medium with coffee oil. The maximum production of 5.46 mg/g dry biomass is reached at 54 hours of cultivation (Graph 14). At this time, the highest ergosterol production of 5.23 mg/g was also recorded.

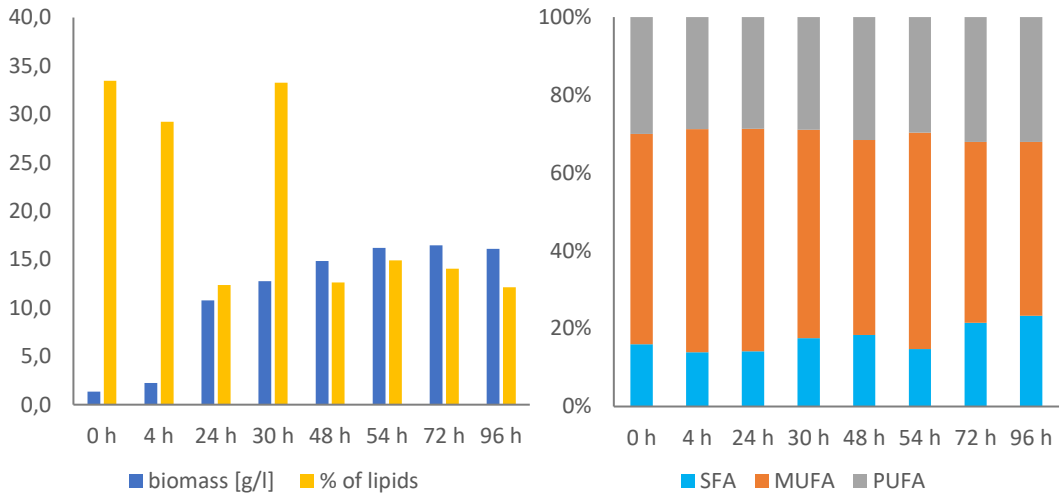
Lipid production data show a classic trend consistent with cultivating the strain in coffee oil. Here, too, in the first days of cultivation, the lipid content decreases, which gradually decreases to 12.15% of lipids in the biomass at the end of the cultivation. An exception here is the sample at 30 hours, where we see a jump in lipid content to 33.27% (Graph 15). The fatty acid profile of *R. mucilaginosa* copies the results of *R. kratochvilovae* yeast. Here, too, we see a high content of monounsaturated fatty acids in waste oil, making up 45-54% of the lipid content. Again, we see a reduced SFA production, which is slightly lesser, but it increases to 23.27% in the end of cultivation. Thus, it can be said that in comparison with the results on coffee oil, the production of unsaturated fatty acids was reversed and overall, their content decreased slightly in favour of saturated fatty acids. From an economic point of view, the most suitable time to harvest the culture is 54 hours, when the culture also contained a very high concentration of ubiquinone, namely 4.07 mg/g.

Table 28. R. mucilaginosa bioreactor cultivation SCG hydrolysate + frying oil

Biomass [g/L]							
0 h	4 h	24 h	30 h	48 h	54 h	72 h	96 h
1.35	2.24	10.78	12.75	14.85	16.21	16.45	16.09



Graph 14. *R. mucilaginosa bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 15. *R. mucilaginosa bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production and fatty acid profile*

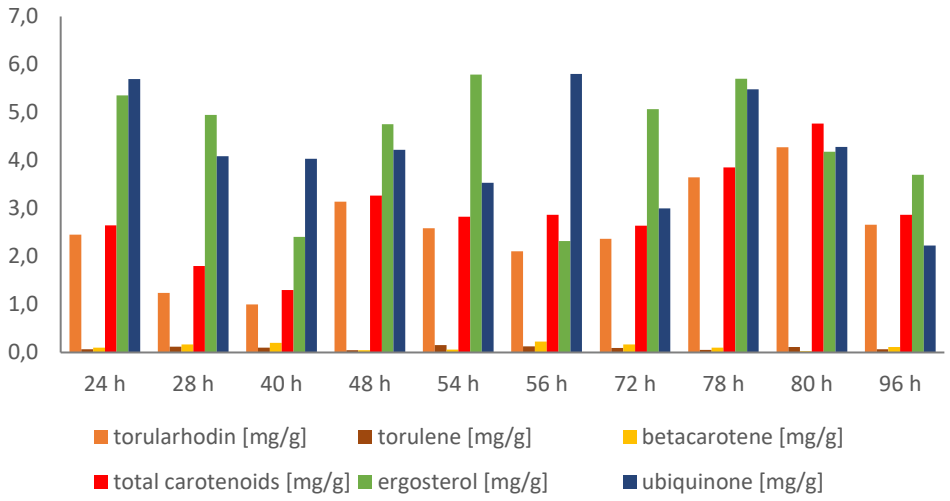
5.1.3.4.3 Rhodosporidium toruloides results

From the results of the biomass growth of the yeast *R. toruloides,* we observe, that the growth is slowed down from the beginning of the cultivation. The yeast reaches a maximum production of 11.08 g/L on the 3rd day of cultivation, and then there is a significant slowdown in growth the next day (Table 29). The maximum of 11.75 g/L is only about 10% higher than in medium with coffee oil. From the HPLC analysis data, we see a relatively large fluctuation in the production of individual metabolites. Multiple maxima can be identified for all metabolites during cultivation, but they do not overlap in one sample. Maximum carotenoid production of 4.77 mg/g reached culture at 80 hours (Graph 16). Here, too was torularhodin the primary pigment produced. In contrast, a maximum ergosterol production of 5.79 mg/g was measured at 54 hours. Maxima of ubiquinone production 5.80 mg/g of dry biomass culture reached at 56th hour.

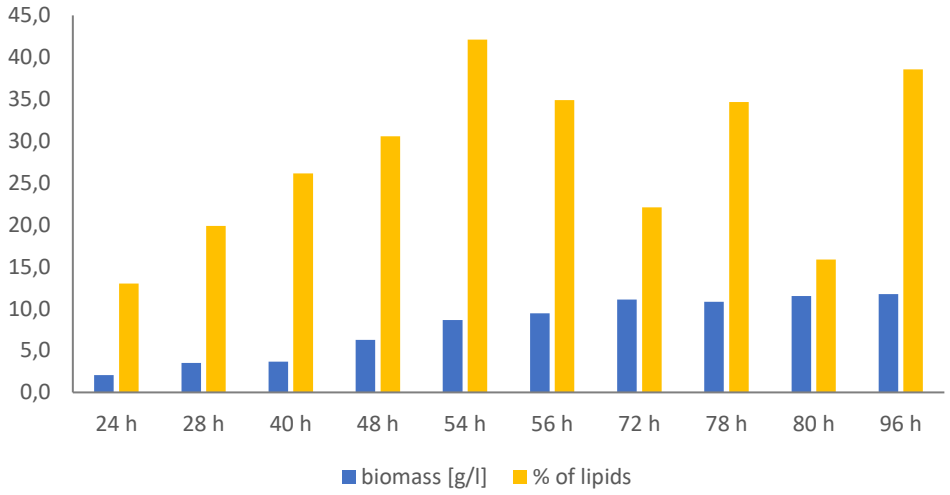
Chromatographic analysis of lipid production and fatty acid profiles indicates the potential of this yeast for lipid production. During the cells' cultivation, the lipid content increases to 42.12% at 54 hours, and after a decrease in the following hours, it reaches two more maxima 34.65% at 78 hours and 38.56% at 96 hours of cultivation (Graph 17). The fatty acid profile of *R. toruloides* shows a very stable production of unsaturated fatty acids, which, with a few exceptions, reach more than 90% of the total fatty acid content throughout the cultivation (Graph 18). From this point of view, it is possible to use this strain to produce lipids with a very high content of unsaturated fatty acids. From the overall economic point of view, the best time to collect is the end of cultivation at 78th hour with sufficient biomass production. Simultaneously, even at this time, the culture achieved an average of high production of all studied metabolites and even lipids.

Table 29. R. toruloides bioreactor cultivation SCG hydrolysate + frying oil

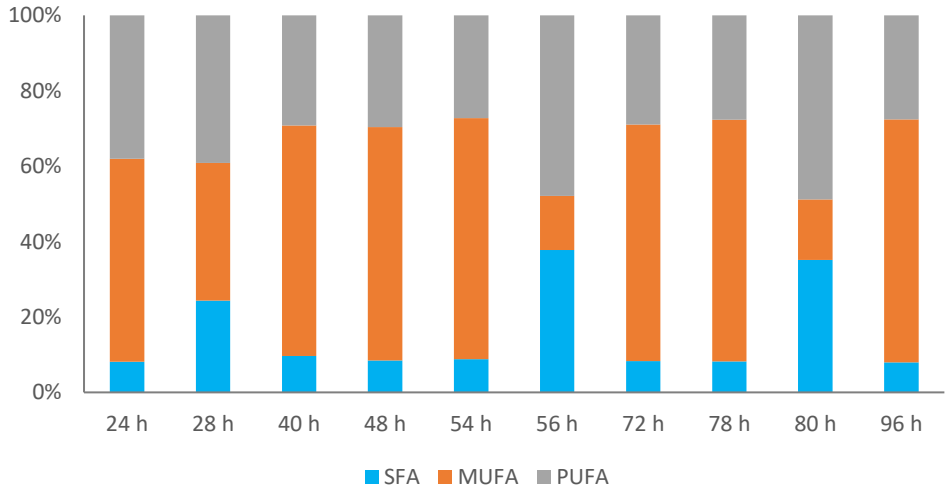
Biomass [g/L]									
24 h	28 h	40 h	48 h	54 h	56 h	72 h	78 h	80 h	96 h
2.06	3.50	3.68	6.25	8.63	9.45	11.08	10.80	11.50	11.75



Graph 16. *R. toruloides bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 17. *R. toruloides bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production*



Graph 18. *R. toruloides bioreactor cultivation on SGC hydrolysate and waste frying oil: fatty acid profile*

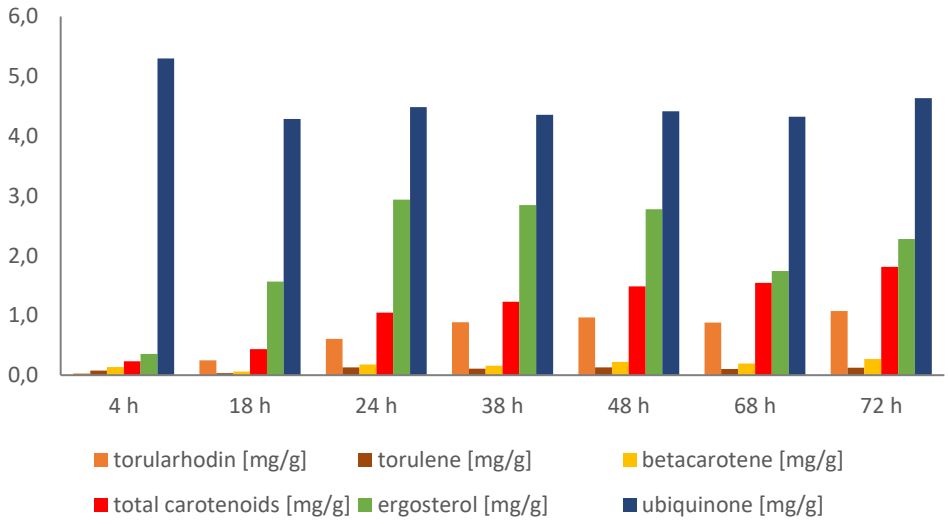
5.1.3.4.4 Cystofilobasidium macerans results

C. macerans performed incomparably better in the medium with waste frying oil. While on the coffee oil medium, the yeast biomass production was only 7.48 g/L. In this experiment, the biomass production reached 19.40 g/L at 72 hours (Table 30). This experiment was terminated prematurely due to incipient contamination of the culture, which was recorded by microscopic inspection of a sample from 72 hours of cultivation. However, we can say with certainty that in a classical 96-hour experiment, yeast culture would increase biomass production and exceed the limit of 21-22 g/L. Rapid growth and biomass production were at the expense of the carotenoid production. In this experiment, yeast's carotenoid content is very low and is maintained in the range of 1.5-1.7 mg/g of dry biomass, which is slightly lower than the medium with coffee oil. Likewise, ergosterol production has decreased and does not exceed 3.0 mg/g of dry biomass. (Graph 19). On the other hand, there was a significant increase in the ubiquinone content inside the cell in this cultivation, which was around 4.2 mg/g of dry biomass throughout the cultivation. Maximum production of 4.63 mg/g is reached at 72 hours of cultivation.

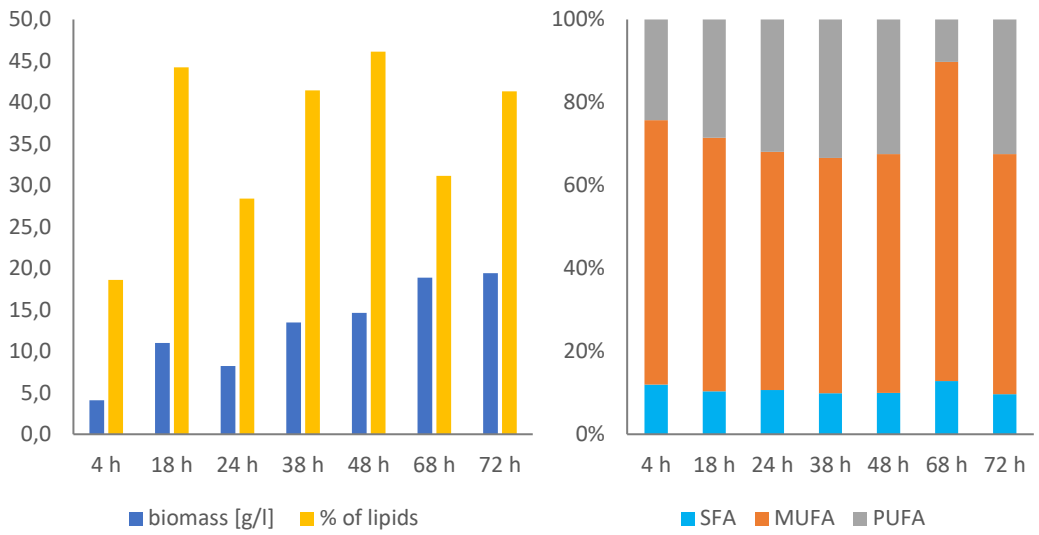
The positive effect of waste frying oil is also observed in *C. macerans* cultivation (Graph 20). We observe a reduction in the SFA content compared to the coffee oil medium. The production of saturated fatty acids is stable in the range of 10-13%. A high amount of MUFA accumulates, and their production reaches 55-60% of total lipids. Together with PUFA, they make up more than 85% of unsaturated fatty acids (Graph 20). As in the case of cultivation the *R. toruloides* strain, there is a high production of lipids in the cell biomass. In this case, the yeast was able to produce a high amount of biomass and at the same time, high content of lipids. At 72 hours of cultivation, the production reaches 41.34% of lipids in the biomass and is the most economically appropriate time to end the cultivation (within the cultivation time interval). Within this sample, the yeast also achieved high productions of ergosterol, ubiquinone I and carotenoids. We see that this medium is the best for this yeast, of all the tested media.

Table 30. C. macerans bioreactor cultivation SCG hydrolysate + frying oil

Biomass [g/L]						
4 h	18 h	24 h	38 h	48 h	68 h	72 h
4.12	11.01	8.23	13.48	14.63	18.87	19.40



Graph 19. *C. macerans bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 20. *C. macerans bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production and fatty acid profile*

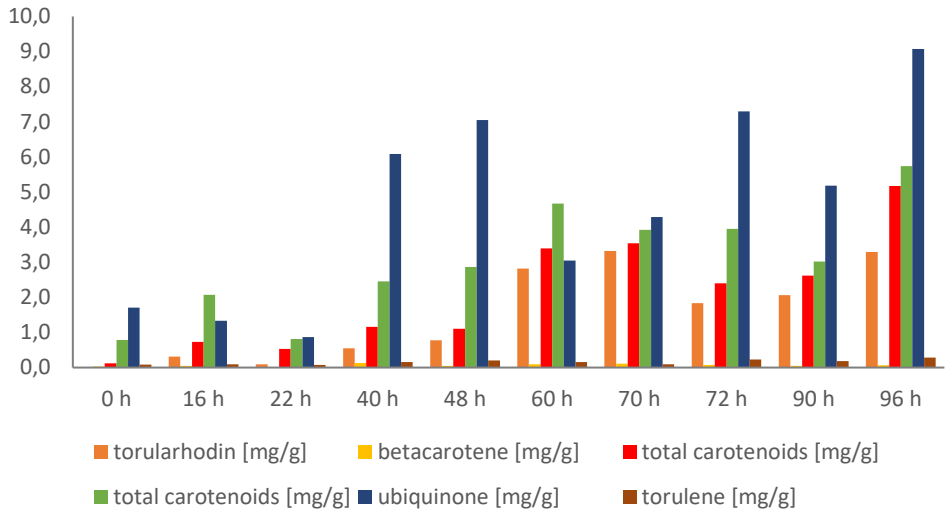
5.1.3.4.5 Sporidiobolus pararoseus results

In Table 31, we see the results of the yeast *S. pararoseus* cultivation. As in the previous experiment with coffee oil, the yeast achieved by far the highest biomass productivity, 21.360 g/L of media in this experiment. On the first day of cultivation, we see a very rapid increase in biomass production. After depletion of the carbohydrate source, the metabolism quickly adapted to oil, and from the beginning of the 3rd day of cultivation, we observe a practically continuous growth of biomass. In terms of the production of lipid substances, we see very low production on the first day. On the second day, there is a gradual increase in all analyzed substances. Carotenoid production reached a maximum of 5.17 mg/g in the last sample at 96 hours. The highest production of ergosterol (5.73 mg/g) and ubiquinone (9.08 mg/g) was also determined in the same sample (Graph 21). Otherwise, the production of ergosterol and especially ubiquinone shows a relatively large fluctuation.

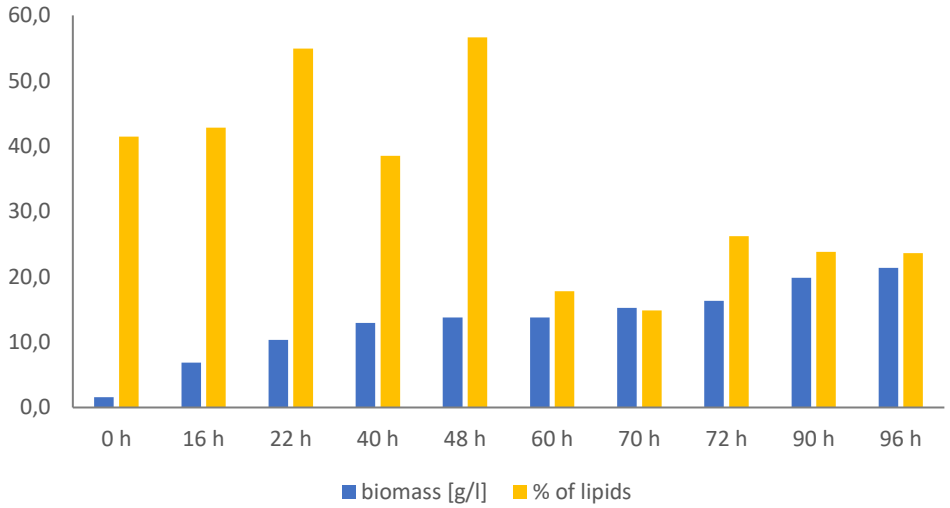
The lipid analysis results of the *S. pararoseus* strain showed a very high increase in the first 48 hours of cultivation, where the lipid content reached 56.65% of the lipids in the biomass (Graph 22). In the second half of the cultivation, there is a dramatic decrease of lipid content. At the end of the cultivation, the lipid production reaches 23.60% of lipids in the biomass. Even in this case, the fatty acid profile during cultivation consists of more than 90% unsaturated fatty acids (Graph 23). Only during the 3rd day of cultivation, we see an increase in SFA production to 33.24%. Immediately afterwards, the fatty acid profile stabilizes again. Within the production of lipids, the most suitable is the second day of cultivation, where the yeast reached the peak of lipid production and at the same time high biomass production of 13,78 g/L of media. The best time to produce the remaining metabolites is 96th hour. Here, the yeast reached the maximum production of biomass and lipid metabolites (carotenoids, ergosterol and ubiquinone).

Table 31. S. pararoseus bioreactor cultivation SCG hydrolysate + frying oil

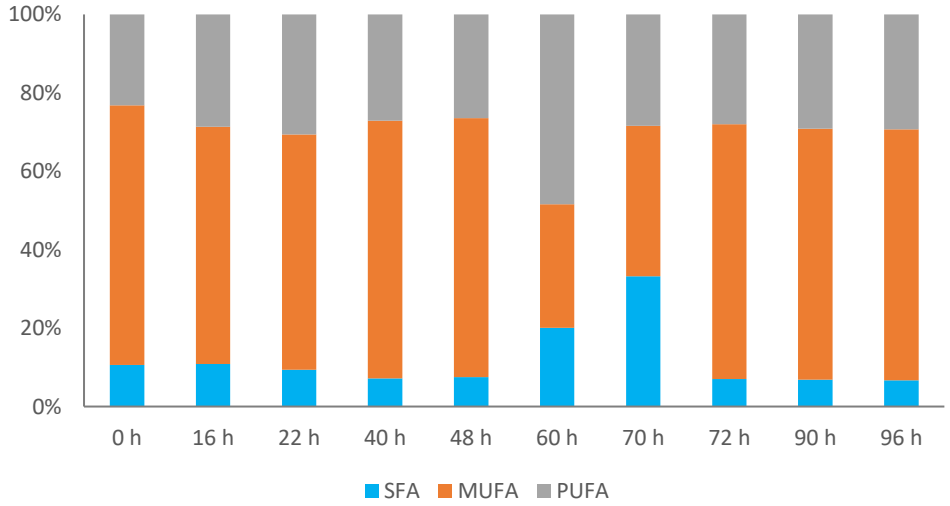
Biomass [g/L]									
0 h	16 h	22 h	40 h	48 h	60 h	70 h	72 h	90 h	96 h
1.59	6.87	10.33	12.93	13.80	13.75	15.25	16.34	19.84	21.36



Graph 21. *S. pararoseus bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 22. *S. pararoseus bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production*



Graph 23. *S. pararoseus bioreactor cultivation on SGC hydrolysate and waste frying oil: fatty acid profile*

By comparing the results of individual yeast strains, it was found that on media containing waste frying oil, except for the strain *R. kratochvilovae*, higher yields of biomass and the studied metabolites were achieved. Thus, it can be said that the presence of antimicrobial phenolic substances had a certain effect on the growth of yeast. However, this inhibition's strength cannot be unambiguously determined here, because, as can be seen from the results, each strain reacted differently. From the overall point of view, the best strain was a representative of the genus *Sporidiobolus*, which achieved the highest increase in biomass on both media and at the same time the production of carotenoids, ergosterol and ubiquinone. By suitable optimization of the cultivation conditions and the composition of the medium, it would be possible to increase further the production of this strain and other representatives of the tested yeast strains.

5.1.4 Partial conclusions - Yeast cultivation

Experimental cultivations of carotenogenic yeasts focused on optimizing the culture conditions, medium composition and selection of suitable strains to find optimal producers of carotenoids, microbial lipids and other substances. This work aimed to test further the possibilities of replacing nitrogen and carbon sources with waste from the food and agricultural industries. The following wastes were selected for testing: animal fat, dairy whey, glycerol and spent coffee grounds. The following representatives of *Rhodotorula kratochvilovae*, *Rhodotorula mucilaginosa*, *Rhodosporidium toruloides*, *Sporidiobolus pararoseus* and *Cystofilobasidium macerans* were tested.

Pilot cultivations of yeast on untreated substrates indicated insufficient or missing enzymatic equipment capable of processing these materials. Only glycerol was able to be processed by yeast without problems and achieved comparable or higher biomass production compared to the control medium. The production of carotenoids, ergosterol and ubiquinone was slightly reduced on glycerol media. In terms of lipid production, it was found that this substrate is not suitable and the yeast generally achieved very low production. The remaining waste substrates were then unable or only very slowly to process the substrate. Based on these data, three basic types of hydrolysis were tested in order to modify the substrate into a form on which the yeast could grow very quickly and produce the desired metabolites.

Cultivations on waste animal fat took place on two hydrolysates: enzymatic and basic hydrolyzate. In these experiments, it was found that yeast processes hydrolyzed fat very well. Cultivations on enzymatic hydrolyzate were characterized by high production of biomass and metabolites in all studied strains. In cultivations on basic hydrolyzed fat, it has been found that fully hydrolyzed fat is not a suitable substrate because of too high concentration of fatty acids formed in the medium, which strongly inhibits yeast growth and productivity. It was found that the concentration of fatty acids in the medium is tolerable for selected yeast strains only up to C/N ratio 50. Only in yeasts of the genus *Rhodotorula* a higher degree of resistance to this effect was found. Thus, partially hydrolyzed fat was used in the following cultivations. In general, higher production of all metabolites was observed on all fat media compared to the control glucose medium, at the expense of biomass production. Lipid production was positively induced on the fat media, and the strains achieved higher lipid production compared to the control glucose medium. The best results of lipid production were achieved by the *S. pararesus* strain, which accumulated more than 45% of the lipid content in biomass on fat media.

Cultivation with whey hydrolysate showed relatively stable trends within the studied strains. For yeast, this substrate was an excellent source, as confirmed by the results of biomass production, where all strains reached at least comparable production with the control medium, and in many cases, we observe 1.5 to 3 times higher production of biomass. With increasing C/N ratio, biomass production continued to increase. In terms of metabolite production, whey was a suitable substrate for the production of sterols, ubiquinone and carotenoids. All strains showed higher production of these metabolites, especially in media with a C/N ratio of 13 and 25. In terms of lipid production, the results show that whey is not a suitable substrate. On average, all strains achieved very low lipid production.

The last substrate tested was coffee grounds hydrolyzate. The results of the flask cultivations clearly showed that this treated material is very good source of organic carbon, due to high content of monosaccharides. Biomass production was higher in all strains compared to the control medium. The best strain was *S. pararoseus*, which produced more than 10 g/L in all flask cultivations on the coffee hydrolyzate. The maximum production is then 17 g/L, which is a very high value in flask cultures. The C/N ratio strongly influenced metabolite production. From the results, we see that with increasing C/N ratio the total production of all metabolites decreases significantly. As with the whey hydrolyzate, lipid production was negatively affected and, in general, the strains showed 5-8% less lipids.

Based on data from cultivations with partially hydrolyzed fat, the procedure was modified and combined carbon sources were used in high-volume cultivations, one substrate for rapid biomass growth and the other inducing increased metabolite production in the stationary phase. Large-scale cultivations began with a combination of whey hydrolyzate and animal fat. Biomass production and growth rate were very different within the strains. At the beginning of cultivation, all strains had a rapid increase in biomass due to the carbohydrate source, resulting in very high total biomass production. Cross-strain differences were observed in the subsequent phase of adaptation to the fat substrate. Metabolite production increased linearly during growth in all strains studied, except for the *C. macerans* strain. However, the total metabolite production was low. From the overall point of view, it is necessary to optimize the cultivation conditions on this substrates' combination.

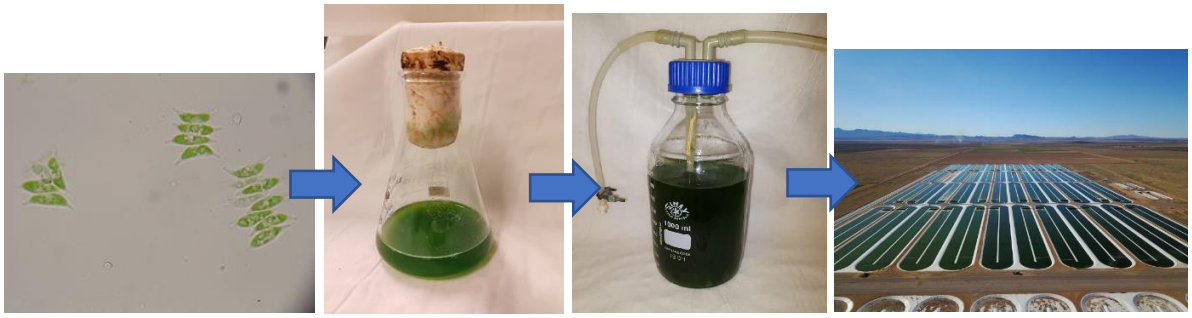
In another series of cultivations, whey hydrolyzate was replaced by coffee hydrolyzate. In these experiments, larger differences were observed between the tested strains. In general, the yeast strains were limited by the presence of phenolic substances and achieved lower production biomass, which was compensated by higher production of carotenoids, ergosterol and ubiquinone. Compared to the first cultivations, coffee hydrolyzate had a positive effect on yeast lipid production. In general, cultivation on combined fat substrates had limitations in the reduced production of biomass and metabolites of interest compared to the experiment with other fat sources. Limitations may be caused by the fat insolubility, and it would be appropriate to optimize this issue.

In the following tests, based on fat media results, coffee oil and waste frying oil were tested, which are liquid under the given cultivation conditions and their accessibility to yeast is thus greater. Cultivations on these media generally show higher production and can therefore be said to be a more suitable combination. The presence of coffee oil increased the concentration of antimicrobials in the medium and reduced yeast biomass production. By replacing coffee oil with waste frying oil, increased biomass production was achieved in all strains. At the same time, frying oil had a significant effect on the composition of fatty acids. Whereas, in the presence of coffee oil, the yeast preferred the production of PUFA, which contained 45-55% of the fatty acid content and together with MUFA made up approximately 75-85% of all fatty acids. A significant turnaround was observed in waste frying oil experiments, where the production of MUFA increased to 60-65% of the fatty acid content and together with PUFA they formed on average 96-91% of all fatty acids.

In both cases, the yeast showed very high lipid production and based on these data, we can say that for biotechnological production of lipids, these two media are most suitable for yeast, with the possibility of overproduction of a certain group of fatty acids by simply choosing a substrate. Besides, yeasts also showed very high production of other metabolites (carotenoids, ergosterol, ubiquinone) in these media. Overall, the best strain on coffee hydrolysates was the *S. pararoseus* strain, which produced more than 20 g/L of biomass on both types and also the highest production of monitored metabolites (9.0 mg/g ubiquinone, 5.1 mg/g carotenoids and 5.73 mg/g ergosterol per medium with frying oil).

In conclusion, we can summarize that the studied yeasts were able to utilize the treated waste substrates. Experimental data show that under selected conditions, the production of biomass and metabolites was high. Further optimization of cultivation conditions (medium composition, ratios of waste substrates, temperature) can achieve higher yields on all types of cultivations. Overall, the best combination was media with coffee hydrolysate and coffee/frying oil. Yeast grown on waste has a huge potential for the processing of waste from the food and agricultural industries and producing biomass enriched with carotenoids, lipids and many valuable substances.

5.2 Microalgae cultivation results



Scheme 15. *Microalgae cultivation process graphical scheme*

5.2.1 Microalgae cultivation in Brno

The following chapter shows a summary of results of microalgae and cyanobacteria cultivation performed in the laboratories of Faculty of Chemistry Brno Unversity of Technology. Cultivation of microalgae and cyanobacteria was performed in several types of cultivation flasks. In addition to classical cultivation in closed Erlenmeyer flasks, in selected experiments, the strains were cultivated in aerated Pyrex flasks, a bioreactor and a multicultivator. The experiment was focused on the study of the influence of physical, chemical and biological stresses on the growth and production of the studied metabolites. Cultivation of microalgae and cyanobacteria strains took place according to the following scheme:

1. Preparation of stock culture
2. Growth testing on various media
3. Selection of a suitable nitrogen source
4. Cultivation at different nitrogen concentrations
5. Cultivation at different concentrations of phosphorus
6. Cultivation under various stress conditions (salt, oxidative, light)
7. Temperature stress, cultivation
8. Mixotrophy testing - cultivation without access to light with an organic carbon source (glucose, glycerol)
9. Tests of tolerance of the presence of an organic carbon source in the medium under classical conditions

As the experiments progressed, the medium's composition and the cultivation conditions were optimized based on the results. The following section shows selected results from all cultivations performed. The following graphs show the results of microalgae and cyanobacteria cultivation of cultivated in a multicultivator on BBM medium with two different nitrogen sources. Unless otherwise stated, the term microalgae hereinafter refers to cultivation experiments performed on microalgae and cyanobacteria.

5.2.1.1 *Microalgae cultivation on different media types*

In this experiment, microalgae were tested on different media (BBM, BG11, *Spirulina*) and their modified versions in order to find the best medium for a given strain. Cultivations were performed in Erlenmeyer flasks at room temperature. Biomass growth and optical density were monitored during the experiment. Culture samples were inspected microscopically to exclude contamination. The obtained biomass was then analyzed chromatographically to determine the lipid content and the pigment's production. Based on this simplified experiment, it was observed that all tested microalgal strains were able to grow best on BBM medium. In *Chlorella* and *Chlamydomonas*, the growth and production of metabolites in BG11 medium were significantly lower.

Only the genera *Desmodesmus* and *Scenedesmus* cultivation results on both BBM and BG11 were practically comparable. The results of cyanobacterial cultivation had a similar trend. The *Synechococcus nidulans* strain had comparable results on both BBM and BG11 medium. Exceptions here are representatives of the genus *Arthrospira*, who strictly require *Spirulina* medium because there was a rapid death of the cyanobacteria observed on other tested media.

Based on the results, the BBM medium was chosen as the compromise solution for most strains (Except for *Arthrospira*). Thanks to this, the growth and productivity of individual strains can then be better compared. After the cultivation medium was selected, the experiment moved further towards testing the medium's basic components. The nitrogen source in the media of microalgae and cyanobacteria consists of an inorganic form. The choice of a suitable nitrogen source is extremely important in terms of growth and production of microalgae and the economics of the whole process. Therefore, three nitrogen sources were chosen: ammonium sulphate, urea and sodium nitrate. Furthermore, simple organic sources of the amino acid were also tested. In the experiment, cultivations with glycine as a nitrogen source were performed. All strains were then cultivated on BBM medium with different nitrogen sources. The results show that each tested strain utilised all forms of inorganic nitrogen and some of them to a small extent, also nitrogen in the organic form .

The cultivation results show that a large effect on the overall increase and production of metabolites in microalgae has a pH of the medium associated with the nitrogen source. Most microalgae prefer a pH in the neutral to the slightly basic range. Ammonium sulphate cultivations initially showed identical growth characteristics to the other two nitrogen sources. Unfortunately, in the long course of cultivation, the medium's pH decreased, and in many strains, growth inhibition was observed, and eventually death of the culture, or contamination by foreign microorganisms [221]. This phenomenon is due to the ammonium sulphate structure, the processing of which (removal of the ammonium group) releases the sulphate anion into the medium. It gradually reduces the pH of the medium outside the pH optimum of the microalgae. The best results were obtained with representatives of the genus *Scenedesmus*, who showed the greatest resistance to pH changes.

Microalgae better utilized the other two nitrogen sources. Urea had virtually zero effect on pH change because microalgae consume both components (NH^+ and HCO_3^-). In the case of sodium nitrate, a slight increase in the medium's pH has been observed in the long term cultivation. Thus, in other experiments dealing with the effect of the medium's nitrogen concentration, only sodium nitrate and urea were used.

5.2.1.2 *Multicultivator cultivation with different nitrogen sources*

In these tests, microalgae and cyanobacteria were cultivated in a multicultivator according to the following scheme. The eight culture tubes were divided into two sets of four, each set of tubes containing 60, 80, 100 and 150% of the given nitrogen source. Cultivation was performed for 14 days at room temperature and alternating ligh cycle (light: dark 16:8 hours) with gradually increasing light intensity. Optical absorbance data at 680 nm were recorded during cultivation. After cultivation, the centrifuged biomass was lyophilized and then analyzed by liquid and gas chromatography. The following graphs show selected biomass and metabolite production results of *Limnospira maxima*, *Desmodesmus armatus* and *Desmodesmus velitaris* strains cultivation. From the biomass production table (Table 32), we see different growth rates between strains. Here the best results were achieved by *D. velitaris* in total, 3.020 g/L of media. We see different effects of nitrogen sources within individual strains. The cyanobacterium *Limnospira maxima* prefer more sodium nitrate in higher concentrations. In contrast, members of the genus *Desmodesmus* show balanced growth on both types of nitrogen source. *D. armatus* grew better on medium with lower nitrogen concentration in the medium than *D. velitaris*, where the medium with the highest nitrogen content clearly dominated.

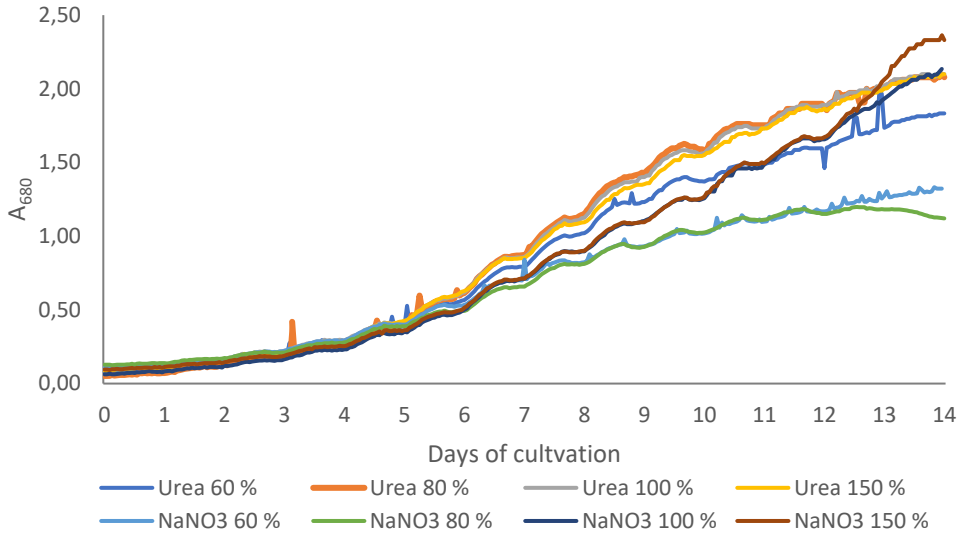
Table 32. Multicultivator experiments: biomass production

Biomass [g/L]								
Strain	$NaNO_3$ 60%	$NaNO_3$ 80%	$NaNO_3$ 100%	$NaNO_3$ 150%	Urea 60%	Urea 80%	Urea 100%	Urea 150%
D. velitaris	1.320	1.400	2.880	3.020	2.188	2.550	2.830	2.860
D. armatus	2.425	2.350	2.300	2.113	2.188	2.550	2.038	1.850
L. maxima	1.156	1.198	1.679	1.224	1.514	1.430	1.055	1.189

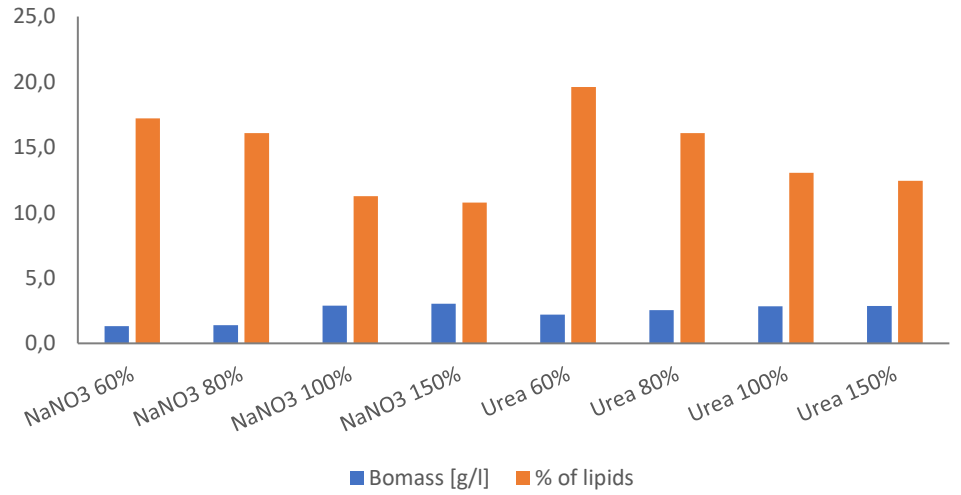
5.2.1.2.1 Desmodesmus velitaris cultivation results

The following graph (Graph 24) shows the growth curve of *D. velitaris* during multicultivator cultivation. We see here that in the first five days of cultivation, all flasks' growth is comparable. On the sixth day of cultivation, higher concentrations of nutrients are already beginning to appear, and we observe an increased growth rate in these media. The best medium here was medium with 150% $NaNO_3$, in which the final absorbance value of the culture was A_{680}=2.25. The chromatographic analysis results show an apparent effect of the medium's reduced nitrogen concentration, which led to increased lipid production (Graph 25 and Graph 26). Maximum yields were achieved on both media with 60% nitrogen with a maximum of 19.62% lipids in urea medium. We do not see any significant differences in terms of fatty acid profile. Thus, the various nitrogen sources and their concentrations have no observable effect here. The microalgae maintain a stable ratio of fatty acids with a predominance of polyunsaturated fatty acids with a 45-48% content. The remaining 55-52% is then evenly distributed between saturated and monounsaturated fatty acids.

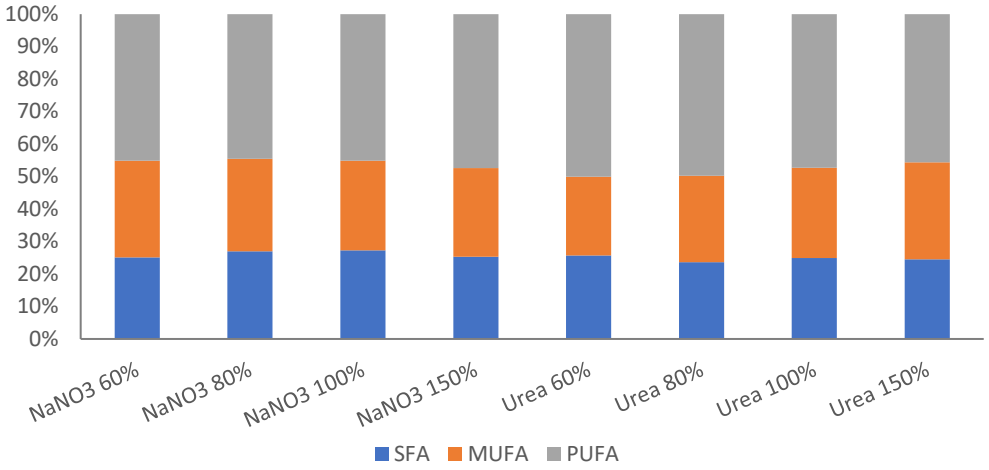
The results of the HPLC analysis of the pigments copy the results of biomass production. We see that with increasing nitrogen concentration in the medium, monitored pigments' level increases. Here, too, the highest production is achieved on a medium with 150% NaNO3, namely 7.90 mg/g carotenoids and approximately 7 mg/g dry of chlorophylls. Interestingly, as the medium's nitrogen content decreases, chlorophyll B production decreases and thus the chlorophyll A/B ratio changes. The primary carotenoid in all samples is β-carotene, followed by lutein. In this experiment's case, no compromise can be found that would allow increased production of all metabolites analyzed.



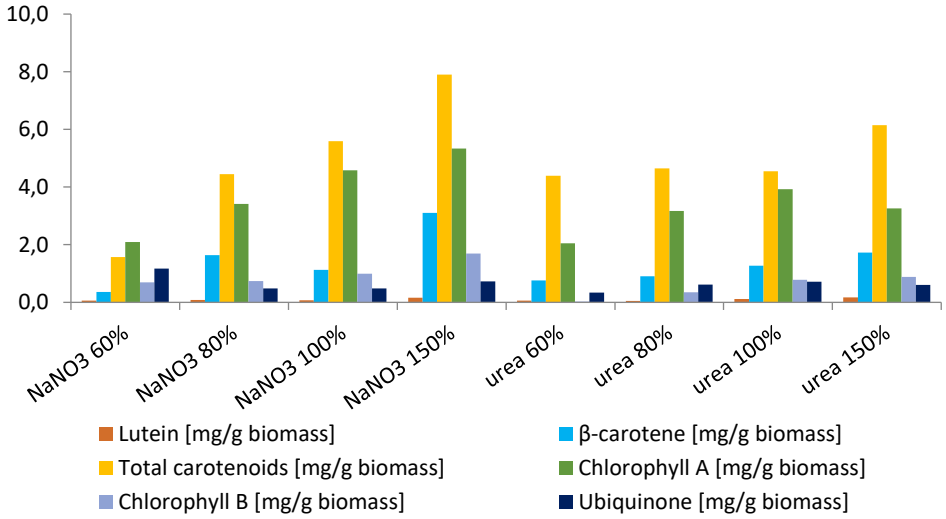
Graph 24. *Desmodesmus velitaris growth curve in multicultivator MC1000 – experiment with different nitrogen concentrations*



Graph 25. *Desmodesmus velitaris multicultivator cultivation on urea and sodium nitrate: lipid production results*



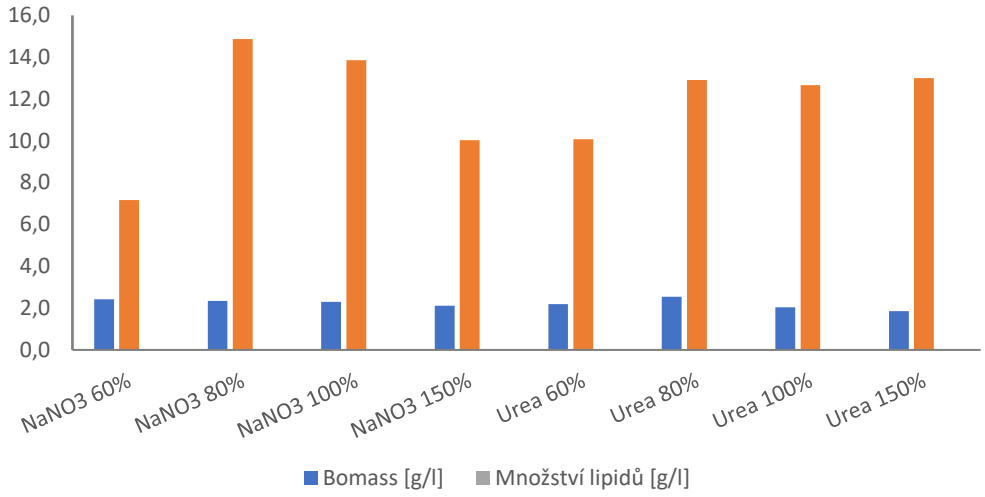
Graph 26. *Desmodesmus velitaris multicultivator cultivation on urea and sodium nitrate: fatty acid profile results*



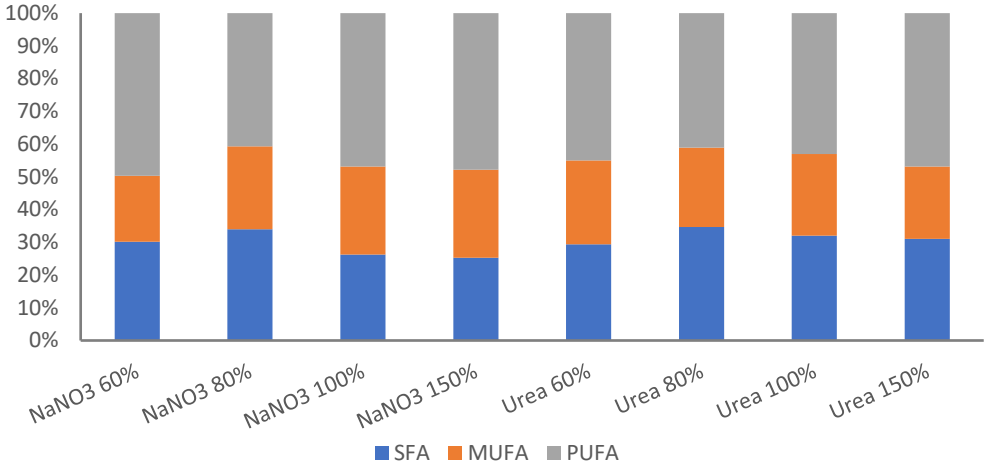
Graph 27. *Desmodesmus velitaris multicultivator cultivation: HPLC analysis results*

5.2.1.2.2 Desmodesmus armatus cultivation results

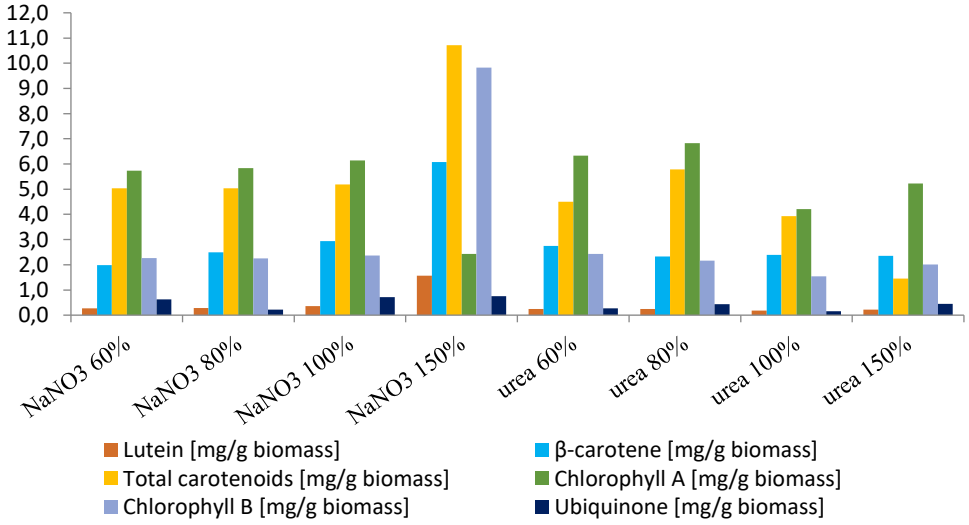
The chromatographic analysis results shown in the graphs below show a particular trend (Graph 28 and Graph 29). In media with 60% nitrogen, this algae produces the least amount of lipids, due to the unusually high growth rate. The maximum of lipid production is reached here only in media with 80% nitrate in a medium (14.87% of lipids), which is an overall more suitable medium for lipid production. The fatty acid profile is no longer as stable here as in the previous case. We see differences here within one nitrogen source and between the two nitrogen sources too. In both media with 80% nitrogen, an increased SFA content is seen at the expense of PUFA. Pigment production reaches a maximum on the medium with the highest sodium nitrate content. There was also an interesting metabolic change in this medium when the ratio of chlorophylls produced was reversed, and there is a high overproduction of chlorophyll B. In urea medium, however, we do not see the expected high production of pigments, and conversely, the production is by far the lowest. From the overall results, it can be clearly stated that the medium with sodium nitrate was the most suitable for this microalgae in terms of biomass and metabolite production.



Graph 28. *Desmodesmus armatus multicultivator cultivation on urea and sodium nitrate: lipid production results*



Graph 29. *Desmodesmus armatus multicultivator cultivation on urea and sodium nitrate: fatty acid profile results*

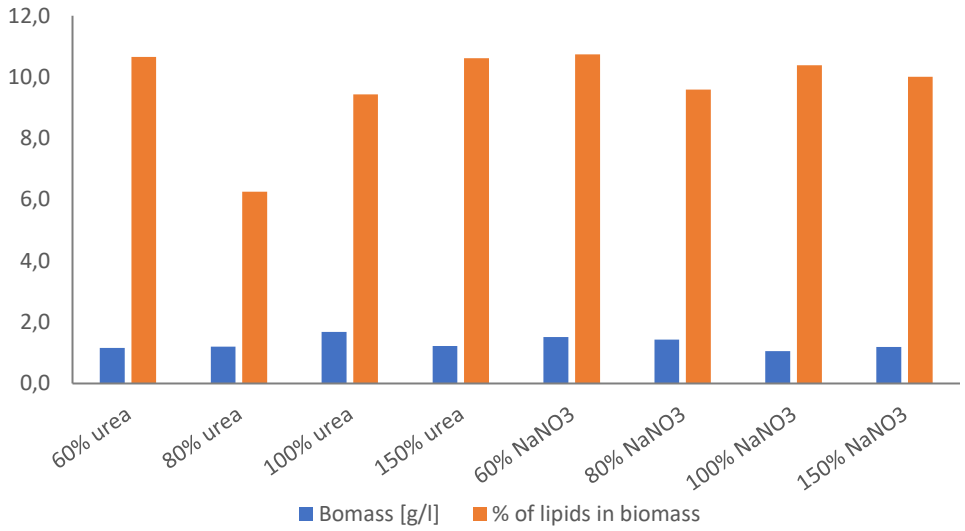


Graph 30. *Desmodesmus armatus multicultivator cultivation: HPLC analysis results*

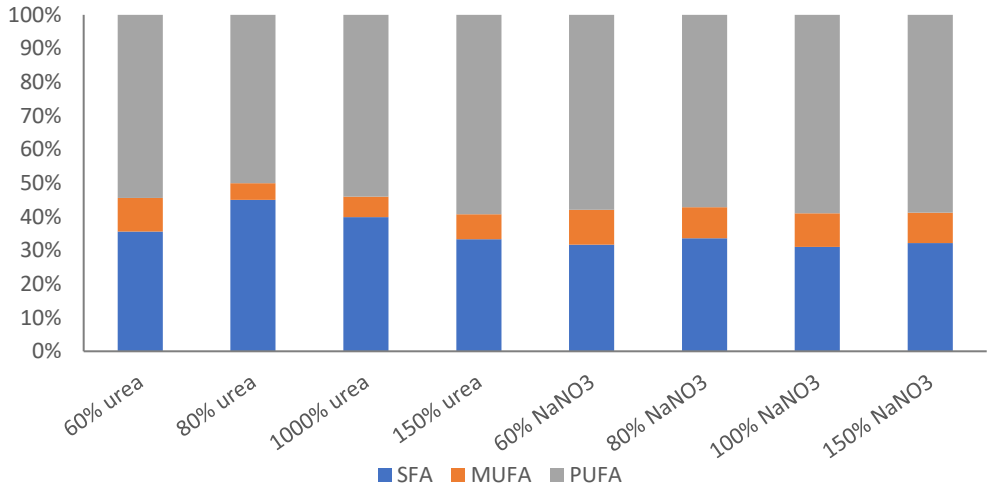
5.2.1.2.3 Limnospira maxima cultivation results

The last results of experiments with different nitrogen sources shown are the cultivation of the cyanobacterium *Limnospira maxima*. Lipid production analysis data show that this cyanobacterium maintains relatively stable lipid production in the range of 9.5-11% of lipids under all conditions studied, except for 80% urea medium (Graph 31). Compared to *Desmodesmus*, monounsaturated media concentration in all media is very low between 8-11%. The cyanobacterium *Limnospira m*. prefers the production of polyunsaturated fatty acids. We also see that in the sodium nitrate media, all fatty acids' production is very balanced.

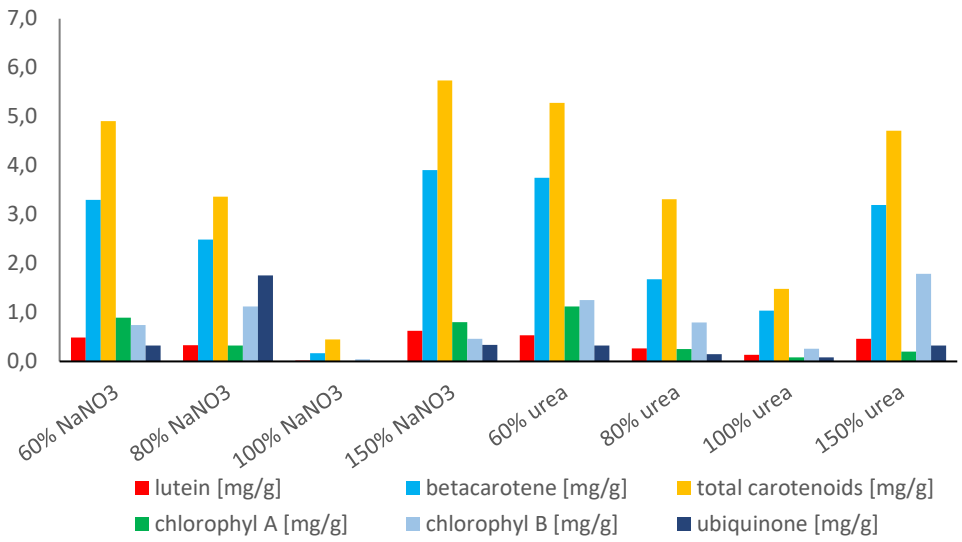
The production of cyanobacterial pigments shows a particular trend (Graph 33), with high production of metabolites being achieved only in media with extreme concentrations of urea and sodium nitrate (60% and 150%). The maximum was the production of 5.74 mg/g of carotenoids in a medium with 150% sodium nitrate, followed by a medium with 60% urea. On the other hand, we see low chlorophyll production, which suggests that the culture was not in an ideal state at the time of cultivation, and therefore these results may be partially skewed. It would be advisable to repeat the experiment for sure.



Graph 31. *Limnospira maxima multicultivator cultivation on urea and sodium nitrate: lipid production results*



Graph 32. *Limnospira maxima multicultivator cultivation on urea and sodium nitrate: fatty acid profile results*



Graph 33. *Limnospira maxima multicultivator cultivation: HPLC analysis results*

The cultivation of microalgae and cyanobacteria from the overall point of view confirms the basic assumptions. In media with a lower amount of nitrogen, microalgae metabolically prefer higher lipid production. In the lack of basic nutrients and continuous lighting, the metabolism of cells is redirected, and the energy generated by photosystems is stored in the storage molecules of lipids or carbohydrates as part of their protection. In contrast, in nutrient-rich media, microalgal metabolism gradually increases chlorophylls' concentration for optimal chloroplast function. It can be assumed that with a sufficient prolongation of the cultivation, the culture would deplete the nutrients from the medium to such a level that the metabolism would be redirected towards lipid production. High production of chlorophylls is associated with increased carotenoid concentration, which serves as antenna pigments in the photosynthetic apparatus. In this experiment, it was shown that representatives of the genera *Chlorella*, *Chlamydomonas* and *Coccomyxa* prefer urea for higher biomass production, on the other hand, higher production of pigments and lipids was achieved on the medium with sodium nitrate.

Representatives of the genera *Desmodesmus* and *Scenedesmus* do not have significant preferences and are capable of comparable growth on both types of inorganic nitrogen tested, with a different concentration being preferred in each. For pigment production, sodium nitrate is a more suitable source. Based on the obtained data, it is impossible to find an exact point/concentration at which the cultivation would produce a higher concentration of biomass, pigments and lipids. Depending on the biotechnological process's needs, it is necessary to seek a compromise or focus primarily on one type of metabolites produced. Thus, each strain has its.

5.2.1.3 *Cultivation on media with different phosphorus concentration*

The optimization of the BBM medium composition continued in the following experiment, where different phosphorus concentrations in the medium were tested. Phosphorus is one of the essential components of the nutrient medium of all microorganisms. It is an essential component of biomembranes, nucleic acids, energy transporters, and the mechanism of phosphorylation/dephosphorylation is one of the basic pathways of enzyme activation. Finding the optimal amount for each strain is an indispensable part for a future successful biotechnological process. We observed that under the conditions of Erlenmeyer flasks cultivation, the tested strains of microalgae and cyanobacteria preferred lower phosphorus concentrations in which the highest increases in biomass and lipids were generally achieved.

On average, the highest production of carotenoids by the tested strains was obtained in media with a standard concentration of phosphorus. These results are in contrast to the data obtained from cultivations of the microalgae *Coccomyxa onubensis* and *Botryococcus braunii* v. Showa-Bielefeld, where the increased concentration of phosphorus in the medium had an overall positive effect. It should be added that these microalgae were cultivated on a medium aerated with air enriched with CO_2, and due to sufficient carbon, their metabolism and growth were much faster. Thus, it can be assumed that by cultivating these algae under comparable conditions with sufficient carbon, a higher amount of phosphorus in the medium would be better utilized and the production maxima would be shifted to higher concentrations of phosphorus in the medium. The second possible answer is the simple fact that the standard concentration of phosphorus in the BBM medium is already sufficient for them.

5.2.1.4 *Cultivation of microalgae with the application of stress factors*

To increase the production of the required metabolites, various physicochemical stress factors are used in biotechnology, the presence of which in the medium elicits an adequate cell protective response. Appropriately chosen stresses can specifically force the culture to overproduce a particular group of metabolites. One of the basic stresses is the lack of nutrients when cells stop multiplying and switch their metabolism towards storing nutrients and energy. Microalgae were subjected to a series of cultivations under various stress conditions. The results of these cultures are summarized here.

5.2.1.4.1 High salinity

The presence of a high concentration of salt in the medium leads to a change in the osmotic pressure that is exerted on the cell. The cells of the microorganisms adapt and, for example, change the composition of the fatty acids forming the membranes to ensure sufficient strength and functionality under the given conditions. The high salt content can further decrease the growth/propagation of the culture, and the cells are oriented towards the synthesis of storage molecules (lipids, polysaccharides, carotenoids). In the experiment, cultivations with a salt content of 0.0-2.0M NaCl were tested. The results of cultivation at high salt concentrations inevitably led to the death of the culture. Some strains were only able to tolerate salt concentrations that were less than 0.5M NaCl. Cultivations with an increased salt concentration in this interval (0.0-0.5M NaCl) led to reduced biomass production. On the other hand, growth inhibition led to a slightly increased lipid content in the biomass. The accumulation of carotenoids and chlorophylls was negatively affected by salt, and higher production of pigments was never achieved in the samples of tested algae due to the increased salt concentration. Representatives of the genus *Scenedesmus* in overall gave the best results. Tested strains of microalgae are classified as freshwater algae, and for this reason, the long-term presence of salt in the medium had a lethal effect for them. As part of the application of salt stress, it would be appropriate to test its application in the later phase of growth, preferably in the stationary phase, when the culture is sufficiently.

5.2.1.4.2 Light intensity

Different light intensities are one of the fundamental possibilities of modulating metabolites' production, especially pigments. Sufficient light intensity is necessary for optimal use of the photosynthetic apparatus and cell growth. With increasing intensity of light radiation, the possibility of damage to the photosynthetic apparatus's basic components increases, and the cell responds with increased production of pigments, which serve as protection. A microalgae cell growing under these conditions processes a larger amount of energy and is able to store it in storage molecules. Thus, the optimally chosen radiation intensity can lead to an increase in pigments and lipids/polysaccharides production. In the experiment, cultivations were performed in a multicultivator with different light intensities in the range of 200-800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photons. Even in the initial cultivations, it was found that this light intensity was too high and in the first days of cultivation led to inhibition and death of the culture. Therefore, the experiments were performed by setting the illumination of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photons for all tubes in the first 3 days of cultivation and then increasing the intensity. The cultivation results show a different tolerance of light intensity between the different strains tested. The microalgae cultivation at the highest intensity of 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photons led to photohibition in all tested strains under the given conditions and biomass production decreased.

Furthermore, pigments and lipids production also decreased. Optimal results were achieved at 200 and 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photons, where we observe an increased production of the studied metabolites in comparison with the classical cultivation under the illumination of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. An average increase in the carotenoid pigment's production by 10-15% was achieved in these cultures. The chlorophyll content increased to a lesser extent, in the range of 1-6%. Thus, it can be stated that for most of the tested strains, the optimum of light intensity lies in this range. An exception was the genus *Chlorella*, whose representatives showed higher resistance to light and the best results and production was achieved at the highest light intensities used in the test.

In this experiment, the second possibility of light stress was partially used, and that is a gradual increase. As with previous salt stress experiments, it would be possible to cultivate microalgae and cyanobacteria under optimal conditions and, in the final phase of cultivation, increase light intensity, suppress cell growth, and redirect metabolism toward storage molecules production.

5.2.1.4.3 Oxidation stress

The presence of iron ions or other metal elements with similar properties causes a higher oxidation potential in the medium, to which the cells must respond. In this case, the basic defence is a production of antioxidants that scavenge radicals and other reactive particles and protect essential cell components. In this experiment, microalgae and cyanobacteria were cultivated in the presence of various concentrations of $FeCl_3$. It can be concluded from the results that in most cases, the production of biomass increases with increasing iron concentration, but the percentual proportion of lipids decreased. In terms of the fatty acid profile, iron ions in the medium lead to a reduction in the content of unsaturated fatty acids.

As in the phosphorus experiments, the production of carotenoids did not show a uniform trend over time. The *Chlorella vulgaris* strain had the highest production of carotenoids (9.79 mg/g) in a sample with a concentration of 0.5 mM/L $FeCl_3$. In the case of *Desmodesmus quadricauda*, the final production of carotenoids in all samples was comparable. In contrast, the *Desmodesmus obliquus* strain shows the highest production (4.93 mg/g) in a sample with a concentration of 0.5 mM/L. *Scenedesmus acutus* strain shows the highest production of carotenoids (6.55 mg/g) at a concentration of 0.1 mM/L, while the *Scenedesmus dimorphus* strain at a concentration of 1 mM/L. In the case of the strain *Coccomyxa sp*. we observe a significant increase in carotenoids (15.12 mg/g) in a sample with a concentration of 0.1 mM/L. Iron ions presence affects the content of the two major pigments lutein and β-carotene. Experiments with media enriched with iron ions have shown that oxidative stress successfully increases carotenoid pigments' content in cyanobacteria and microalgae biomass. The results show that each strain responds to different concentrations of iron. Further optimization of the iron concentration or testing of other metals would achieve even better results.

5.2.1.5 *Conclusions: microalgae cultivation in Brno*

Microalgae and cyanobacteria cultivation results indicate the great potential of these strains. In a number of experiments, the strains achieved high production of valuable substances (carotenoids, lipids, sterols, etc.). Cultivations were performed under standard air aeration conditions, i.e. not under optimal conditions. If the cultures were aerated with carbon dioxide-enriched air, the growth of microalgae would be accelerated, which is incomparably smaller than the yeasts studied in this work. In this experiment, it was further demonstrated that the appropriate combination and timing of applying stress factors can be used to shift the metabolism and overall behaviour of microalgal cells to overproduce selected metabolites. Unfortunately, the rule also applies here that it is impossible to get everything at once and it is always necessary to choose a suitable compromise. Of all the microalgae tested, representatives of the genera *Scenedesmus* and *Desmodesmus* achieved the best results overall. The mentioned potential of microalgae was further used in biological stress tests in the form of co-cultivation with carotenogenic yeasts. The results of these experiments are in the following chapter of this work.

5.2.2 Microalgae cultivation in Huelva

5.2.2.1 *Coccomyxa onubensis salt adaptation experiments*

Laboratory experiments focused on the adaptation of *Coccomyxa onubensis* to salt stress for the future possible use of seawater as a fundamental component of the medium for large-scale cultivation. The adaptation took place by gradually increasing the salt content in the medium. During the adaptation, the morphology, quantum fluorescence yield and overall culture state were monitored. Production of lipids and pigments (carotenoids and chlorophylls) was then determined in the produced biomass. The pigments were determined spectrophotometrically, according to the Lichtenthaler method.

The following chapter summarizes the results of all tests performed on K9 medium enriched with 0.5M NaCl. The first experiment was to test the effect of different nitrogen sources on the growth and production properties of *Coccomyxa onubensis.* In this experiment, the absorbance of the culture and the quantum yield of fluorescence were measured continuously. The measured absorbance results, quantum fluorescence yield, and observations found that the most suitable nitrogen source for the microalgae *Coccomyxa onubensis* is urea. The results of a duplicate experiment also supported this conclusion. In the case of ammonium sulphate, we observed a lesser biomass production and a slight decrease in media pH. In the case of sodium nitrate, the medium's pH increased, and the growth gradually slowed down.

Only urea did not affect the pH of the medium, and thus, the culture was able to maintain its pH optimum throughout the experiment. Based on these data, urea was used as the sole nitrogen source in the following experiments. At the end of the experiment, the production of pigments was determined spectrophotometrically and lipid production by gas chromatography.

5.2.2.1.1 Cultivation on media with different pH

One of the first experiments needed was to determine the microalgae pH optimum for cultivation on media with salt stress. Due to the fact that *Coccomyxa o.* is one of the extremophilic acidophilic microalgae, the optimal pH of the medium is an essential factor for growth, production properties and de facto possible future application on an industrial scale. The previous research found that on the classical K9 medium *Coccomyxa o*. grows best in the pH range 2-2.5. As part of the adaptation of the culture to salt stress, it was found that at a given pH, the growth of microalgae is slow and there is a decrease in the quantum yield of fluorescence, i.e. a decrease in photosynthetic activity. Based on this, a series of flasks with different pH values of the medium in the range of 2-7 were prepared. The following table below shows the results of the determination of total biomass. The results do not show values for a medium with pH = 2, because a few days after inoculation with the stock culture, the growth of the culture was stopped under these conditions and the QY fell below 0.25. Thus, it is clear that in the case of a medium with added salt, the microalgae is not able to grow under the same conditions as of a classical K9 medium without added salt.

From the table below (Table 33), we can see further that in media with pH> 6 the growth stops and the culture dies relatively quickly. In these experiments, the originally symbiotic strains of microorganisms gradually multiplied at *Coccomyxa o.* cells' expense. The best pH interval for growth under salt stress conditions is pH=3-4. Under these cultivation conditions, the highest biomass production was measured. A gradual increase in the pH of the medium was also observed in this experiment. It can be concluded that, under these conditions of salt stress, *Coccomyxa o.* partially loses its ability to regulate the pH of the medium, compared to cultivation on a medium without salt stress. Therefore, it can be assumed that during long-term cultivation, it would be necessary to regulate the pH by external intervention.

Table 34 shows the results of spectrophotometric analysis of carotenoid and chlorophyll A and B pigments. The determination was performed three times by the Lichtenthaler method. The results of pigment production correspond to the results of the measured quantum fluorescence yield and biomass production. In media with pH outside the pH optimum of *Coccomyxa o*. we observe a very rapid decrease in chlorophyll pigments production from the initial days of cultivation. In these media, we also observe a decrease in the production of carotenoids. In the medium with pH=6 in the first week of cultivation, carotenoids' content increases to 5.12 mg/g of biomass (Table 34). Subsequently, however, we observe a linear decrease in the content as the microalga's culture conditions in the given medium deteriorate. In a medium with pH=5, carotenoid production during cultivation is relatively stable in the interval 4.7-5.0 mg/g of dry biomass. The last two flasks with lower media pH show a linear increase in carotenoid production in both cases. The highest production of carotenoids 7.54 mg/g dry biomass was reached at pH=3. Here too, chlorophylls' production increases practically linearly and reaches a peak on the last day of cultivation (10.25 mg/g chlorophyll A and 6.46 mg/g chlorophyll B). From the point of view of all pigments' production, a medium with pH=3 is the most suitable. We can see from the data that if the cultivation time were extended, the accumulation of pigments would reach even higher values.

Table 33. Biomass production of Coccomyxa onubensis cultivated under different pH levels

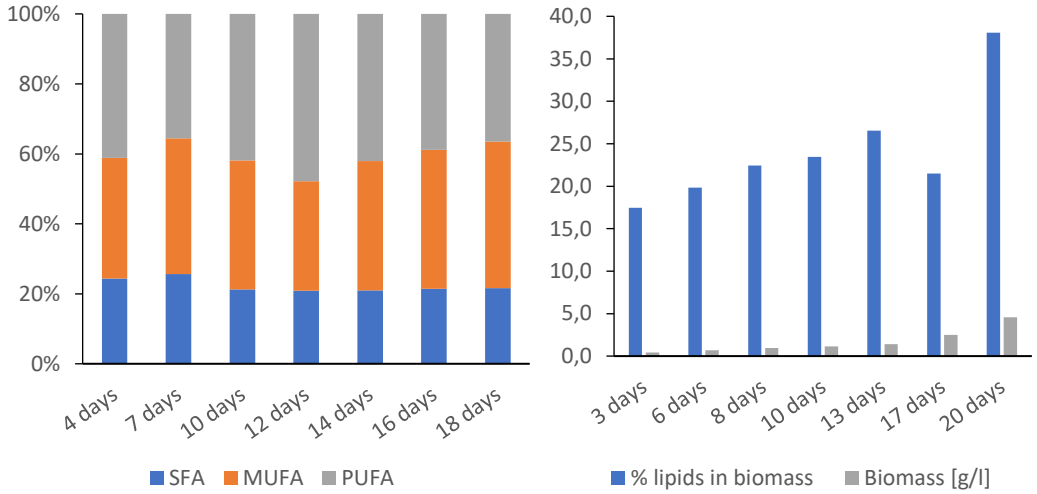
	Biomass [g/L]						
Sample	3 days	6 days	8 days	10 days	14 days	17 days	20 days
pH=3	0.41	0.67	0.97	1.15	1.39	2.48	4.58
pH=4	0.49	0.62	0.84	1.02	1.37	2.81	3.73
pH=5	0.40	0.54	0.68	0.81	0.97	1.74	2.64
pH=6	0.35	0.40	0.48	0.51	0.55	0.53	-
pH=7	0.38	0.43	0.44	0.45	-	-	-

Table 34. Coccomyxa o experiment with different nitrogen concentration – pigment analysis results

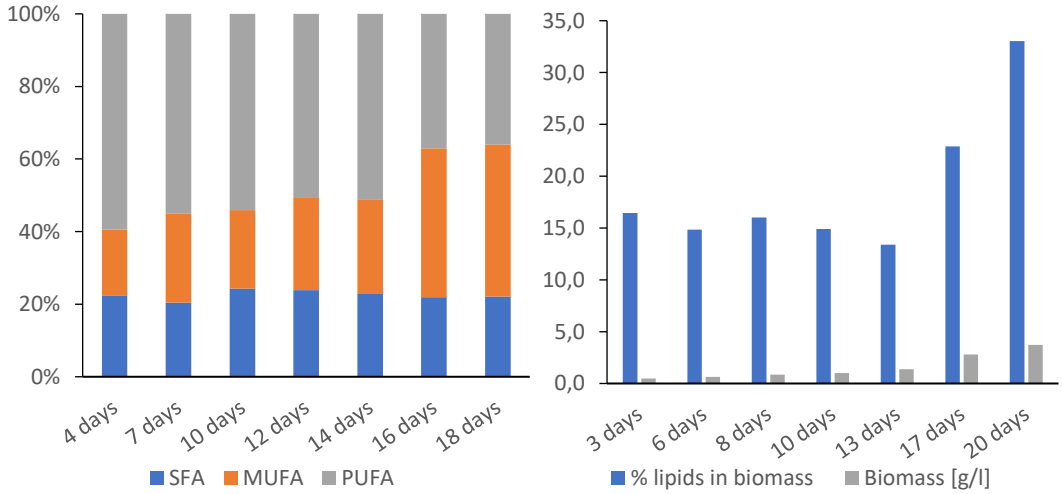
Pigment production [mg/g of dry biomass]						
	Salt K9 media pH=3			Salt K9 media pH=4		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
3	4.59	6.23	3.88	4.54	6.04	3.68
6	5.22	7.65	3.99	4.98	6.98	3.88
8	5.88	8.32	4.57	5.49	7.46	4.32
10	6.48	8.72	4.97	5.99	8.03	4.77
14	7.13	9.07	5.47	6.65	8.55	5.16
17	7.46	9.77	6.01	6.85	9.23	5.99
20	7.55	10.25	6.46	7.05	9.68	6.23
	Salt K9 media pH=5			Salt K9 media pH=6		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
3	4.72	3.68	2.83	4.36	3.46	2.49
6	4.98	4.01	2.72	4.88	3.33	2.75
8	5.33	3.90	3.02	5.12	3.12	2.89
10	5.08	3.72	2.67	4.99	2.98	2.76
14	4.86	2.79	2.01	4.76	2.66	2.42
17	4.62	2.13	1.87	4.49	2.78	1.96
20	4.76	2.24	1.35	4.03	2.35	1.23

The results of the lipid production analysis copy the production of biomass The biomass's total lipid content and the largest increase in biomass production are observed on the medium with pH=3 (Graph 34).. In this medium, we see from the beginning an increase in the lipid content of the biomass from the initial 17.45% to 38.06% on the 20th day of the experiment. In the second flask with a medium with pH=4 (Graph 35), slightly lower lipid production was achieved, namely 33.03%. Unlike the first cultivation, here during the experiment, the production is relatively balanced and only at the end, there is an exponential increase in the biomass's lipid content. In a medium with a higher pH, we already clearly know the strong negative effect of pH on growth and lipid production. Relatively good results were obtained by cultivation on a medium with pH=5 (Graph 36), where the highest production was 24.71%. In other flasks, we already see a gradual decrease in lipid production below 10%.

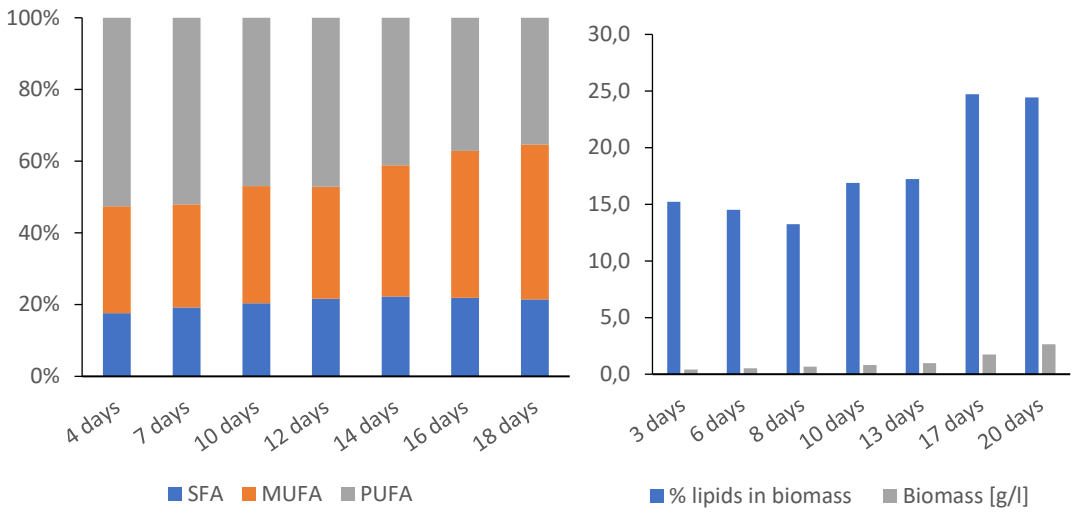
From the point of view of the fatty acid profile, we see that gradually during the cultivation, there is a decrease in the content of polyunsaturated fatty acids in favour of monounsaturated fatty acids. If we do not consider the medium with the highest pH, the production of PUFA stabilized in the range of 40-35%. Here, as the medium's initial pH increases, the percentage of PUFA decreases to a lower limit of that interval. This decrease is most evident on media with pH=4 and pH=5. The total MUFA content is then stabilized in all cultures in the range of 38-42%. Even under different pH media conditions, *Coccomyxa* o. maintains a stable SFA production, which is here in the range of 18-22%. Only the test medium with the highest initial pH shows a gradual increase in SFA content. This phenomenon can be partly attributed to the gradual increase of symbiotic bacteria in the sample.



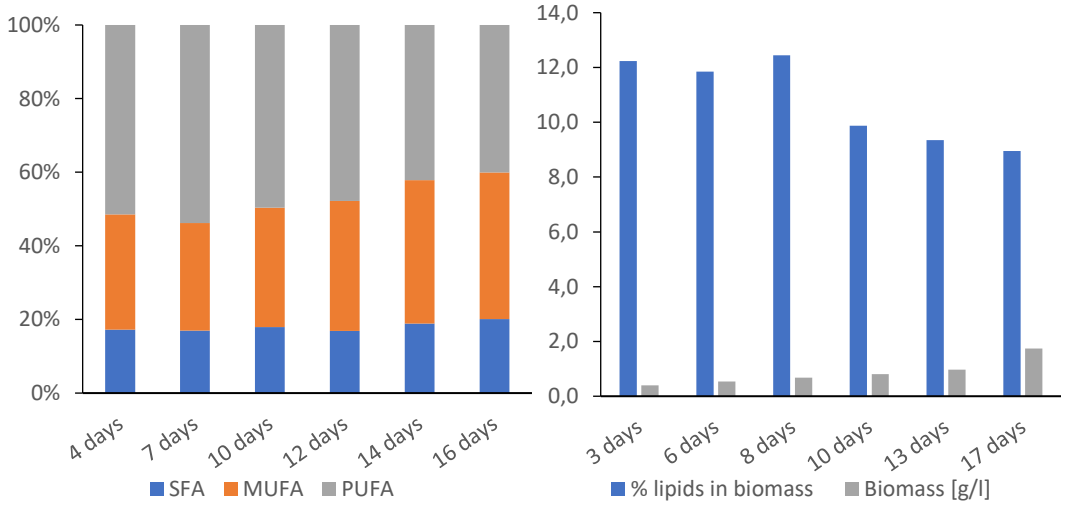
Graph 34. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with pH=3*



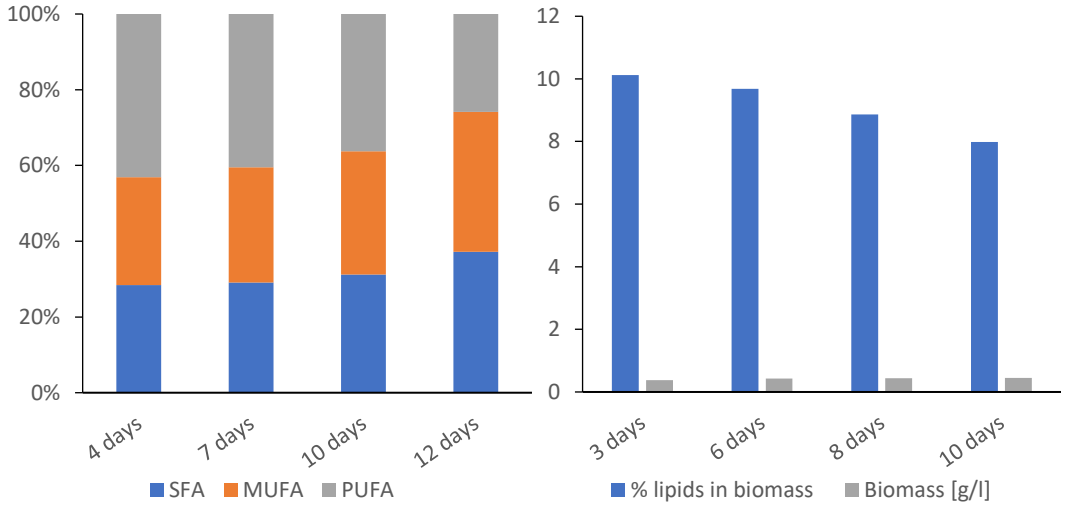
Graph 35. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with pH=4*



Graph 36. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with pH=5*



Graph 37. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with pH=6*



Graph 38. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with pH=7*

5.2.2.1.2 Cultivation on media with different N concentration in media

The following experiment was done to monitor the effect of nitrogen source concentration on selected metabolites' production. In a 3-week experiment, 6 Erlenmeyer flasks with nitrogen concentrations between 20-150% were prepared. A duplicate experiment was performed simultaneously. The data shown below (Table 35) are the average values of the two experiments. From the beginning, we see a difference in the growth rate of biomass. In media with the lowest nitrogen concentrations, the culture grows slowly from the beginning. Even in a medium with a high nitrogen concentration, we see an initial slow growth rate, which gradually increases. In an overall comparison, we see that the best biomass production results, 4.17 g/L, were achieved on a medium containing 80% nitrogen. Followed by 60% nitrogen with 4.10 g/L. Analysis of the accumulation of the pigment in the biomass shows the effect of a medium's nitrogen concentration on individual pigments production (Table 36).

In the case of chlorophyll pigments, we see that with decreasing nitrogen concentration, the production of both types of chlorophyll pigments decreases. This trend is observed in media with 60% nitrogen or less. The production of chlorophyll in this medium decreases by 20%, and with a decreasing amount of nitrogen, this trend deepens further. We observe the same trend in the production of carotenoids. In this case, however, the decline in total production is not so significant. On the contrary, in media with standard or higher nitrogen concentration, we see a positive effect on the increased production of all studied pigments. Carotenoid production was most affected in excess of nitrogen, increasing by approximately 25% compared to the standard medium to value 9.234 mg/g of dry biomass. In the case of the main chlorophyll pigment A, the production in this medium was increased by 10%. The increased nitrogen concentration had no significant effect on the production of chlorophyll B. Summarizing the results, we see that increased nitrogen concentration had a very positive effect on pigment production.

Table 35. Biomass production of Coccomyxa onubensis cultivated on media with different N concentration

	Biomass [g/L]								
Sample	4 days	7 days	10 days	12 days	14 days	16 days	18 days	20 days	21 days
20 % N	0.34	0.45	0.58	0.71	0.83	0.90	0.91	0.98	1.12
40 % N	0.40	0.51	0.69	0.88	0.94	1.02	1.35	1.40	1.39
60 % N	0.44	0.65	0.93	1.33	1.83	2.36	2.85	3.18	4.10
80 % N	0.45	0.89	1.13	2.32	2.44	2.85	3.01	3.31	4.17
100 % N	0.52	0.99	1.35	1.86	2.34	2.80	3.12	3.54	3.45
150 % N	0.21	0.51	0.79	1.21	1.75	2.24	2.55	2.71	3.59

Table 36. Coccomyxa o experiment with different nitrogen concentration – pigment analysis results

Pigment production [mg/g of dry biomass]						
	Media with 60 % N			Media with 80 % N		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.14	5.79	3.02	4.38	6.13	3.43
7	4.43	6.03	3.40	4.79	6.73	3.82
10	4.90	5.96	3.87	5.02	6.81	4.49
12	5.09	6.49	4.32	5.42	7.47	4.98
14	5.30	7.19	4.78	5.86	8.22	5.52
16	5.78	7.65	5.01	6.08	8.33	5.46
18	5.67	8.03	4.82	6.43	8.82	5.79
20	5.82	8.23	5.23	6.72	9.18	6.30
21	5.78	8.40	5.76	6.96	9.02	6.43
	Media with 100 % N			Media with 150 % N		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.23	6.49	4.02	4.76	6.48	4.31
7	4.62	7.12	4.97	5.33	7.33	4.67
10	5.14	8.13	5.24	5.98	7.42	5.43
12	5.65	9.27	5.98	6.49	8.23	6.02
14	6.03	9.96	5.85	7.06	9.08	6.53
16	6.49	9.87	6.44	7.27	9.89	6.69
18	6.89	10.13	6.78	8.25	10.48	7.08
20	7.24	10.54	6.81	8.80	11.05	6.73
21	7.40	10.76	7.10	9.23	11.85	7.03

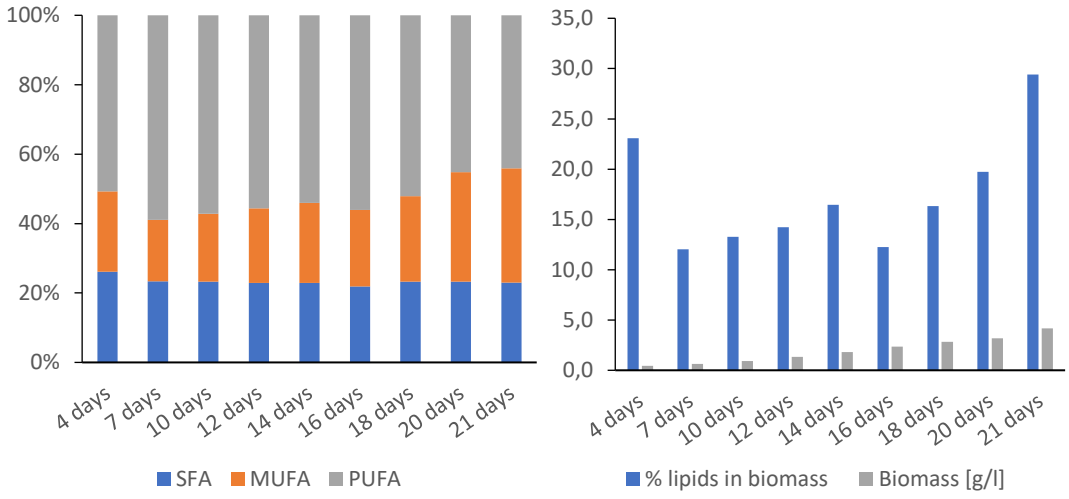
From the beginning of the cultivation, we observe deficient lipid production in flasks with 20% and 40% nitrogen in the medium. On average, the lipid content was below 10% and rather decreased at the end of the cultivation. The fatty acid profile replicated data showing results from cultivation on media with higher nitrogen concentrations. These cultivations were also characterized by a relatively low quantum yield of fluorescence in the range of 0.30-0.40, which indicates that the given composition of the medium and culture conditions (nitrogen content) were insufficient.

The following graphs summarize the results of the GC analysis of lipids. Graph 39 shows the results of cultivation on medium containing 60% nitrogen. We see a relatively stable trend in SFA production in the range of 22-26% in terms of fatty acid profile. In the first two weeks of cultivation, the production of unsaturated fatty acids is also stable. Only in the last week of cultivation does the MUFA content increase at the expense of PUFA. The final ratio is then approximately 44% PUFA, 33% MUFA and 23% SFA. In terms of total lipid production, the percentage of lipids decreased from 23.07% in the first week, followed by a gradual increase in the following ten days of cultivation. In conclusion, we see a virtually exponential increase in lipid content to a final value of 29.41%.

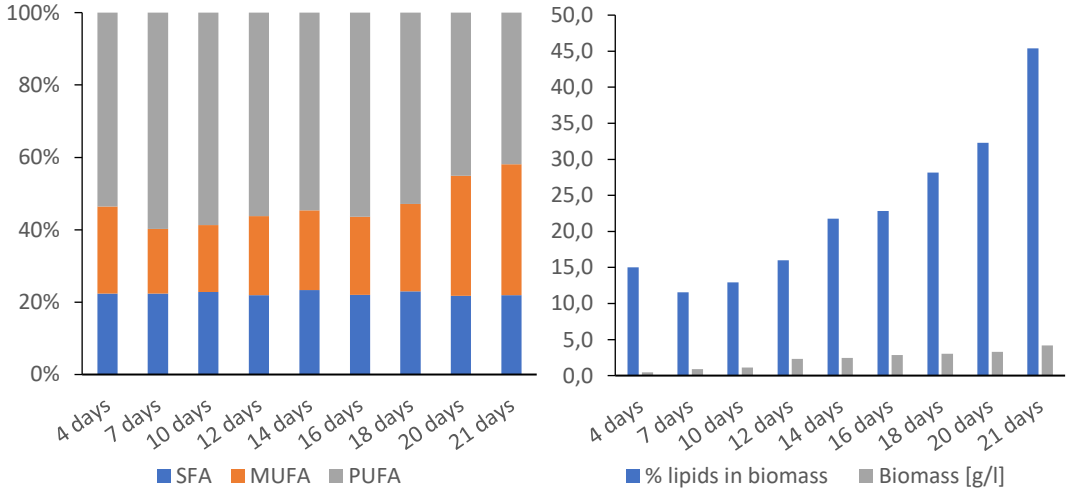
Cultivation with 80% nitrogen in the medium copies the same trends as in the previous case. Again, we see stable SFA production in the range of 22-24% (Graph 40). MUFA production is stable here and rises again to 36.14% in the last four days of cultivation. The highest content again applies to polyunsaturated fatty acids 41.91%. Total lipid production has been gradually increasing since the beginning of cultivation, producing 45.41% in the final exponential growth, which is a tenth of a percent more than the following experiment with the standard K9 medium. Compared to all media in this experiment, this medium was by far the most successful in lipid production, because even in biomass production, it surpassed others.

In the control medium (Graph 41) with standard nitrogen concentration, we see the same trends as in the previous media. Thus, stable SFA production, a linear increase in MUFA content in the final stage of cultivation. Compared to all media in this experiment, this medium was as successful in terms of lipid production as the medium with 80% nitrogen, achieving total production of 45.31% lipids in the biomass. Unfortunately, purely in terms of PUFA production, this culture in the final phase of the experiment was worse than media with 60% and 150% nitrogen.

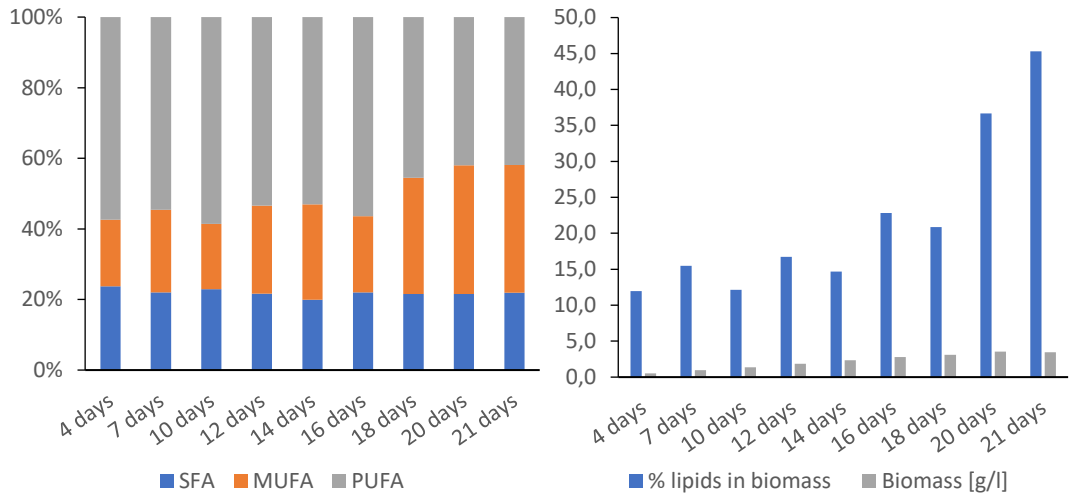
The last results shown here are cultures with an excess of nitrogen in the medium (Graph 42). The fatty acid profile here again shows the same characteristics as in the previous experiments. It is worth mentioning mainly the different production of lipids. Here, the percentage of lipids is lower compared to other cultivations. At the same time, we see a much greater fluctuation in the percentage of lipids during the measurement. The highest value of 26.95% was reached here on the 18th day of cultivation, and then there is only a decrease in production.



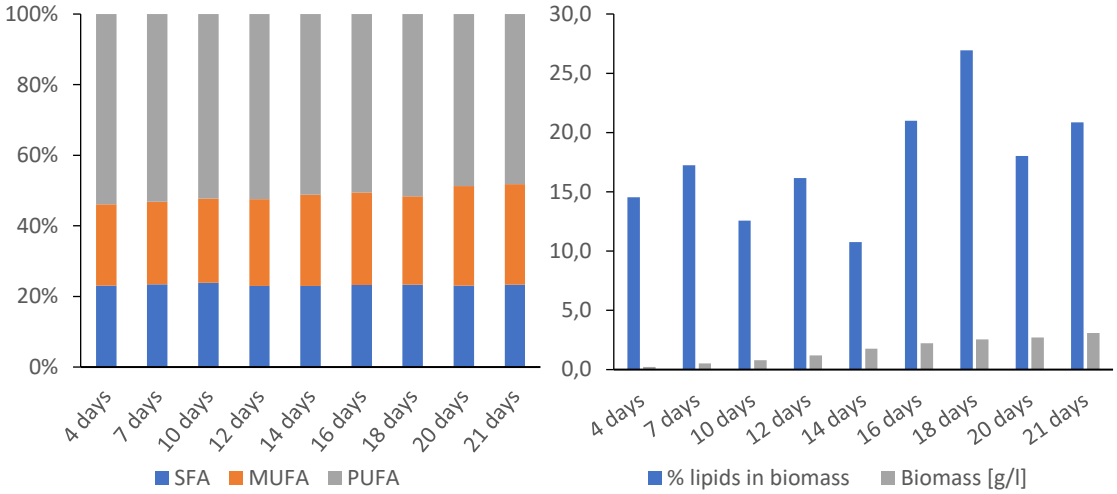
Graph 39. *Nitrogen deficiency experiment: Lipid production and fatty acid profile of Coccomyxa o. cultivated on K9 media with 60% concentration of nitrogen source*



Graph 40. *Nitrogen deficiency experiment: Lipid production and fatty acid profile of Coccomyxa o. cultivated on K9 media with 80% concentration nitrogen source*



Graph 41. *Nitrogen deficiency experiment: Lipid production and fatty acid profile of Coccomyxa o. cultivated on K9 media with 100% concentration nitrogen source (standard media)*



Graph 42. *Nitrogen deficiency experiment: Lipid production and fatty acid profile of Coccomyxa o. cultivated on K9 media with 150% concentration nitrogen source*

This part of the experiment shows that a slight reduction in nitrogen content (80% of N) has a positive effect on the production of biomass and lipids. The highest percentage of lipids of 45.41% was measured in this medium, and at the same time, the highest biomass production of 4.17 g/L was achieved here. The lower nitrogen content already had rather negative effects on the production of pigments. No optimal compromise can be found here for the increased production of all metabolites. Within acceptable losses of lipid and biomass production, it would be appropriate to cultivate under conditions with 150% nitrogen, where increased pigment production would streamLine the economic efficiency of the cultivation process.

5.2.2.1.3 Cultivation on media with different P/N ratio in media

The following experiment focused on the effect of the ratio of the two primary medium components phosphorus and nitrogen. Nitrogen is an essential component of all nucleotides, proteins and many other molecules. Phosphorus plays an irreplaceable role in, e.g. the synthesis of nucleic acids, ATP, cell membranes and protein phosphorylation. Optimal levels of phosphorus in the medium can lead to increased production of biomass or some metabolites. From the previous experiment, an 80% nitrogen level was chosen as the baseline nitrogen level. Furthermore, four P/N ratios were selected, where a chosen amount of phosphorus source was put into the individual flasks after sterilization. The P/N ratio 0.15 is the standard ratio here and used as a control medium.

In terms of biomass production, we see very fast growth on the medium with the lowest P/N ratio in the first ten days of cultivation, and then the growth rate slows down. The lowest biomass production was achieved at a P/N ratio of 0.10 (Table 37), where the growth rate was very low from the beginning. The lower growth rate was accompanied by the medium with the highest P/N ratio in the first seven days of cultivation. Then we observe a rapid increase and in the final day of experiment *Coccomyxa o.* produced highest biomass on medi this media with P/N 0.30. From the data of the measured pigment production (Table 38), we observe significant differences in the production of pigments. In the case of chlorophyll pigments, the concentration of major chlorophyll A and chlorophyll B gradually increases with increasing P/N ratio. In contrast, carotenoid production shows the opposite trend, and the maximum (8.53 mg/g) was reached on the medium with the lowest ratio. In all flasks, pigments' production fluctuates in the first half, which is stabilized only in the final phase of cultivation.

Table 37. Biomass production of Coccomyxa onubensis cultivated on media with different P/N ratio

	Biomass [g/L]								
Sample	4 days	7 days	10 days	12 days	14 days	16 days	18 days	20 days	21 days
P/N=0.05	0.59	0.72	0.82	1.20	1.70	2.08	2.33	2.54	2.62
P/N=0.10	0.21	0.27	0.37	0.40	0.57	0.74	0.81	0.95	1.12
P/N=0.15	0.39	0.66	0.89	0.84	1.53	1.63	2.16	2.44	2.79
P/N=0.30	0.44	0.63	0.82	0.92	1.58	2.01	2.59	2.98	3.42

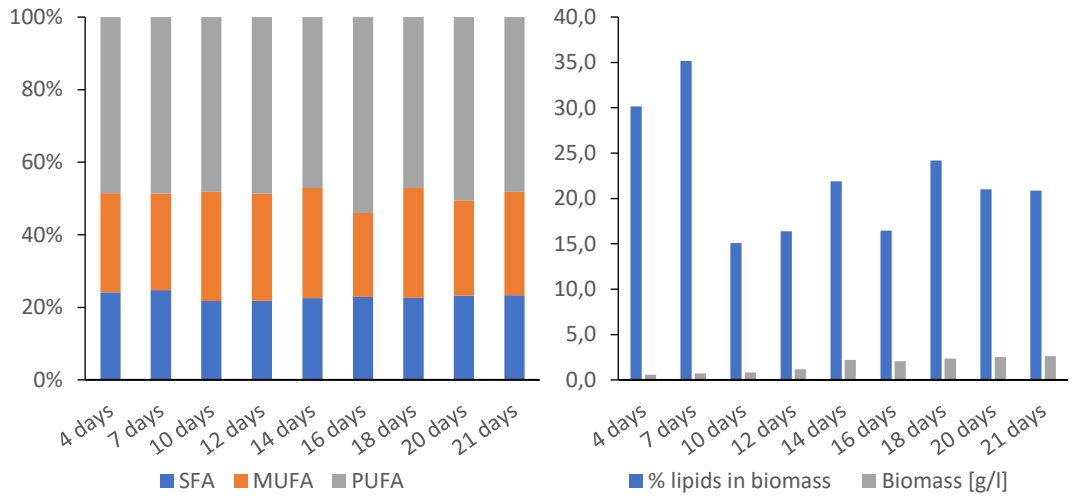
The chromatographic analysis of lipids produced by *Coccomyxa o.* cultivated on media with different P/N ratios has several identical properties. The fatty acid profile is again relatively stable here. The percentage of SFA in all samples is in a stable range of 20-25% throughout the cultivation. In contrast to previous experiments with different amounts of nitrogen, there is a relatively stable production of MUFA in each flask in these experiments, the values for each flask being different. At the lowest P/N ratio (Graph 43), the MUFA content is in the range of 28-31%. As the ratio increases, the MUFA content increases on average to 45% for a ratio of 0.10 (Graph 44) and 40-42% for a standard ratio of 0.15 (Graph 45). Unfortunately, the same cannot be said about the highest tested ratio of 0.30 (Graph 46), where we see a decrease in the MUFA content to values comparable to the ratio of 0.05, i.e. 28-32%. Decreased or increased production is always at the expense of fatty acid production with a larger number of double bonds (PUFA). An exception is the sample on the 16th day of cultivation when there is a metabolic change, and we see large differences in the otherwise stable profile of fatty acids. The media with the highest ratios show, a sharp, 15% increase in MUFA content.

Table 38. Coccomyxa o experiment with different P/N ratio – pigment analysis results

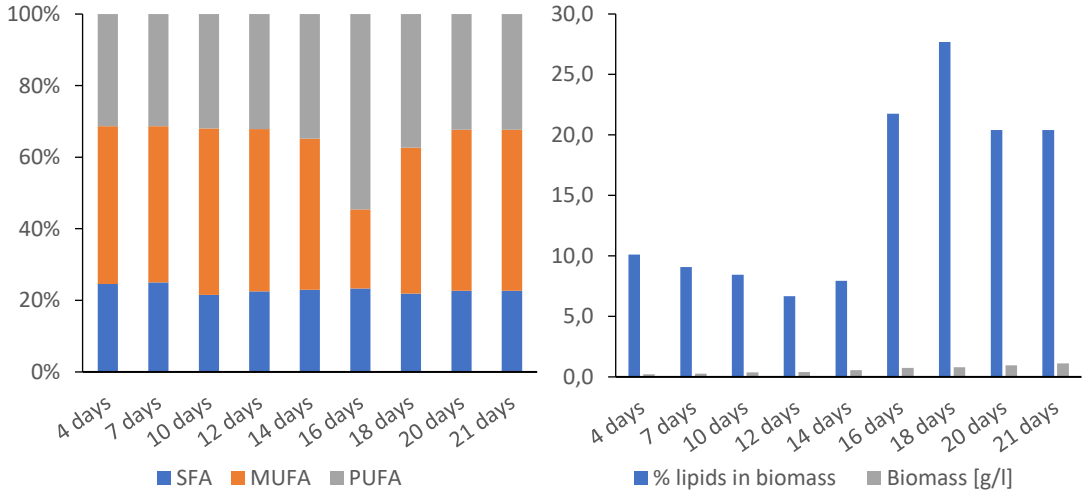
Pigment production [mg/g of dry biomass]						
	Media with P/N 0.05			Media with P/N 0.10		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.27	6.23	3.02	4.58	6.18	3.21
7	4.97	7.20	3.52	5.02	6.23	3.42
10	5.42	6.97	3.18	5.66	7.01	3.99
12	6.09	7.42	3.79	6.17	8.01	4.83
14	6.98	7.99	4.18	7.01	7.89	4.72
16	7.80	8.33	4.50	6.98	8.42	5.00
18	7.47	9.00	4.78	7.36	9.20	5.41
20	8.26	9.20	5.01	7.72	9.00	5.70
21	8.53	8.94	5.21	8.03	9.15	5.50
	Media with P/N 0.15 STD			Media with P/N 0.30		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.71	6.77	4.01	5.23	6.34	3.88
7	5.42	6.49	3.99	5.03	6.83	3.47
10	6.20	7.01	4.53	6.22	7.72	4.08
12	6.72	7.75	4.71	6.75	7.85	4.78
14	7.51	8.51	5.47	7.30	8.03	5.36
16	7.43	8.42	6.00	7.72	8.87	5.88
18	7.78	8.89	5.85	8.08	9.34	6.12
20	8.02	9.77	6.02	7.92	9.78	6.23
21	8.23	9.49	5.79	8.12	9.89	5.98

Conversely, in two media with a lower P/N ratio, we see the opposite trend of a decrease in MUFA in favour of PUFA, which is very evident in the case of a sample from a medium with a P/N ratio 0.10. From these data, we see that in media where microalgae grow faster, *Coccomyxa* o. cells prefer a higher proportion of polyunsaturated fatty acids. In terms of total lipid production, the lipid content in all media was 20-23% at the end of the experiment, with a maximum in the standard ratio medium (P/N 0.15). In general, there are significant fluctuations in lipid production in all media during cultivation. We see this most markedly on the medium with a standard ratio, where at the beginning of the 3rd week of cultivation there is a decrease in lipid content with a subsequent significant increase at the end of cultivation.

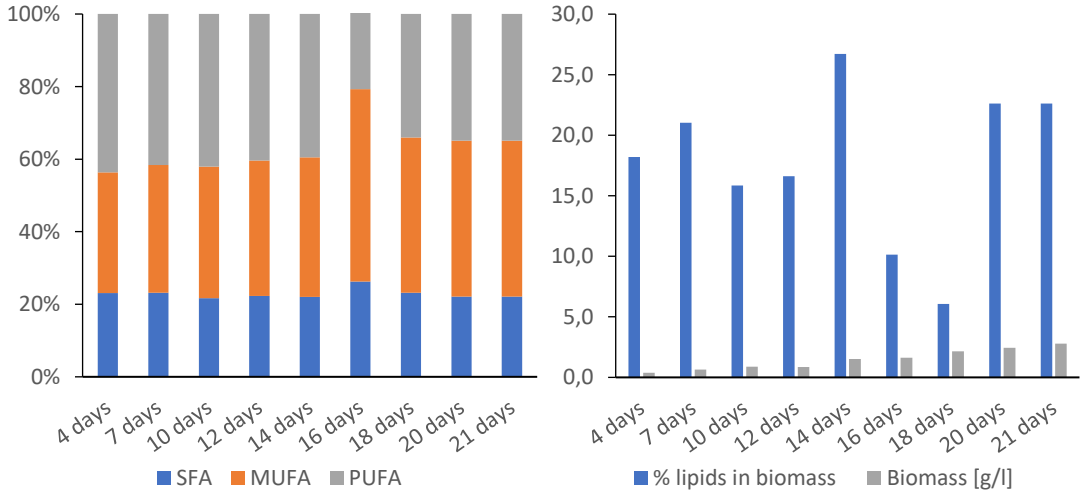
Furthermore, on medium with a P/N ratio 0.1, we see a jump from 7.93% to 21.77% of lipids in biomass during two days of cultivation after two weeks of low lipid production. From the overall comparison, if we consider biomass production, the most effective medium was the highest P/N ratio, where the percentage of lipids in biomass was 20.27%. The highest lipid content measured in this experiment was 35.15% on the seventh day of cultivation on the lowest ratio medium. On the other hand, if we include higher biomass production, a medium with a P/N ratio = 0.3 is a better option here as well.



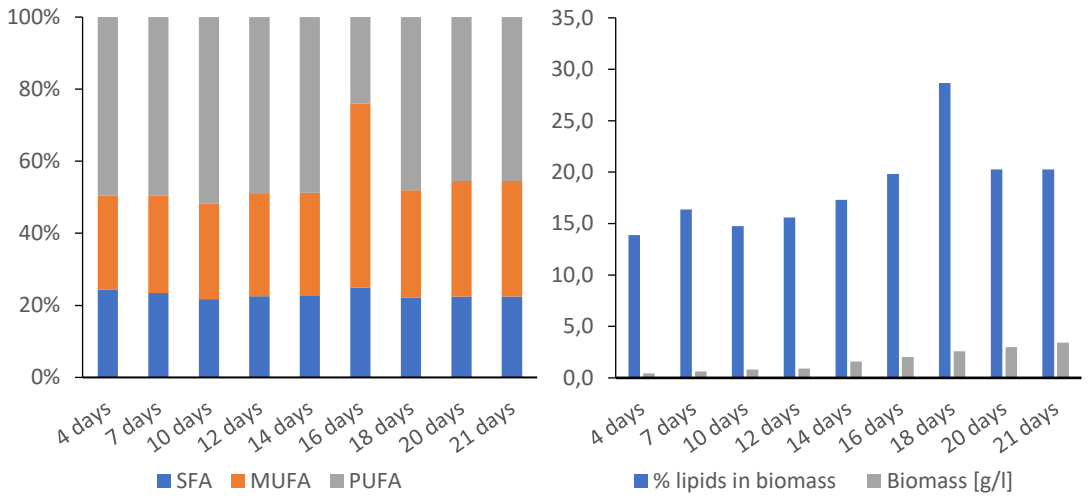
Graph 43. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with P/N ration 0.05*



Graph 44. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with P/N ration 0,10*



Graph 45. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with P/N ration 0,15*



Graph 46. *Fatty acid profile and total lipid production of Coccomyxa o. cultivated on salt K9 media with P/N ration 0,3*

5.2.2.1.4 Oxidation stress – cultivation with Fe^{3+}

The last part of results shown in this chapter are tests of the effect of oxidative stress caused by the presence of iron ions in the medium. In this experiment, four flasks were cultivated, the first served as a control medium without the addition of iron and then the other three with increasing concentrations of iron ions. Due to lack of time, this cultivation lasted only 14 days (Table 39). From the biomass production data, it is not clear whether iron ions affect biomass production. In a flask with a minimum concentration of biomass, reduced growth rate occurs. On the other hand, in the remaining two flasks, we see increased biomass production. The best results were then obtained on a medium with 200 mM iron ions.

The table below shows (Table 40) the results of pigment production analysis by the microalgae *Coccomyxa o.* under oxidative stress. The results show a particular trend where this microalga prefers a narrow range of iron ion concentration in the medium (within the tested conditions) in terms of carotenoid production. Thus, in an environment with a 200mM concentration of iron ions, the highest production of carotenoids of 8.72 mg/g of dry biomass was achieved, which is a 16% increase compared to the control medium.

In media with lower or higher iron concentration, carotenoids' production was lower than the best medium and the control K9 medium. The presence of iron ions in the medium affected the profile of accumulated carotenoid pigments, where under the given conditions, the content of carotenes was increased at the expense of xanthophylls. The production of chlorophylls in all media containing iron ions decreased overall. The measured values show a slow increase in chlorophyll content. However, compared to the control medium, it is significantly smaller and, in general, the decrease in production is proportional to the concentration of iron in the medium.

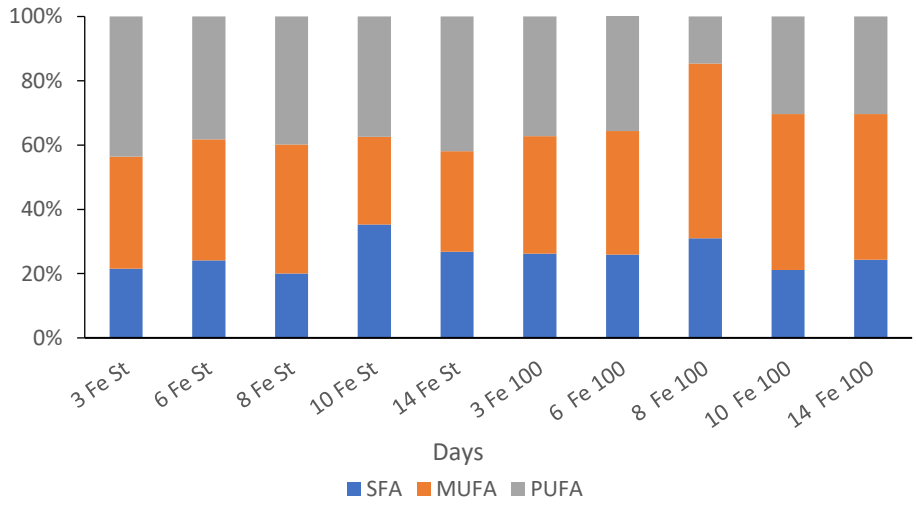
Table 39. Biomass production of Coccomyxa onubensis cultivated on media with different Fe^{3+} ion concentration

	Biomass [g/L]				
Sample	3 days	6 days	8 days	10 days	14 days
Fe ST	0.70	1.08	1.05	1.20	1.31
Fe 100	0.61	0.66	0.87	0.96	1.11
Fe 200	0.71	1.68	1.73	1.98	2.12
Fe 300	0.68	0.97	1.24	1.42	1.61

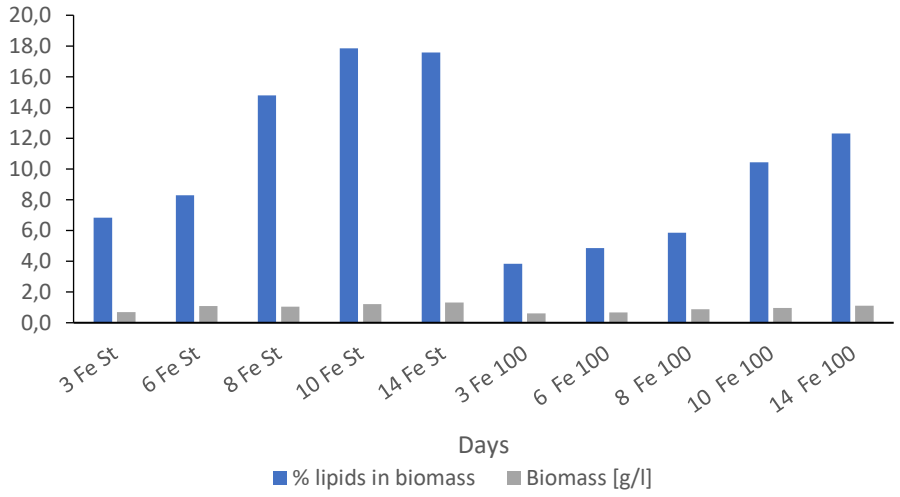
Table 40. Coccomyxa o. cultivation under oxidative stress – pigment analysis results

Pigment production [mg/g of dry biomass]						
	Standard salt K9 media			Salt K9 + 100 mM Fe^{3+}		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
3	4.587	4.816	4.799	3.487	4.415	3.844
6	5.126	5.977	5.607	4.012	4.232	3.943
8	6.623	7.102	5.498	4.716	5.011	4.011
10	7.024	8.015	6.311	5.426	5.856	4.784
14	7.526	8.646	6.785	5.230	6.458	4.648
	Salt K9 + 200 mM Fe^{3+}			Salt K9 + 300 mM Fe^{3+}		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
3	5.234	4.415	2.715	3.758	4.655	2.485
6	6.523	4.788	2.986	4.254	4.412	3.022
8	7.254	5.426	3.283	5.716	4.718	2.989
10	8.065	6.238	4.318	6.234	5.018	3.214
14	8.716	6.026	4.121	6.526	5.228	3.502

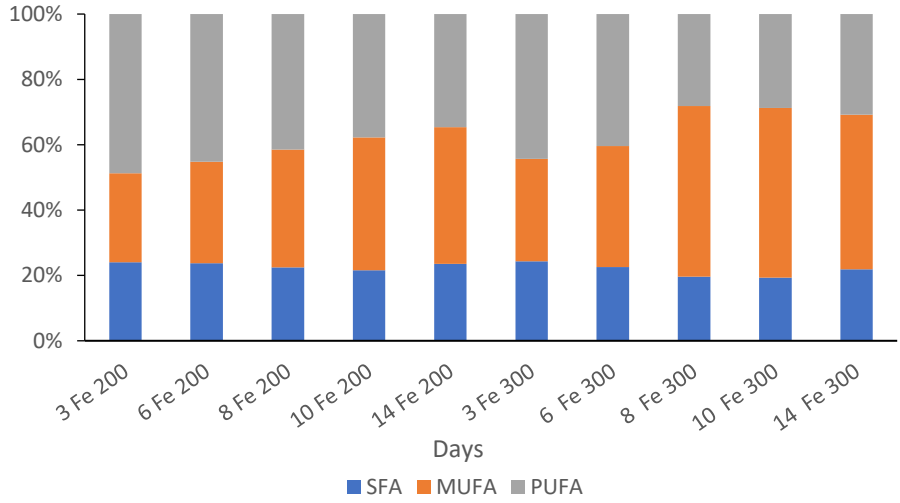
The following four graphs summarize the results of the chromatographic analysis of lipids. Due to the shorter cultivation time and the smaller number of samples, the two cultivation flasks' results are combined in the graph. In the results of the total lipid content, we see that, as in the production of biomass, comparable (300 mM Fe^{3+}) or higher lipid production (200 mM Fe^{3+}) was achieved here as well. Only in the case of cultivation with the lowest iron ions concentration a reduced lipid production was achieved. In all cultivations, we see a gradual increase in the content of lipids in the biomass, so it can be assumed that prolonging the cultivation would achieve a higher lipid content in all media. We again see stable production of saturated fatty acids in the interval of 21-25% in terms of fatty acid profile. Only in the case of the control medium, a higher value of 35.29% was measured on the 10th day of cultivation and 31.03% on the 8th day of cultivation on the medium with 100 mM Fe^{3+} (Graph 48). However, we see here that iron's presence had a negative effect on the PUFA content, which decreased with the continuing days of cultivation (Graph 47)(Graph 49). On the other hand, the presence of iron, induced the synthesis of monounsaturated fatty acids, which is on average, 10-15% higher compared to the control medium. Overall in terms of oxidative stress, the best medium was a medium with 200 mM iron ions.



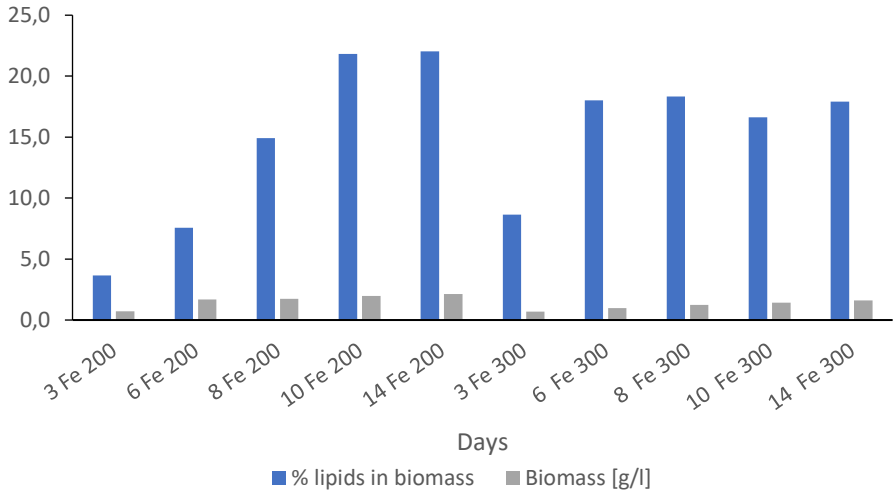
Graph 47. *Fatty acid profile Coccomyxa o. cultivated on salt K9 media and K9 media with 100mM Fe^{3+}*



Graph 48. *Total lipid production of Coccomyxa o. cultivated on salt K9 media and K9 media with 100mM Fe^{3+}*



Graph 49. *Fatty acid profile Coccomyxa o. cultivated on salt K9 media with 200mM Fe^{3+} and 300mM Fe^{3+}*



Graph 50. *Total lipid production of Coccomyxa o. cultivated on salt K9 media with 200mM Fe^{3+} and 300mM Fe^{3+}*

5.2.2.1.5 Salt adaptation experiment results summary

This experiment aimed to adapt the acidophilic microalgae *Coccomyxa onubensis* to a medium with a high content of sodium chloride and subsequently optimize the medium's composition to increase the production of the studied substances. After a successful adaptation, a series of cultivations were performed, which began with selecting a suitable nitrogen source. Three sources were tested in the experiment, namely ammonium sulphate urea and sodium nitrate. Experiments have shown that urea is the optimal source of nitrogen. This nitrogen source can be fully processed by the microalgae and does not further affect the pH of the medium, unlike other nitrogen sources.

Determining the pH optimum for cultivation *Coccomyxa o.* was the primary task to which all other experiments were linked. The increased concentration of salts increases the osmotic stress exerted on the cells in the medium, and they are forced to adapt to it. With increased osmotic stress, the cells may no longer be able to tolerate the pH optimum of standard cultivation conditions. Flasks with different pH in the range of pH=2-7 were tested in the experiment. The pH experiment results clearly showed that the pH optimum of microalgae cultivated under salt stress conditions lies in the range of pH=3-4. Under these conditions, the largest increase in biomass and lipids was achieved. Simultaneously, the value of the quantum yield of fluorescence in these conditions was stable in the range of 0.6-0.75, indicating a good condition of the cell's photosynthetic apparatus. Under these conditions, the highest production of lipids was achieved, namely 38.05% of lipids in biomass, the production of which was set at 4.59 g/L. At lower or higher pH values, the culture either grew very slowly, or there was a rapid growth of symbiotic organisms, which outgrew the microalgae. The production of carotenoids and chlorophylls in individual media copies data from biomass production. Outside the pH optimum, the production of pigments is strongly negatively affected and gradually decreases rapidly. In the pH optimum, the production of pigments is stable and increases during cultivation.

Subsequently, cultivations were performed using six different concentrations of nitrogen. From the performed experiments, it was found that in the case of low concentrations of nitrogen in the medium (20% and 40%), biomass and lipids production is very low. With increasing nitrogen concentration, biomass and lipid production is already stable and increasing. Overall, the best results were obtained on medium with 80% nitrogen concentration and then on standard K9 medium. In both of these media, we observe a linear increase in lipid content, which turns into exponential growth in the final phase. The highest production in both cases exceeded the limit of 45% lipids in dry matter. Different nitrogen concentration had virtually no effect on the fatty acid composition, as no change was observed depending on the medium's different nitrogen content. Increased MUFA production at the expense of PUFA was observed in the final phase in all flasks. Analysis of the pigment production under these conditions has shown that decreasing nitrogen content in the medium leads to reduced chlorophyll pigment production. The same trend was observed in the production of carotenoid pigments, but not with such intensity. On the contrary, the increased nitrogen concentration led to a higher production of all pigments.

With these results, experiments were continued with different P/N ratios, i.e. different phosphorus concentrations in the medium. In this experiment set, the highest P/N ratio = 0.3, i.e. the medium with a higher phosphorus content, clearly won. In this medium, the highest biomass production and 28.67% lipids in biomass were achieved. The increasing P/N ratio then further led to an increase in the production of chlorophyll A. The opposite trend was observed in carotenoids production, where the most suitable conditions for production were achieved at the lowest ratio. Various sulphur concentrations were also tested in the experiment, but the results did not show clear effects on the production of the studied metabolites, and it would be appropriate to modify or repeat the experiment. Therefore, these results are not reported here. The last experiment was to test the effect of oxidative stress induced by the presence of iron ions. In these experiments, it was found that the optimal level of iron in terms of biomass and lipid production is 200 mM. The presence of iron had a more significant effect on the fatty acid profile compared to previous experiments. We see here a general trend of increasing the content of monounsaturated fatty acids in the cell at the expense of PUFA production. Oxidative stress in the medium affected the production of the studied pigments. The results show that *Coccomyxa o.* prefers a certain level of iron ions in the medium in terms of biomass and carotenoid production. In this experiment, it was 200 mM Fe^{3+}. On the other hand, the increased concentration of iron ions has a strong inhibitory effect on chlorophylls' production.

From the fatty acid profiles data, we can see that in an environment with 0.5 M NaCl, *Coccomyxa o.* maintains the stable production of saturated fatty acids in the range of 20-25%. Moreover, any differences depending on the changing cultivation conditions are reflected only in mono and polyunsaturated fatty acids' distribution. In cultivations where there was a faster growth of microalgae, we observed a slightly increased preference for the production of polyunsaturated fatty acids. The analysis also shows that even in flasks where there was a high production of biomass, the microalgae maintained high lipid production, which is a perfect prerequisite for future use in industrial applications based on biomass production with high lipid content. Under many conditions, the microalgae was able to maintain relatively stable carotenoid production. Their increase was achieved by increasing the concentration of nitrogen and iron in the medium. The production of chlorophylls by microalgae can be increased in this case by increasing the concentration of nitrogen and phosphorus.

5.2.2.2 *Raceway pond cultivations of Coccomyxa onubensis*

Experimental cultivation in raceway ponds was designed to study *Coccomyxa* resistance to contamination, outdoor growth properties, pigment, and lipid production. And finally, the whole feasibility of the process. In the beginning, three different conditions were prepared, which are shown in chapter 4.4.3.1.2. The final volume in each pond was set to 320 L (300 L media + 20 L inoculum). To mimic the light wind condition, two raceway ponds were aerated. The culture was mixed with a paddlewheel set to 20 revolutions per minute. Samples from each raceway pond were taken every other day (Monday – Wednesday – Friday) to measure these parameters: Absorbance 680 nm + 720 nm, media pH and temperature, Quantum Yield, and biomass production. Biomass was then analysed for carotenoid, chlorophyll, and lipid production. Culture samples were periodically checked for microbial contamination. This study focused on basic culture characteristics, which can easily and fast represent the overall state of *Coccomyxa onubensis* in raceway ponds.

Measuring culture turbidimetry is a widely used technique for determining growth speed, welfare, and possible contamination of the culture. It can also tell how a strain is adapting to new media conditions and other valuable information. For this study, NPK fertiliser media with low pH was used as a mineral source. NPK media was chosen to fulfil one of this study's primary aim to find a suitable and cheapest sources and cultivation conditions.

5.2.2.2.1 Biomass production and growth kinetics in different raceway ponds.

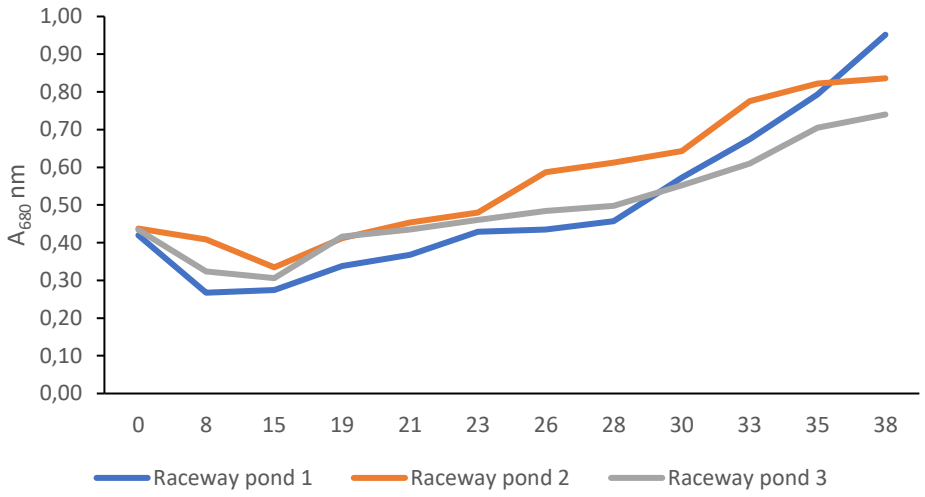
Table 41 describes the overall biomass productivity of *Coccomyxa* onubensis in all three ponds. The beginning of the experiment is practically comparable for all monitored ponds. After inoculating the culture into the production medium, a visible lag phase occurs, when the microalgae adapt to the new conditions. These conditions are relatively different from the inoculum medium, especially in the amount of dissolved carbon dioxide, lighting, and medium temperature. The loss of biomass observed in the second week of cultivation - between 8 and 15 days- is associated with the culture's sedimentation, which firmly adhered to the vessel's walls and bottom. In this phase, the final adaptation of *Coccomyxa* to the cultivation conditions took place, and in the following samples, we already see a gradual increase in biomass production.

Table 41. Biomass production of Coccomyxa o. cultivated in open raceway ponds

	Biomass [g/L]										
Days of cult.	8	15	19	21	23	26	28	30	33	35	38
Race 1	0.46	0.36	0.27	0.53	0.65	0.72	0.83	0.93	1.00	1.10	1.18
Race 2	0.44	0.32	0.26	0.43	0.64	0.66	0.70	0.73	0.81	0.93	1.01
Race 3	0.45	0.35	0.30	0.49	0.61	0.71	0.73	0.75	0.75	0.82	0.87

Figure 23. Coccomyxa onubensis cultivated in raceway pond 1

In raceway pond 1, which contains twice the amount of nutrients, we observe a linear increase in absorbance from day 23rd to 38th day of cultivation (Graph 51). Due to the slight acceleration of the increase in absorbance between the 35th and 38th day of cultivation, it can be assumed that the linear growth curve would continue in the following days. In raceway pond 2, *Coccomyxa* growth showed comparable characteristics as in the first case. We see a decrease in total biomass in the second week of cultivation, associated with a decrease in absorbance at 680 nm to the lowest measured value compared to other experiments. Then there is a transition to linear culture growth, and as in the case of the first raceway pond, it can be assumed that in the following days of continuation of the experiment there would be a linear growth of the culture. Cultivation in the third algae reactor represented the most basic conditions, with standard low nutrient content, without aeration. After the same adapt period as in the previous cases, we see a gradual increase in absorbance and biomass production, which is lower than the total production in the first and second ponds. In the final phase of the experiment, there is a slight acceleration of the increase in biomass production, and the same applies to absorbance.

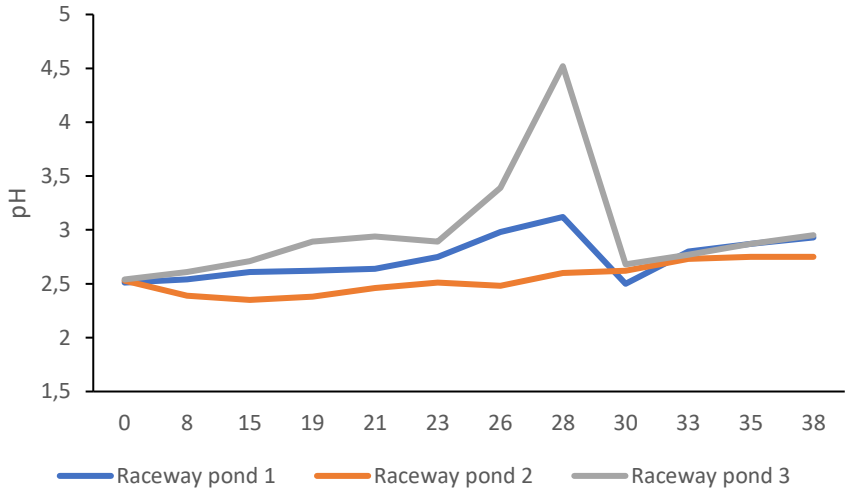


Graph 51. *The absorbance A_{680} of Coccomyxa cultures measured throughout the experiment*

5.2.2.2.2 pH measurements

The pH of the medium was crucial in this experiment, because cultivated *Coccomyxa onubensis* is an extremophilic acid-tolerant microalga. The low pH of the medium is a huge advantage for this type of cultivation, as it is one of the main parameters that prevent contamination of the medium by other types of microorganisms. As already mentioned in the materials and methods section, the inoculum medium's initial pH was 2.5. This value was chosen based on previous experiments and is the value at which the reproduction rate of *Coccomyxa* reaches a compromise in correlation with possible contamination using a higher pH value of the medium. In all cases, we see that *Coccomyxa* can maintain a low pH in the range of 2.5-3.0 for a long time, preventing contamination by other strains. An exception is a short section of cultivation in the third pond where between the 26th and 28th day of cultivation, we observed a sudden increase in pH to 4.5. There was probably a metabolic shunt or culture response to an external stimulus. Microscopic observation revealed no significant contamination with another type of microorganism. Most of the *Coccomyxa* cells were aggregated, and floating colonies formed visible to the naked eye. Due to the experiment's continuation, the medium's pH value was slowly adjusted back below pH=3 in the next two days (Graph 52).

A similar case was observed in the first raceway pond. Between the 23rd and 28th day, the pH of the medium increased to 3.12. In this case, however, no aggregate formation was observed, and without external intervention, the media's pH returned to 2.5 in the following days. There is a slight increase in the medium's pH in the second half of the cultivation in all experiments. This phenomenon is due to the metabolic products of *Coccomyxa* and the gradual consumption of nutrients in the medium.



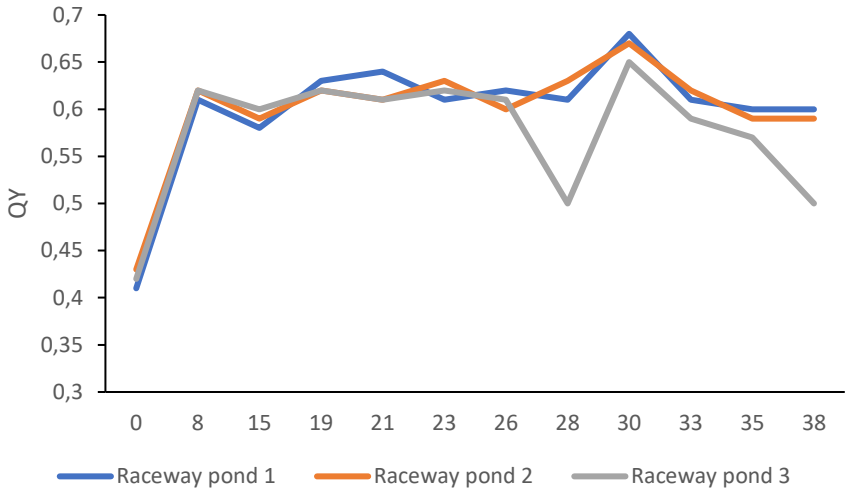
Graph 52. *Schematic diagram depicting pH measurements during the whole experiment*

5.2.2.2.3 Quantum yield

Measuring the quantum yield provides quick information about the overall state of microalgae. From this value, it is possible to deduce how chloroplasts work, i.e. photosystems in the microalgae cell. In the case of *Coccomyxa*, the value of the quantum yield of fluorescence under optimal conditions is in the range of 0.75-0.80. The data shown in the graph below show the development of QY during cultivation (Graph 53). When inoculating the inoculum culture from large bags, the optical density of the culture was very high, and for this reason, the quantum yield of the sample was lower at 0.4. With a high increase in biomass, the transmission of light throughout the culture is reduced, and the culture adapts by reducing the production of chlorophylls and other molecules needed for photosynthesis. Another reason was that the culture was already in the stationary phase, and most of the nutrients in the medium had already been consumed.

After inoculating the inoculum into raceway ponds, we see a rapid increase in the quantum yield of fluorescence. There was a much higher light transmission in the fresh medium with sufficient nutrients and low optical density, and the cultures in all ponds quickly adapted to these conditions. For all ponds, the value during cultivation was on average 0.6-0.65, which is approximately 0.1-0.15 lower than the value measured under lab conditions in the optimised K9 media. Cultivation in the first and second ponds in terms of QY value shows a comparable course with a maximum of 0.68 on the 30th day of cultivation. Then QY stabilises again at an average value of 0.6. The first significant changes in QY values are observed in the third raceway pond, where there is a significant decrease to 0.5 on the 28th day of cultivation, followed by a return to the original values.

This decrease in QY is associated with an increase in the culture medium's pH and thus, a deviation from stable cultivation conditions. After adjusting the pH in the medium, the QY value returns to normal. In the last days of the experiment, we observe a slight decrease in QY values in all monitored ponds. The culture reached the most significant decrease in the 3rd pond, and it can be concluded that in the following days of the experiment there would be a decrease in the growth rate of the culture and an increase in the difference compared to the first and second pond. From the total QY values, it can be concluded that even under these cultivation conditions, *Coccomyxa* retained a relatively high photosynthetic efficiency, and with further optimisation of the conditions, the growth rate could increase significantly.



Graph 53. *Schematic diagram depicting quantum yield of Coccomyxa culture.*

5.2.2.2.4 Microbiological observations

Coccomyxa onubensis as a microalga in natural conditions coexists in a symbiotic relationship with other microorganisms. In this alga case, it is mainly a symbiosis with strains of bacteria. This symbiotic relationship maintains suitable conditions for growth, such as pH and limitation of the presence or overgrowth of other non-symbiotic species of microorganisms. Maintaining a suitable pH is made possible by exchanging chemicals, where algae are thought to produce organic compounds that are processed by heterotrophs to form molecules that lower the pH of the medium. There is also a mutual exchange of gases. The oxygen produced by the alga is processed by the heterotroph and recovered in carbon dioxide. The original medium from which *Coccomyxa* was isolated is characterised by a very low pH and a high content of salts and metals. Under these conditions, the solubility of carbon dioxide in the medium is very low and therefore, the path of symbiosis with the heterotroph is very advantageous for algae.

On the other hand, the heterotroph acquires a stable source of nutrients. During cultivation, samples of culture were periodically checked for contamination under the microscope. At the beginning of the experiment, practically axenic culture was inoculated, no significant contamination was observed, and this trend was maintained throughout the cultivation in all three culture ponds. Various dust particles were observed in the samples, which were clogged by the wind. Overall, however, there was no significant contamination of the culture in any culture tank, which would lead to reduced growth or killing of the culture.

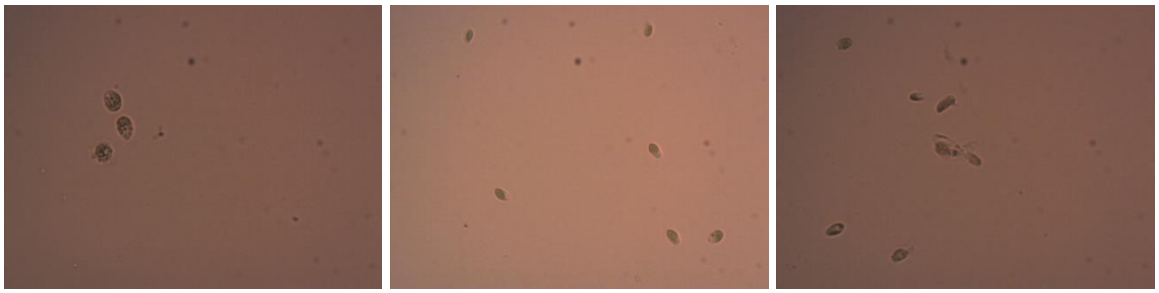


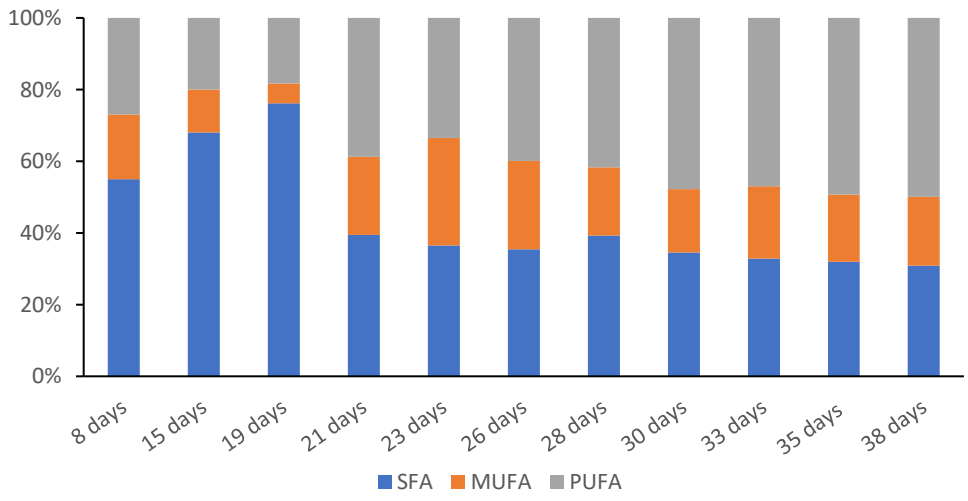
Figure 24. Microscopic sample of Coccomyxa o. cultivated in raceway ponds 1 to 3.

5.2.2.2.5 Lipid production and fatty acid composition

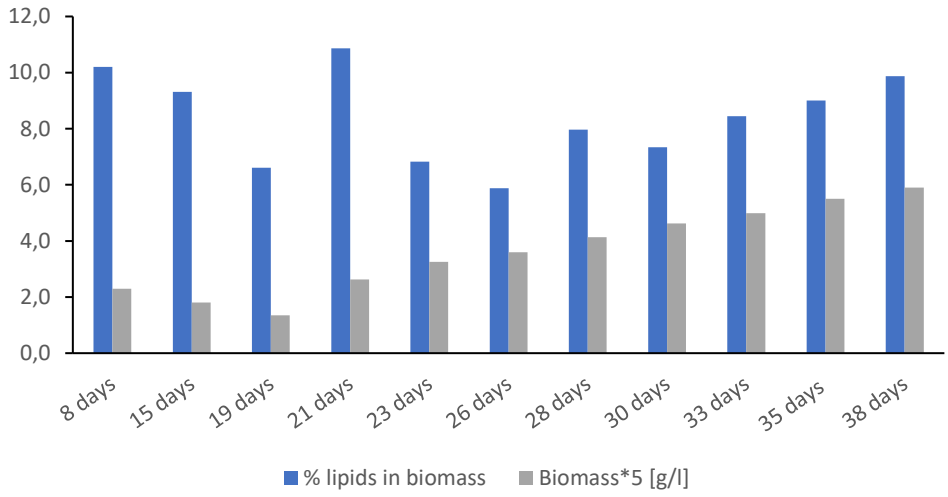
As mentioned in the introductory chapters, microalgae's lipids can best be used in the food and pharmaceutical industries. During the experiment, biomass samples were subjected to GC analysis to determine the fatty acid profile and total lipid production. Microorganisms maintain an optimal fatty acid composition that allows fluidity of all membranes and structural integrity. Microalgae are characteristic for producing a large spectrum of polyunsaturated fatty acids compared to yeasts and bacteria. The application of various stress factors can influence the fatty acid profile. Furthermore, the composition of fatty acids may change at different stages of the cell growth cycle.

5.2.2.2.5.1 Raceway pond 1

In the case of cultivation in the first pond (Graph 54), we see an increase in the production of saturated fatty acids after inoculation in the first 19 days with the peak of SFA production at 76.2% of fatty acids. In the following days, the SFA content decreased to 40%, where this value increased slightly for seven days and then decreased linearly to the final 31% at the end of the cultivation. After an initial decrease in unsaturated fatty acids, a significant increase in production was observed on the 21st day of cultivation. In the following days of cultivation, MUFA production ranged from 20-24%. In PUFA production, there was a linear increase in production from day 21 to a final 49.92%. From the data of total lipid production (Graph 54), we see that in the first phase the content of lipids in biomass decreases, this can be attributed to the adaptation of the culture to new cultivation conditions and the use of stored energy. The total percentage of lipids after the adaptation phase slowly increases linearly to 10% of the biomass content. It can be assumed that the lipid content would continue to increase as culture continues.



Graph 54. *Coccomyxa onubensis: Fatty acid profile of raceway pond 1 cultivation*

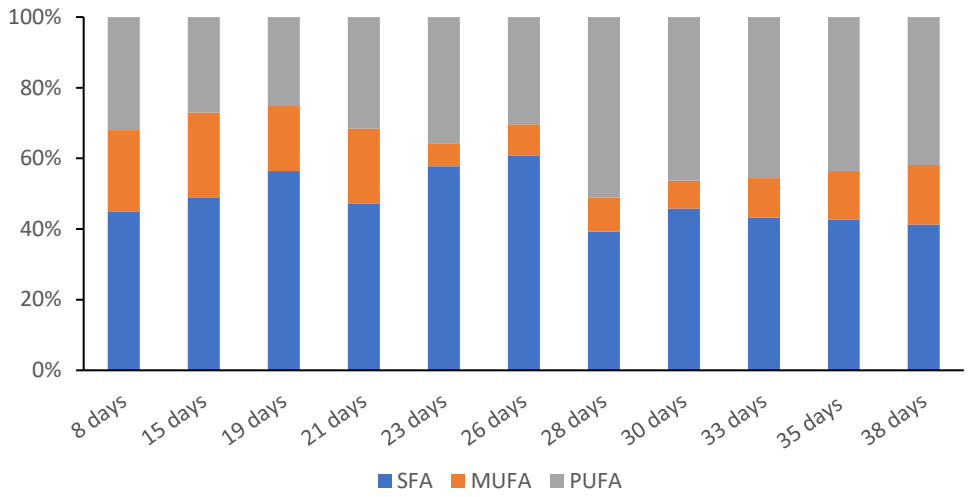


Graph 55. *Coccomyxa onubensis raceway pond 1 cultivation: % of lipids in biomass*

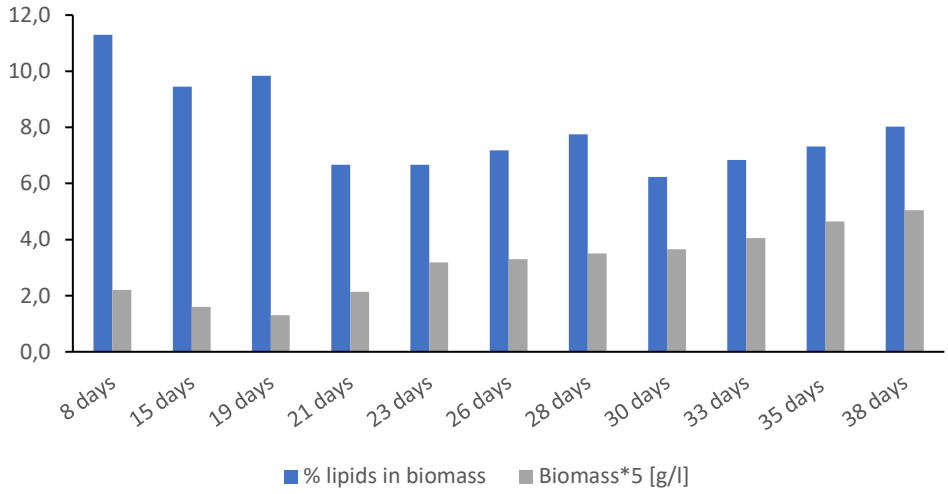
5.2.2.2.5.2 Raceway pond 2

From the GC analysis of the culture, we see the same trend as in the previous case, when the SFA content increases in the first three weeks of the cultivation. In the following week of cultivation, the SFA content initially decreases in favour of PUFA production. This decline is offset in the following days, so we see the same trend as at the beginning of cultivation. In the 26th day, we observe the second peak of SFA production, 60,81%. In next days, we observe a significant decrease in SFA production by 20% in favour of PUFA. In the following days, we observe the stabilisation of the fatty acid composition in the biomass. The highest production of monounsaturated fatty acids, namely 19-24%, is observed only in the first three weeks of cultivation. This is followed by a trend of gradual reduction of MUFA content in biomass. Unlike the first raceway pond, the content of unsaturated fatty acids increases more slowly. In the final phase of cultivation, the PUFA content lowers by 8%. The biomass's total lipid content decreases from the original 11.3% to 6.7% at the beginning of the cultivation (Graph 57). In this experiment, we see two lipid production peaks, where the first one is (7.8%) on the 28th day of cultivation. After a subsequent reduction, the lipid content increases linearly to a final 8.0%.

Graph 56. *Coccomyxa onubensis cultivated in raceway pond 2*



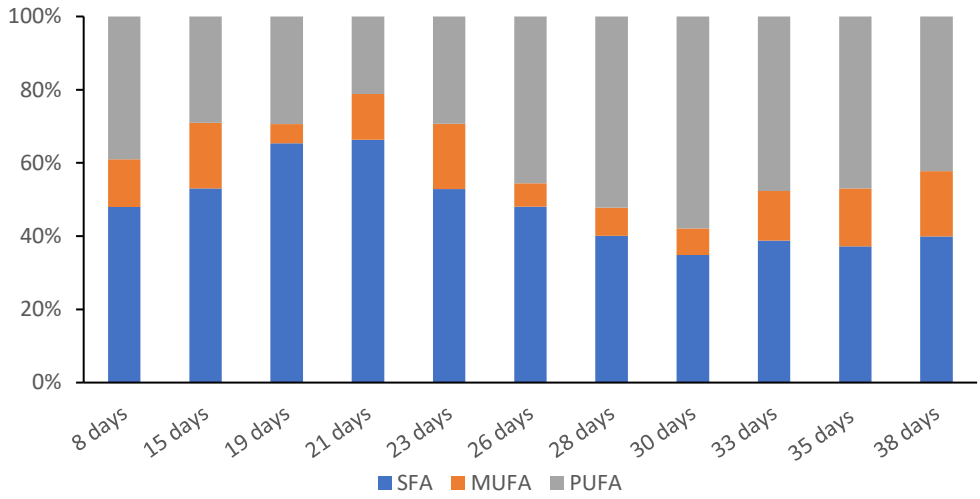
Graph 57. *Coccomyxa onubensis: Fatty acid profile of raceway pond two cultivation*



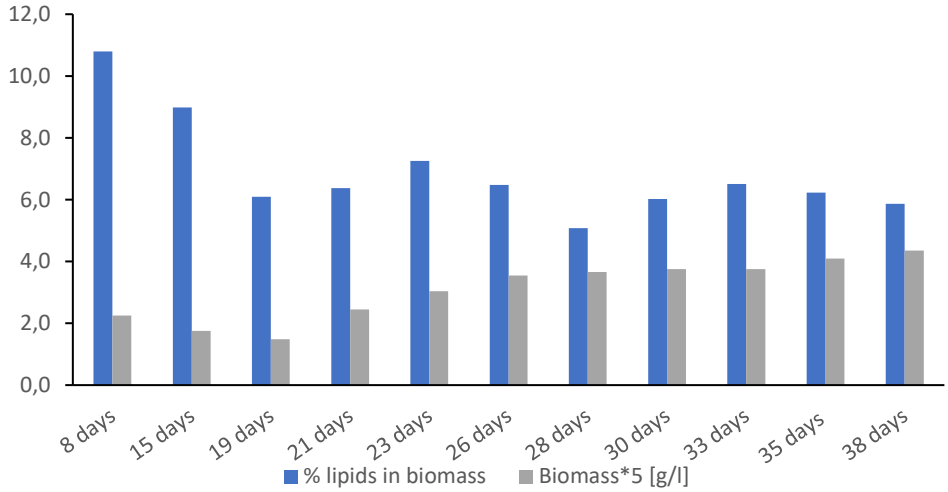
Graph 58. *Coccomyxa onubensis raceway pond two cultivation: % of lipids in biomass*

5.2.2.2.5.3 Raceway pond 3

The measured cultivation data in the 3rd raceway pond show the same trend as previous cultivations. Again we see a linear increase in the SFA content of biomass at the expense of PUFA production initially. The maximum production of SFA occurs on the 21st day of cultivation. Compared to previous cultivations, we see that this trend is two days longer in the case of this cultivation. Subsequently, production decreases and stabilises at 39.9% at the end of the experiment. The production of monounsaturated fatty acids was lower compared to previous cultivations. The maximum production of MUFA was observed at the end of the experiment, namely 17.85%. The average PUFA content in the biomass was comparable to previous experiments. The highest value of production was measured on the 30th day of cultivation. Total lipid production again shows the same trends as the previous cultivation. After an initial decline, we see two production peaks on the 23rd and 33rd days of cultivation. In the final phase of the experiment, we see a gradual decrease in lipid content. The highest achieved production was on the 23rd day, namely 7.23%.



Graph 59. *Coccomyxa onubensis: Fatty acid profile of raceway pond three cultivation*



Graph 60. *Coccomyxa onubensis raceway pond three cultivation: % of lipids in biomass*

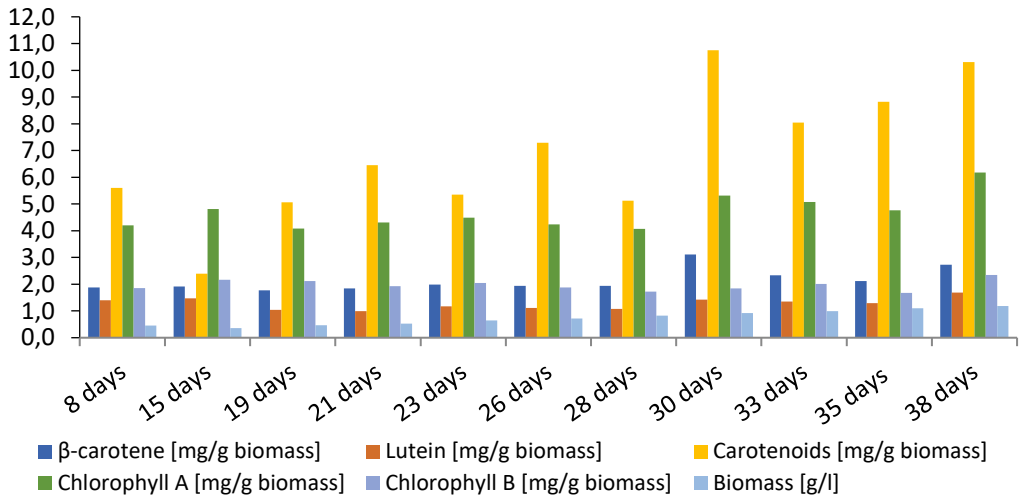
The results show that the total lipid production is low under all studied conditions. In all three experiments, the percentage of lipids decreases in the initial phase, which increases slightly after stabilisation. In the first and second cultivation, we see a linear increase in lipid content in the last ten days of cultivation. The best results were obtained in the first cultivation, namely 9.87% of lipids in biomass. All three cultivations are further characterised by a high content of PUFA and SFA. The low content of lipids is mainly due to the low content of dissolved CO_2 in the medium due to its low pH and absence of artificial CO_2. Increased lipid production was also achieved by aeration. Further optimisation of conditions would achieve higher production.

5.2.2.2.6 Pigment production

In thi study, the effect of different outdoor conditions on carotenoids production and its profile was investigated as well.

5.2.2.2.6.1 Raceway pond 1

The following graph below shows the chromatographic analysis of pigments produced by the microalgae Coccomyxa onubensis cultivated in raceway pond one. We see a drop from 7.93 to 4.58 mg/g of dry biomass in the second week of cultivation in carotenoid production (Graph 61). Furthermore, during cultivation, we observe alternating fluctuations in increased and decreased carotenoid production. The peak of production in this experiment occurred on the thirtieth day of cultivation when the measured production was 10.76 mg/g. At the end of the experiment, 11.32 mg/g of dry biomass was then measured. The profile of carotenoids in this culture was different compared to the classical production of this alga. The lutein content decreased and ranged from 0.9 to 1.7 mg/g of dry biomass, 10-15% of the carotenoid content. The β-carotene content accounted for 20-25% of the total production. In general, carotenes rather than xanthophylls predominate in this cultivation. Chlorophyll production shows a much more stable character. The highest production of both types of chlorophyll A and B was measured at 38 days of cultivation, namely 6.18 mg/g chlorophyll A and 2.34 mg/g of dry biomass of chlorophyll B. It can be assumed that higher production of pigments would be achieved with more prolonged cultivation. In terms of all pigments' production, the last day of cultivation is most suitable for harvesting.

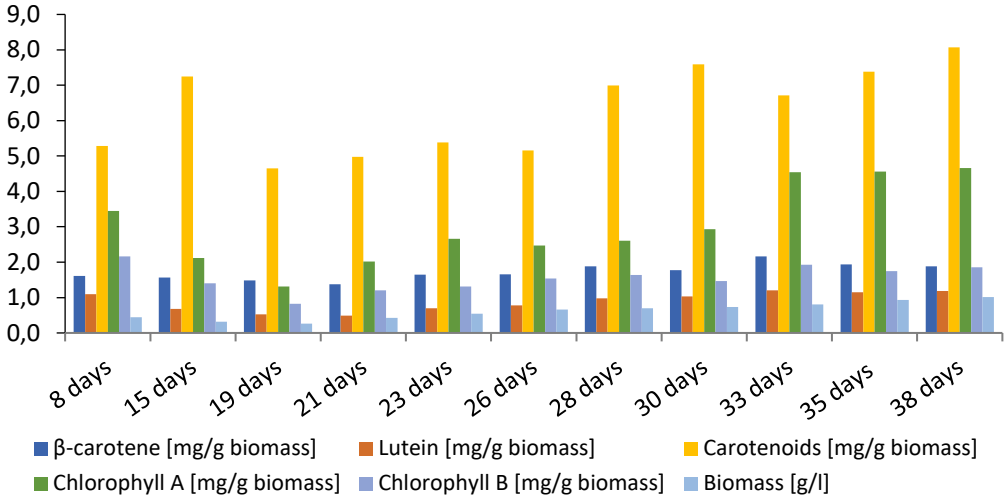


Graph 61. *Raceway pond 1: HPLC analysis results*

5.2.2.2.6.2 Raceway pond 2

The analysis results from the second pond (Graph 62) show a direct effect of the concentration of nutrients in the medium. Half of the medium's nutrient content compared to the first culture had a negative effect on all observed pigments' production. The highest production of carotenoids 8.07 mg/g of dry biomass on the 38th day of cultivation is 3 mg/g lower than the first cultivation vessel. The carotenoid profile remains virtually unchanged here, and again the microalgae prefer carotene production, with the highest proportion being β-carotene. During cultivation, we again see fluctuations in carotenoid production. After an initial increase in the second week of cultivation, a slow increase followed until the end of cultivation. We see the predominant production of chlorophyll A in overall chlorophyll production.

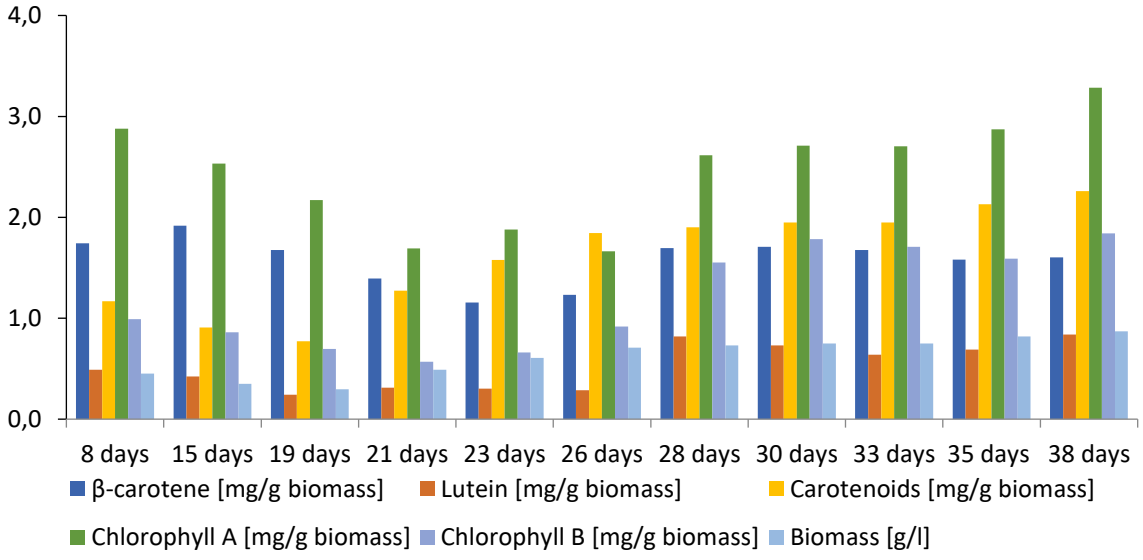
The initial decrease associated with culture adaptation led to reduced chlorophyll production, which ends on the 19th day of cultivation, then production stabilises, and there is a gradual increase in both chlorophyll types in biomass. Even in this case, the most suitable day of harvest is the last day of cultivation.



Graph 62. *Raceway pond 2: HPLC analysis results*

5.2.2.2.6.3 Raceway pond 3

Low nutrient content in the medium and the absence of aeration had a strong negative effect on pigment production. The results below (Graph 63) show that carotenoid production is lower than in previous experiments. With the highest production of carotenoids 6.93 mg/g of dry biomass on the last day of cultivation. In terms of carotenoid profile, we see the same trend as described for the previous two samples. After an initial decline in the first three weeks of cultivation, there is then a gradual increase. The results also show a reduction in chlorophyll production, especially chlorophyll A. This cultivation again shares the best time to harvest the culture, which is on the 38th day of cultivation.



Graph 63. *Raceway pond 3: HPLC analysis results*

By summarizing the results so far, we see that even slight aeration of the medium (2 l/min) in such a large culture volume affects the overall production. Furthermore, a doubling of the concentration of nutrients in the medium led to increased valuable substances' production. A gradual increase in all monitored metabolites' production was observed in all three raceway ponds in the last ten days. It can be assumed that greater production of carotenoids and chlorophylls could be achieved by prolonging the cultivation time.

5.2.2.2.7 Raceway pond cultivation conclusions

Overall data show that *Coccomyxa onubensis* could survive and grow in open pond cultivation, in cheap media under non-sterile conditions. Microalgae was able to control and sustain stable pH in interval 2.5-4.0 throughout the cultivation. Thus, preventing the contamination by other microorganism species. Microalgae was able to produce large quantities of pigments mainly in first two raceway ponds. The best results were obtained from raceway pond one with twice the amount of nutrients and aeration in the final day of the experiment, with carotenoid production 10.76 mg/g, 4.66 mg/g chlorophyll A and 1.85 mg/g of dry biomass. This also applies to lipids production, which reached 9.87% while reaching the highest proportion of PUFA yields, namely 49.92%. Other two cultivations produced less metabolites than the first one. Overall the lipid production was deficient, due to cultivation conditions (low carbon dioxide content, high media temperature and high irradiation. Although biomass production has increased linearly, its productivity is still low. This is one of the main shortcomings of this open cultivation of phototrophic microalgae. Another negative feature of outdoor cultivation is high water evaporation. If the evaporation is slow enough and the culture can grow sufficiently until harvest, this is a positive thing that reduces costs. However, in this experiment, water evaporation was high and will cause an increase in operating costs. Subsequent optimization of cultivation conditions, especially aeration of the medium, the content of individual macro and microbiogenic elements is needed to increase the yield of the cultivation and changes the yield/cost ratio.

5.2.2.3 *Botryococcus braunii v. Showa-Bielefeld cultivation*

The second strain that was tested during the internship abroad was the *Botryococcus braunii* v. Showa-Bielefeld strain. This genus is known to form colonies coated with a protective coating consisting of either polysaccharides or terpenoid lipids, referred to as botryoterpenes. The variety Showa-Bielefeld belongs to the second group, i.e., its colonies' envelope is made up of botryoterpenes and other lipid substances. Furthermore, these microalgae are capable of high production of carotenoid pigments, especially in adverse conditions, there is a significant induction of carotenoid production. This part of the research aimed to optimize the medium and cultivation conditions in order to increase the production of carotenoids, accelerate slow growth and the possibility of extraction of the lipid envelope of the culture. Chu medium was used as a cultivation medium, which is successfully used for members of the genus *Botryococcus*. The following tests were performed as part of the experimental work:

1. Optimization of nitrogen source
2. Cultivation at different pH
3. Cultivation on media with different nitrogen content
4. Cultivation on media with different phosphorus contents
5. Cultivation in the presence of oxidative stress (Co, Fe)
6. Cultivation with diluted media
7. Botryoterpenes cyclic extraction

The first experiments with the microalga *Botryococcus braunii* were focused on finding the most suitable source of nitrogen. Again, three nitrogen sources were tested, as in the previous chapter of *Coccomyxa onubensis* microalgae cultivation. Media with ammonium sulphate, urea and sodium nitrate were prepared and adjusted to normal pH of Chu media, pH=7.2. In the first days of cultivation, a strong negative effect of ammonium sulphate was observed, when its consumption in the medium led to a rapid decrease in pH in the first week of cultivation. When the pH dropped below 6, the growth of microalgae was inhibited, the quantum yield of fluorescence decreased, and the culture gradually died. In the case of sodium nitrate, the effect on the medium's pH was also observed, but it was not as significant, and the culture was able to grow further during the cultivation, but unfortunately not fast enough compared to the medium containing urea. In the long run, however, the nitrate medium's pH also increased outside the optimum level. Therefore, urea was further used as the sole nitrogen source in subsequent experiments.

A follow-up experiment, which was linked to the previous one, was the study of the effect of the medium's pH. Due to the relatively low salt concentration, the medium has a low buffering capacity, and therefore a buffer at a concentration of 100 mM was added to the medium to ensure greater pH stability during the experiment. A series of media with a pH in the range of 5-9 was prepared. Even in this experiment, the microalgae *Botryococcus* was found to have a relatively narrow pH optimum. *Botryococcus* was able to grow optimally in the range of pH=6.5-7.5. At higher pH, growth was slightly inhibited. At pH<6, there was again a rapid inhibition of growth and slight contamination of the culture. In terms of lipid production, production in the range of 8-11% of lipids in biomass was measured in all media.

5.2.2.3.1 Cultivation on media with a different nitrogen concentration

The first results shown here are cultivations studying the effect of the amount of nitrogen present in the medium. In general, the same observations apply here as for other strains of microalgae. At low nitrogen levels in the medium, we observe a minimal increase in biomass production in media with 20-40% nitrogen. The total biomass production reaches a maximum of 1.34 g/L in a medium with 40% nitrogen. From 60% nitrogen in the medium, we can already see a stable growth of biomass, which gradually increases with increasing nitrogen content. The largest increase in biomass was then achieved on the medium with the largest amount of nitrogen, namely 2.67 g/L. From the point of view of lipid production, the opposite trend is observed, we see the highest production on media with low nitrogen content (Graph 64)(Graph 65). The maximum production of 15.85% was measured in a medium with 20% nitrogen.

With increasing nitrogen content and faster growth, lipid production steadily decreases to an average value in the interval of 8-10% (Graph 66). We see here that under optimal conditions, the microalgae maintains stable lipid production, which appears to be low, only around 10% (Graph 67). However, we must also take into account the production of extracellular lipids in the form of botryoterpenes. The fatty acid profile of the microalgae is very stable under the given cultivation conditions. If we focus on saturated fatty acids content, these acids stably make up at least 19-21% of the total fatty acid content. A slightly increased SFA content was measured in the low nitrogen content medium. The production of unsaturated fatty acids then makes up the remaining 79-81%. From the results, we see the microalgae *Botryococcus* prefers to produce the mostly monounsaturated fatty acids. Results show a gradual increase in the content of monounsaturated fatty acids at the expense of polyunsaturated fatty acids during all cultivations. The final MUFA content makes up 58-63% of the biomass's fatty acid content, but it is not clear whether the amount of nitrogen in the medium has a direct effect on the total content of monounsaturated fatty acids. The percentage of PUFA gradually decreases during cultivation with increasing biomass. From the overall point of view, the best medium was the one with the highest nitrogen content.

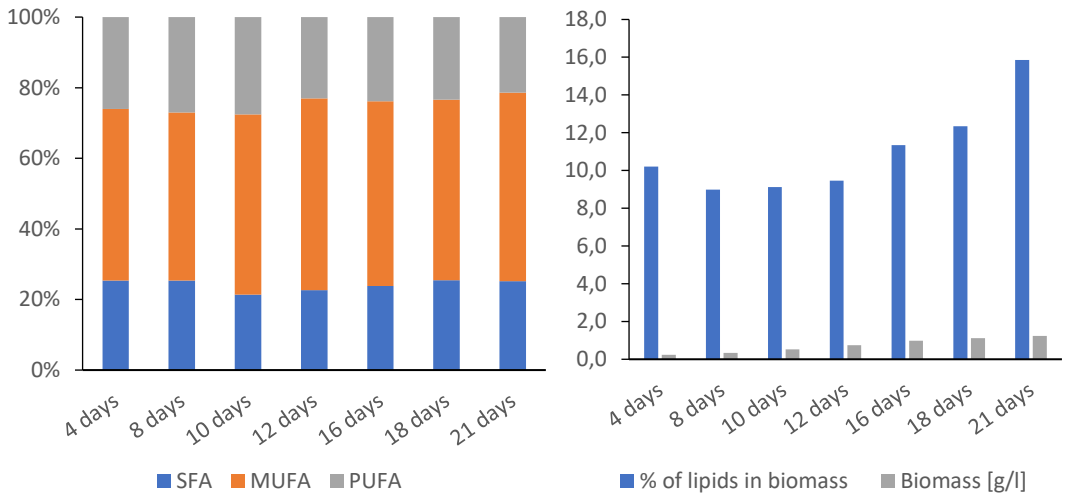
From the pigment analysis results (Table 43), we see a stable trend of increasing carotenoid pigments production with increasing nitrogen concentration in the medium. In more detail, we can see that the limit here is a concentration of 80% nitrogen in media. Here we see a significant jump in the production of carotenoids, but also chlorophylls. Thus, the primary assumption was confirmed that at a higher concentration of nitrogen in the medium, the cells are capable of greater biomass production and higher production of carotenoids, i.e. by reducing the C/N ratio. The same can be said about the production of chlorophylls. Thus, under the given cultivation conditions, the most suitable combination is 150% nitrogen, when carotenoid production 8.03 mg/g, chlorophyll A 5.15 mg/g and chlorophyll B 10.15 mg/g of dry biomass was achieved. Furthermore, the results did not show that a change in the nitrogen concentration affected the change in chlorophyll pigments' content. In all cultivation flasks, the microalgae *Botryococcus braunii* retains chlorophyll B as the major green pigment. Furthermore, as chlorophyll B concentration increases, the ratio of chlorophyll B/A concentrations gradually increases.

Table 42. B. braunii experiment with different nitrogen concentration – biomass production

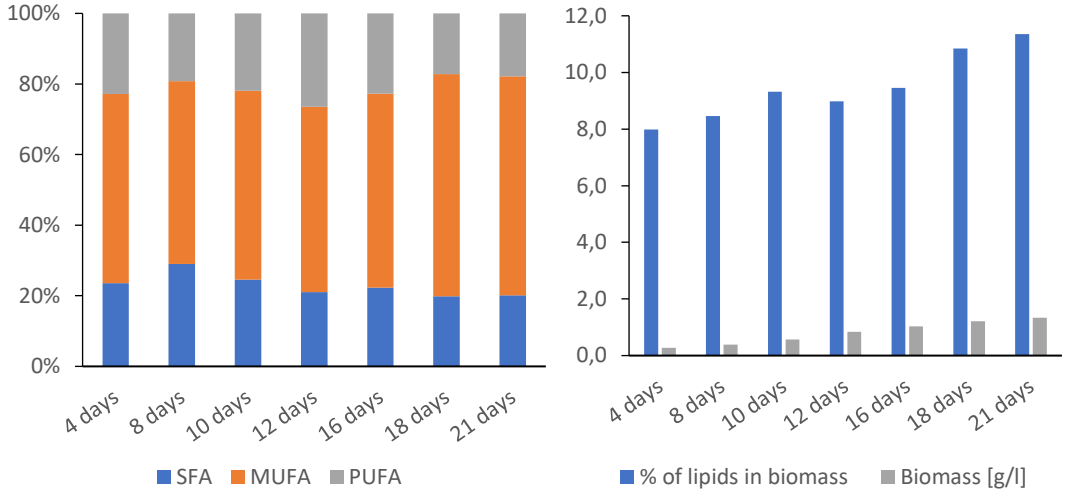
Biomass [g/L]							
	4 days	8 days	10 days	12 days	16 days	18 days	21 days
20 % N	0.23	0.34	0.52	0.75	0.98	1.12	1.23
40 % N	0.28	0.39	0.57	0.84	1.03	1.21	1.34
60 % N	0.29	0.42	0.63	1.01	1.36	1.57	1.96
80 % N	0.34	0.52	0.78	1.11	1.39	1.77	2.14
100 % N	0.41	0.65	1.02	1.33	1.64	1.98	2.45
150 % N	0.45	0.76	1.12	1.61	1.93	2.28	2.67

Table 43. B. braunii experiment with different nitrogen concentration – pigment analysis results

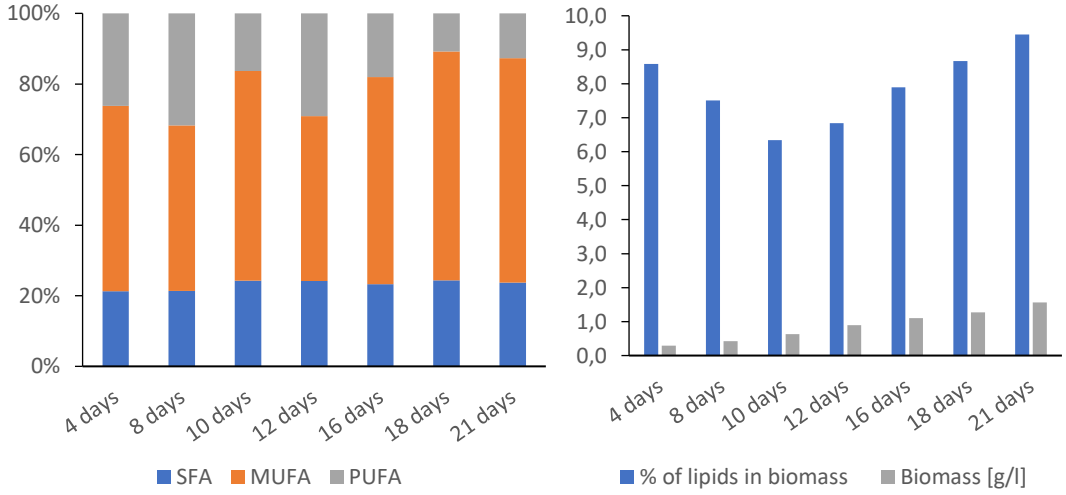
Pigment production [mg/g of dry biomass]						
	Media with 20 % N			Media with 40 % N		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.09	2.80	4.02	4.16	3.59	4.64
8	4.65	3.00	3.78	4.65	3.49	4.65
10	4.87	3.15	3.83	4.98	3.40	4.11
12	5.12	3.24	4.09	5.16	3.02	4.35
16	5.02	3.02	4.12	4.84	2.86	4.58
18	4.90	2.99	4.02	5.08	2.97	4.35
21	5.04	2.87	3.81	4.80	3.40	4.69
	Media with 60 % N			Media with 80 % N		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	3.81	3.85	4.71	4.96	3.98	5.03
8	4.02	4.04	5.26	5.19	4.33	6.03
10	4.26	4.33	5.65	5.47	4.57	6.85
12	4.89	4.19	6.35	5.88	4.77	7.56
16	5.02	4.62	6.85	6.13	4.70	7.23
18	4.93	4.43	7.01	6.03	4.72	7.35
21	5.10	4.05	6.80	6.23	4.97	7.40
	Media with 100 % N			Media with 150 % N		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	5.03	4.01	5.54	4.33	4.21	5.24
8	5.84	4.42	5.65	5.00	4.48	6.03
10	6.41	4.25	6.48	5.87	4.71	7.26
12	6.80	4.64	6.62	6.72	4.98	7.89
16	7.15	4.92	7.68	7.08	5.20	8.73
18	7.40	5.11	8.41	7.46	5.01	9.41
21	7.35	5.06	8.69	8.03	5.15	10.15



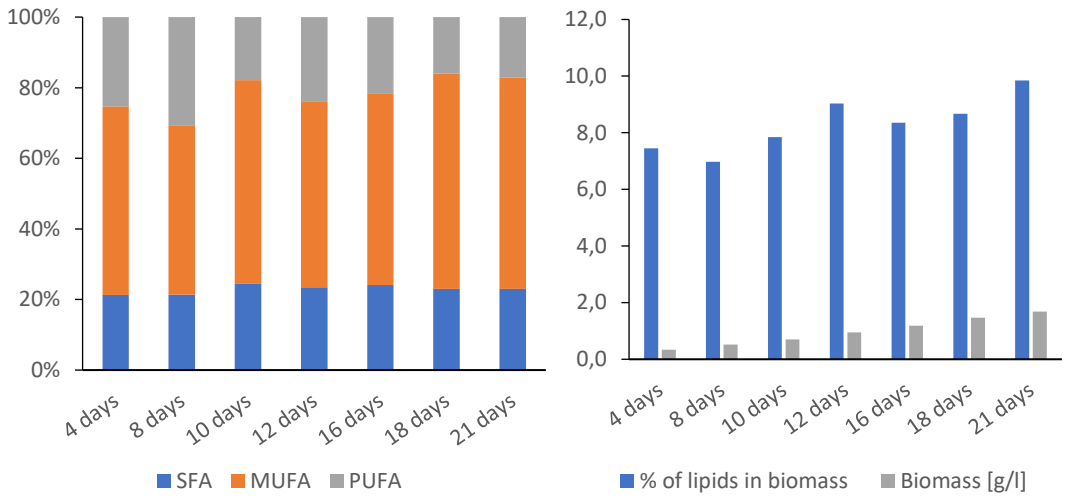
Graph 64. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 20 % of nitrogen*



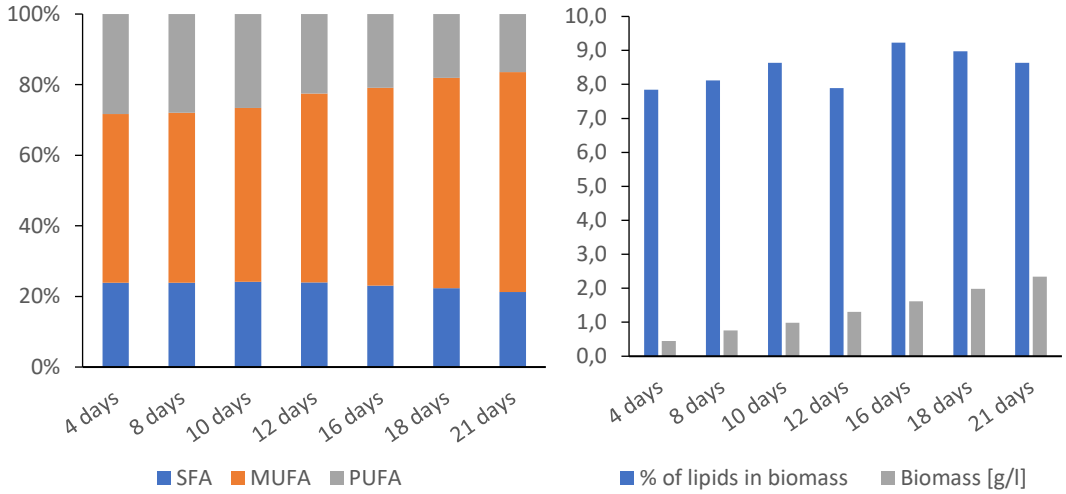
Graph 65. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 40 % of nitrogen*



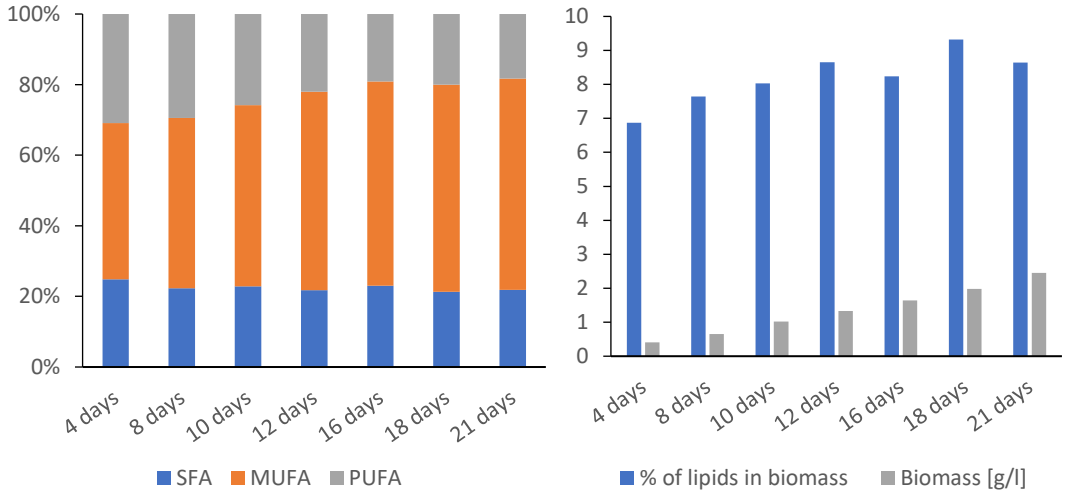
Graph 66. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 60 % of nitrogen*



Graph 67. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 80 % of nitrogen*



Graph 68. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with standart nitrogen concentration*



Graph 69. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 150 % of nitrogen*

5.2.2.3.2 Cultivation on media with a different phosphorus concentration

The fourth experiment in a row was to test different concentrations of phosphorus in the medium. The microalgae was tested in media with six different concentrations in the range of 25-300%. In terms of biomass production, phosphorus level does not have an as significant effect as in the case of nitrogen. In all media, more than 3.4 g/L of dry biomass (Table 44) in the medium was achieved at the end of the experiment. With increasing phosphorus concentration, a gradual increase in biomass productivity is observed, and the medium then reached the highest production with doubled and tripled phosphorus concentration. The pigment production data in the table below (Table 45) show that in higher phosphorus concentration in media, the microalgae is able to produce more carotenoid pigments and chlorophylls. However, a significant increase in pigment production is observed in media with 2 times the original phosphorus concentration. At the same time, the results show that this increase in pigment production is not proportional to the phosphorus concentration. Conversely, in media with a lower phosphorus content, there is no significant decrease in the concentration of carotenoid pigments in the biomass. However, the production of chlorophyll pigments is very negatively affected. Thus, a medium with twice the phosphorus is more suitable for obtaining a higher amount of pigments (carotenoids 7.03 mg/g, chlorophyll A 6.49 mg/g and chlorophyll B 8.71 mg/g of dry biomass).

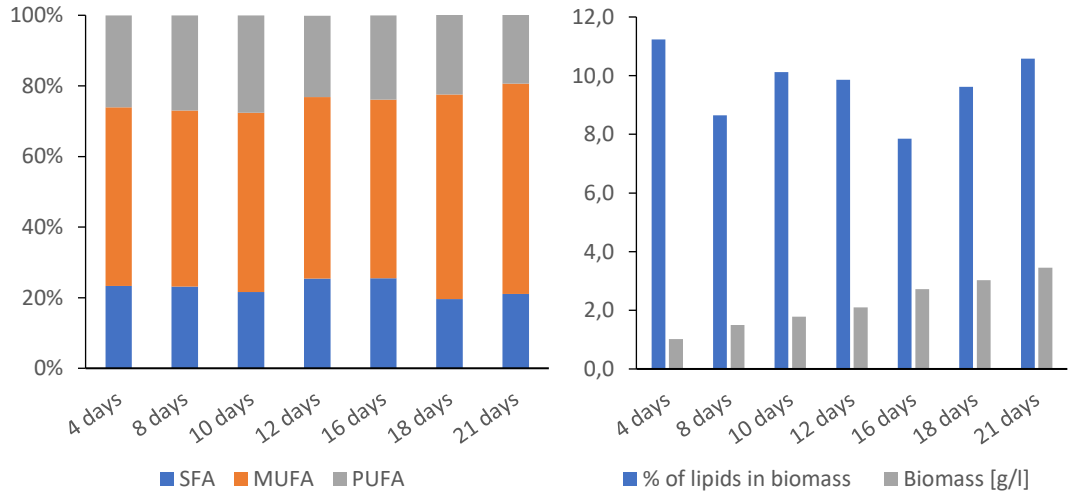
Different concentrations of phosphorus in the medium had a relatively ambiguous effect on the biomass's lipids accumulation, as we can see in graphs below. The average production of lipids here is again in the range of 9-11% of lipids in dry biomass. An exception is a medium with 50% phosphorus concentration (Graph 71), where 14.15% lipids in biomass were reached on the 18th day of cultivation. We also observe a gradual linear increase in biomass lipid content in media with a higher phosphorus content. The fatty acid profile produced in all bottles shows several identical properties. The basic information here is a stable production of SFA in the range of 20-24%. In monounsaturated fatty acids production, we see a slightly increased production in media with a higher phosphorus content than standard media. In general, the MUFA content slowly increases during cultivation at the expense of PUFA until 18th days of cultivation. Then, there is a slight turnaround on the last days of cultivation, and the PUFA content increases slightly.

Table 44. B. braunii experiment with different phosphorus concentration – biomass production

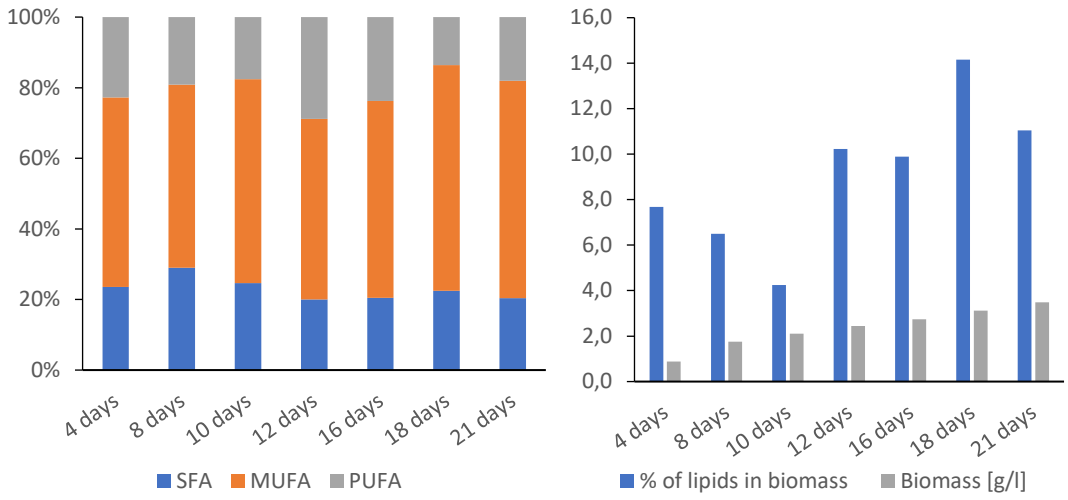
	Biomass [g/L]						
Media type	4 days	8 days	10 days	12 days	16 days	18 days	21 days
25 % P	1.02	1.50	1.78	2.10	2.72	3.03	3.45
50 % P	0.88	1.76	2.10	2.44	2.74	3.12	3.49
100 % P	0.76	1.67	2.16	2.54	2.89	3.24	3.78
150 % P	0.81	1.74	2.44	2.71	2.99	3.43	3.81
200 % P	0.90	1.88	2.54	2.74	3.12	3.54	4.08
300 % P	1.06	1.80	2.47	2.86	3.23	3.63	4.14

Table 45. B. braunii different phosphorus concentration – pigment analysis results

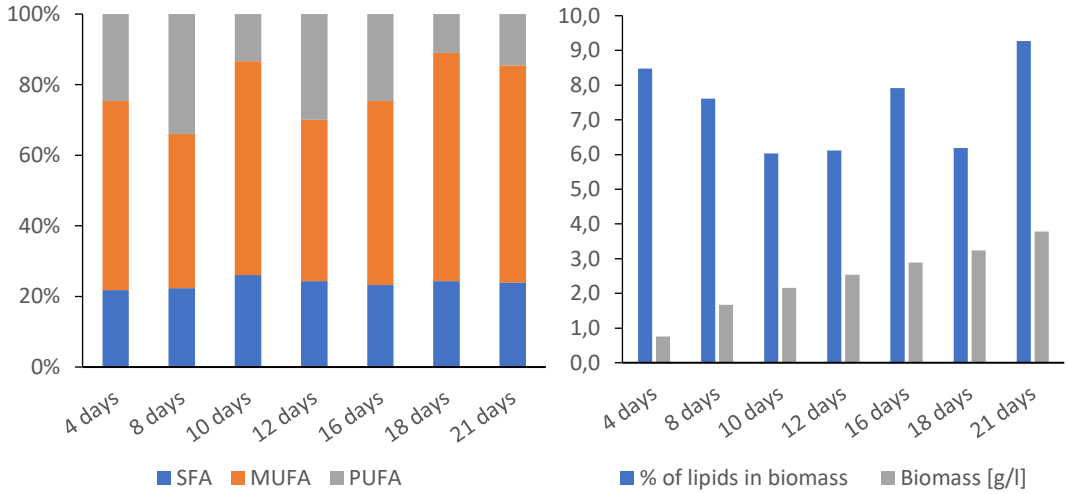
Pigment production [mg/g of fry biomass]						
	Media with 25 % P			Media with 50 % P		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.39	2.90	4.62	4.76	2.80	4.54
8	4.47	3.03	4.42	4.90	2.99	4.43
10	4.93	3.22	4.53	5.23	3.28	4.21
12	5.27	3.19	4.78	5.68	2.98	4.04
16	5.53	3.29	4.24	5.44	3.11	4.43
18	5.70	2.98	4.68	5.68	3.35	4.32
21	5.68	2.98	4.02	5.60	3.43	4.50
	Standart media			Media with 150 % P		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.82	3.95	5.73	4.96	3.85	5.98
8	5.44	4.14	5.92	5.19	4.13	6.33
10	5.23	4.44	6.19	5.47	4.52	6.73
12	5.63	4.69	6.49	5.88	4.83	7.02
16	5.98	4.98	6.78	6.13	4.63	7.23
18	6.33	5.01	7.01	6.03	4.73	7.54
21	6.03	4.86	7.30	6.23	4.85	7.36
	Media with 200 % P			Media with 300 % P		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	4.68	4.66	5.43	4.72	5.33	6.47
8	5.37	4.83	5.78	5.54	5.35	6.85
10	6.13	4.70	6.02	5.47	5.43	7.26
12	6.42	4.99	6.55	5.98	5.73	7.51
16	6.82	5.43	7.06	6.24	5.99	8.63
18	6.79	5.79	8.32	6.55	6.02	8.49
21	7.03	6.49	8.71	6.97	6.35	8.57



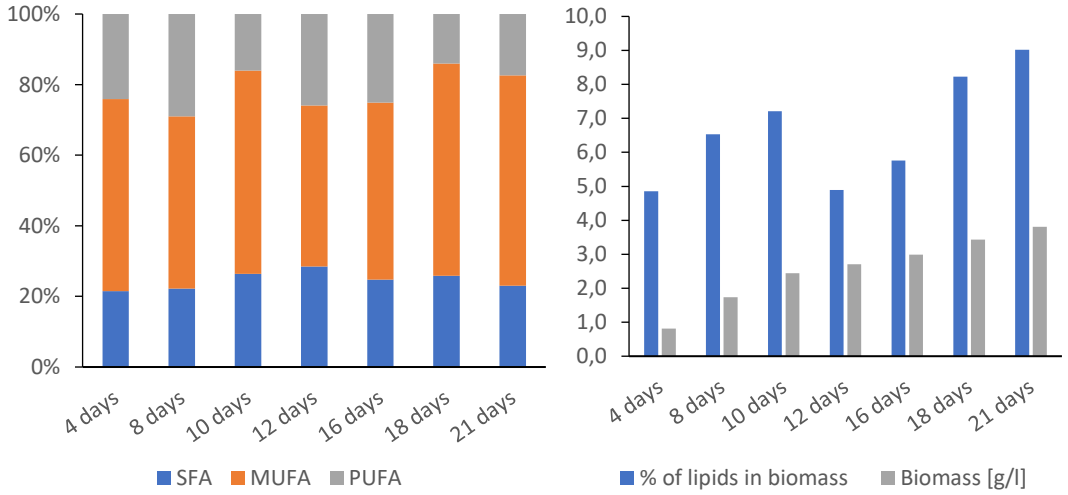
Graph 70. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 25 % phosphorus*



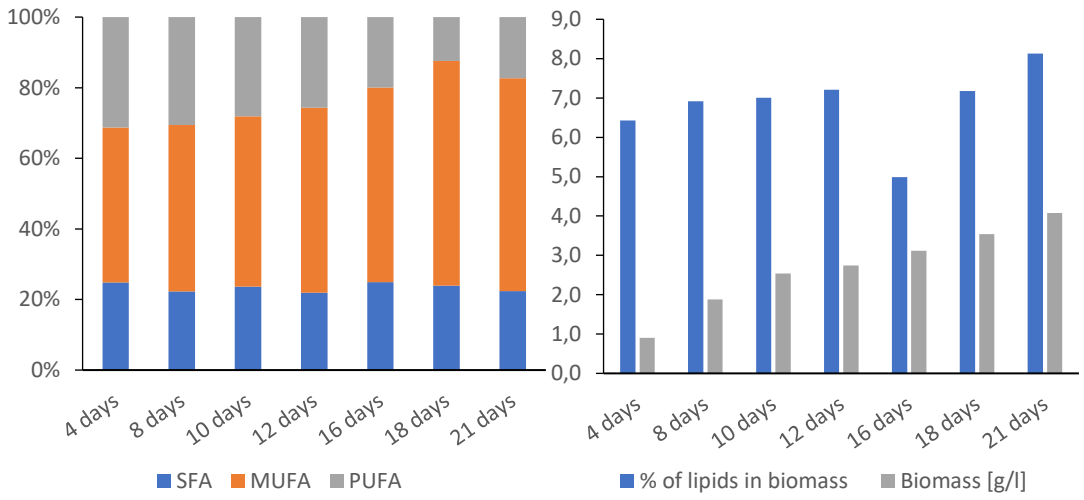
Graph 71. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 50 % phosphorus*



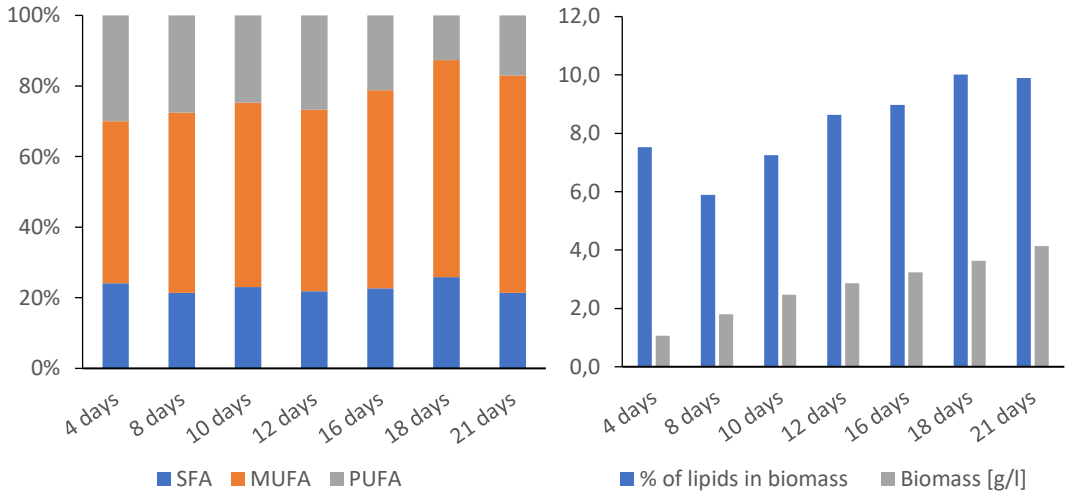
Graph 72. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with standart phosphotus concenrtation*



Graph 73. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 150 % phosphorus*



Graph 74. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 200 % phosphorus*



Graph 75. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 300 % phosphorus*

5.2.2.3.3 Cultivation under oxidation stress

Simultaneously with the phosphorus experiment, cultivations were performed to test the effect of oxidative stress induced by the presence of iron ions and cobalt ions. The prepared media contained 200 mM Fe^{3+}, 300 mM Fe^{3+}, 2.5 mM Co^{2+} and 5.0 mM Co^{2+}, respectively. The standard Chu medium served as a control medium, the results of which are shown in the graph (Graph 72). As the concentration of iron in the medium increases, the inhibitory effect on the growth of the culture increases. In the flask with the highest iron concentration, biomass production is reduced by one third. In the presence of a lower concentration of iron, this negative effect is much weaker, biomass production is reduced only by 15% and reached 3.24 g/L on the last day of cultivation (Table 46). Experiments with the presence of a small amount of cobalt in the medium were focused on the possibility of inducing the production of a specific carotenoid - lutein. In these experiments, we also see reduced biomass production by about 20%. Both 2.5mM and 5.0mM cobalt concentrations achieve comparable production of 3.15 g/L.

The oxidative stress experiment's main goal was to study the effect on carotenoid production in *B. braunii* microalgae cells (Table 47). Compared to the standard medium, the presence of iron has a significant effect on the production of carotenoids. In both cases, increased carotenoid production is observed; in a medium with 200 mM Fe^{3+}, carotenoid production increased by 21% to 7.23 mg/g of dry biomass. In the medium with a higher concentration of Fe^{3+}, the induction of production was only half. In this case, the concentration of Fe^{3+} ions was probably already on the verge of tolerability for microalgae, and it can be assumed that a further increase in concentration would already lead to inhibition and the overall result would be negative, compared to the control medium.

The production of chlorophylls was without distinction negatively affected by the presence of a higher concentration of Fe^{3+}. From the beginning of cultivation, results show gradual decrease in chlorophylls' content in the biomass (Table 47). In this case, different concentrations of Fe^{3+} in the flasks had a comparable effect. The second set of flasks containing the addition of cobalt salt has, at first glance, a very positive effect on the microalgae *B. braunii*. In both media, with the addition of cobalt, we see an increase in all measured pigments. The production of carotenoids in these media was 33% higher compared to the control medium. Simultaneously, in contrast to iron-added flasks, the presence of cobalt also slightly increased the production of both types of chlorophylls. The best results in this experiment were obtained on medium with 5.0mM cobalt salt. This is closely followed by a medium with a lower cobalt content.

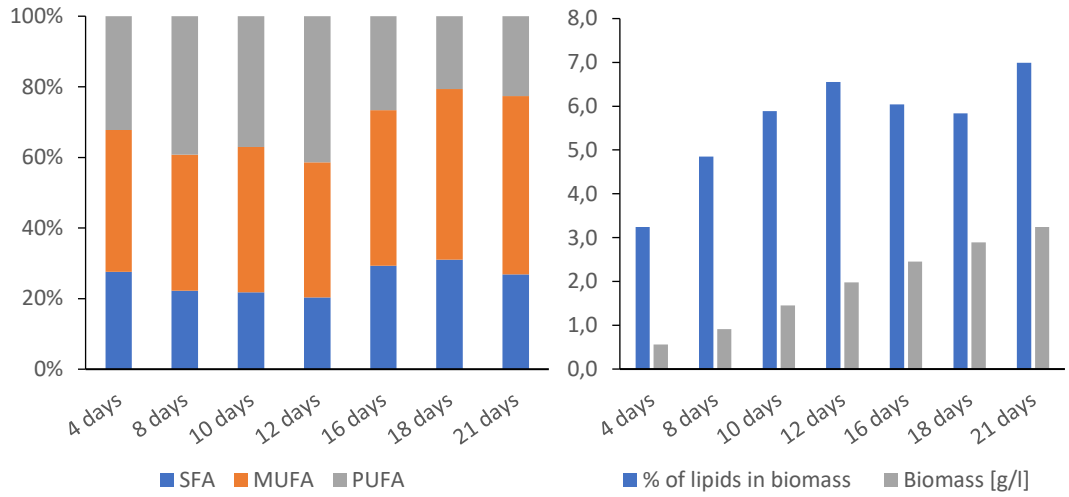
The production of lipids in the presence of iron shows a slightly lower production in the range of 6-10% compared to the control medium (Graph 72). The lower biomass production in a medium with a higher iron concentration is compensated here by a higher lipid production than a medium with a lower iron concentration (Graph 77). We see practically the same situation in the cobalt media. Here, the medium with a higher concentration of cobalt produced more lipids, namely 13.24% (Graph 79). However, the presence of oxidative stress has already affected the composition of fatty acids. In all cultivations, we see a slight increase in the production of saturated fatty acids. The presence of iron or cobalt had a more significant effect on the composition of unsaturated fatty acids. *Botryococcus* generally produces more MUFA acids (55-60% at the end of cultivation) (Graph 78). Here, however, in the presence of oxidative stress, a shift towards greater production of polyunsaturated fatty acids occurs. We observe this at the beginning of cultivation on all media types, where PUFA production reached 35-40% of lipid content. With a higher concentration of iron in the medium, we see a stronger effect on the composition of unsaturated fatty acids compared to cobalt. In the following days of cultivation, there is then an equilibration towards the preferred ratio of MUFA and PUFA.

Table 46. B. braunii oxidation stress experiment: biomass production

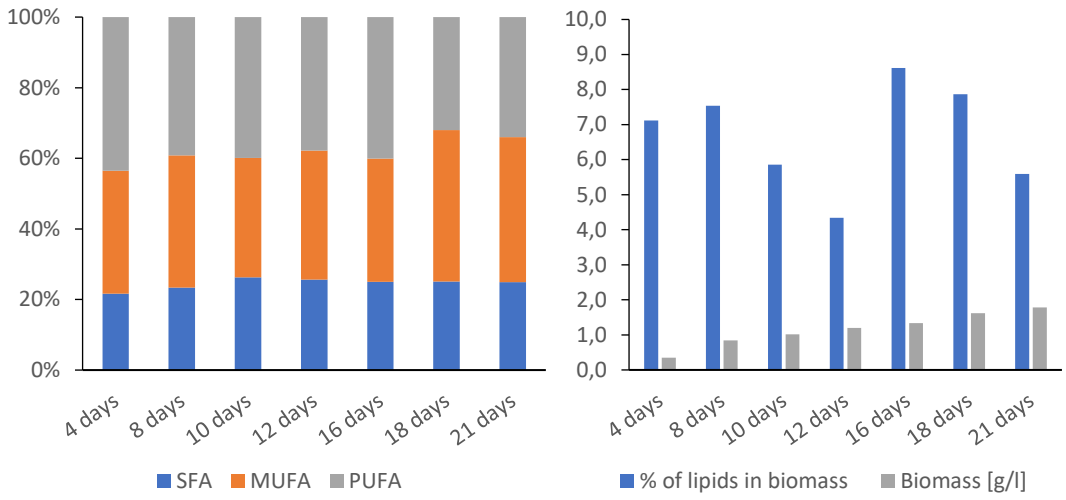
	Biomass [g/L]						
Media type	4 days	8 days	10 days	12 days	16 days	18 days	21 days
Chu media	0.76	1.67	2.16	2.54	2.89	3.24	3.78
200 mM Fe	0.56	0.91	1.45	1.98	2.45	2.89	3.24
300 mM Fe	0.45	0.84	1.11	1.45	1.78	2.12	2.45
2.5 mM Co	0.61	1.12	1.65	2.01	2.51	2.78	3.18
5.0 mM Co	0.59	1.14	1.58	1.98	2.34	2.81	3.15

Table 47. B. braunii oxidation stress – pigment analysis results

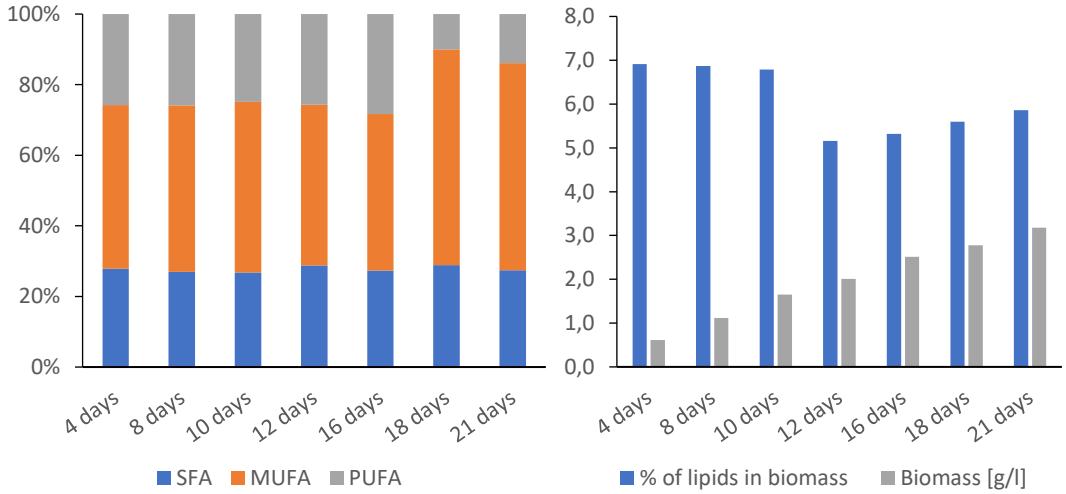
Pigment production [mg/g of fry biomass]						
	Media with 200mM Fe^{3+}			Media with 300mM Fe^{3+}		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	5.27	2.88	4.78	5.30	2.44	4.45
8	6.00	3.02	4.85	6.01	2.66	4.32
10	6.45	3.00	4.43	5.90	2.58	4.45
12	6.90	3.15	4.56	6.75	2.79	4.30
16	7.03	3.29	4.33	7.12	3.01	4.28
18	7.19	2.98	4.44	7.02	2.78	4.03
21	7.23	2.88	4.02	6.78	2.66	3.98
	Media with 2.5 mM Co^{2+}			Media with 5.0 mM Co^{2+}		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
4	5.63	4.09	5.93	5.78	4.33	5.03
8	6.38	4.24	6.23	6.33	4.02	5.88
10	6.86	4.45	5.88	6.98	4.55	6.68
12	7.32	4.32	6.35	7.35	4.35	6.73
16	7.65	4.90	6.87	7.95	4.79	6.97
18	7.85	5.02	7.03	8.26	5.02	7.22
21	8.02	5.34	7.46	8.34	5.47	7.85



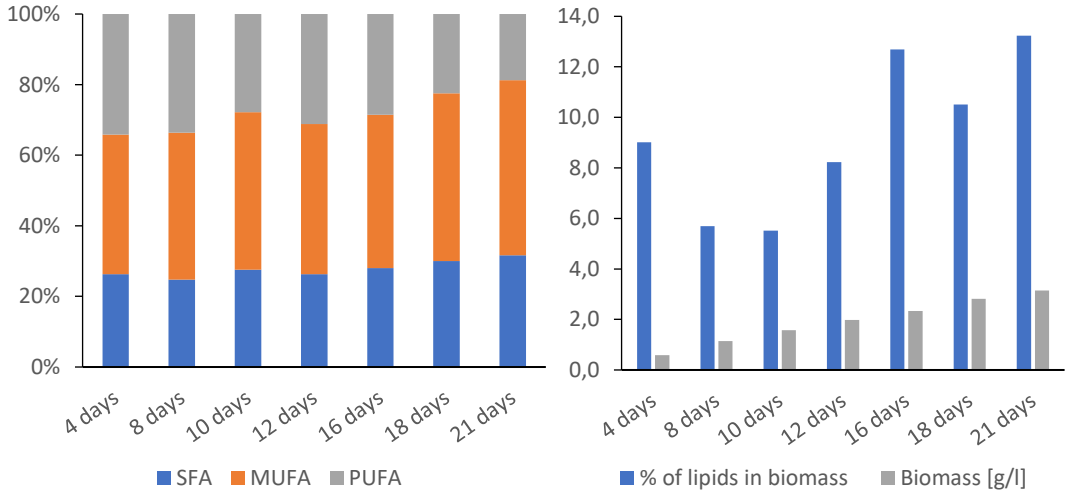
Graph 76. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on Chu media with doubled Fe concentration*



Graph 77. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on Chu media with tripled Fe concentration*



Graph 78. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 2.5 mM Co*



Graph 79. *Fatty acid profile and total lipid production of Botryococcus braunii cultivated on chu media with 5.0 mM Co*

5.2.2.3.4 Diluted media experiment:

After the gradual optimization of the nitrogen and phosphorus composition in the medium, experiments were performed with the diluted medium to determine under which cultivation conditions the formation of cells with a high content of carotenoids occurs in the culture. In this experiment, *Botryococcus braunii* was inoculated into Roux cultivation flasks and cultivated for four weeks. Cultivation medium was prepared according to updated concentrations of nitrogen, phosphorus and cobalt. Initially, the culture was inoculated at a higher optical density of A_{680}=0.200 (0.10 g/L), due to the new cultivation vessel with a shorter optical path and more intense illumination. The table below (Table 48) clearly shows the biomass production during the experiment. Samples were processed at weekly intervals.

From the results we see that high dilution in the medium led to low biomass production, on the other hand already at the beginning of the third week of cultivation in the 4th bottle there was a gradual change in colour of the culture and the formation of higher amounts of carotenoids to protect against adverse conditions. This process with a one-week delay also occurred in the third bottle with 5 times diluted medium. In the second bottle, with half the concentration of nutrients, a fading of the culture's deep green colour and a change towards orange was observed in the last days of cultivation. No such change was observed in the first bottle throughout the experiment. A comparable growth rate was observed in the first two bottles, although a larger increase was expected in the standard medium with more nutrients (Table 48). This was probably due to the lower intensity of bubbling at some point in the experiment. Insufficient supply of carbon dioxide could lead to the slower growth of this bottle. Unfortunately, two flasks were contaminated when performing a duplicate experiment, and the cultivation was terminated prematurely. It was not possible to perform this experiment again within the internship's time frame, and it would be appropriate to repeat it

We observe a significant increase in lipid content in the most dilute medium within this strain in lipid production (Graph 83), which corresponds to increased carotenoid production as protection under adverse conditions. At the end of the cultivation, 14.14% of lipids in the biomass were measured in this medium, which is unusual for this strain. With gradually decreasing dilution, the total lipid content in the biomass decreases proportionally (Graph 81) (Graph 82). The standard lipid content in standard Chu medium was 8.51%, which corresponded to previous cultivation results. We see a slight trend of increasing SFA content in the first two media with continued cultivation. In very dilute media (5 and 10 times) the SFA content has a balanced or rather decreasing tendency (Graph 82)(Graph 83). As in previous experiments, *Botryococcus* tends to gradually increase the MUFA content in biomass at the expense of PUFA. It can be assumed for all cultivations that if the cultivation extended, the percentage of lipids in the biomass would further increase. In this strain, this increase could be interesting in the case of dilute media, as more and more cells would be gradually filled with carotenoids dissolved in lipids. Here, the percentage of content is estimated to exceed 15 and 20%.

The effect of nutrient dilution on pigments production is shown in the table below (Table 49). The control medium shows a stable character of pigment production during cultivation. During cultivation, the concentrations of pigments in the biomass gradually increase. The maximum production is measured on the last day of cultivation, and from the obtained data it can be said that in the case of prolonged cultivation, the culture would continue to grow and with it the accumulation of pigments in the cells. If we compare the results of this cultivation with previous cultivations in media with the same composition in Erlenmeyer flasks, we see that especially the production of carotenoids is lower in this experiment in cultivation Roux bottles. It is necessary to consider the different cultivation conditions, the different light transmission and its intensity, the intensity of the bubbling and the proportionally dissolved amount of carbon in the medium, which is greater with respect to the path of the enriched air bubbles.

The presumption of the ability to increase the accumulation of carotenoids in the lack of nutrients was confirmed relatively quickly, as can be seen from the results of the analysis of culture samples in 10x and 5x diluted medium. In the most dilute medium, we see already on the 10th day of cultivation the beginning of a decrease in chlorophyll pigments' content in the biomass and, conversely, the beginning of intensive production of carotenoids. As the cultivation time progressed, the cells accumulated more and more carotenoids and maximum 11.65 mg/g dry biomass was reached on the last day of culture. The results of *Botryococcus braunii* cultivation in 5x diluted medium copy the same character. With a delay of about four days, there is again a decrease in chlorophyll pigment production and an increased production of carotenoids to a maximum of 9.08 mg/g dry biomass. In both of these cases of significant increase, the majority of the increased content was comprised by lutein.

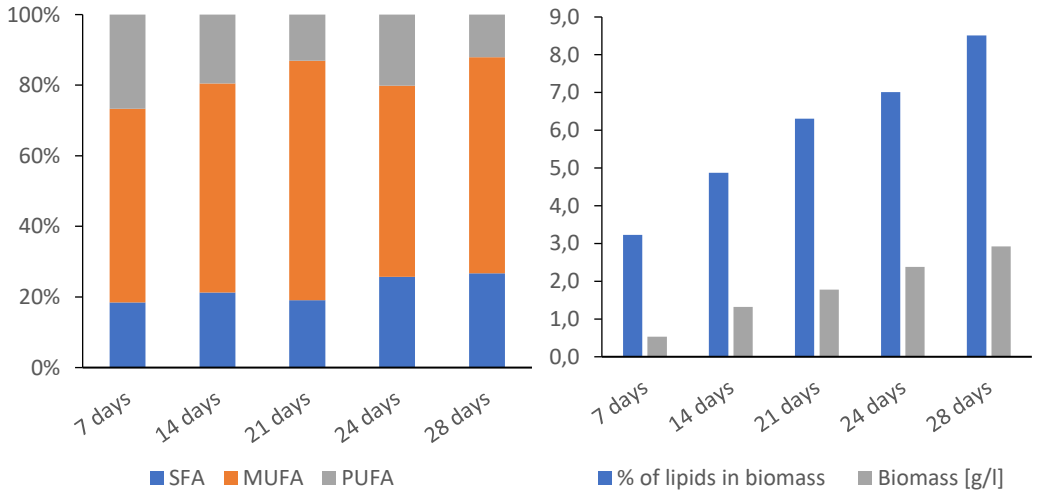
The concentration of nutrients in the second bottle (2 times diluted) was sufficient throughout the cultivation, so there was no change of metabolism towards increased carotenoid production. The production of pigments here thus shows the characteristics of standard cultivation without significant stress factors. The maximum production of chlorophylls is reached on the 21st day of cultivation, namely 4.63 mg/g of chlorophyll A and 6.24 mg/g of chlorophyll B. Then there is a slight decrease in chlorophyll content, based on which it can be assumed that in the following days' depletion of nutrients and a change in metabolism to higher production of carotenoids, which has been steadily increasing throughout the cultivation. As part of the dilute media study, it would be appropriate to address further the influence of individual components responsible for the change in metabolism and optimize their level in the medium so that a compromise is reached between the increase in biomass and the production of desired substances.

Table 48. Biomass production of B. braunii in diluted media experiment

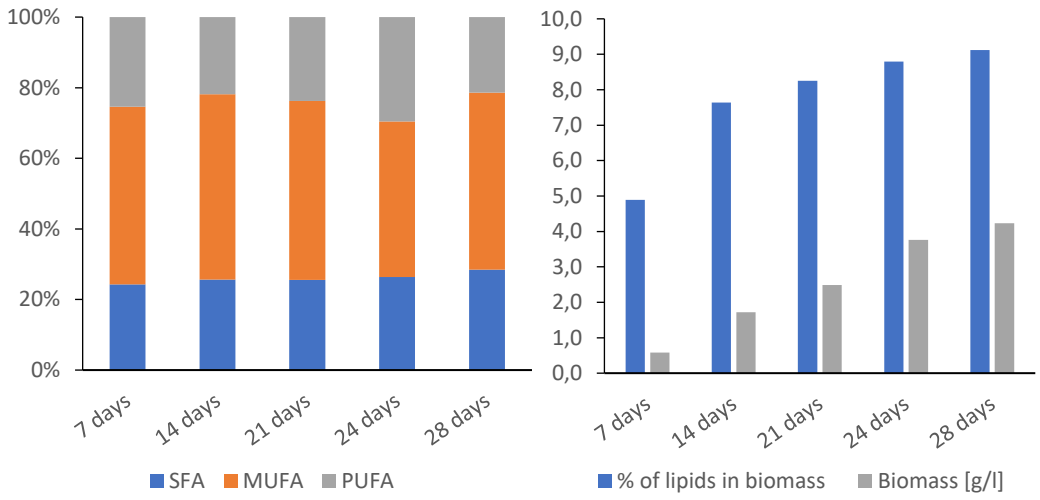
Biomass [g/L]					
Media	7 days	14 days	21 days	24 days	28 days
Standard	0.53	1.32	2.48	3.38	3.92
2 times dil	0.58	1.72	2.49	3.76	4.23
5 times dil	0.58	0.98	1.48	1.98	2.54
10 times dil	0.43	0.81	1.23	1.32	1.66

Table 49. Diluted media experiment – pigment analysis results

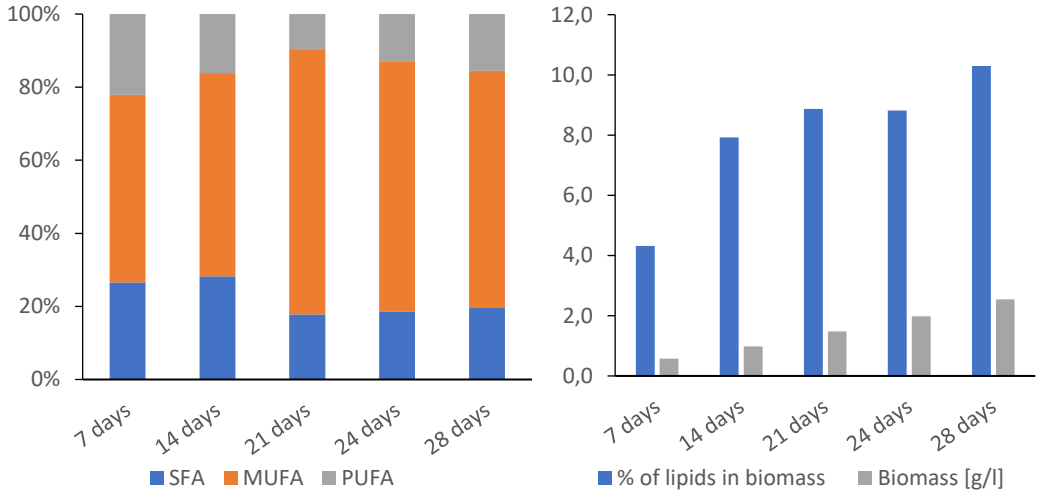
Pigment production [mg/g of fry biomass]						
	Standard Chu media			2 times diluted media		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
3	4.21	4.20	6.30	4.13	3.55	6.70
7	4.46	4.61	6.99	4.40	3.83	6.33
10	4.84	4.28	6.47	3.85	3.15	6.56
12	4.38	3.90	6.57	4.74	3.67	6.79
14	4.92	4.06	6.38	4.58	4.18	6.90
16	5.02	4.32	6.56	4.69	4.33	6.13
18	4.96	4.54	6.74	4.82	4.21	5.97
21	5.05	5.76	7.14	5.34	4.63	6.24
24	5.68	4.50	7.89	5.58	3.85	5.92
28	5.90	5.22	8.55	6.38	3.38	5.65
	5 times diluted media			10 times diluted media		
Days	Carotenoids	Chlorophyll A	Chlorophyll B	Carotenoids	Chlorophyll A	Chlorophyll B
3	4.13	3.55	4.70	2.15	4.09	4.45
7	3.70	2.83	4.33	3.02	4.98	4.33
10	3.74	3.67	4.79	3.85	5.07	3.45
12	4.01	3.01	4.23	3.71	4.37	2.97
14	3.98	2.18	3.54	4.50	3.89	2.26
16	4.50	2.40	2.23	5.99	1.88	1.65
18	5.32	2.21	1.97	6.25	0.64	1.23
21	6.34	1.63	1.34	7.78	0.60	0.98
24	7.58	0.85	1.62	9.32	0.55	0.55
28	9.08	0.74	1.15	11.65	0.50	0.42



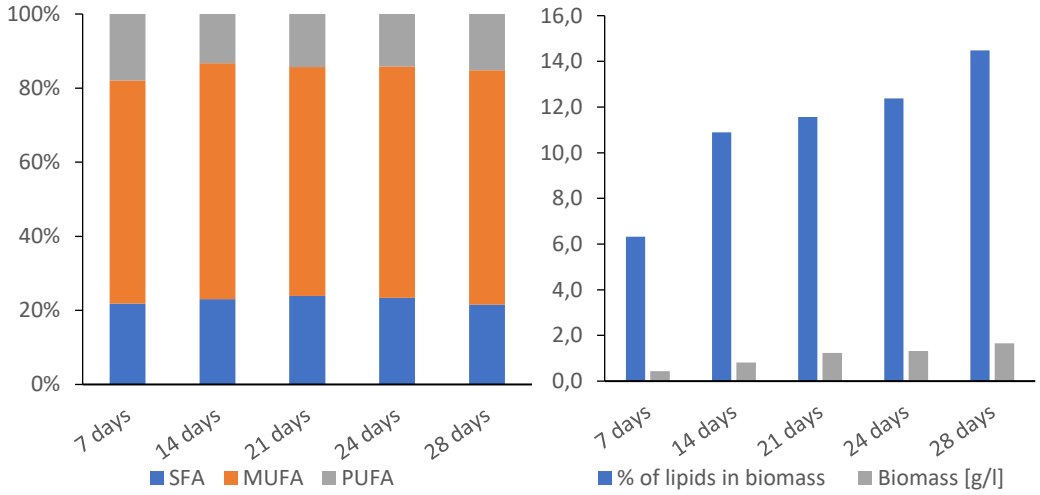
Graph 80. *B. braunii diluted media experiment: Standard Chu media*



Graph 81. *B. braunii diluted media experiment: 2 times diluted Chu media*



Graph 82. *B. braunii diluted media experiment: 5 times diluted Chu media*



Graph 83. *B. braunii diluted media experiment: 10 times diluted Chu media*

5.2.2.3.5 Botryococcus braunii botryoterpenes cyclic extraction

Botryococcus microalgae are characterized by the production of large amounts of lipids or carbohydrates that form the colony's shell. The basic experimental/biotechnological scheme assumes the cultivation of microalgae in a medium to the stationary growth phase, harvesting and the subsequent extraction of these valuable substances. This procedure is suitable for common algae, which grow much faster than members of the genus *Botryococcus*. This slow growth is one of the fundamental problems of greater use of this microalgae. One of the possibilities that offers to overcome this primary problem is the idea of gentle cyclic extraction of lipids or polysaccharides from the envelope of the microalgae colony while maintaining the viability of the culture. By this mechanism, valuable raw materials could be extracted repeatedly. The principle of cyclic extraction encounters several pitfalls that need to be addressed. We can mention here finding an effective and at the same time safe extraction reagent, optimizing the time and period of extraction, the ratio of reagents.

Several experiments with various members of the genus *Botryococcus* can be found in the literature regarding classical extraction, but not many with the idea of cyclic extraction. In this experiment, preliminary extraction experiments were performed with *Botryococcus braunii* v. Showa-Bielefeld microalgae. The experiment was focused on testing the effect of hexane used for extraction, extraction conditions and cycle length. The following table summarizes the tested conditions of this experiment. In the experiment, culture samples were taken to determine the main parameters of the culture. The most important factor here was the value of the quantum yield of fluorescence, which informs about the state of the culture cells' photosynthetic apparatus and its viability.

Table 50. Botryoterpenes cyclic extraction experiment conditions

	Experiment 1	Experiment 2
Optical density A_{680}	2.0	2.0
Culture volume [mL]	400	400
Hexane volume [mL]	100	50
Extraction time [min]	10	10
Magnetic stirrer [rpm]	100	100
Temperature [°C]	25	25
Cycle period	Mon Wed Fri	Mon Wed Fri

The following table (Table 51) summarizes the results of cyclic extraction tests of botryoterpenes. In this work, only two experiments were performed, which differed in the amount of hexane used. As we can see from the gravimetric data, although more terpenes were extracted in the first experiment with a larger amount of hexane, on the other hand, the culture was weakened in the first extraction, as we see in a relatively rapidly declining quantum fluorescence yield. In the second extraction cycle, we already see a significant decrease in the extract yield. The third extraction cycle continues with decreasing yield of the extract.

In the second experiment, half the amount of hexane was used; the extract's weight is lower compared to the first experiment. However, the lower amount of hexane had a positive effect on the culture's overall condition, as shown by the measured quantum fluorescence yield data. Overall, a lower amount of extractant thus appears to be a better alternative, allowing more cycles to be performed. In the case of the second experiment, we can therefore assume that it would be possible to perform the 4th extraction cycle. From the result of this simple experiment, we see that there is a possibility of multiple extractions of terpenes from the culture of the microalgae *Botryococcus*.

Unfortunately, we see that this process cannot be performed indefinitely, and with increasing extraction cycles, the viability of the culture is further reduced, and the photosynthetic apparatus and culture growth rate is gradually inhibited. In this experiment, the culture was extracted after every 48 hours. It can be assumed that by prolonging the cycle and optimizing other parameters, it would be possible to perform more cycles in a more extended period, ensuring greater viability of the culture. On the other hand, we come across the need for a compromise solution concerning the overall economics of the process.

Table 51. Botryococcus b. cyclic extraction experiment results

First experiment			
Cycle number	Qy	A_{680}	Extract weight [mg]
1	0.74	2.010	167.011
2	0.56	2.103	57.023
3	0.40	1.983	28.871
Second experiment			
Cycle number	Qy	A_{680}	Extract weight [mg]
1	0.76	2.054	137.031
2	0.64	2.139	64.061
3	0.51	2.084	54.072

5.2.2.3.6 Botryococcus braunii v. Showa-Bielefeld experiment conclusions

As part of experimental work with the *Botryococcus braunii* strain, cultivations were performed to optimise Chu media's composition. The aim was to increase the production of lipid metabolites - carotenoids. Chu medium was chosen as the optimal medium based on previous experiments. The optimization of the medium composition proceeded as follows: Selection of a suitable pH medium > nitrogen source > nitrogen concentration > phosphorus concentration > oxidative stress (Fe^{3+}, Co^{2+}) > effect of diluted media. The results showed that *Botryococcus braunii* has a pH optimum in the range of 6.5-8.0. When media pH shifts to a slightly acidic pH <6.0, rapid inhibition of culture growth occurs. Likewise, if the pH of the medium rises above 8.5. Urea and sodium nitrate appear to be the most suitable sources for this microalgae. The experimental work continued with urea, as it has virtually zero effect on the medium's pH change. Selection of a suitable nitrogen source was followed by testing of its concentration. In terms of metabolites and biomass production, the most suitable medium was with 150% nitrogen concentration, where an increase in the production of all metabolites and biomass was observed.

Unfortunately, the performed experiments with different concentrations of phosphorus have already shown different results. For increased lipids production in biomass, a medium with 50% of the original phosphorus concentration is more suitable. Conversely, in pigments and biomass production, a higher phosphorus concentration is preferred, namely 200%. The higher tested concentration of 300% phosphorus was practically no different from the previous result. Thus, 200 % of phosphorus in the medium was chosen as the optimal compromise in this area. In the experiment, cultivations with different concentrations of sulphur in the medium were further tested. The analyses showed that under the tested conditions (50-150%) no significant effect on the production of biomass or analyzed metabolites was observed.

The increased concentration of iron ions in the medium increased carotenoid pigments' production by more than 20%. On the other hand, the content of chlorophylls and lipids decreased significantly. The experiment also tested the presence of cobalt and the possible induction of carotenoid production. In both cultivation flasks (2.5 mM and 5.0 mM), despite a slight decrease in biomass production, a very positive effect of cobalt on the production of all analyzed pigments and lipids was observed. Based on these data, the used Chu medium was modified, and cultivation was performed to induce the production of protective substances by microalgae in case of nutrient deficiency. In the experiment, three bottles with different degrees of nutrient dilution were cultivated. Cultures in 5-fold and 10-fold diluted media showed a fast change in microalgal metabolism towards higher carotenoid production. Chlorophyll production decreased very rapidly, and the whole culture began to turn brown-orange. The final content of carotenoids in the 5-fold diluted medium reached 9.08 mg/g and in the 10-fold diluted even 11.65 mg/g of dry biomass. Unfortunately, the medium with 2.5 times diluted nutrients did not reach the same point during cultivation as the more diluted media. In the last phase of cultivation, there was already a gradual decrease in the chlorophyll content. It can be assumed that this metabolic change would occur in the following days of cultivation. The most suitable variant, taking into account compromise between biomass production and carotenoids would be an optimized Chu medium, which will be diluted 3 to 4 times.

5.3 Co-cultivation of microalgae and carotenogenic yeasts results

The following chapter summarizes the results of the co-cultivation experiments divided according to the individual phases. The results' graphs included the total production of carotenoids, major carotenoid pigments, chlorophylls, sterols, ubiquinone, and biomass. Graphs showing the results of GC lipid analysis were divided into two parts. Results from the first to the third phases are listed in a table describing the percentage composition of each type of fatty acid and the percentage of lipids and biomass. In the last part of the experiment, the results of the GC analysis are shown in graphs. The first graph describes the % composition of fatty acid groups (SFA, MUFA, PUFA). The second graph shows the percentage of lipids in biomass and biomass production

Scheme 16. *Co-cultivation process graphical abstract*

5.3.1 The first phase – yeast cultivation on BBM and standard media

In this phase of the experiment, basic cultivations were performed to compare yeast growth in the simple mineral medium and BBM medium used for microalgae cultures. Furthermore, the influence of different combinations of carbon and nitrogen sources on yeast's growth and production properties was tested here. At the beginning of the experiment, two sets of 4-day cultivations were performed, followed by an experiment with 10-day cultivations on BBM medium. The extended cultivation time was chosen in order to be able to compare the growth with the microalgae in the subsequent phase of the experiment. The following table (Table 52) compares the biomass production of selected strains of the yeasts *R. kratochvilovae*, *R. toruloides* and *P. rhodozyma*. The results show that we see fundamental differences in biomass production within one strain due to the selected carbon and nitrogen source in all three cultivations.

Classical cultivation on a simple mineral yeast medium showed significant differences in growth rate and biomass production. Overall, the highest biomass production was achieved in the *R. kratochvilovae* strain on media containing yeast autolysate as a nitrogen source. The worst results were achieved by the strain *Phaffia rhodozyma*. This result was expected for this strain because it has much slower growth than other carotenogenic yeasts. We also see differences here within the selected carbon source, where higher biomass production has always been observed on glucose media compared to glycerol media. The only exception is the *P. rhodozyma* strain, which was characterized by better growth on glycerol media.

We see a change in individual strains' biomass production on a shorter 4-day cultivation of yeasts on BBM medium. In the case of the best strain *R. kratochvilovae*, the best growth was obtained on urea-containing media. Here again, glucose is a better carbon source than glycerol, except for cultivation with ammonium sulphate. For this strain, it is unclear whether BBM media's cultivation was better or worse than the classical yeast medium. In the case of the *R. toruloides* strain, we see that cultivation on BBM medium generally had a positive effect on biomass production, except for cultivation on medium with glucose and yeast autolysate, where biomass production stopped at 6.87 g/L, which is approximately 80% compared to the mineral yeast medium. Cultivation on BBM medium led to higher biomass production on average in the *P. rhodozyma* strain. Here we can highlight the medium with glycerol and urea, where the increase in production was more than 2.5 times. It can be stated that the medium containing more microelements had a positive effect on this strain.

The last experiment at this stage was prolonged cultivation of yeast on BBM medium, aiming to simulate the length of cultivation for microalgae. For the *R. kratochvilovae* strain, it cannot be said unequivocally that there were positive changes in the extended experiment. In the case of glucose media, we see a decrease in biomass production, and in glycerol media, we see a positive effect in media containing ammonium sulphate or urea. Unfortunately, we see a decrease in the *R. toruloides* strain's biomass production, except for the medium with glycerol and yeast autolysate. With more prolonged cultivation, the culture partially dies. On the other hand, prolonged cultivation had a very positive effect on the *P. rhodozyma* strain, where we see more than two-fold increases compared to short-term experiments.

From the overall comparison, we see that the most suitable strain in terms of biomass production is the strain *R. kratochvilovae*, which was the only one that managed to get over the limit of 10 g/L biomass in Erlenmeyer flasks on BBM medium containing urea. We see that, in general, the yeast on the selected BBM co-cultivation medium was able to achieve the same or higher biomass production. From the results, it is impossible to say unambiguously for all strains which source of carbon or nitrogen is the most suitable. Ammonium sulphate appears to be the least suitable source of nitrogen for cultivation. During its treatment by yeast, a sulphate group is released into the medium, which lowers the pH value. As a result, there is a faster deviation from the optimum pH values, and thus gradual inhibition of growth occurs until it stops. Yeast autolysate proved to be a suitable source of nitrogen, and the results show high biomass production. However, one of the disadvantages is the high cost, which can drastically increase the whole process's cost.

Table 52. Biomass production of yeasts cultivated on different media [g/L of media]

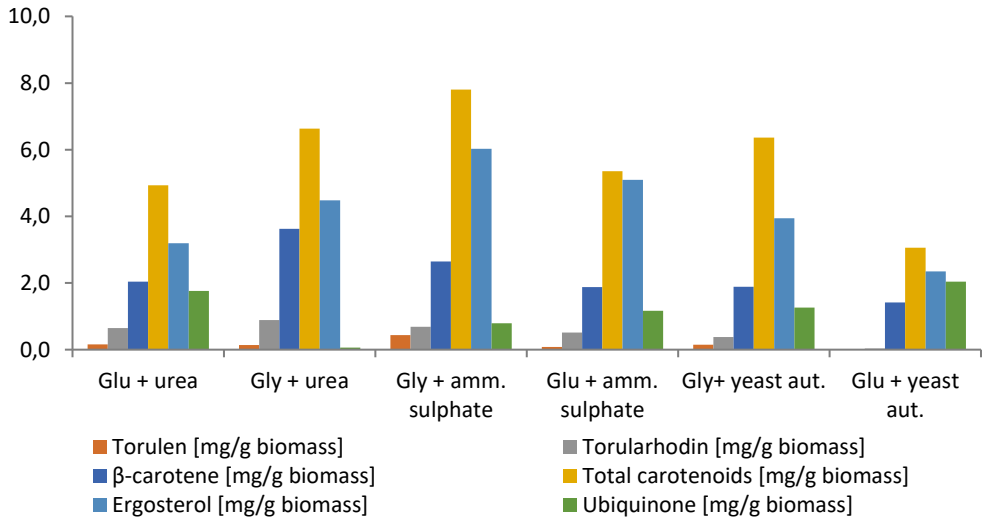
	Yeast mineral media: 4 days cultivation					
Strain	Glucose + urea	*Glycerol + urea*	*Glucose + $(NH_4)_2SO_4$*	*Glycerol + $(NH_4)_2SO_4$*	*Glucose + yeast aut.*	*Glycerol + yeast aut.*
R. kratochvilovae	12.17	4.78	9.32	6.19	13.38	11.42
R. toruloides	6.56	4.99	6.35	6.11	8.41	6.15
P. rhodozyma	2.69	2.69	2.39	2.66	3.11	3.48
	BBM media: 4 days cultivation					
R. kratochvilovae	18.42	12.47	3.98	8.85	5.85	5.89
R. toruloides	7.86	7.26	6.23	5.98	6.87	6.42
P. rhodozyma	2.53	6.88	2.59	2.02	3.96	4.34
	BBM media: 10 days cultivation					
R. kratochvilovae	*10.19*	*9.21*	*5.84*	*10.46*	*13.57*	*6.83*
R. toruloides	*5.46*	*4.76*	*4.60*	*5.01*	*7.72*	*2.31*
P. rhodozyma	*4.72*	*5.89*	*5.57*	*5.30*	*4.68*	*5.44*

5.3.1.1 *Carotenoid, sterol and ubiquinone production*

This chapter shows results of HPLC analysis from experiments performed in the first phase of the co-cultivation experiment. The first graph (Graph 84) shows the results of comparative cultivation of *R. kratochvilovae* on a standard mineral yeast medium. The results show that yeast's highest carotenoid production of 7.81 mg/g biomass is achieved on a medium with glycerol and ammonium sulphate. In general, glycerol media were more suitable for carotenoid production because the other two highest productions were achieved on urea-glycerol media and yeast autolysate. The carotenoid profile in all cultures looks very similar; the largest proportion here is β-carotene, which always makes up at least 30-35% of the carotenoid content, except for the medium containing yeast autolysate and glucose.

Furthermore, we see the production of lycopene and torularhodine everywhere. In terms of ergosterol production, production of 6 mg/g of biomass was achieved on both media containing ammonium sulphate. Ubiquinone was identified in all samples, the production of which ranged from 1.0 to 2.0 mg with a maximum in the medium with glucose and yeast autolysate. Overall, the most successful medium was a combination of glycerol and ammonium sulphate.

In the case of *P. rhodozyma*, lower production of the metabolites of interest was generally achieved. The *P. rhodozyma* strain produced higher carotenoids 1.5 mg/g biomass on only three media (Gly + urea, Gly-Glu + ammonium sulphate). Increased ergosterol and ubiquinone production was also observed in the same media. Overall, the best medium was glucose medium with ammonium sulphate. In contrast, the *R. toruloide*s strain achieved similar productions with the *R. kratochvilovae* strain. The highest production of carotenoids was achieved on glucose media, where the production of total carotenoids was on average at 8 mg/g of biomass. The highest production was achieved on a combination of glucose and urea. This medium was evaluated as the best overall because the production of other monitored metabolites was again high; ubiquinone production reached 4 mg/g biomass. Ergosterol production was relatively stable in the range of 4.5-5.5 mg/g biomass in all monitored media in this strain. The results also show that this strain prefers glucose as a carbon source.



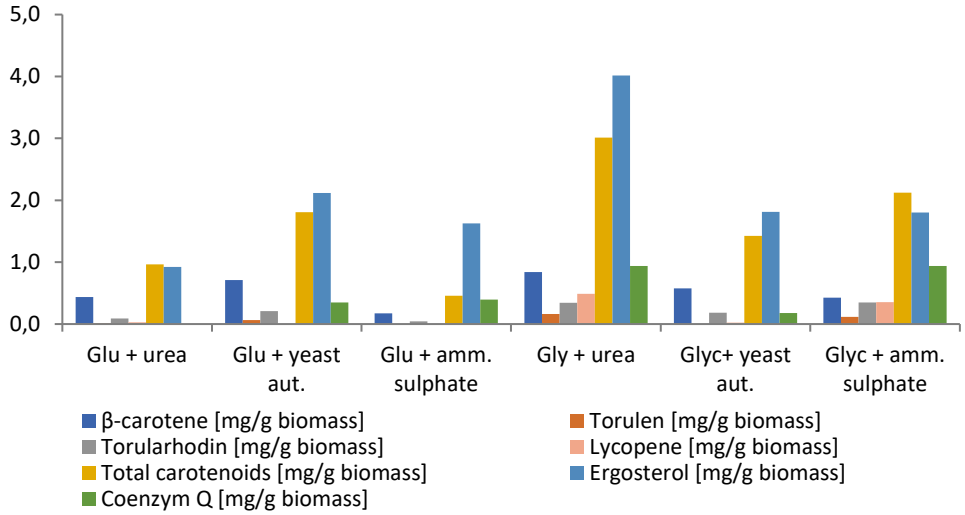
Graph 84. *R. kratochvilovae 4-day cultivation on standard yeast media HPLC results*

The experiment continued with cultivation tests on mineral BBM medium with a combination of carbon and nitrogen sources. This part was crucial for further developing the co-cultivation experiment. The graphs below summarize the results of the chromatographic analysis of lipid metabolites (pigment, ergosterol, ubiquinone). Comparing the overall results of cultivated yeast on both types of media, we see a significant decrease in BBM medium production compared to yeast mineral medium. However, it should be noted that there are differences between the media that lead to different productions. The yeast medium has an optimal pH for their growth, which is around pH = 5.6. In contrast, BBM medium has a pH of 7.0-7.1 and is therefore outside the optimal pH value that yeast prefers.

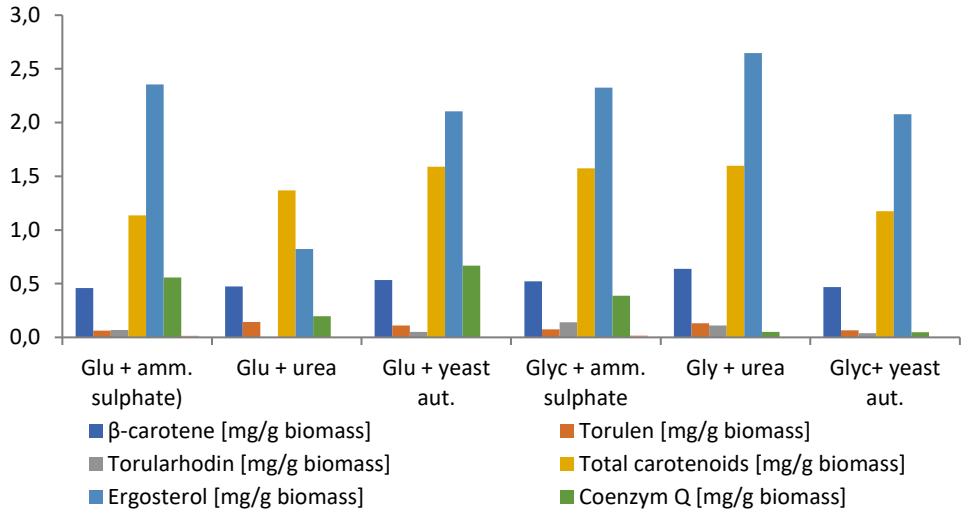
Furthermore, other media components besides the carbon and nitrogen source were not optimized in the pilot experiments. Of the essential macrobiogenic elements, it is not mainly P, S and Mg. Their different value will affect the production of a given strain. Furthermore, these cultivations serve primarily to determine the strains' response to this medium and their growth. Therefore, the comparison of productions between these media is only indicative. It can be assumed that by optimizing individual component media, higher productions would be achieved. However, the optimization will take place only in the final phase of cultivation in bioreactors until the determination of optimal combinations of strains for co-cultivation.

Results of strain *R. kratochvilovae* (Graph 85) showed a decrease in the monitored metabolites' production compared to the classical yeast medium. The results show that glycerol is a more suitable carbon source in the BBM medium for this strain. The highest production of the monitored metabolites was achieved in combination with urea, followed by the medium with ammonium sulphate and both media with yeast autolysate. Significant production of ergosterol 4.02 mg/g of dry biomass on medium with glycerol and urea can be noticed compared to other media. Glycerol in the medium was again most suitable for the production of yeast ubiquinone. Carotenoid production in *R. toruloides* (Graph 86) shows no significant differences and ranges from 1.0-1.6 mg/g of dry biomass. This yeast prefers glucose as a more suitable carbon source for ubiquinone production. Except for media containing glucose and urea, ergosterol production is greater than 2 mg/g biomass in all media with a maximum Glycerol + urea media. From an overall perspective, it is not clear which combination of carbon and nitrogen sources is the most suitable.

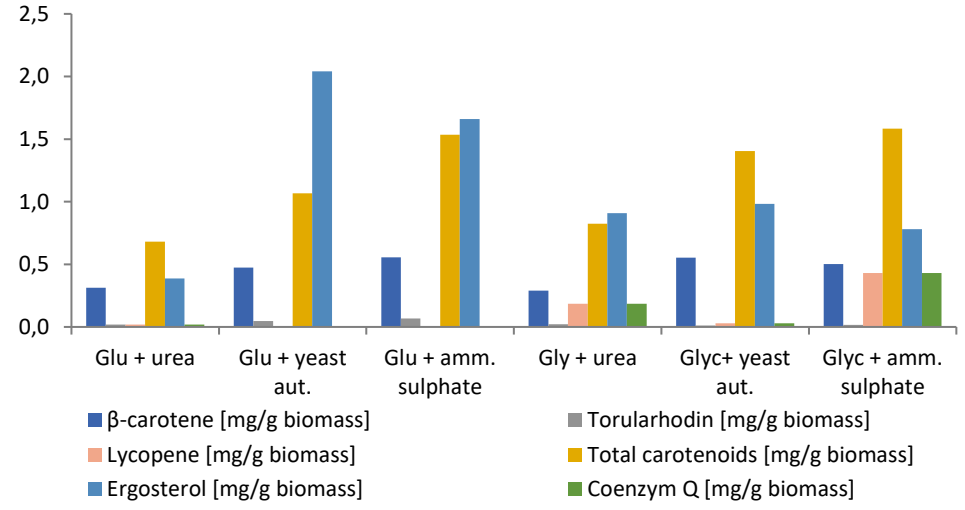
In the case of the *P. rhodozyma* strain, worse results were generally achieved in the production of lipid substances and the production of biomass, which is limited by a slower growth rate. The results show that the cultivation on yeast autolysate and ammonium sulphate performed best. The different effect of the carbon source is not observed here at all. In absolute results, the highest production was achieved on ammonium sulphate media, where the production of carotenoids was 1.54 mg/g biomass, with a majority of β-carotene (> 30%). The highest concentration of ubiquinone was measured in a medium with glycerol and ammonium sulphate. Only ergosterol production differed, and the maximum production of 2.04 mg/g of dry biomass was measured on glucose medium with yeast autolysate. From the overall comparison of all three tested strains, *R. kratochvilovae* and *R. toruloides* are the best aspirants for co-cultivation. The *P. rhodozyma* strain then follows with a slight deficit. All named strains were further tested in co-cultivation experiments with selected microalgal strains.



Graph 85. *R. kratochvilovae 10-day cultivation on BBM media: HPLC results*



Graph 86. *R. toruloides 10-day cultivation on BBM media: HPLC results*



Graph 87. *P. rhodozyma 10-day cultivation on BBM media: HPLC results*

5.3.1.2 *Fatty acid profile and lipid production*

As with HPLC analysis, samples of the biomass produced were subjected to GC-FID analysis to determine the fatty acid profile and total lipid production. It was also studied whether different combinations of carbon and nitrogen sources affect the composition of fatty acids in biomass. In the following chapter, the tables clearly show the GC analysis results of cultivated yeasts *R. kratochvilovae* and *P. rhodozyma*, divided by one strain each.

Table 54 shows the results for *R. kratochvilovae*. In the first part showing the results of cultivation on mineral yeast medium, we see a relatively stable 15% lipid content in the biomass, with the exception of the medium with glycerol and urea, where the maximum for this experiment was measured at 19.94%. The highest SFA content was also measured in this medium, reaching 39%. The highest PUFA production of 34.61% was measured in the glycerol and yeast autolysate media. Long-term cultivation on BBM media harmed lipid production in all samples, and the total lipid content of all samples is below 10%. However, except for media with yeast autolysate, we see a significant increase in PUFA content than yeast media. Thus, under these conditions, the BBM media is more suitable for PUFA production. In most cases, however, the increased PUFA production was at the expense of MUFA production, which is very low and almost no MUFA was measured in a sample of BBM media with glycerol and yeast autolysate.

Analysis of the lipids produced by the *P. rhodozyma* strain (Table 55) shows no stable media trends in lipid percentage. The highest lipid content of 19.51% on yeast mineral medium was measured on glucose media with urea. In contrast on BBM media, the highest production was determined for media containing ammonium sulphate. A very high PUFA content characterizes this strain, reaching 72.64% (glucose + $(NH_4)_2SO_4$) and 70.50% (glycerol + yeast. aut.) On the mineral yeast medium. We see high production on BBM media with a PUFA content of more than 40%. Another characteristic feature is the low SFA content, which is less than 20% in most samples. Cultivation of *R. toruloides* were characterized by a low PUFA content on BBM media (less than 15%) in favour of MUFA production. The total lipid content of the *R. toruloides* strain was always less than 10% in all cultures. The SFA content was very variable, and a general trend for medium choice could not be established.

Table 53. Rhodotorula kratochvilovae lipid production and fatty acid profile

Yeast mineral media 4 days						
	Glucose + urea	Glycerol + urea	Glucose + $(NH_4)_2SO_4$	Glycerol + $(NH_4)_2SO_4$	Glucose + yeast a.	Glycerol + yeast a.
Biomass [g/L]	12.17	4.78	9.32	6.19	13.38	11.42
Lipids in bio. [%]	13.08	19.94	15.12	14.94	14.56	8.79
SFA [%]	22.88	38.87	26.31	31.60	23.95	25.54
MUFA [%]	56.78	32.55	55.76	42.43	52.03	39.85
PUFA [%]	20.34	28.58	17.93	25.97	24.02	34.61
BBM media 4 days						
Biomass [g/L]	6.87	13.57	9.21	5.84	10.46	10.20
Lipids in bio. [%]	34.17	20.19	22.83	62.48	6.04	6.43
SFA [%]	39.51	41.00	27.07	32.09	10.40	25.46
MUFA [%]	53.58	52.36	59.19	47.36	57.04	47.02
PUFA [%]	6.90	6.63	13.74	20.55	32.56	27.52
BBM media 10 days						
Biomass [g/L]	5.44	4.68	5.89	5.57	5.30	4.72
Lipids in bio. [%]	5.17	3.20	9.20	2.72	8.52	1.45
SFA [%]	18.07	55.70	18.12	33.02	24.34	76.53
MUFA [%]	37.44	13.07	35.43	21.46	51.38	0.05
PUFA [%]	44.49	31.22	46.44	45.52	24.28	23.42

Table 54. Phaffia rhodozyma lipid production and fatty acid profile

Yeast mineral media 4 days						
	Glucose + urea	Glycerol + urea	Glucose + $(NH_4)_2SO_4$	Glycerol + $(NH_4)_2SO_4$	Glucose + yeast a.	Glycerol + yeast a.
Biomass [g/L]	2.69	2.69	2.39	2.66	3.11	3.48
Lipids in bio. [%]	19.51	10.16	5.20	12.68	11.94	6.95
SFA [%]	18.70	17.55	13.57	16.27	20.29	12.21
MUFA [%]	50.15	37.66	13.79	44.70	25.63	17.29
PUFA [%]	31.15	44.79	72.64	39.04	54.08	70.50
BBM media 4 days						
Biomass [g/L]	5.44	4.68	5.89	5.57	5.30	4.72
Lipids in bio. [%]	5.17	3.20	9.20	2.72	8.52	1.45
SFA [%]	18.07	55.70	18.12	33.02	24.34	76.58
MUFA [%]	37.44	13.07	35.43	21.46	51.38	0.00
PUFA [%]	44.49	31.22	46.44	45.52	24.28	23.42
BBM media 10 days						
Biomass [g/L]	4.72	5.89	5.57	5.30	4.68	5.44
Lipids in bio. [%]	8.27	15.51	17.56	17.08	5.80	10.63
SFA [%]	15.63	23.64	18.58	24.76	15.55	27.48
MUFA [%]	39.18	28.49	40.83	28.09	26.96	17.01
PUFA [%]	45.19	47.87	40.59	47.15	57.49	55.51

5.3.2 The second phase – microalgae cultivation on BBM media

The co-cultivation experiment continued with testing the strains of microalgae and cyanobacteria on BBM medium. In this experiment, microalgae were inoculated into a medium containing glucose and glycerol and various nitrogen sources. This section aimed to identify if the selected strains are capable of a mixotrophic lifestyle. In total, the two series of experiments were performed. In the first, the microalgae were cultivated in the dark for ten days. It was found that none of the selected strains of microalgae is capable of a mixotrophy. From a continuous measurement of the growth curve at 680 nm, a linear decrease in absorbance after three days was observed for all strains, continuing until the culture's death. Chromatographic analysis of the medium indicated the consumption of embedded carbon sources. Therefore, the experiment was repeated with the addition of a small amount of antibiotic to rule out that the bacteria present were responsible for the consumption of organic carbon. In this experiment, it was confirmed that the strains we selected could not assimilate glucose and glycerol from the medium. In the 2nd series, the microalgae were cultivated under light for ten days. The presence of glucose in the medium, as the final product of photosynthesis, could inhibit photosynthesis even under light access. The aim was to determine whether microalgae are capable of photosynthesis in the presence of organic carbon sources and whether they can tolerate it and do not inhibit their growth. This experiment's result was that both sources at a given concentration slightly inhibited the growth of microalgae. In a long-term experiment, the culture was stopped or killed. Due to this, no analyzes of lipid composition and pigment production were performed. Based on these results, the co-cultivation procedure was changed in the next phase of the experiment.

5.3.3 Third phase – small scale co-cultivation

Based on previous experiments, the cultivation procedure was modified. As mentioned in chapter 4.4.4.3, when inoculating into the media, the microalgae was first inoculated and then the yeast in a given ratio. The cultivation was performed on reciprocal shakers. Co-cultivation continued with a single source of nitrogen. Urea was chosen as a compromise. In cultivations with yeast, excellent productions were achieved, and even experiments with microalgae showed compatibility. The advantage of urea is that it does not significantly affect the pH of the culture medium, and after removal of the amine groups, CO_2 is released into the media, which serves the phototrophs as a carbon source. Yeast autolysate appeared to be a suitable nitrogen source for yeast but negatively affected microalgae experiments. In the case of microalgae, ammonium sulphate did not work at all. The accelerated decrease of the medium's pH has manifested itself much more in microalgae cultivation because microalgae are more susceptible to changes in the pH of the media and at the same time their pH optimum lies more in neutral and slightly basic pH.

A comparison of biomass production is given in table 55. In the case of *R. kratochvilovae*, co-cultivation was successful. The microalga *D. quadricauda* always achieved a greater biomass growth during co-cultivation than pure yeast. In the experiment with glycerol in the medium, the biomass production was less than 3 g/L. Similarly, when cultivated with *Coccomyxa sp*. on glycerol, higher production of 7 g/L was achieved. In general, all micro-algal co-cultivations were successful, and glycerol was a more suitable carbon source. Co-cultivation of *R. toruloides* did not reach the same yields as in the previous case. Overall, glycerol was again a better source of carbon. Biomass production in co-cultivation has always been lower. An exception is co-cultivation with *D. acutus* on glucose, where slightly higher production was achieved than in the control cultivation of pure yeast. From all co-cultivation experiments, the best results were obtained on glycerol media with *D. quadricauda* and *D. acutus*.

As in previous cases, we also observe higher production on glycerol media with the yeast *P. rhodozyma* (Table 55). The best co-cultivation experiment was co-cultivation with *D. acutus* where biomass production reached almost the same values as control cultivation. Positive results were obtained on glucose media only in co-cultivation with the cyanobacterium *S. nidulans*. In other cases, the total biomass was always lower. The overall data show a practically clear trend, in which in most cases co-cultivation on glycerol media produced more biomass than glucose media. We see here the effect of a stronger effect growth imhibition of phototrophic microorganisms. However, the biomass production data alone do not have a complete informative value about the success of co-cultivation, because the measured data include the entire combined biomass of yeast and microalgae. For a better understanding, it was necessary to perform chromatographic analyses, which are shown in the following chapters.

Table 55. Biomass production of small-scale co-cultivation experiments [g/L]

	Microalgae strains					*Media*
Yeast strain	*D. quadricauda*	*S. nidulans*	*D. acutus*	*Coccomyxa sp.*	*Yeast*	BBM C + N
Rhodotorula kratochvilovae	*9.58*	*8.92*	*7.69*	*8.97*	*9.17*	*Glucose + urea*
Rhodotorula kratochvilovae	*7.02*	*5.49*	*4.72*	*11.37*	*4.18*	*Glycerol + urea*
Rhodosporidium toruloides	*0.90*	*1.56*	*2.17*	*1.75*	*2.12*	*Glucose + urea*
Rhodosporidium toruloides	*4.05*	*3.77*	*4.97*	*3.88*	*5.70*	*Glycerol + urea*
Phaffia rhodozyma	*2.69*	*3.28*	*2.63*	*2.55*	*3.12*	*Glucose + urea*
Phaffia rhodozyma	*4.07*	*3.89*	*8.43*	*2.51*	*8.71*	*Glycerol + urea*

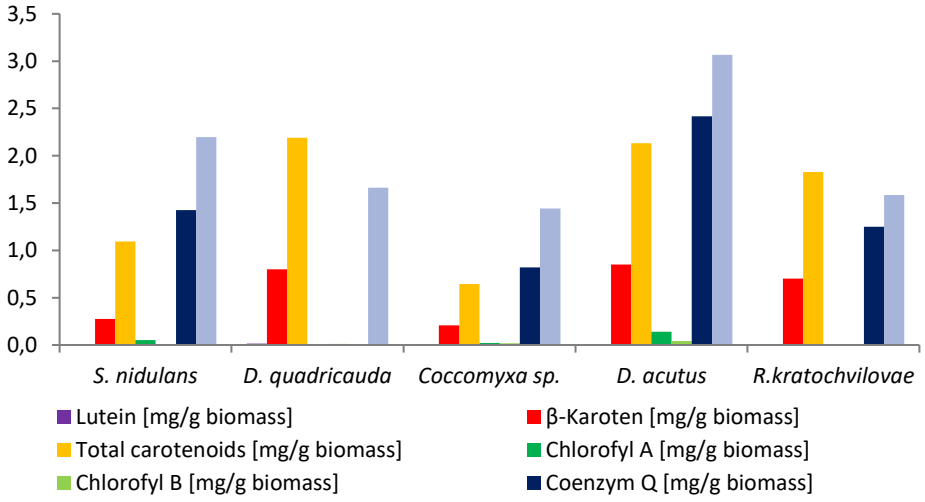
5.3.3.1 *Third phase: Carotenoid, chlorophyll, sterol and ubiquinone production*

Another essential evaluation factor was the liquid chromatography results focused on the total production of pigments, sterols and ubiquinone. The media had the same composition, based on BBM media with a nitrogen source of urea. The only difference was in the carbon source, where glucose or glycerol was used. The following graphs show the major carotenoid pigments lutein and β-carotene, total carotenoids, chlorophylls A and B, ergosterol and ubiquinone. Other identified carotenoid pigments were excluded from the plot due to low production. These were violaxanthin, neoxanthin, torulene, torularhodin, astaxanthin and lycopene. Only in the case of higher productions are they shown in the graph. The co-cultivation part of the results compared the production of yeasts and algae both on their own and co-cultivations. The graphs are divided so that its name indicates co-cultivation with given microalgae. The name of the yeast indicates a control culture of pure yeast. From each strain, the best cultivation is always depicted here.

5.3.3.1.1 HPLC analysis co-cultivation of the yeast R. kratochvilovae

The co-cultivation of the *R. kratochvilovae* strain with selected microalgal strains is shown in the following graph (Graph 88). Cultivation was performed in Erlenmeyer flasks in standard BBM media with glycerol and urea. The data show that the largest biomass production was achieved in the co-cultivation of yeast with the microalgae *D. quadricauda* (Table 55). Comparable production was further obtained in co-cultivation with microalgae *Coccomyxa sp.* and cyanobacteria *S. nidulans*. By comparing the chromatographic data with the control cultivation, we observe increased ergosterol production in all cultivation except Coccomyxa *sp.* Carotenoid production increased in two cases in co-cultivations with *D. quadricauda* and *D. acutus*, where the production of 2.19 mg/g and 2.13 mg/g of dry biomass was achieved to control 1.83 mg/g of dry biomass. Increased ubiquinone production is observed in co-cultivations with *D. acutus* and *S. nidulans*. In terms of the production of metabolites produced by microalgae, we see deficient production of chlorophylls.

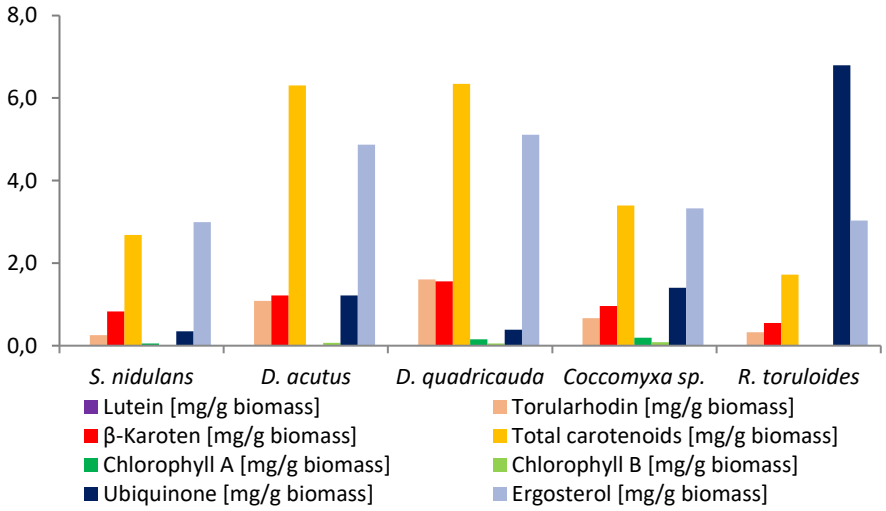
The experiment's highest values were achieved with the microalga *D. acutus*, namely 140 µg/g biomass. No chlorophyll was identified when cultivating with *D. quadricauda*. The low production of algal metabolites was caused by a small increase in microalgae and the fact that they served as a source of nutrients for the yeast in the later experiment stages. For this reason, the vast majority of biomass was made up of yeast biomass. From the overall point of view, we see that co-cultivation positively affects the total production of the studied metabolites and biomass.



Graph 88. *R. kratochvilovae small-scale 10-day co-cultivation on BBM media (glucose + urea)*

5.3.3.1.2 HPLC analysis co-cultivation of the yeast R. toruloides

The results of a co-cultivation experiment with the yeast *R. toruloides* show the same properties as in the yeast *R. kratochvilovae*. From the point of view of pure biomass production, co-cultivation on glycerol was more successful. However, higher production of the observed lipid metabolites was achieved by any co-cultivation. The best lipid production results were obtained on glucose and urea media, the results of which are shown in the graph below (Graph 89). The results show a significant difference in the production of carotenoids, wherein the case of co-cultivation with representatives of the genus *Desmodesmus*, the production reached more than 6.30 mg/g of dry biomass. Overall, all co-cultivations always produced more carotenoids and ergosterol. We see a fundamental difference in the production of carotenoids compared to other yeasts, namely in torulene production. In co-cultivations with the genus *Desmodesmus*, the yeast produced a comparable amount of torulene and β-carotene, where these two pigments accounted for more than 50% of the total carotenoids. Only ubiquinone production was much higher in pure yeast cultivation, namely 6.79 mg/g of dry biomass. In all experiments, small amounts of chlorophylls and lutein were again identified. Thus, it can be stated that in all experiments, the microalga survived and was part of the final analyzed biomass.

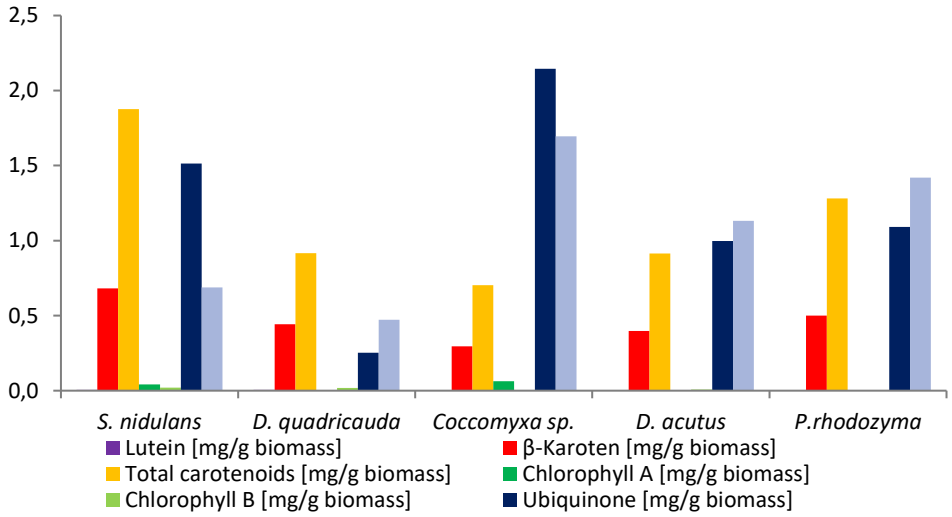


Graph 89. *R. toruloides small-scale 10-day co-cultivation on BBM media (glucose + urea)*

5.3.3.1.3 HPLC analysis co-cultivation of the yeast P. rhodozyma

P. Rhodozyma cultivation results show, that BBM medium containing glycerol (Graph 90) was better in all respects than medium with glucose. Biomass production in both cases did not reach the control culture's values, except co-cultivation of *S. nidulans* on glucose. However, the chromatographic data show that co-cultivation had a favourable effect on the studied metabolites' production. Especially in co-cultivations with *S. nidulans* and *Coccomyxa sp.*, where higher production of carotenoids was measured. In other metabolites, the production was lower in all co-cultivation experiments. The primary pigment here is again β-carotene, which makes up more than 30% of the carotenoid content. A significant increase in ubiquinone production is further observed in these co-cultivations as well. The production of algal metabolites was recorded in only three co-cultivations. In the case of *D. acutus* co-cultivations, the culture was killed during cultivation.

The best co-cultivation was the already mentioned experiments with the cyanobacterium *S. nidulans* and the microalga *Coccomyxa sp*. The overall low production of biomass and the control medium was measured on glucose medium. In this case, the best co-cultivation was cultivation with *D. quadricauda*, where high productions of ergosterol 6.14 mg/g, and ubiquinone 5.78 mg/g of dry biomass were achieved.



Graph 90. *P. rhodozyma small-scale 10-day co-cultivation on BBM media (glycerol + urea)*

In the overall evaluation, the most suitable microalgal strains in these experiments were genus *Desmodesmus* and the *Coccomyxa* strain for both the yeast *Rhodotorula kratochvilovae.* and *Rhodosporidium toruloides.* In both cases, glucose was a better source of carbon. The yeast *P. rhodozyma* achieved better results on a medium with glycerol combined with the microalgae Coccomyxa sp. and cyanobacteria S. nidulans.

5.3.3.2 *Third phase: Fatty acid profile and lipid production*

The results of the GC analysis of biomass samples from co-cultivation experiments are shown in the following tables. The data are again sorted according to the cultivated carotenogenic yeast and the corresponding co-cultivation experiments. The main monitored parameters were again the composition of individual groups of fatty acids, the percentage of lipids in biomass, and lipids' total production.

5.3.3.2.1 GC analysis co-cultivation of the yeast R. kratochvilovae

Table 56 shows co-cultivation results with the *R. kratochvilovae* strain on BBM media with glycerol and glucose. Overall, lower production of lipids in biomass was achieved in all co-cultivations. Only in glucose media did co-cultivation with *D. quadricauda* and *S. nidulans* achieve approximately the same production as the pure control medium. The unifying parameter for both types of media is the low PUFA content, except for cultivation with *D. acutus* on glucose, which was always below 30%. In comparison with the production of MUFA on yeast media, a larger production of MUFA was achieved in all co-cultivations, with the exception of co-cultivation with *Coccomyxa sp.* From the overall results, it is generally impossible to determine the direct effect of co-cultivation on the change in the ratio of fatty acids in the produced biomass. It should be added that the biomass produced was made by a majority of carotenogenic yeast cells and therefore we do not see significant differences in the proportion of MK compared to the control, with the exceptions mentioned above.

Table 56. R. kratochvilovae lipid production and fatty acid profile in small-scale co-cultivation experiments

	BBM media glycerol + urea				
	R. kratochvilovae	*D. quadricauda*	*D. acutus*	*S. nidulans*	*Coccomyxa sp.*
Biomass [g/L]	4.18	7.02	4.72	5.49	11.37
Lipids in bio. [%]	18.50	6.69	10.42	11.76	3.51
SFA [%]	19.26	18.68	23.60	18.40	52.03
MUFA [%]	53.46	58.36	51.39	59.93	22.86
PUFA [%]	27.28	22.96	25.01	21.67	25.10
	BBM media glucose + urea				
	R. kratochvilovae	*D. quadricauda*	*D. acutus*	*S. nidulans*	*Coccomyxa sp.*
Biomass [g/L]	9.17	9.58	7.69	8.92	8.97
Lipids in bio. [%]	20.23	19.05	9.68	19.67	17.95
SFA [%]	21.16	26.09	14.97	25.47	30.88
MUFA [%]	49.40	46.34	48.63	47.35	41.90
PUFA [%]	29.44	27.57	36.40	27.18	27.22

5.3.3.3 *GC analysis co-cultivation of the yeast R. toruloides*

The following cultivation data from *R. toruloides* co-cultivations (Table 57) indicate a very low production of lipids in both media types. In all experiments, the percentage of lipids in the biomass was less than 8%. The fatty acid profile was relatively stable in the experiments. The only exceptions were co-cultivation with *Coccomyxa sp.*, where very high SFA production was achieved at the expense of PUFA. In other cases, SFA production was low. The PUFA content was around 40% and did not show significant fluctuations. The highest content was measured during co-cultivation with *D. quadricauda* on both types of media.

Table 57. R. toruloides lipid production and fatty acid profile in small-scale co-cultivation experiments

	BBM media glycerol + urea				
	R. toruloides	*D. quadricauda*	*D. acutus*	*S. nidulans*	*Coccomyxa sp.*
Biomass [g/L]	5.70	4.97	3.77	4.05	3.88
Lipids in bio. [%]	6.79	5.10	6.10	6.88	3.21
SFA [%]	8.55	10.04	15.51	12.87	63.55
MUFA [%]	46.31	44.32	53.29	47.69	36.45
PUFA [%]	45.14	45.63	31.19	39.44	0.00
	BBM media glucose + urea				
	R. toruloides	*D. quadricauda*	*D. acutus*	*S. nidulans*	*Coccomyxa sp.*
Biomass [g/L]	2.12	0.90	2.17	1.56	1.75
Lipids in bio. [%]	2.28	3.28	4.45	2.20	1.59
SFA [%]	12.08	14.32	11.63	24.84	49.32
MUFA [%]	52.12	39.45	47.53	33.58	34.29
PUFA [%]	35.81	46.23	40.84	41.58	16.39

A comparison of the results of all three strains shows that the best producer here was the yeast *P. rhodozyma*, which achieved high lipid production in general in all media. The worst was the *R. toruloides* strain. If extreme cases are not considered, we observe an unsaturated fatty acid content of more than 75% in all carotenogenic yeasts. From the obtained results, we see that there is no significant influence on the production of individual groups of fatty acids during co-cultivation. In the case of *P. rhodozyma*, the co-cultivation experiment had a positive effect on the overall lipid production. In the *R. kratochvilovae* strain case, we can speak of a slightly positive effect, and in the yeast *R. toruloides* cultivation, we observe a somewhat negative effect under the given conditions.

5.3.3.4 *GC analysis co-cultivation of the yeast P. rhodozyma*

The GC analysis results of *P. rhodozyma* biomass cultivated on BBM media with glycerol show several identical properties, the composition of fatty acid groups is very similar, and there are no significant fluctuations (Table 58). We also see a high production of total lipids, the content of which is always greater than 20%. In co-cultivation with *Coccomyxa sp.* and *S. nidulans,* the highest production of 29.12% and 32.06% was obtained. Unsaturated fatty acids predominate in all co-cultivation experiments, making up 75-80% of all lipids. On the other hand, co-cultivation on glycerol, except for the *D. acutus* strain, was unsuccessful in lipid production. Only co-cultivation with *D. acutus* produced a slightly higher lipid content compared to the control. In the other three, production was deficient. In the fatty acid profile, we see a decrease in PUFA content compared to glucose medium. On the contrary, the SFA content increased significantly. Low MUFA content was measured in co-cultivations with *D. quadricauda* and in co-cultivations with *Coccomyxa sp.* was not measured at all.

Table 58. P. rhodozyma lipid production and fatty acid profile in small-scale co-cultivation experiments

	BBM media glycerol + urea				
	P. rhodozyma	*D. quadricauda*	*D. acutus*	*S. nidulans*	*Coccomyxa sp.*
Biomass [g/L]	8,71	4,07	8,43	3,89	2,51
Lipids in bio. [%]	23,23	3,53	24,74	3,23	2,94
SFA [%]	30,63	60,62	30,05	28,17	83,83
MUFA [%]	45,57	19,21	47,79	52,30	0,00
PUFA [%]	23,80	20,16	22,15	19,53	16,17
	BBM media glucose + urea				
	P. rhodozyma	*D. quadricauda*	*D. acutus*	*S. nidulans*	*Coccomyxa sp.*
Biomass [g/L]	3,12	2,69	2,63	3,28	2,55
Lipids in bio. [%]	21,81	20,93	21,66	32,06	29,12
SFA [%]	21,82	21,16	22,20	20,32	22,73
MUFA [%]	42,62	40,01	42,51	46,42	45,53
PUFA [%]	35,57	38,84	35,29	33,26	31,74

5.3.4 Fourth phase – co-cultivation in aerated flasks

From the data obtained from previous experiments, an experimental scheme for phase four was proposed. In this experiment, cultivations were performed in aerated 1L Pyrex flasks. In each cultivation, yeasts were cultivated separately on BBM media with carbon sources, microalgae on BBM medium with and without carbon source, and then co-cultivated with yeasts in three inoculation ratios: microalgae 1:1, 1:2 and 1:4. Because the yeast growth was always significantly larger than the microalgae biomass in previous experiments, the yeast inoculation ratio was reduced in these experiments. In the experiments, the yeast was inoculated so that the initial absorbance of the yeast culture was A_{580} = 0.100. The addition of a microalgae bottle with BBM medium without a carbon source was chosen as a second control to compare the effect of the carbon source on the growth of the microalgae and further study the effect of bacterial contamination on the microalgae if any. The basic goals of this phase of the experiment were to test the effect of aeration on the production of biomass and metabolites in co-cultivations and separate cultures. Furthermore, the different inoculation rates allowed the study of the compatibility of yeast and microalgae with different amounts of each strain. All results gained here were compared to laboratory small scale experiments (phase three 5.3.3).

Table 59 summarizes the biomass production in co-cultivation of *R. kratochvilovae* and *R. toruloides* with different microalgae strains. Results show that co-cultivation of *R. kratochvilovae* with *S. obliquus* and *D. acutus* produced lesser amounts of biomass compared to the control yeast cultivation. Overall comparable results were obtained only in co-cultivation with *S. Obliquus* in a ratio of 1:2, where 3.12 g/L of dry biomass was produced. Furthermore, in co-cultivation with *D. acutus* in a ratio of 1:4, where the final biomass growth was 3.42 g/L. Contamination and death of the culture occurred during the sole cultivations of *D. acutus* on both BBM media. Overall, the most successful experiment was with *D. quadricauda* microalgae, where a higher growth of biomass was achieved in all co-cultivation conditions compared to the control medium. The maximum was the production of 7.21 g/L in a ratio of 1:2, which exceeded the control cultivation by more than 3 g/L.

Co-cultivation experiments with the yeast *R. toruloides* achieved the best results on glycerol medium. No significant effect of the culture ratio on biomass production was observed during co-cultivation with *D. acutus*. In all cases, the production was about half compared to the control cultivation of sole yeast. Compared to the yeast *R. kratochvilovae*, co-cultivation with this alga was significantly better. In co-cultivation with *Chlamydomonas reinhardtii,* we observe the inoculation ratio's effect, where the best ratio was 1:2. Co-cultivation with microalgae on glucose medium in this yeast was rather negative and is not shown here. Unfortunately, no positive effect on biomass production or monitored metabolites was observed in the yeast *P. rhodozyma*. Overall lower biomass production in these experiments was expected due to a lower inoculation ratio in yeast.

Table 59. Biomass production of R. kratochvilovae and R. toruloides in co-cultivation with different microalgae in aerated Pyrex flasks

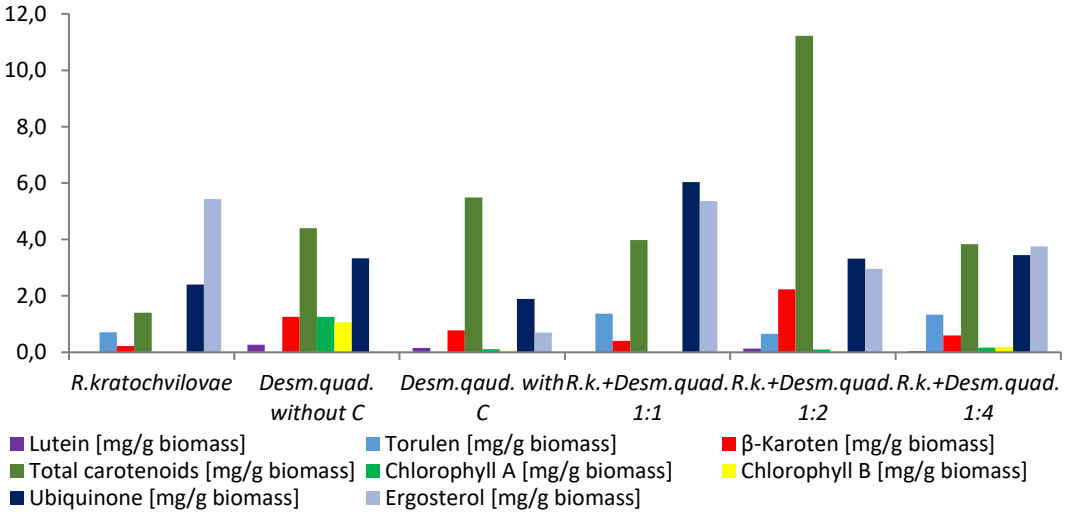
R. kratochvilovae + *Scenedesmus obliquus* BBM media + glucose + urea					
R. k.	*Scen. obl.* - C	*Scen. obl.* - C	Y+ Al 1:1	Y+ Al 1:2	Y+ Al 1:4
4.32 g/L	0.63 g/L	0.22 g/L	0.49 g/L	3.12 g/L	0.92 g/L
R. kratochvilovae + *Desmodesmus acutus* BBM media + glucose + urea					
R. k.	*Desm. ac.* - C	*Desm. ac.* + C	Y+ Al 1:1	Y+ Al 1:2	Y+ Al 1:4
4.32 g/L	Died	Died	0.65 g/L	1.24 g/L	3.42 g/L
R. kratochvilovae + *Desmodesmus quadricauda* BBM media + glucose + urea					
R. k.	*Desm. q.* - C	*Desm. q.* + C	Y+ Al 1:1	Y+ Al 1:2	Y+ Al 1:4
4.48g/L	0.23 g/L	2.75 g/L	4.61 g/L	7.21 g/L	4.59 g/L
R. toruloides + *Desmodesmu acutus* BBM media + glycerol+ urea					
R. t.	*Desm. ac.* - C	*Desm. ac.* + C	Y+ Al 1:1	Y+ Al 1:2	Y+ Al 1:4
5.41 g/L	0.95 g/L	0.64 g/L	2.65 g/L	2.76 g/L	2.66 g/L
R. toruloides + *Chlamydomonas reinhardtii* BBM media + glycerol + urea					
R. t.	*Ch. reinh.* - C	*Ch. reinh.* + C	Y+ Al 1:1	Y+ Al 1:2	Y+ Al 1:4
3.07 g/L	Died	0.29 g/L	1.347 g/L	3.27 g/L	2.467 g/L

5.3.4.1 *Fourth phase: Carotenoid, chlorophyll, sterols and ubiquinone production*

The following graphs show the production of major carotenoid pigments lutein and β-carotene, total carotenoids, chlorophylls A and B, ergosterol and ubiquinone. Other identified carotenoid pigments were excluded from the plot due to low production. These were violaxanthin, neoxanthin, torulene, torularhodin, astaxanthin and lycopene. Only in the case of higher productions are they shown in the graph. The graphs contain the results from cultivation of sole yeast, algae on media with and without carbon source, and then co-cultivation with different ratios. In the event of a culture death, the data are excluded from the graph. The following graphs show only the best co-cultivations from all performed experiments. During the experiment, other species of microalgae, such as *Chlamydomonas reinhardtii* and *Scenedesmus Obliquus*, were also tested experimentally in large bottles. The necessary previous experimental steps for these cultivations were not completely done at the time of writing and are therefore not listed in the results section.

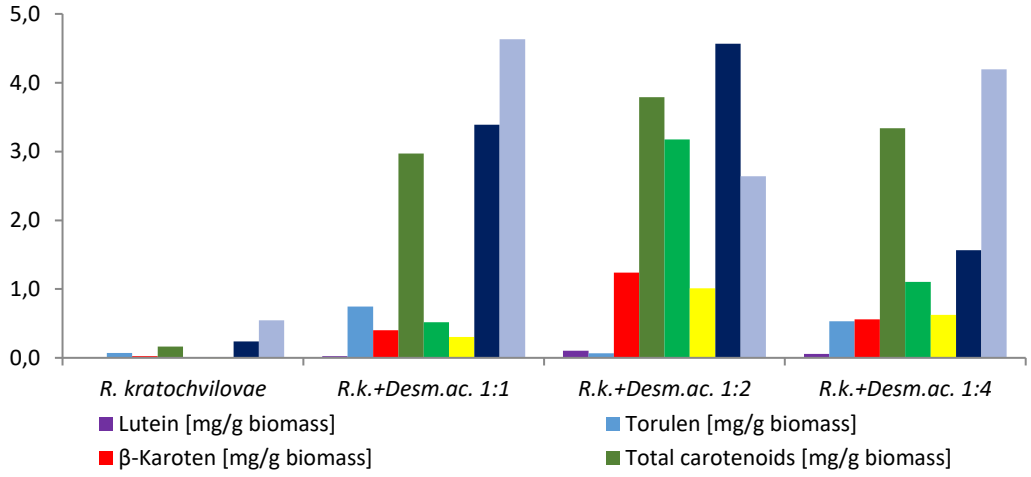
5.3.4.1.1 HPLC analysis Rhodotorula kratochvilovae co-cultivation

As we already saw from biomass production data that in the case of this yeast, the best partners for co-cultivation are representatives of the genus *Desmodesmus.* Chromatographic analysis of the pigment and other lipid substances confirmed this assumption. In the graph below (Graph 91) we see a positive effect of co-cultivation on the monitored metabolites' production. The most successful co-cultivation was a 1:2 ratio, where carotenoid production of 11.22 mg/g dry biomass was measured. In general, all co-cultivations always had higher carotenoid production. However, the carotenoid profile is different. There is a reduction in the production of the primary carotenoid pigments β-carotene and torulene favouring other pigments. At the same time, we see a low production of chlorophylls (less than 1 mg/g biomass) than conventional microalgae cultivation. At the end of the cultivation, there was probably a reduction in chlorophylls production by algae and an accumulation of carotenoid pigments. Co-cultivation also positively affected ubiquinone production, where the highest values of 6.04 mg/g of dry biomass were reached at a co-cultivation ratio of 1:1. At the same co-cultivation ratio, the highest ergosterol production was also achieved (5.37 mg/g of dry biomass).



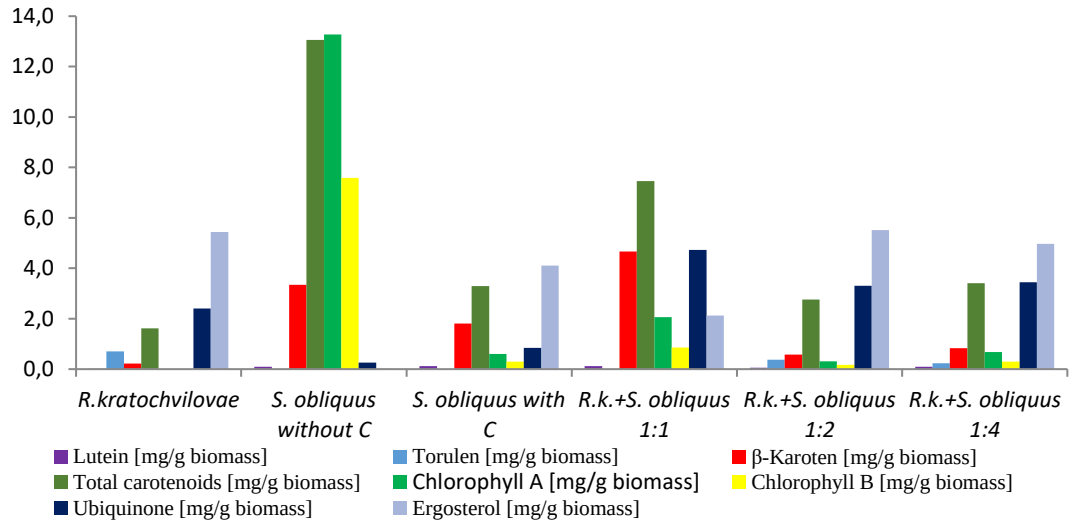
Graph 91. *R. kratochvilovae co-cultivation with Desmodesmus quadricauda HPLC results*

The experiment continued with co-cultivation with the microalgae *Desmodesmus acutus*, where even in repeated experiments, death occurred in bottles with pure algal culture. Interestingly, in the case of co-cultivation under given conditions, the microalgae survived, as evidenced by the measured data of chlorophyll A and B production (Graph 92). Compared to classical BBM medium without carbon source, the second bottle with carbon source had an inhibitory effect on glucose. This phenomenon was probably due to the rapid depletion of the carbon source from the medium and a reduction in glucose's inhibitory effect. In terms of biomass production, the best ratio was 1:4, which together with the ratio 1:2 reached the best production of monitored metabolites. In the case of all experiments, the co-cultivations overcame the low production of the sole yeast medium. If we compare it with the data from previous experiments, the results obtained are still very good.



Graph 92. *R. kratochvilovae co-cultivation with Desmodesmus acutus HPLC results*

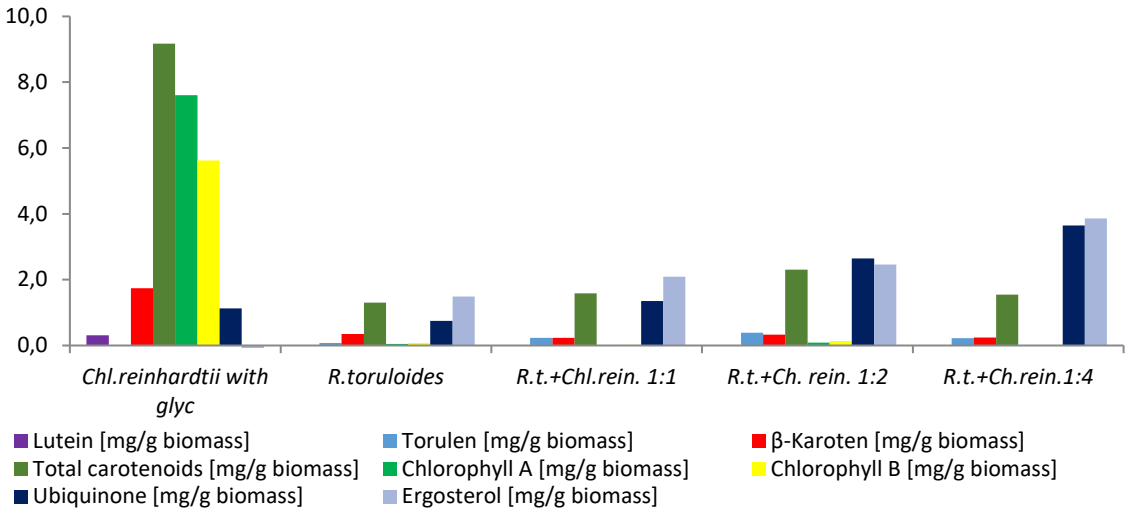
Based on the data obtained from co-cultivation with representatives of the genus *Desmodesmus*, a test co-cultivation with microalgae *Scenedesmus obliquus* was performed. Due to the morphological similarities between the genera *Desmodesmus* and *Scenedesmus*, a representative of the genus *Scenedesmus* was also tested in this experiment. The reasons are summarized in the final summary of this chapter. From the results, we see a positive effect of co-cultivation on studied metabolites' production (Graph 93). The control cultivation of pure microalgae has the highest production of monitored metabolites, but on the other hand, the production of biomass was very low, only 0.63 g/L. In an overall comparison, the production of lipid substances was higher in co-cultivation experiments, in which the highest production was achieved at a cultivation ratio of 1:1. Here, carotenoids' production was 7.46 mg/g of biomass, which comprised of 60% β-carotene. The highest ubiquinone production was also achieved in the same medium, namely 4.73 mg/g biomass. This smallest inoculation ratio was most suitable for the microalgae itself. Compared to the control of sole yeast culture, we see that the co-cultivation were more productive in all proportions. If we include biomass production, the largest profits were achieved at a ratio of 1:2.



Graph 93. *R. kratochvilovae co-cultivation with Scenedesmus obliquus HPLC results*

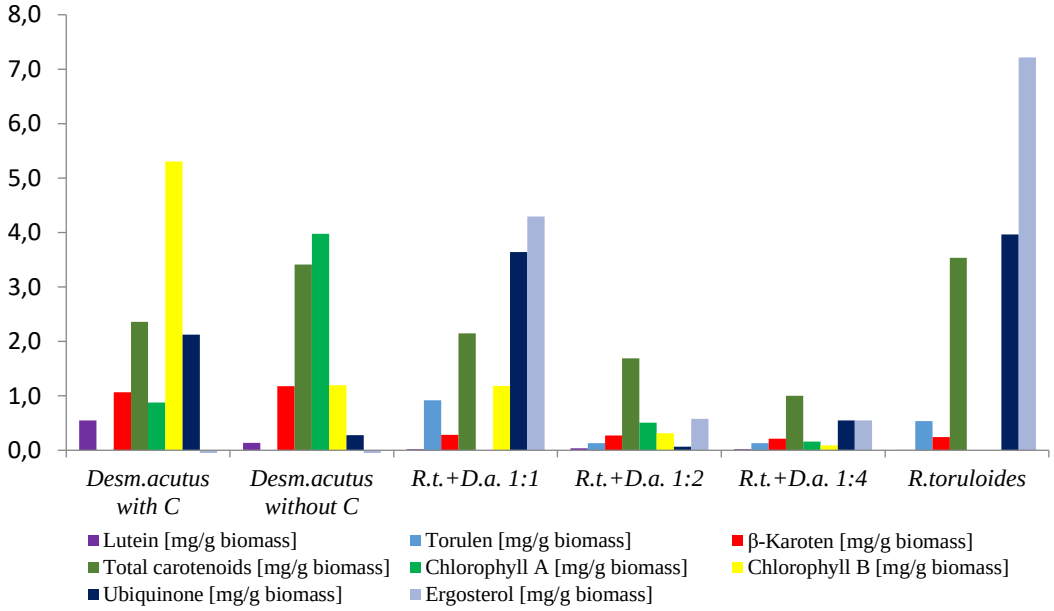
5.3.4.1.2 HPLC analysis Rhodosporidium toruloides co-cultivation

The following graphs show selected results of co-cultivation experiments of *R. toruloides* in aerated bottles. In the first graph, we see the results of co-cultivation with *Ch. reinhardtii* chromatographic analysis of lipid metabolites (Graph 94). Microalgae was contaminated during cultivation in BBM medium without glycerol, and therefore these results are not listed here. The highest production was achieved by the sole microalgae itself cultivated on a media with glycerol. However, this increased metabolite production was at the expense of biomass production, which was only 0.29 g/L. Data from co-cultivation show one similar trend, and that is practical absence of chlorophylls, which means that the microalgal cells were assimilated by the yeast cells in the medium. From the comparison with the yeast control, we see that this affected the production of the monitored substances, which are higher in all proportions. If we do not consider the assimilation of algal cells, the best ratio was the 1:2 ratio, which reached the highest biomass production of 3.27 g/L and the highest carotenoid production level. It produced second-best results of ergosterol and ubiquinone production, behind the 1:4 ratio.



Graph 94. *R. toruloides co-cultivation with Chlamydomonas reinhardtii HPLC results*

The second graph (Graph 95) summarizes the results of co-cultivation with *D. acutus* microalgae. Biomass production in co-cultivation was approximately half that of yeast in all three ratios. The presence of chlorophylls was measured in all three samples. Thus, we can state that the microalgae survived in this medium. From the HPLC results, we see that higher lipid metabolites' production was not achieved in the co-cultivations than the control cultivations. The results show that this strain can tolerate the presence of glucose and glycerol in the media. The presence of organic carbon in the media had an interesting effect on the production of chlorophylls. In the absence of carbon in the medium, chlorophyll B's production predominates (5.30 mg/g), but the addition of organic carbon changes and then chlorophyll A predominates (3.98 mg/g).



Graph 95. *R.toruloides co-cultivation with Desmodesmus acutus HPLC results*

5.3.4.1.3 Fourth phase: Fatty acid profile and lipid production

The results of the GC analysis of biomass samples from co-cultivation experiments are shown in the following table 60. The main monitored parameters were the composition of individual fatty acid groups, the percentage of lipids in the biomass and the total lipid production. As in the previous chapters, it is not possible to evaluate and determine the general trend. In the case of co-cultivation of the yeast *R. kratochvilovae*, the highest content of lipids in co-cultivation with *D. quadricauda* was achieved. As in the case of pigment production, the cultivaton ratio of 1:2 was the best here with a total content of 30.5% lipids in the biomass and thus approached the control medium. Another effect in all co-cultivation experiments of this yeast was the production of PUFA at the expense of the SFA content. MUFA production was relatively balanced in the range of 43-50% except for cocultivation of *S. obliquus* ratio 1:1 and *D. acutus* ratio 1:2.

Table 60. R. kratochvilovae lipid production and fatty acid profile in co-cultivation experiments in aerated flasks

Co-cultivation *R. kratochvilovae* + *S. obliquus*						
	Yeast	Algae - C	Algae + C	Y + Al 1:1	Y + Al 1:2	Y + Al 1:4
Biomass [g/L]	4.47	0.63	0.22	0.49	3.12	0.92
Lipids in bio. [%]	32.06	6.43	3.64	2.84	17.27	11.07
SFA [%]	36.77	20.07	45.54	39.79	28.05	29.50
MUFA [%]	49.70	17.45	38.41	32.83	49.42	47.68
PUFA [%]	13.53	62.48	16.05	27.38	22.53	22.82
Co-cultivation *R. kratochvilovae* + *D. quadricauda*						
	Yeast	Algae - C	Algae + C	Y + Al 1:1	Y + Al 1:2	Y + Al 1:4
Biomass [g/L]	4.43	0.24	1.05	8.70	4.49	5.88
Lipids in bio. [%]	32.06	3.02	23.96	22.91	30.46	28.04
SFA [%]	36.77	54.37	24.31	35.03	37.55	39.09
MUFA [%]	49.70	45.63	51.84	47.92	45.83	43.41
PUFA [%]	13.53	0.00	23.84	17.05	16.63	17.49
Co-cultivation *R. kratochvilovae* + *D. acutus*						
	Yeast	Algae - C	Algae + C	Y + Al 1:1	Y + Al 1:2	Y + Al 1:4
Biomass [g/L]	4.32	Died	Died	0.65	1.24	3.42
Lipids in bio. [%]	32.06	-	-	16.89	3.38	18.06
SFA [%]	36.77	-	-	28.47	25.96	28.98
MUFA [%]	49.70	-	-	47.28	36.64	48.53
PUFA [%]	13.53	-	-	24.26	37.40	22.49

Table 61 shows the GC results of selected co-cultivation experiments with the yeast *R. toruloides*. Total lipid production is deficient in all co-cultivation experiments, except for only two cases, co-cultivation with *Chlamydomonas reinhardtii* in a ratio 1:4 and *Desmodesmus acutus* in a ratio 1: 1. In both cases, the lipid content approached that of the control yeast culture. MUFA production has a relatively stable trend of % content 40-47% in co-cultivation and in control yeast cultivation. In the production of SFA, in all co-cultivation experiments, there is always a higher production in comparison with the control cultivation.

Table 61. R. toruloides lipid production and fatty acid profile in co-cultivation experiments in aerated flasks

Co-cultivation *R. toruloides* + *D. acutus*						
	Yeast	Algae - C	Algae + C	Y + Al 1:1	Y + Al 1:2	Y + Al 1:4
Biomass [g/L]	5.41	0.95	0.64	2.65	2.76	2.66
Lipids in bio. [%]	25.69	2.96	2.89	23.98	6.91	8.47
SFA [%]	25.95	64.66	52.98	33.08	42.66	41.29
MUFA [%]	44.56	21.20	47.02	47.19	42.71	43.00
PUFA [%]	19.50	14.14	0.00	19.74	14.63	15.71
Co-cultivation *R. toruloides* + *Ch. reinhardtii*						
	Yeast	Algae - C	Algae + C	Y + Al 1:1	Y + Al 1:2	Y + Al 1:4
Biomass [g/L]	5.41	0.29	1.10	1.35	3.27	2.47
Lipids in bio. [%]	25.69	5.77	3.22	5.77	7.86	25.33
SFA [%]	25.95	40.24	53.69	48.53	70.62	34.67
MUFA [%]	44.56	14.30	43.21	40.38	5.24	44.24
PUFA [%]	19.50	45.45	3.10	11.09	24.14	21.09

5.3.5 Summary of the results of the 1st to 4th phase

The following paragraph summarises the results of previous co-cultivation experiments performed in the 1st to 4th phase. The first phase of the experiments shows that the selected carotenogenic yeasts are able to grow on an algal mineral medium. Compared to the control mineral yeast medium, biomass production on some combinations of nitrogen and carbon sources is an even higher. The *R. kratochvílovae* strain achieved the absolute best results of the three strains studied, followed by the *R. toruloides* strain. The worst strain was the *P. rhodozyma* strain, which is limited by its slower growth, but this handicap is no longer so significant than the production in 4-day cultivation. The results showed that the best source of nitrogen is yeast autolysate. However, this is also due to the fact that this material contains a number of other substances, vitamins and minerals that further promote growth. Unfortunately, its price is too high compared to other tested nitrogen sources. In terms of the production of carotenoids and other lipid substances, the order of succession of the strains was again the same as in biomass production, i.e. *R. kratochvilovae* > *R. toruloides* > *P. rhodozyma*. Cultivation on glycerol media have been more successful in lipid production.

In the second phase, selected strains of microalgae and cyanobacteria were tested in an experiment to determine whether these strains are capable of mixotrophy or can tolerate the presence of an organic carbon source and grow under the access of light. The experiments show that no strain is capable of a mixotrophy, both in light and in the dark. Experiments have shown that selected microalgae cannot tolerate long-term tolerance of organic carbon in the medium. Especially glucose, has the most potent inhibitory effect. So, we can say that these microalgae strains are strict phototrophs.

In the third phase of the experiment, pilot laboratory co-cultivation tests were performed in Erlenmeyer flasks. Even at this stage of the experiment, the best strain was generally *R. kratochvilovae*. The trend then applies to all strains, where more biomass was produced in co-cultivations with glycerol in the media. The best strains of microalgae for co-cultivation were representatives of the genus *Desmodesmus* and cyanobacteria *S. nidulans*. In lipids' production, co-cultivation had a positive effect, especially in the *P. rhodozyma* strain, where more lipids were always achieved in these media than in the control medium. In terms of the production of lipid metabolites, co-cultivation with the genus *Desmodesmus* had a very positive effect on the production of carotenoids and ubiquinone in particular.

In experiments, it was further found that co-cultivation with the yeast strains *R. toruloides* and *R. kratochvilovae* has one major weakness: the morphology of microalgal cells. With rapid yeast growth, nutrient sources are consumed, and the yeast then uses microalgae cells as another source of nutrients, which in most cases leads to higher production of monitored metabolites. However, the whole experiment's primary goal is to prepare and test the possibility of co-cultivation of microalgae and yeast to produce combined biomass, which contains both yeast cells and microalgae. The obtained biomass should thus benefit the consumer by the presence of products of both types of microorganisms. Initial results from co-cultivation showed that microalgae, which are live in large colonies/coenobium composed of multiple cells, or cells that form outer envelopes resistant to yeast enzymes, can defend against aggressive growth. The genus *Desmodesmus* is characterized by both properties and therefore, in general, the results of co-cultivation with this strain were among the best.

This phenomenon was further confirmed in co-cultivation in aerated bottles, where clearly the best results were achieved by representatives of the genus *Desmodesmus*. Identical results were also obtained in a test cultivation of the *Scenedesmus obliquus* strain. In these experiments, the results from the second phase were further confirmed, when in a series of experiments, the alga itself could not grow for a long time in a medium with the addition of an organic carbon source. Furthermore, it was found that for most of the selected co-cultivations, the best co-cultivation ratio clearly does not apply. Based on these results, the yeasts *Rhodotorula kratochvilovae* and *Rhodosporidium toruloides* were selected for the experiment's final 5th phase.

5.3.6 Fifth phase – bioreactor cultivation

The final phase of the experiment consisted of bioreactor co-cultivation. In these co-cultivations, a selected microalga was inoculated into the prepared standard medium, which was left for 24 hours, and then a yeast inoculum was added to the medium. Due to the organic carbon source's inhibitory effect on algae, the carbon source was put into the system by a peristaltic pump with a fed-batch system. During the cultivation, biomass samples were taken regularly, which were further processed and ignited. According to individual bioreactor cultivation, the results are sorted here and include a biomass productivity table, complete GC and HPLC analysis. Chromatographic analysis of lipids is divided into two graphs. The first graph plots the percentage of lipids in biomass and biomass production in g/L. The second graph shows the percentage of fatty acid groups in the columns. The third graph shows the results of a chromatographic analysis of lipid substances (carotenoids, chlorophylls, ubiquinone, ergosterol).

5.3.6.1 *Bioreactor co-cultivations of Rhodosporidium toruloides*

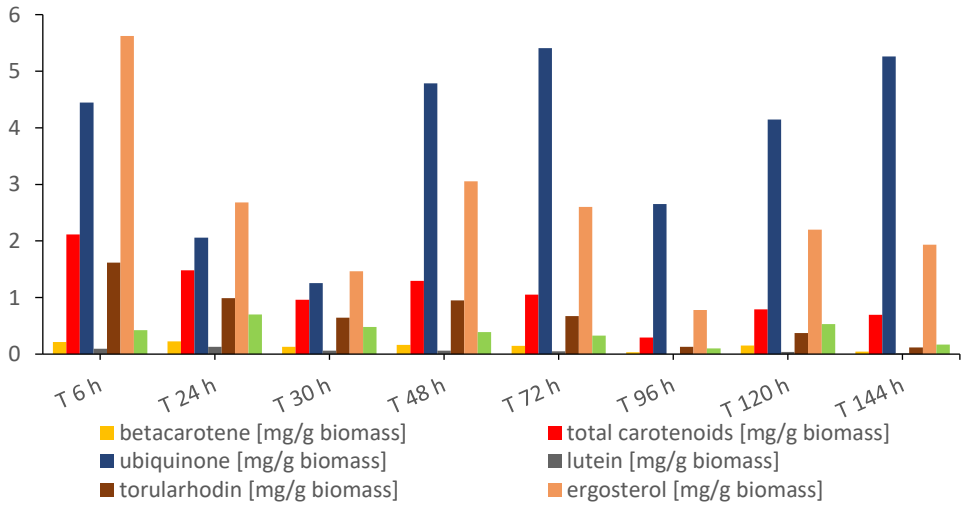
5.3.6.1.1 Bioreactor co-cultivations of Rhodotorula kratochvilovae + Desmodesmus quadricauda C/N = 100 – first trial

The first bioreactor experiment was co-cultivation of *R. kratochvilovae* and *D. quadricauda* in BBM media with glycerol + urea on C/N ratio 100. The table below (Table 62) shows the biomass production of a co-cultivation experiment. During cultivation, we see an exponential increase in biomass production ending at 144 hours with a final production of 8.78 g/L. We see a somewhat fluctuating trend in carotenoid production over time. During the growth of the culture, three local maxima can be observed in the production of carotenoids (Graph 96) the first was reached in the first stages of culture growth, at six hours, where the production reached 2.11 mg/g of dry biomass, the second maximum was reached at 48 hours, wherethe production was 1.29 mg/g of dry biomass and third at 120 hours with a 0.79 mg/g of dry biomass. Two maxima characterized chlorophyll production at 24 and 120 hours, where at 24 hours production reached 0.70 mg/g biomass, and at 120 hours it was 0.53 mg/g biomass.

Ubiquinone production after an initial decrease in the first 30 hours of cultivation is followed by a high increase in production, which is the first maximum production of 5.41 mg/g at 72 hours. The second maximum is in the final phase, with 5.26 mg/g of dry biomass production. In terms of ergosterol production, there is a linear decrease in the content of biomass dry matter. The highest production, 3.02 mg/g biomass, is reached at 48 hours.

Table 62. Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. quadricauda – first trial

Cultivation time [hours]										
Sample name	T 0H A	T 0h A+Y	T 6H	T 24h	T 30h	T 48h	T 72h	T 96h	T 120h	T 144h
Biomass [g/L]	0.05	0.50	0.60	1.50	2.07	3.00	4.13	6.66	7.53	8.78



Graph 96. *Bioreactor co-cultivation R. kratochvilovae + D. quadricauda HPLC results – first trial*

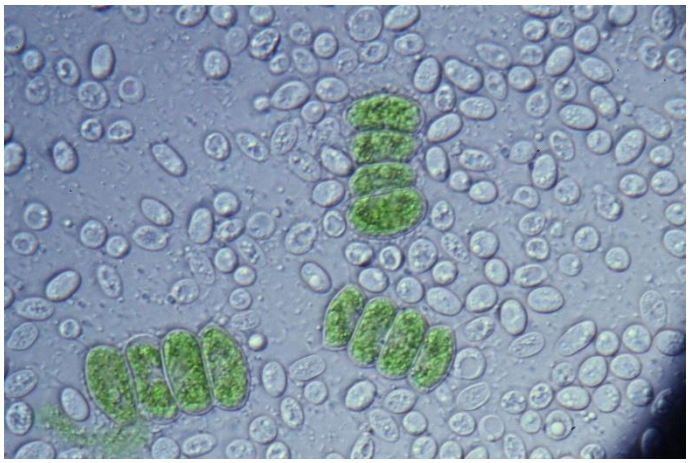
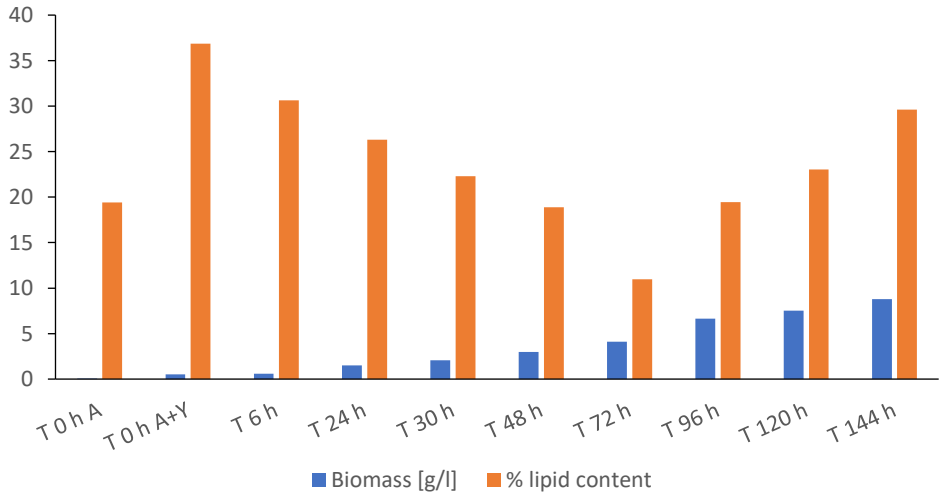
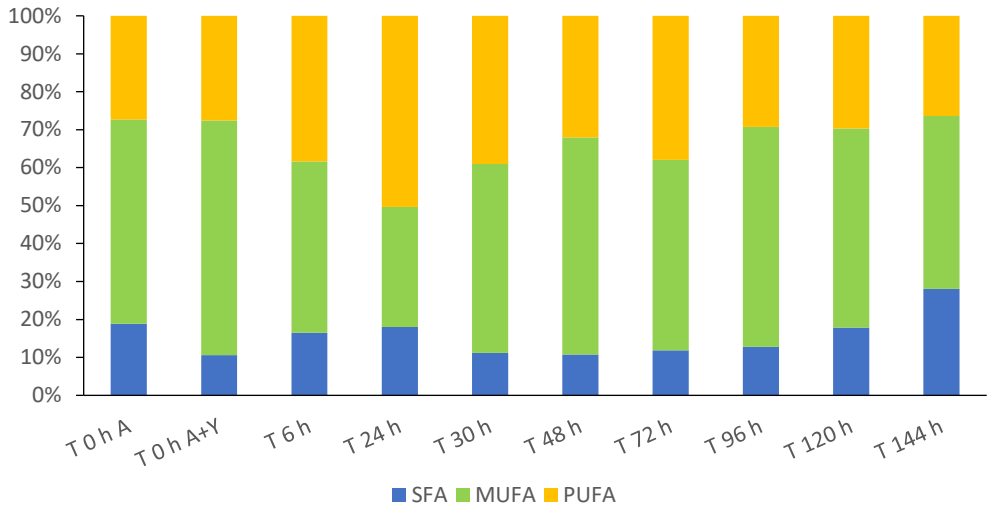


Figure 25. Microscopic image of R. kratochvilovae co-cultivation with D. quadricauda – 96 hours

From the graphs of lipid production (Graph 97 and Graph 98), we see a linear decrease in the percentage of lipid content associated with increased biomass production. After that, however, there is a rapid linear growth in production to the final 29.62%. The fatty acid profile during this cultivation showed relatively stable production of all three types of fatty acids. On the first day, we see a slight increase in PUFA production at the expense of MUFA. In the final phase, we also see a slight increase in SFA content to a total of 28%. Thus, the final lipid content is 28% SFA, 46% MUFA and 26% PUFA. The best harvesting time for the recovery of enriched biomass with all monitored components with emphasis on lipids was 144 hours where the highest values of biomass growth (8.78 g/L) reached with high lipid enrichment (29.62%) with the production of carotenoids (0.69 mg/g of dry biomass) and chlorophylls (0.17 mg/g of dry biomass).



Graph 97. *Total lipid production in bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus quadricauda – first trial*



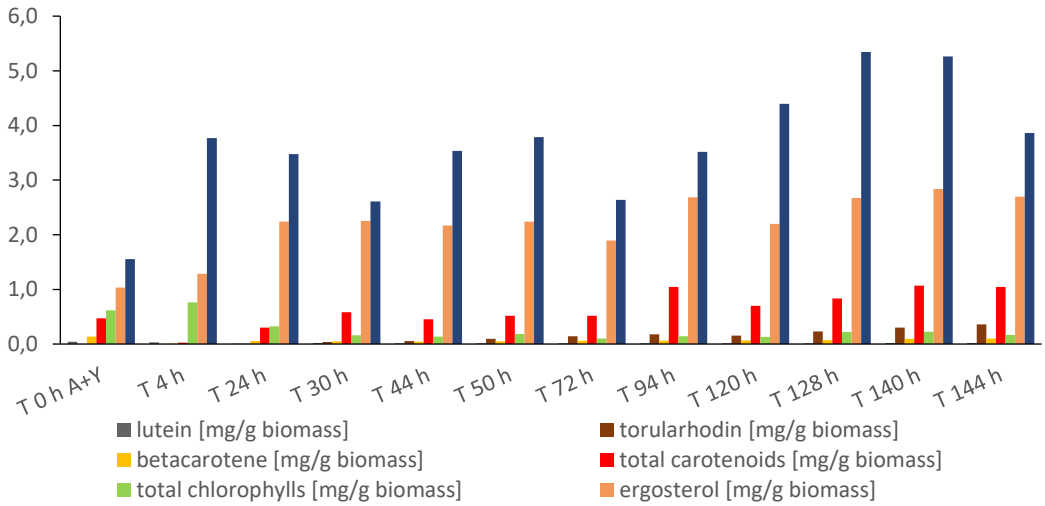
Graph 98. *Fatty acid profile of bioreactor co-cultivation of Rhodotorulak with Desmodesmus quadricauda - second trial*

5.3.6.1.2 Bioreactor co-cultivation Rhodotorula kratochvilovae + Desmodesmus quadricauda C/N = 100 – second trial

In the second co-cultivation experiment, we again see a linear increase in biomass at the beginning of the cultivation, which slowed down in the second phase of cultivation and practically stops and oscillates around 7.5 g/L. In the second bioreactor, during the growth of the culture, carotenoid pigments' production gradually increased until the 72nd hour of cultivation, after which a jump in production to a value of 1.04 mg/g of dry biomass was recorded at 94th hour. The maximum production of carotenoids was reached by co-culture in 140 hours of cultivation with a value of 1.07 mg/g of dry biomass. Overall, however, it is a low production of total carotenoids. From the point of view of chlorophyll production, the highest production values were reached from the beginning of cultivation, precisely at 4 hours after inoculation of the medium with yeast, where the production value reached 0.77 mg/g of dry biomass. Production fell down to its minimum at 72 hours of cultivation at 0.10 mg/g of dry biomass and then gradually increased to its second maximum at 140 hours when it reached 0.23 mg/g of dry biomass. We again see two local production maxima in ubiquinone production at 50 hours (3.78 mg/g) and then followed by a linear increase to 128 hours with the highest production of 5.34 mg/g of dry biomass. Ergosterol production changes significantly when compared to ubiquinone production. We see two production maxima at 94 hours (2.68 mg/g) and a total maximum at 140 hours of 2.84 mg/g of dry biomass.

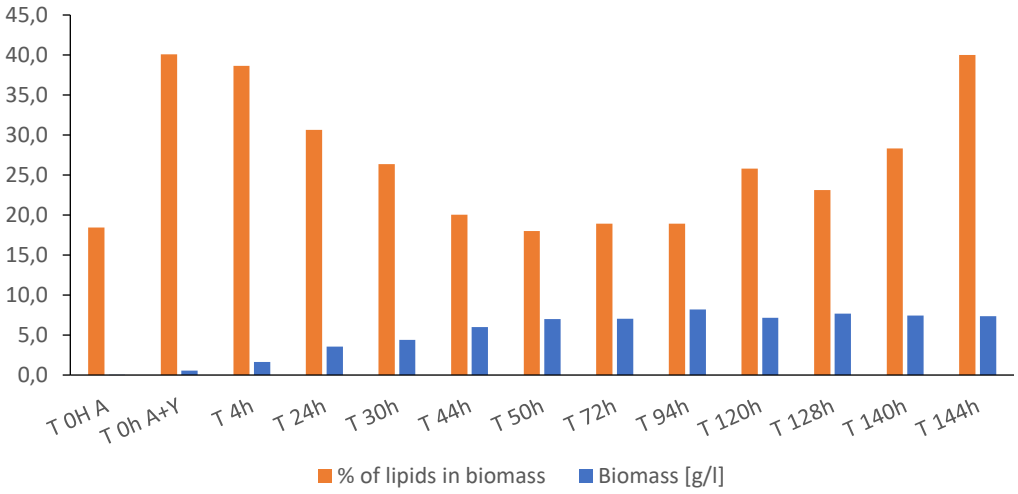
Table 63. Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. quadricauda – second trial

Cultivation time [hours]												
Sample name	T 0 h A	T 0 h A+Y	T 4 h	T 24 h	T 30 h	T 44 h	T 50 h	T 72 h	T 94 h	T 120 h	T 128 h	T 140 h
Biomass [g/L]	0.05	0.55	1.62	3.57	4.39	6.00	6.51	7.02	7.18	7.14	7.67	7,42

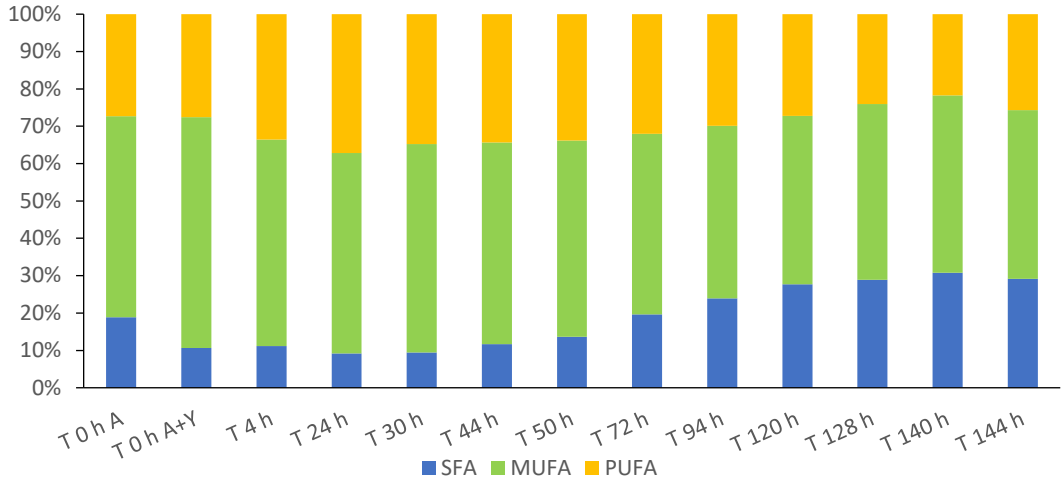


Graph 99. *Bioreactor co-cultivation R. kratochvilovae + D. quadricauda HPLC results – second trial*

If we compare biomass production in the first and second co-cultivation, the second cultivation lags in biomass production by approximately 1.5 g/L. On the other hand, we see a much higher total lipid content in biomass. From the graph (Graph 100), we see the same trend with the first co-cultivation experiment, i.e. a decrease in the initial high percentage of lipids. The decline here is stopped faster. It is then followed by a practically exponential increase in lipid production, especially on the last day of cultivation to 39.99%. The fatty acid profile is stable throughout the cultivation (Graph 101), and there are no significant fluctuations in the products of the individual groups. From the 50th hour of cultivation, the SFA content increases, reaching 29% at the end. The final content of unsaturated fatty acids is 45% MUFA and 26% PUFA. For the highest production of accumulated lipids and pigments, harvesting should be done in the 144th hour, where the lipid concentration reached a high value (39.99%) with a highest biomass growth (7.35 g/L) with carotenoid production (1.05 mg/g of dry biomass) and chlorophylls (0.17 mg/g of dry biomass). This harvest time is also the most suitable for all productions.



Graph 100. *Total lipid production in bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus quadricauda – second trial*



Graph 101. *Fatty acid profile of bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus quadricauda - second trial*

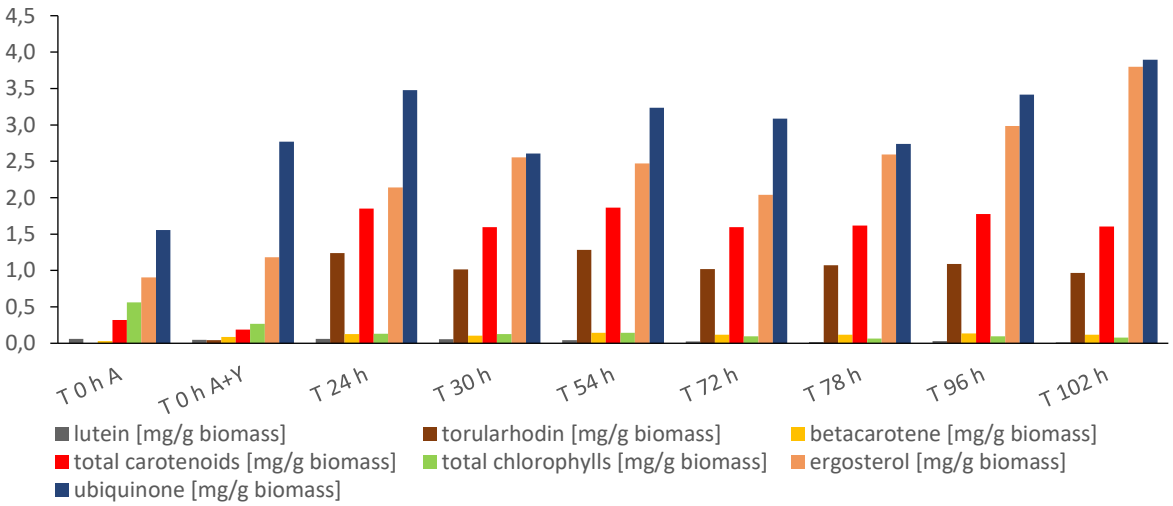
The results of both co-cultivation experiments indicate a high production of lipids in the biomass. The second cultivation also confirmed this phenomenon under the same conditions. On the other hand, we see low carotenoid production at the expense of high lipid production. In general, in the case of an excess of carbon source, the yeasts prefer lipids' production. At lower C/N ratios, the production of carotenoids then increases. Based on this, a cultivation with a lower carbon content was subsequently performed at a C/N ratio of 25.

5.3.6.1.3 Bioreactor co-cultivation Rhodotorula kratochvilovae + Desmodesmus quadricauda C/N = 25

Table 64 shows biomass production results. This experiment was cultivated for only 102 hours due to the onset of bacterial contamination, which was detected at 102 hours. Therefore, the cultivation was stopped at that moment, and the experiment will be repeated in the future. Nevertheless, we managed to obtain valuable data, which are listed below. The chromatographic analysis results confirmed the assumption from the previous experiment that reducing the amount of carbon in the medium will increase the production of carotenoids. Compared to the C/N=100, we see almost doubled production of carotenoids. At the beginning of cultivation, production is very low, but then after 24 hours of cultivation, a jump to 1.85 mg/g of biomass is observed. In further samples, carotenoids' production is in the range of 1.6-1.8 mg/g of biomass. Interestingly, in this experiment, there is a high production of the pigment torularhodine, which makes up more than 60% of the total carotenoids. The 24th and 54th hours are also the points of the highest production of carotenoids. Ubiquinone production initially increases to a first apex at 24 hours (3.48 mg/g of dry biomass). Then there is a slight decrease. In the end, however, production rises again and reaches the peak of production at 102nd hour, namely 3.89 mg/g of dry biomass. Ergosterol production mimics the trend of ubiquinone production. Again, we see two local maxima here, at 30nd hour and then at 102nd hour, where a maximum of 3.79 mg/g biomass was reached.

Table 64. Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. quadricauda C/N=25

Cultivation time [hours]									
Sample name	T 0 h A	T 0 h A+Y	T 24 h	T 30 h	T 54 h	T 72 h	T 78 h	T 96 h	T 102 h
Biomass [g/L]	0.10	1.03	3.775	4.85	6.03	6.08	6.78	7.35	6.63



Graph 102. *Bioreactor co-cultivation R. kratochvilovae + D. quadricauda C/N=25 HPLC results*

5.3.6.1.4 Bioreactor co-cultivation Rhodotorula kratochvilovae + Desmodesmus dimorphus C/N=100

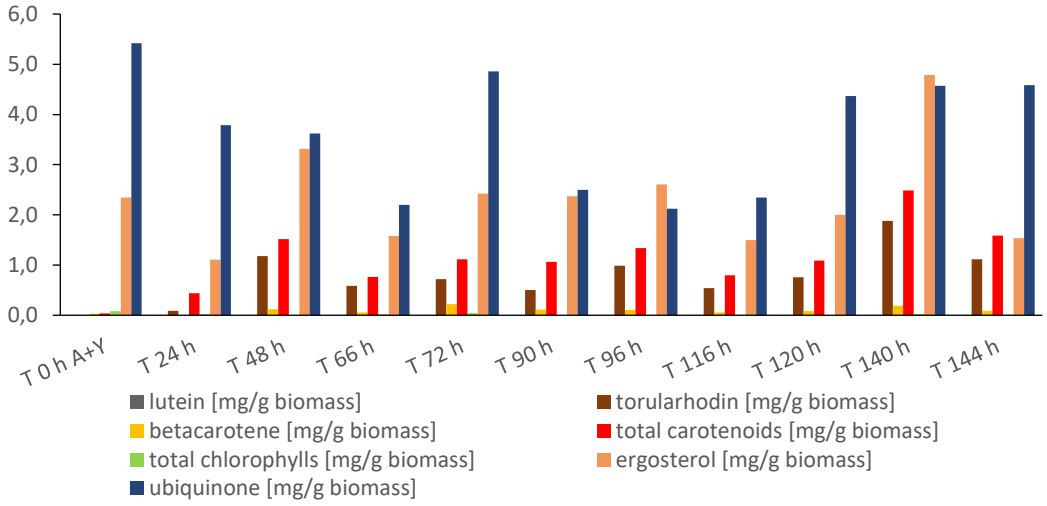
Biomass production in this co-cultivation reached the absolute highest values of all tests performed. At the beginning of cultivation, we see a rapid increase in biomass by the 66th hour of cultivation, slowing down slightly but still growing. In the last sample, the production reaches 11.28 g/L. It can be assumed that it would continue to grow. During cultivation, carotenoid production reached three major local maxima. The first maximum was recorded at 48 hours where carotenoid production reached 1.51 mg/g of dry biomass. The second maximum was observed at 96 hours of cultivation with a production value of 1.34 mg/g of dry biomass. The third and highest maximum was reached in the 140th hour of cultivation and reached the value of 2.49 mg/g of dry biomass, which is also the highest achieved value of carotenoid production in all performed experiments. On the other hand, very low carotenoid production was observed throughout the cultivation, indicating that the microalgae were assimilated by yeast during the cultivation. The highest chlorophylls production was reached at the beginning of cultivation at the beginning of the cultivation and reached 0.08 mg/g of dry biomass.

The graphs below (Graph 104) and (Graph 105) show the results of GC analysis of lipids of co-cultivation of *R. kratochvilovae* and *D. dimorphus*. As in previous experiments, high lipid production was achieved. Unlike other microalgae representatives, in the case of co-cultivation with *D. dimorphus* there is no initial decrease in the percentage of lipids, but on the contrary, we see increasing lipid production from the beginning. During the cultivation, two maxima were reached at 90 and 144 hours, the value of which exceeded 30%.

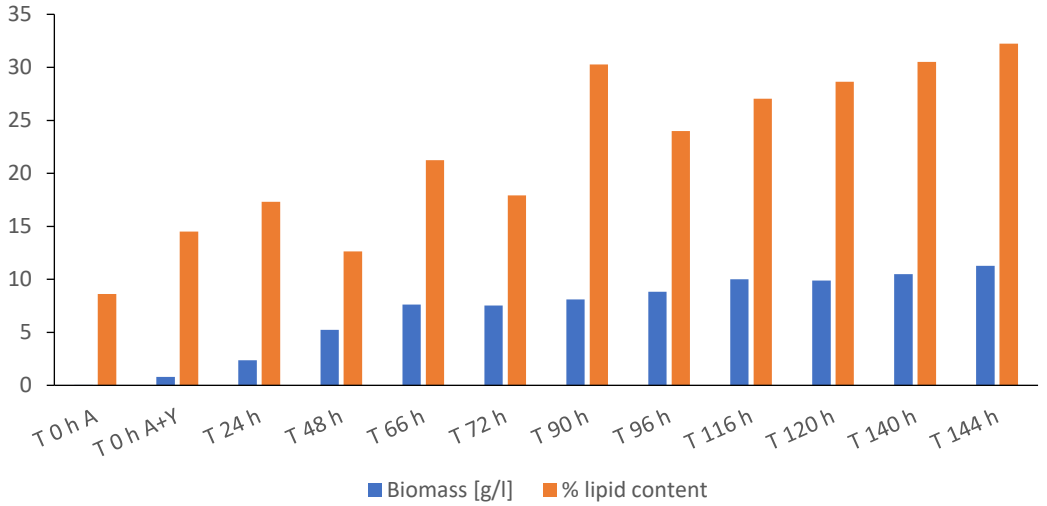
After the first day of cultivation, the fatty acid profile stabilised and remained virtually unchanged until the end of the cultivation. At the last sample, the SFA content jumped to 62% at the expense of PUFA production. The best harvest time for achieving maximum enrichment of biomass, both with lipids and pigments, was 140 hours, during which a significant increase in biomass (10.51 g/L) and at the same time considerable production of lipids (30.51%) and carotenoids (2.49 mg/g of dry biomass) was measured.

Table 65. Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. dimorphus C/N=100

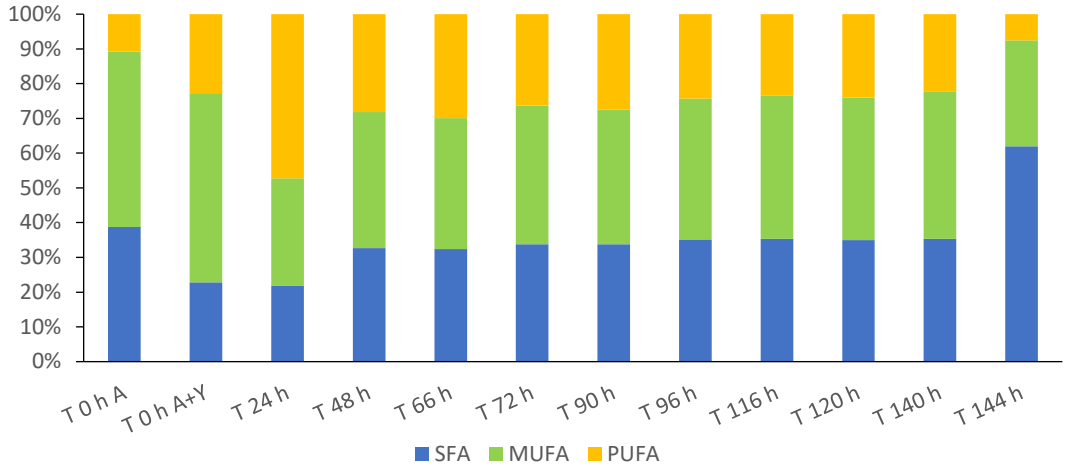
Cultivation time [hours]												
Sample name	T 0 h A	T 0 h A+Y	T 24 h	T 48 h	T 66 h	T 72 h	T 90 h	T 96 h	T 116 h	T 120 h	T 140 h	T 144 h
Biomass [g/L]	0.05	0.79	2.36	5.22	7.63	7.52	8.11	8.84	10.00	9.89	10.50	11.27



Graph 103. *Bioreactor co-cultivation R. kratochvilovae + D. dimorphus C/N=100 HPLC results*



Graph 104. *Total lipid production in bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus dimorphus*



Graph 105. *Fatty acid profile of bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus dimorphus*

5.3.6.1.5 Bioreactor co-cultivation Rhodotorula kratochvilovae + Desmodesmus acutus C/N=100

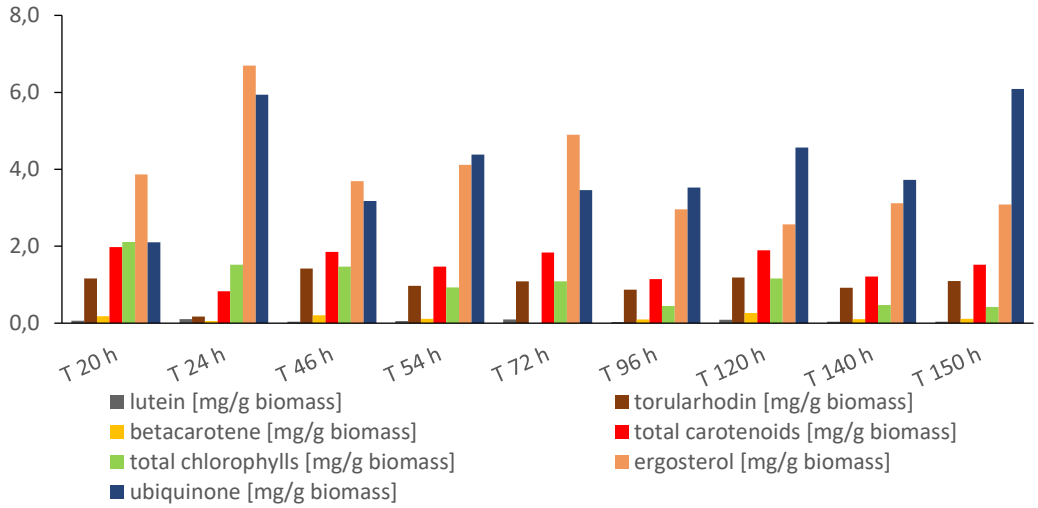
Co-cultivation with the *D. acutus* strain again provided high biomass production. We observe a linear increase in biomass throughout the experiment with a maximum (10.25 g/L) in the experiment's final phase. Again, we can assume that the culture would continue to grow as the experiment was extended.

We again see a high content of pigment torularhodine in all samples from the data of chromatographic analysis of lipid substances (Graph 106). The total production of carotenoids is in the range of 1.5-1.9 mg/g of dry biomass, except for the sample at 24 hours, where the production is very low. Production has two local maxima at 46 and 120 hours. The peak of ubiquinone production occurs in the final phase of the experiment at 150 hours (6.09 mg/g of dry biomass) and then at 24 hours 5.94 mg/g of dry biomass. The maximum ergosterol production of 6.69 mg/g of dry biomass was also measured in this sample. In other samples, we see only a decrease in ergosterol production. Chlorophyll production reached its highest value in the 20th hour of cultivation (2.11 mg/g of dry biomass) decreased from 20 to 54 hours, and then increased until 120 hours, reaching a significant maximum (1.17 mg/g of dry biomass). After 120 hours, chlorophyll production decreased until the end of the cultivation. The combination of *Desmodesmus acutus* and *Rhodotorula kratochvilovae* achieved the highest values of chlorophyll production from all performed experiments.

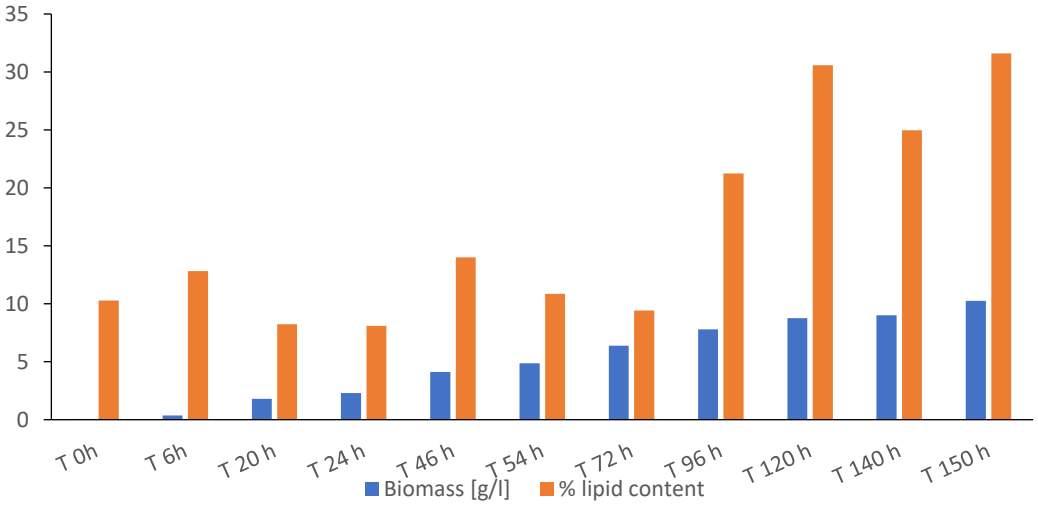
Linear biomass growth was followed by the production of lipids (Graph 107). We see fluctuations in production in the first three days of cultivation, but then there is an intensive production of lipids to a final value of 31.61% in the last sample. The following graph shows a slight, almost linear decrease in PUFA content in favour of SFA. Thus, here we observe a similar trend as in previous cultivations, We see here that in the final stages of culture, more MUFA with SFA accumulates in the cells, rather than PUFA. Ideal harvest time to obtain biomass enriched to the maximum with pigments and at the same time lipids is the 120th hour when the highest production of carotenoids (1.89 mg/g of dry biomass) and chlorophyll (1.17 mg/g of dry biomass) was achieved with a significant increase in biomass (8.76 g/L) with total lipid content (30.59%).

Table 66. Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. acutus C/N=100

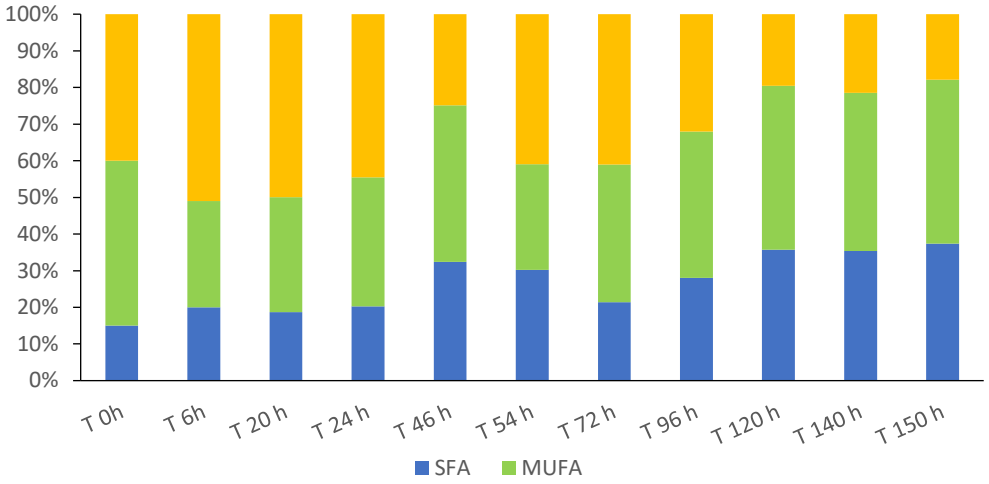
Cultivation time [hours]												
Sample name	T 0 h A	T 0 h A+Y	T 6 h	T 20 h	T 24 h	T 46 h	T 54 h	T 72 h	T 96 h	T 120 h	T 140 h	T 150 h
Biomass [g/L]	0.03	0.27	0.37	1.80	2.30	4.13	4.88	6.38	7.80	8.76	9.01	10.25



Graph 106. *Bioreactor co-cultivation R. kratochvilovae + D. acutus C/N=100 HPLC results*



Graph 107. *Total lipid production in bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus acutus*



Graph 108. *Fatty acid profile of bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus acutus*

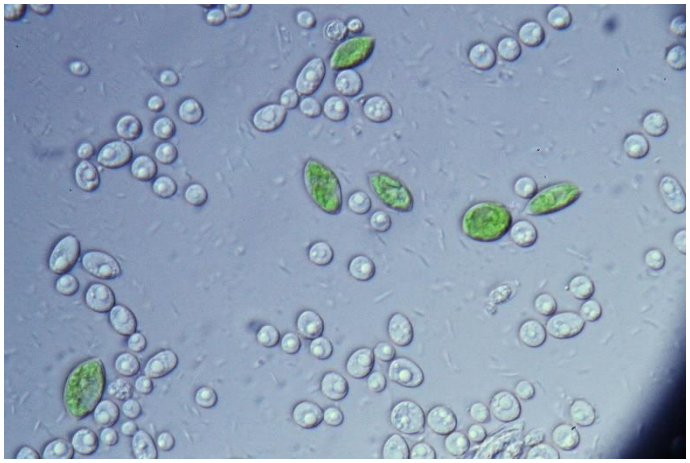


Figure 26. Microscopic image of R. kratochvilovae co-cultivation with D. acutus – 96 hours

5.3.6.2 *Bioreactor co-cultivations of Rhodosporidium toruloides*

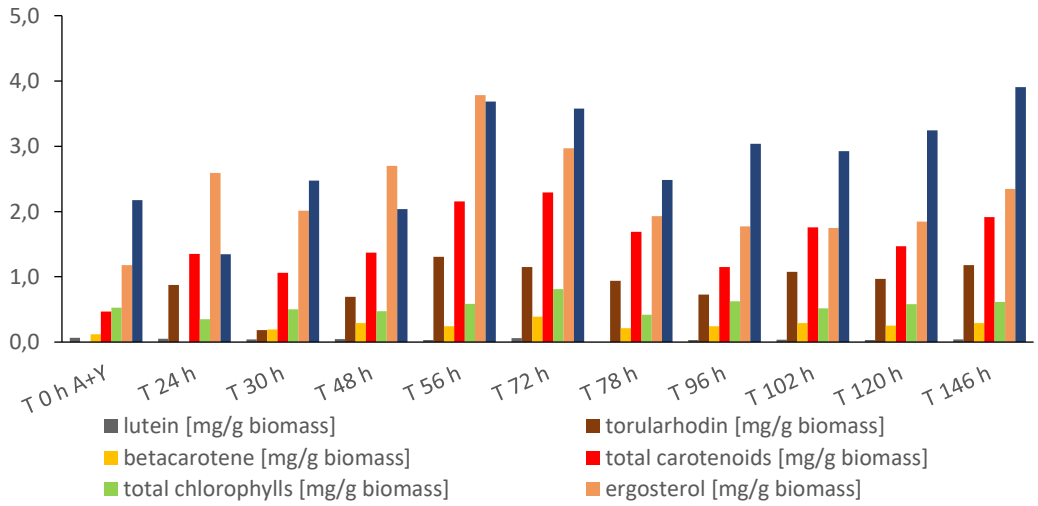
5.3.6.2.1 Bioreactor co-cultivation Rhodosporidium toruloides + Desmodesmus quadricauda C/N=100

The biomass production results by co-cultivation of the yeast *R. toruloides* show comparable results compared to the *R. kratochvilovae* strain. We see an overall linear increase in biomass during fermentation, only between 96 and 102 hours there was a small decrease in biomass production. The maximum biomass production reached 10.5 g/L. The production of carotenoids has the character of a wave line with two local maxima. The first in the 72nd hour, 2.29 mg/g of dry biomass. The second maximum is standardly in the last sample at 146 hours with production 1.91 mg/g of dry biomass (Graph 109). Again, even in this case, the majority of carotenoids is made up by torularhodine. The production of ubiquinone and ergosterol copy the trend of carotenoids with two maxima. The first was at 56 hours with ergosterol production 3.78 mg/g and ubiquinone 3.69 mg/g of dry biomass. The second maxima is in the same sample as in the case of carotenoids. Ergosterol production is lower here, and only ubiquinone production rose to 3.90 mg/g of dry biomass.

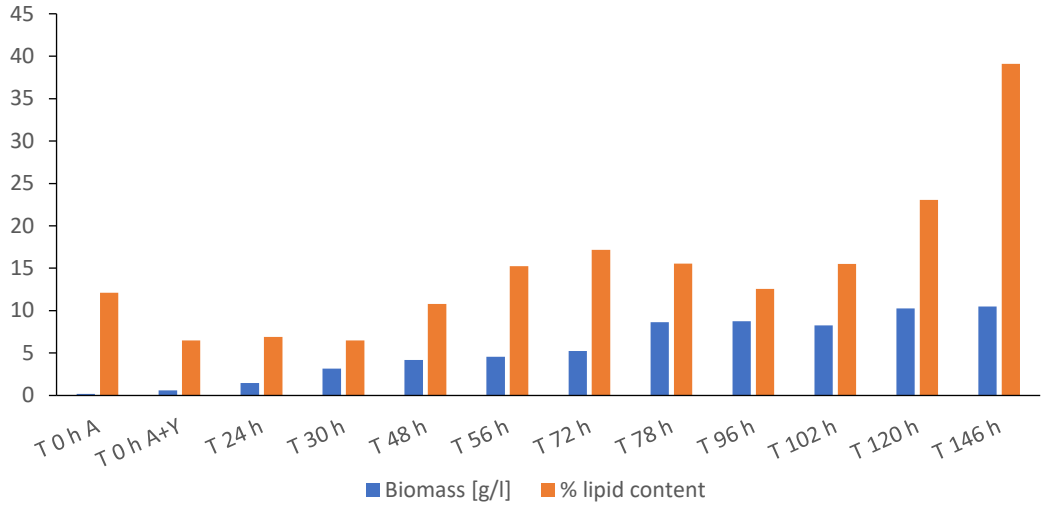
The initial production of lipids decreases in the first two days of cultivation, and then we observe an increase until the first maximum of 17.16% at 72 hours (Graph 110). There is a slight decrease, and from 102nd hour we observe a sharp increase in the percentage of lipids increasing to 39.09% in the last sample. The fatty acid profile is shown in the figure below (Graph 111) initially shows an increase in SFA content by 30 hours of cultivation. In the following days, the SFA percentage then stabilized in the range of 30-37%. In the last sample, we observed a slight decrease to 25% in favour of PUFA production. The initial higher MUFA content gradually stabilized at 45-47% during cultivation. The best harvesting time for achieving maximum enrichment of biomass, both with lipids and pigments fo this cultivation is 140 hours, during which a significant increase in biomass (10.51 g/L) and at the same time significant production of lipids (30.51%) and carotenoids (2.49 mg/g of dry biomass) occurs.

Table 67. Biomass production: Bioreactor co-cultivation R. toruloides + D. quadricauda C/N=100

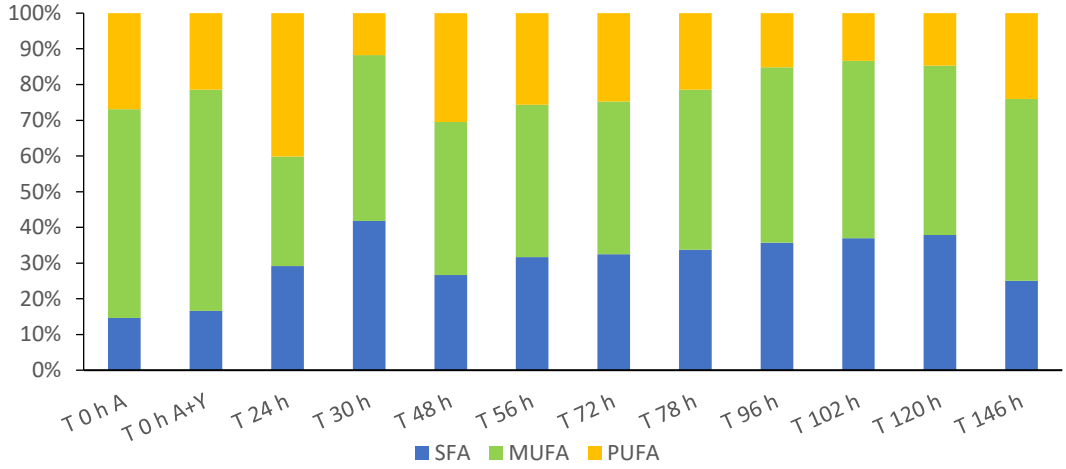
Cultivation time [hours]												
Sample name	T 0 h A	T 0 h A+Y	T 24 h	T 30 h	T 48 h	T 56 h	T 72 h	T 78 h	T 96 h	T 102 h	T 120 h	T 146 h
Biomass [g/L]	0.06	0.61	1.48	3.16	4.18	4.58	5.23	8.63	8.75	8.25	10.25	10.5



Graph 109. *Bioreactor co-cultivation R. toruloides + D. quadricauda C/N=100 HPLC results*



Graph 110. *Total lipid production in bioreactor co-cultivation of Rhodosporidium toruloides with Desmodesmus quadricauda*



Graph 111. *Fatty acid profile of bioreactor co-cultivation of Rhodosporidium toruloides with Desmodesmus quadricauda*

5.3.7 Co-cultivation conclusions

The following chapter summarizes the whole experiment's results, which dealt with pilot tests of symbiotic co-cultivation. The performed tests confirmed that selected microalgae, cyanobacteria, and yeast representatives can grow in a symbiotic growth. The results pointed to significant differences in the level of symbiotic growth and the whole process's final productivity. In the first phase of the experiment, yeast's production abilities on BBM medium were compared with the basic mineral yeast medium, which is much simpler. Furthermore, various combinations of carbon and nitrogen sources were tested. The results showed that the studied yeast strains are able to grow on BBM medium. Each of the yeast tested preferred a different combination of carbon and nitrogen source. The best strain in biomass production, lipid substances production (carotenoids, ergosterol, ubiquinone) and lipid production was the yeast *Rhodotorula kratochvilovae*. The second in order was *Rhodosporidium toruloides*. In the second phase, the microalgae were cultivated in the dark and light in BBM medium with a combination of carbon and nitrogen sources.

The results showed that the studied microalgal strains are not capable of mixotrophy and are capable of only partial tolerance of glucose or glycerol in the medium. In the third phase, laboratory co-cultivation was performed based on the first and second phases. In these experiments, the yeast *R. kratochvilovae* again achieved the best results. For all yeasts tested, glycerol medium was more successful in co-cultivation experiments than glucose. One of the factors that promoted increased production was the lower inhibitory effect of glycerol on microalgae growth. From the all tested microalgae strains, were the best representatives of the genus *Desmodesmus* and cyanobacteria *S. nidulans*. Although the *Phaffia rhodozyma* strain was limited by its slower growth, on the other hand, a higher percentage of lipids was always achieved in co-cultures.

In the fourth phase, the experiments were moved to larger volumes in which the effect of aeration and different inoculation ratios were tested. At this stage, it was again clearly confirmed that the best co-cultivation partner for yeast are representatives of the genus *Desmodesmus.* The experiment further showed that there is no uniform inoculation ratio for all yeasts and microalgae. Significant differences were also observed between representatives of one genus. Microalgae deaths occurred during testing of other microalgae. It has been found out, that some species of microalgae are not able to coexist in a medium with yeast for a long time, because in the absence of resources, predation occurs and microalgae are consumed as another source of nutrients. This phenomenon occurred, for example, in strains of *Chlamydomonas reinhardtii* or *Chlorella sorokiniana*. The success of co-cultivations with representatives of the genus *Desmodesmus i*s based on the microalgae's morphology. The genus *Desmodesmus* is characterized by the formation of cenobias, which are coated with a polysaccharide coating, which is indigestible for yeast. Thus, these representatives were able to coexist with the yeast in the absence of resources. To confirm this assumption, co-cultivation was performed with the *S. obliquus* strain, which shares many morphological features with the genus *Desmodesmus*. Even in this case, this phenomenon was confirmed.

Based on the obtained results, candidates for bioreactor co-cultivation were selected. Among the yeasts were *Rhodotorula kratochvilovae* and *Rhodosporidium toruloides*. Microalgae chosen were the genera *Desmodesmu*s and *Scenedesmus.* The results of the cultivations provided clear information about the possibilities of co-cultivation of yeasts and microalgae. The performed pilot cultivations also showed a positive effect of co-cultivation on the monitored metabolites' total production. They had a positive effect on the production of ubiquinone, ergosterol and carotenoids. In the case of lipids, we see an increased production of lipids and a slightly increased content of unsaturated fatty acids compared to the classical profile in pure yeast.

Total carotenoid production was lower due to the high C/N ratio in the media. Increased carotenoid production was achieved during trial co-cultivation with a lower C/N ratio. The lower results of some metabolites' production compared to the mineral yeast media can be overcome by optimizing the medium's components. At present, experiments are underway to test new strains, optimize media components, and test waste materials to reduce operating costs. There is considerable potential for optimization in this area. The absolute best bioreactor results were obtained in the co-cultivation of the yeast *R. kratochvilovae* and representatives of the genera *Desmodesmus* and *Scenedesmus*. High lipid production was achieved in these co-cultivations, where the percentage of lipids in the biomass was always higher than 30%. The highest production of lipids was achieved by co-cultivation of *R. kratochvilovae* and *D. quadricauda*. In the case of carotenoids, then co-cultivation of *R. toruloides* and *D. quadricauda*. At the end of this chapter, it can be concluded that the goals set for this experiment have been achieved. Pilot experiments have confirmed that carotenogenic yeasts and microalgae are capable of symbiotic coexistence.

6 DISSERTATION CONCLUSIONS

This dissertation thesis was focused on complex study of metabolic activity of selected strains of carotenogenic yeasts, microalgae, and cyanobacteria to optimise the production of microbial biomass enriched with studied substances with added value: carotenoids, single-cell oils, ubiquinone, ergosterol, chlorophylls. The optimisation was focused on the economics of the process and finding the cheapest variant. Microorganisms were exposed to various stress factors, whether biological, physicochemical or nutritional. The experimental work was divided into several parts according to microorganisms studied: i) yeasts, ii) algae and iii) their co-cultivation.

The first step of this work was selecting a suitable medium for microorganisms and the subsequent optimization of the nutrients used and their concentration in the medium to achieve increased production of targeted metabolites. To further increase metabolites' production, various forms of physicochemical, nutritional, and biological stresses (waste substrates) were applied. As part of the work with carotenogenic yeasts, representatives of the genera *Rhodotorula*, *Rhodosporidium*, *Cystofilobasidium* and *Sporidiobolus* were studied, which were cultivated on media with different C/N ratios using food industry wastes (whey, waste animal fat, waste frying oil, coffee oil) and coffee grounds hydrolyzate. These materials served as inexpensive sources of carbon and nitrogen in the medium. The cultivation of microalgae of the genera *Desmodesmus*, *Scenedesmus*, *Chlorella*, *Coccomyxa*, *Chlamydomonas*, *Botryococcus* dealt with the optimization of the cultivation conditions of microalgae using the application of various physicochemical stress factors. As part of experiments carried out as part of an internship abroad, pilot large-volume cultivations with the extremophilic microalgae *Cocomyxa onubensis* in open ponds were also performed. The final part of the dissertation is concluded by tests of biological compatibility of carotenogenic microalgae and yeasts with the aim of symbiotic co-cultivation in the medium, based on the idea of mutual exchange of gases and nutrients in the medium. A series of laboratory and large volume bioreactor cultivations of yeast/microalgae pairs were done.

The experiments showed that carotenogenic yeasts are capable of growth and high production on media with treated waste substrates. In experiments, it was found that to ensure maximum production, the waste substrate needs to be partially hydrolyzed in the medium to release a sufficient amount of simple carbon source into the medium, which induces very rapid growth of biomass in the first phase and second unhydrolysed part induces increased production of the desired metabolites in the latter time of cultivation. In the case of waste animal fat, it can be partially hydrolyzed to obtain the mixture. However, in the case of other waste substrates (whey, spent coffee grounds hydrolyzate), the yeast does not have sufficient enzyme equipment, and it is essential to hydrolyze them whole. As a compromise of this situation, experiments were performed with combined waste substrates, where the first simple source of carbohydrates was coffee hydrolyzate or whey hydrolyzate. The second source was then untreated lipid substrate (animal fat, coffee oil, frying oil). The yeast achieved very good results in the biomass production and metabolites production on this combination of substrates. According to the results, the best combinations were coffee hydrolyzate: coffee oil and coffee hydrolyzate: waste frying oil, on which the best strain of *Sporidiobolus pararoseus* achieved a biomass production of more than 20 g/L and at the same time a high content of all desired metabolites.

The results of microalgae cultivation on optimized media indicated a huge potential of these microorganisms. Experiments have shown that by a suitable choice and ratio of nitrogen and phosphorus, an increase in metabolites' production in the order of units and tens of percent can be achieved. Simultaneously, a further increase in production is possible by a suitable application of oxidative and nutritional stress. Microalgae have been found to be very consistent in the production and profile of fatty acids. Even the application of oxidative stress did not significantly affect the change in the ratio of the group of fatty acids.

As part of an internship abroad, a series of tests were performed with the extremophilic microalgae *Coccomyxa onubensis*. In large-volume open ponds, this acidophilic microalga has been shown to be capable of long-term resistance to contamination by other types of microorganisms. Furthermore, it was shown that even slight aeration with air in the order of units of liters per minute had a positive effect on the growth and productivity of this microalgae. From an economic point of view, these data are very important, because they outline the possibilities of large-volume industrial cultivations of this microalgae with low costs and at the same time with high resistance to possible contamination. Growth and productivity would be further enhanced by enriching aerated air with carbon dioxide, for example from an industrial plant. This microalga is able to tolerate high concentrations of salts and metals in the medium and therefore even waste carbon dioxide with trays of sulphur or other elements would not have such a high negative effect on culture.

The last experimental part performed in this dissertation was the study of co-cultivation and symbiotic growth of carotenogenic yeasts and microalgae. In a series of laboratory cultivations, the growth properties of the yeast were tested on the BBM medium. Furthermore, the utilization or tolerance of the organic carbon source by microalgal strains was tested. The experiment continued with a series of flask co-cultivations of selected yeasts and microalgae performed in order to find symbiotic pairs. Based on the results of flask co-cultivation experiments, the best pairs were cultivated in aerated flasks to confirm the data obtained. It has been found that yeast acts as a strong predator in the medium and, after depletion of simple nutrient sources, reorients to microalgae in the medium. In a series of experiments, compared with flask cultivations, the cultivated yeast thus assimilated and destroyed the microalgae culture. This problem lies in the morphology of microalgae. It has been found that in strains that are able to form large colonies, this phenomenon occurs minimally or not at all.

The best results were achieved here by representatives of the genera *Desmodesmus* and *Scenedesmus*, who live in colonies protected by a cover that is indigestible for yeast and the original premise of symbiotic growth is practically fulfilled here. Yeast and microalgae live here in one medium and exchange mainly metabolic gases (O_2 and CO_2). These compatible pairs were then cultivated in pilot bioreactor cultivations further to explore the possibility of industrial biotechnological use of co-cultivation. The results of these cultivations confirmed the positive effect of symbiotic growth, and high production of biomass and metabolites was achieved. At the same time, it is necessary to add that the biomass prepared in this way contains metabolites of both groups of microorganisms, and it is, therefore, possible to use the positives of both strains. Cultivations were performed predominantly with glycerol because the glucose was found to have a large inhibitory effect on photosynthesis and growth of selected microalgal strains. Based on this, it can be stated that this process could process waste glycerol from biofuels' production. Or further use to process lipid waste substrates containing glycerol in its structure (animal fat, frying oil, coffee oil) and further to make deeper use of the huge potential of co-cultivation of the strains themselves.

The results of the experiments with carotenogenic yeasts and microalgae confirmed the huge potential of their biotechnological use. It was confirmed that by suitable optimization of cultivation conditions, it is possible to modulate metabolites' production and increase the profits of the whole process. Co-cultivation is a potentially very effective method of cultivating yeasts and micro-algae, enabling a reduction in the cost of aeration of the medium providing combined biomass enriched with a number of valuable substances.

7 LIST OF ABBREVIATIONS

SCO	–	Single cell oils
ICDH	–	Isocitrate dehydrogenase
ACP	–	Acyl carrier protein
CoA	–	Coenzyme A group
DXP	–	Deoxy-D-xylulose-5-phosphate pathway
HMG-CoA	–	Hydroxymethylglutaryl Coenzyme A
GPP	–	Geranyl pyrophosphate
FPP	–	Farnesyl pyrophosphate
IPP	–	Isopentenyl pyrophosphate
DMAPP	–	Dimethylallyy pyrophosphate
HMB	–	Hydroxymythyl bilane
CMC	–	Critical micellar concentration
BOD	–	Biochemical oxygen demand
COD	–	Chemical oxygen demand
HPLC	–	High-performance liquid chromatography
GC	–	Gas chromatography
FID	–	Flame ionization detector
DAD	–	Diode array detector
RI	-	Refractive index detector
QY	–	Quantum yield
FA	–	Fatty acids
SFA	–	Saturated fatty acids
MUFA	–	Monounsaturated fatty acids
PUFA	–	Polyunsaturated fatty acids
FAME	–	Fatty acid methyl ester
ATP	–	Adenosine triphosphate
AMP	–	Adenosine monophosphate
NADPH	–	Nicotinamide adenine dinucleotide phosphate
NADH	–	Nicotinamide adenine dinucleotide
$FADH_2$	–	Flavin denine dinucleotide
FAS	–	Fatty acid synthase
BBM	–	Bolds basal media
BG11	–	Type of microalgae cultivation media
K9	–	Type of microalgae cultivation media
C/N ratio	–	Carbon/nitrogen ration

8 REFERENCES

[1] VOET, Donald a Judith G. VOET. *Biochemistry*. 4th ed. Hoboken: Wiley, c2011. ISBN 978-0470570951.

[2] MURRAY, Robert K. *Harperova Biochemie*. 23. vyd., (4. české vyd.), v H. Jinočany: H, 2002. Lange medical book. ISBN 80-731-9013-3.

[3] JEREMY M. BERG, JOHN L. TYMOCZKO a Lubert STRYER. *Biochemistry*. 7th ed., International ed. Basingstoke: Palgrave Macmillan, 2011. ISBN 14-292-7635-5.

[4] SANTOS, Ana L. a Giulio PRETA. Lipids in the cell: organisation regulates function. *Cellular and Molecular Life Sciences*. 2018, 75(11), 1909-1927. ISSN 1420-682X. Dostupné z: doi:10.1007/s00018-018-2765-4

[5] PARSONS, Sophie, Michael J. ALLEN a Christopher J. CHUCK. Coproducts of algae and yeast-derived single cell oils: A critical review of their role in improving biorefinery sustainability. *Bioresource Technology*. 2020, 303. ISSN 09608524. Dostupné z: doi:10.1016/j.biortech.2020.122862

[6] BOROWITZKA, Michael. The physiology of microalgae. New York, NY: Springer Berlin Heidelberg, 2016. ISBN 978-3-319-24943-8.

[7] NIELSEN, Jens. Systems biology of lipid metabolism: From yeast to human. FEBS Letters [online]. 2009, 583(24), 3905-3913 [cit. 2020-07-13]. DOI: 10.1016/j.febslet.2009.10.054. ISSN 00145793. Dostupné z: http://doi.wiley.com/10.1016/j.febslet.2009.10.054

[8] BABA, Masato a Yoshihiro SHIRAIW. Biosynthesis of Lipids and Hydrocarbons in Algae. DUBINSKY, Zvy, ed. Photosynthesis [online]. InTech, 2013, 2013-06-12 [cit. 2018-08- 02]. DOI: 10.5772/56413. ISBN 978-953-51-1161-0. Dostupné z: http://www.intechopen.com/books/photosynthesis/biosynthesis-of-lipids-andhydrocarbons-in-algae

[9] MUNIRAJ, Iniya Kumar, Siva Kumar UTHANDI, Zhenhu HU, Liwen XIAO a Xinmin ZHAN. Microbial lipid production from renewable and waste materials for second-generation biodiesel feedstock. *Environmental Technology Reviews*. 2015, 4(1), 1-16. ISSN 2162-2515. Dostupné z: doi:10.1080/21622515.2015.1018340

[10] GUO, Mingxin, Weiping SONG a Jeremy BUHAIN. Bioenergy and biofuels: History, status, and perspective. *Renewable and Sustainable Energy Reviews*. 2015, 42, 712-725. ISSN 13640321. Dostupné z: doi:10.1016/j.rser.2014.10.013

[11] MAJIDIAN, Parastoo, Meisam TABATABAEI, Mehrshad ZEINOLABEDINI, Mohammad Pooya NAGHSHBANDI a Yusuf CHISTI. Metabolic engineering of microorganisms for biofuel production. *Renewable and Sustainable Energy Reviews*. 2018, 82, 3863-3885. ISSN 13640321. Dostupné z: doi:10.1016/j.rser.2017.10.085

[12] CERTIK, M. a S. SHIMIZU. *Biosynthesis and regulation of microbial polyunsaturated fatty acid production*. *Journal of Bioscience and Bioengineering*. 1999, 87(1), 1-14.

[13] ATHENAKI, M., C. GARDELI, P. DIAMANTOPOULOU, S.S. TCHAKOUTEU, D. SARRIS, A. PHILIPPOUSSIS a S. PAPANIKOLAOU. Lipids from yeasts and fungi: physiology, production and analytical considerations. Journal of Applied Microbiology. 2018, 124(2), 336-367. DOI: 10.1111/jam.13633. ISSN 13645072. Dostupné z: http://doi.wiley.com/10.1111/jam.13633

[14] AGEITOS, Jose, Juan VALLEJO, Patricia VEIGA-CRESPO a Tomas VILLA. Oily yeasts as oleaginous cell factories. Applied Microbiology and Biotechnology. 2011, 90(4), 1219- 1227. DOI: 10.1007/s00253-011-3200-z. ISSN 0175-7598. Dostupné z: http://link.springer.com/10.1007/s00253-011-3200-z

[15] ANSCHAU, Andréia. Lipids from oleaginous yeasts: production and encapsulation. Nutrient Delivery. Elsevier, s. 749-794. DOI: 10.1016/B978-0-12-804304-2.00020-2. ISBN 9780128043042. Dostupné z: https://linkinghub.elsevier.com/retrieve/pii/B9780128043042000202

[16] PARMAR, Asha, Niraj SINGH, Ashok PANDEY, Edgard GNANSOUNOU a Datta MADAMWAR. Cyanobacteria and microalgae: A positive prospect for biofuels. Bioresource Technology. 2011, 102(22), 10163-10172. DOI: 10.1016/j.biortech.2011.08.030. ISSN 09608524. Dostupné z: https://linkinghub.elsevier.com/retrieve/pii/S0960852411011187

[17] ZHU, L., Z. LI a E. HILTUNEN. Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock. BioMed Research International. 2016, 2016, 1-8. DOI: 10.1155/2016/8792548. ISSN 2314-6133. Dostupné z: https://www.hindawi.com/journals/bmri/2016/8792548/

[18] TRAN, N.H., J.R. BARTLETT, G.S.K. KANNANGARA, A.S. MILEV, H. VOLK a M.A. WILSON. Catalytic upgrading of biorefinery oil from micro-algae. Fuel. 2010, 89(2), 265- 274. DOI: 10.1016/j.fuel.2009.08.015. ISSN 00162361. Dostupné z: https://linkinghub.elsevier.com/retrieve/pii/S0016236109003767

[19] KLUG, Lisa a Günther DAUM. Yeast lipid metabolism at a glance. FEMS Yeast Research [online]. 2014, 14(3), 369-388 [cit. 2020-07-13]. DOI: 10.1111/1567-1364.12141. ISSN 15671356. Dostupné z: https://academic.oup.com/femsyr/articlelookup/doi/10.1111/1567-1364.12141

[20] FAKAS, Stylianos. Lipid biosynthesis in yeasts: A comparison of the lipid biosynthetic pathway between the model nonoleaginous yeast Saccharomyces cerevisiae and the model oleaginous yeast Yarrowia lipolytica. Engineering in Life Sciences [online]. 2017, 17(3), 292-302 [cit. 2020-07-13]. DOI: 10.1002/elsc.201600040. ISSN 16180240. Dostupné z: http://doi.wiley.com/10.1002/elsc.201600040

[21] ATHENAKI, M., C. GARDELI, P. DIAMANTOPOULOU, S.S. TCHAKOUTEU, D. SARRIS, A. PHILIPPOUSSIS a S. PAPANIKOLAOU. Lipids from yeasts and fungi: physiology, production and analytical considerations. Journal of Applied Microbiology [online]. 2018, 124(2), 336-367 [cit. 2020-07-13]. DOI: 10.1111/jam.13633. ISSN 13645072. Dostupné z: http://doi.wiley.com/10.1111/jam.13633

[22] Molecular mechanisms in yeast carbon metabolism. New York: Springer, 2014. ISBN 978- 3-642-55012-6.

[23] WANG, Yanan, Sufang ZHANG, Zhiwei ZHU, Hongwei SHEN, Xinping LIN, Xiang JIN, Xiang JIAO a Zongbao Kent ZHAO. Systems analysis of phosphate-limitation-induced lipid accumulation by the oleaginous yeast Rhodosporidium toruloides. Biotechnology for Biofuels [online]. 2018, 11(1) [cit. 2020-07-13]. DOI: 10.1186/s13068-018-1134-8. ISSN 1754-6834. Dostupné z: https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-018-1134-8

[24] SANTEK, Mirela Ivancic, Suncica BELUHAN a Bozidar SANTEK. Production of Microbial Lipids from Lignocellulosic Biomass. NAGESWARA-RAO, Madhugiri a Jaya R. SONEJI, ed. Advances in Biofuels and Bioenergy [online]. InTech, 2018, 2018-07-04 [cit. 2020-07-13]. DOI: 10.5772/intechopen.74013. ISBN 978-1-78923-286-8. Dostupné z: http://www.intechopen.com/books/advances-in-biofuels-and-bioenergy/production-ofmicrobial-lipids-from-lignocellulosic-bioma

[25] CHEIRSILP, B. a Y. LOUHASAKUL. *Industrial wastes as a promising renewable source for production of microbial lipid and direct transesterification of the lipid into biodiesel. Bioresource Technology*. 2013, 142(1), 329-337.

[26] DAVOLI, P., V. MIERAU a R. W. S. WEBER. Carotenoids and Fatty Acids in Red Yeasts Sporobolomyces roseus and Rhodotorula glutinis. *Applied Biochemistry and Microbiology*. 2004, 40(4), 392-397. DOI: 10.1023/B:ABIM.0000033917.57177.f2. ISSN 0003-6838.

[27] TEHLIVETS, Oksana, Kim SCHEURINGER a Sepp D. KOHLWEIN. Fatty acid synthesis and elongation in yeast. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2007, 1771(3), 255-270. DOI: 10.1016/j.bbalip.2006.07.004. ISSN 13881981.

[28] GIPSON, P., D. J. MILLS, R. WOUTS, M. GRININGER, J. VONCK a W. KUHLBRANDT. Direct structural insight into the substrate-shuttling mechanism of yeast fatty acid synthase by electron cryomicroscopy. *Proceedings of the National Academy of Sciences*. 2010, 107(20), 9164-9169. DOI: 10.1073/pnas.0913547107. ISSN 0027-8424.
[29] MAIER, T., M. LEIBUNDGUT a N. BAN. The Crystal Structure of a Mammalian Fatty Acid Synthase. *Science*. 2008, 321(5894), 1315-1322. ISSN 0036-8075. Dostupné z: doi:10.1126/science.1161269
[30] ASTURIAS, Francisco J, James Z CHADICK, Iris K CHEUNG, Helga STARK, Andrzej WITKOWSKI, Anil K JOSHI a Stuart SMITH. *Structure and molecular organization of mammalian fatty acid synthase*. 2005, 12(3), 225-232. ISSN 1545-9993. Dostupné z: doi:10.1038/nsmb899
[31] JOSHI, Anil K, Vangipuram S RANGAN, Andrzej WITKOWSKI, Stuart SMITH, Andrzej WITKOWSKI, Anil K JOSHI a Stuart SMITH. *Engineering of an Active Animal Fatty Acid Synthase Dimer with Only One Competent Subunit*. 2003, 10(2), 169-173. ISSN 10745521. Dostupné z: doi:10.1016/S1074-5521(03)00023-1
[32] *Science-Business eXchange*. 2. 2009. ISSN 1945-3477. Dostupné také z: http://link.springer.com/10.1038/scibx.2009.430
[33] LOMAKIN, Ivan B., Yong XIONG a Thomas A. STEITZ. The Crystal Structure of Yeast Fatty Acid Synthase, a Cellular Machine with Eight Active Sites Working Together. *Cell*. 2007, 129(2), 319-332. ISSN 00928674. Dostupné z: doi:10.1016/j.cell.2007.03.013
[34] QIU, Xiao, Xi XIE a Dauenpen MEESAPYODSUK. Molecular mechanisms for biosynthesis and assembly of nutritionally important very long chain polyunsaturated fatty acids in microorganisms. *Progress in Lipid Research*. 2020, 79. ISSN 01637827. Dostupné z: doi:10.1016/j.plipres.2020.101047
[35] BLACK, Paul N. a Concetta C. DIRUSSO. Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2007, 1771(3), 286-298. ISSN 13881981. Dostupné z: doi:10.1016/j.bbalip.2006.05.003
[36] TEHLIVETS, Oksana, Kim SCHEURINGER a Sepp D. KOHLWEIN. Fatty acid synthesis and elongation in yeast. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2007, 1771(3), 255-270. ISSN 13881981. Dostupné z: doi:10.1016/j.bbalip.2006.07.004
[37] AL-FEEL, W., S. S. CHIRALA a S. J. WAKIL. Cloning of the yeast FAS3 gene and primary structure of yeast acetyl-CoA carboxylase. *Proceedings of the National Academy of Sciences*. 1992, 89(10), 4534-4538. ISSN 0027-8424. Dostupné z: doi:10.1073/pnas.89.10.4534
[38] BYERS, David M. a Huansheng GONG. Acyl carrier protein: structure–function relationships in a conserved multifunctional protein family. *Biochemistry and Cell Biology*. 2007, 85(6), 649-662. ISSN 0829-8211. Dostupné z: doi:10.1139/O07-109
[39] FICHTLSCHERER, Fabienne, Christian WELLEIN, Maria MITTAG a Eckhart SCHWEIZER. A novel function of yeast fatty acid synthase. *European Journal of Biochemistry*. 2000, 267(9), 2666-2671. ISSN 00142956. Dostupné z: doi:10.1046/j.1432-1327.2000.01282.x
[40] MERCHANT, Sabeeha S, Janette KROPAT, Bensheng LIU, Johnathan SHAW a Jaruswan WARAKANONT. TAG, You're it! Chlamydomonas as a reference organism for understanding algal triacylglycerol accumulation. *Current Opinion in Biotechnology*. 2012, 23(3), 352-363. ISSN 09581669. Dostupné z: doi:10.1016/j.copbio.2011.12.001
[41] LA RUSSA, M., C. BOGEN, A. UHMEYER, A. DOEBBE, E. FILIPPONE, O. KRUSE a J.H. MUSSGNUG. Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga Chlamydomonas reinhardtii. *Journal of Biotechnology*. 2012, 162(1), 13-20. ISSN 01681656. Dostupné z: doi:10.1016/j.jbiotec.2012.04.006

[42] FAN, Jilian, Carl ANDRE a Changcheng XU. A chloroplast pathway for the de novo biosynthesis of triacylglycerol in Chlamydomonas reinhardtii. *FEBS Letters*. 2011, 585(12), 1985-1991. ISSN 00145793. Dostupné z: doi:10.1016/j.febslet.2011.05.018
[43] SCHNURR, Judy A., Jay M. SHOCKEY, Gert-Jan DE BOER a John A. BROWSE. Fatty Acid Export from the Chloroplast. Molecular Characterization of a Major Plastidial Acyl-Coenzyme A Synthetase from Arabidopsis. *Plant Physiology*. 2002, 129(4), 1700-1709. ISSN 0032-0889. Dostupné z: doi:10.1104/pp.003251
[44] HÖLZL, Georg a Peter DÖRMANN. Chloroplast Lipids and Their Biosynthesis. *Annual Review of Plant Biology*. 2019, 70(1), 51-81. ISSN 1543-5008. Dostupné z: doi:10.1146/annurev-arplant-050718-100202
[45] ARMSTRONG G. A., and HEARST J. E.: *Carotenoids 2: Genetics and molecular biology of carotenoid pigment biosynthesis.* The FASEB Journal, 1996. 10(2): p. 228-237. ISSN 0892-6638
[46] BRITTON G., LIAAEN-JENSEN S., and PFANDER H.: *Carotenoids, Volume 4:Natural Functions*. 2008, Basel – Boston - Berlin: Birkhäuser Verlag. 370 p. ISBN 978-3-7643-7498-3
[47] SANDMANN, G.. *Carotenoid biosynthesis in microorganisms and plants. European Journal of Biochemistry*. 1994,223(1), 7-24.
[48] TAKAICHI, Shinichi. Carotenoids in Algae: Distributions, Biosyntheses and Functions. *Marine Drugs*. 2011, 9(6), 1101-1118. DOI: 10.3390/md9061101. ISSN 1660-3397.
[49] GONG, Mengyue a Amarjeet BASSI. Carotenoids from microalgae: A review of recent developments. *Biotechnology Advances*. 2016, 34(8), 1396-1412. DOI: 10.1016/j.biotechadv.2016.10.005. ISSN 07349750.
[50] SATHASIVAM, Ramaraj a Jang-Seu KI. A Review of the Biological Activities of Microalgal Carotenoids and Their Potential Use in Healthcare and Cosmetic Industries. *Marine Drugs*. 2018, 16(1). DOI: 10.3390/md16010026. ISSN 1660-3397.
[51] NOVOVESKÁ, Lucie, Michael E. ROSS, Michele S. STANLEY, Rémi PRADELLES, Virginie WASIOLEK a Jean-François SASSI. Microalgal Carotenoids: A Review of Production, Current Markets, Regulations, and Future Direction. *Marine Drugs*. 2019, 17(11). DOI: 10.3390/md17110640. ISSN 1660-3397.
[52] Markets and Markets Carotenoid Market: By Type (Astaxanthin, B-carotene, Canthaxanthin, Lutein, Lycopene, and Zeaxanthin), Source (Synthetic and Natural), Application (Supplements, Food, Feed, and Cosmetics), and by Region—Global Trends and Forecast to 2026 Available online: https://www.marketsandmarkets.com/Market-Reports/carotenoid-market-158421566.htmL.
[53] AMBATI, Ranga Rao, Deepika GOGISETTY, Ravishankar Gokare ASWATHANARAYANA, Sarada RAVI, Panduranga Narasimharao BIKKINA, Lei BO a Su YUEPENG. Industrial potential of carotenoid pigments from microalgae: Current trends and future prospects. *Critical Reviews in Food Science and Nutrition*. 2018, 59(12), 1880-1902. ISSN 1040-8398. Dostupné z: doi:10.1080/10408398.2018.1432561
[54] JOHNSON, Elizabeth J. The Role of Carotenoids in Human Health. *Nutrition in Clinical Care*. 2002, 5(2), 56-65. ISSN 1096-6781. Dostupné z: doi:10.1046/j.1523-5408.2002.00004.x
[55] IRWANDI JASWIR. Carotenoids: Sources, medicinal properties and their application in food and nutraceutical industry. *Journal of Medicinal Plants Research*. 2011, 5(33). ISSN 19960875. Dostupné z: doi:10.5897/JMPRX11.011
[56] COOPERSTONE, J.L. a S.J. SCHWARTZ. Recent Insights Into Health Benefits of Carotenoids. *Handbook on Natural Pigments in Food and Beverages*. Elsevier, 2016, 2016, , 473-497. ISBN 9780081003718. Dostupné z: doi:10.1016/B978-0-08-100371-8.00020-8

[57] LORENZ, R.Todd a Gerald R CYSEWSKI. Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends in Biotechnology*. 2000, 18(4), 160-167. DOI: 10.1016/S0167-7799(00)01433-5. ISSN 01677799.
[58] SURAI, P. F., B. K. SPEAKE a N. H. C. SPARKS. Carotenoids in Avian Nutrition and Embryonic Development. 1. Absorption, Availability and Levels in Plasma and Egg Yolk. *The Journal of Poultry Science*. 2001, 38(1), 1-27. DOI: 10.2141/jpsa.38.1. ISSN 1346-7395.
[59] ZAKYNTHINOS, G a T VARZAKAS. Carotenoids: From Plants to Food Industry. *Current Research in Nutrition and Food Science Journal*. 2016, 4(1), 38-51. ISSN 2347467X. Dostupné z: doi:10.12944/CRNFSJ.4.Special-Issue1.04
[60] Englmaierová M., Skřivan M., Bubancová I. A comparison of lutein, spray-dried *Chlorella*, and synthetic carotenoids effects on yolk colour, oxidative stability, and reproductive performance of laying hens. Czech J. Anim. Sci. 2013;58:412–419. doi: 10.17221/6941-CJAS
[61] STAHL, Wilhelm a Helmut SIES. B-Carotene and other carotenoids in protection from sunlight. *The American Journal of Clinical Nutrition*. 2012, 96(5), 1179S-1184S. DOI: 10.3945/ajcn.112.034819. ISSN 0002-9165.
[62] STAHL, Wilhelm, Ulrike HEINRICH, Olivier AUST, Hagen TRONNIER a Helmut SIES. Lycopene-rich products and dietary photoprotection. *Photochem. Photobiol. Sci*. 2006, 5(2), 238-242. DOI: 10.1039/B505312A. ISSN 1474-905X.
[63] CHO, Kyoung Sang, Myeongcheol SHIN, Sunhong KIM, Sung Bae LEE a Helmut SIES. Recent Advances in Studies on the Therapeutic Potential of Dietary Carotenoids in Neurodegenerative Diseases. *Oxidative Medicine and Cellular Longevity*. 2018, 2018(2), 1-13. DOI: 10.1155/2018/4120458.
[64] FIEDOR, Joanna a Květoslava BURDA. Potential Role of Carotenoids as Antioxidants in Human Health and Disease. *Nutrients*. 2014, 6(2), 466-488. DOI: 10.3390/nu6020466. ISSN 2072-6643.
[65] GRIFFITHS, Keith, Bharat AGGARWAL, Ram SINGH, Harpal BUTTAR, Douglas WILSON a Fabien DE MEESTER. Food Antioxidants and Their Anti-Inflammatory Properties: A Potential Role in Cardiovascular Diseases and Cancer Prevention. *Diseases*. 2016, 4(4). DOI: 10.3390/diseases4030028. ISSN 2079-9721.
[66] SLUIJS, I., E. CADIER, J.W.J. BEULENS, D.L. VAN DER A, A.M.W. SPIJKERMAN a Y.T. VAN DER SCHOUW. Dietary intake of carotenoids and risk of type 2 diabetes. *Nutrition, Metabolism and Cardiovascular Diseases*. 2015, 25(4), 376-381. DOI: 10.1016/j.numecd.2014.12.008. ISSN 09394753.
[67] EGGERSDORFER, Manfred a Adrian WYSS. Carotenoids in human nutrition and health. *Archives of Biochemistry and Biophysics*. 2018, 652, 18-26. DOI: 10.1016/j.abb.2018.06.001. ISSN 00039861.
[68] GAMMONE, Maria a Nicolantonio D'ORAZIO. Anti-Obesity Activity of the Marine Carotenoid Fucoxanthin. *Marine Drugs*. 2015, 13(4), 2196-2214. DOI: 10.3390/md13042196. ISSN 1660-3397.
[69] TORREGROSA-CRESPO, Javier, Zaida MONTERO, Juan FUENTES, Manuel REIG GARCÍA-GALBIS, Inés GARBAYO, Carlos VÍLCHEZ a Rosa MARTÍNEZ-ESPINOSA. Exploring the Valuable Carotenoids for the Large-Scale Production by Marine Microorganisms. *Marine Drugs*. 2018, 16(6). DOI: 10.3390/md16060203. ISSN 1660-3397.
[70] MIRKOVIC, Tihana, Evgeny E. OSTROUMOV, Jessica M. ANNA, Rienk VAN GRONDELLE, GOVINDJEE a Gregory D. SCHOLES. Light Absorption and Energy Transfer in the Antenna Complexes of Photosynthetic Organisms. *Chemical Reviews*. 2016, 117(2), 249-293. DOI: 10.1021/acs.chemrev.6b00002. ISSN 0009-2665.
[71] GRUDZINSKI, Wojciech, Lukasz NIERZWICKI, Renata WELC, Emilia RESZCZYNSKA, Rafal LUCHOWSKI, Jacek CZUB a Wieslaw I. GRUSZECKI. Localization and Orientation of Xanthophylls in a Lipid Bilayer. *Scientific Reports*. 2017, 7(1). DOI: 10.1038/s41598-017-10183-7. ISSN 2045-2322.

[72] KIOKIAS, S., C PROESTOS a T VARZAKAS. A Review of the Structure, Biosynthesis, Absorption of Carotenoids-Analysis and Properties of their Common Natural Extracts. *Current Research in Nutrition and Food Science Journal*. 2016, 4(1), 25-37. ISSN 2347467X. Dostupné z: doi:10.12944/CRNFSJ.4.Special-Issue1.03
[73] YOUNG, Andrew J. a Gordon M. LOWE. Antioxidant and Prooxidant Properties of Carotenoids. *Archives of Biochemistry and Biophysics*. 2001, 385(1), 20-27. ISSN 00039861. Dostupné z: doi:10.1006/abbi.2000.2149
[74] G. Britton, S. Liaanen-Jensen, a H. Pfander, Carotenoids. Handbook. 2004.
[75] BRITTON G., LIAAEN-JENSEN S., and PFANDER H.: *Carotenoids, Volume 4:Natural Functions*. 2008, Basel – Boston - Berlin: Birkhäuser Verlag. 370 p. ISBN 978-3-7643-7498-3
[76] DELLAPENNA, D.. *Carotenoid Synthesis and Function in Plants: Insights from Mutant Studies in Arabidopsis thaliana. The Photochemistry of Carotenoids*. Dordrecht: Kluwer Academic Publishers, 2004, 21.
[77] LANDRUM, J. T.. *Carotenoids: physical, chemical, and biological functions and properties*. Boca Raton: CRC Press, c2010.
[78] ARMSTRONG G. A., and HEARST J. E.: *Carotenoids 2: Genetics and molecular biology of carotenoid pigment biosynthesis.* The FASEB Journal, 1996. 10(2): p. 228-237. ISSN 0892-6638
[79] LIAO, Pan, Andréa HEMMERLIN, Thomas J. BACH a Mee-Len CHYE. The potential of the mevalonate pathway for enhanced isoprenoid production. *Biotechnology Advances*. 2016, 34(5), 697-713. DOI: 10.1016/j.biotechadv.2016.03.005. ISSN 07349750.
[80] CHANDRAN, Sunil S., James T. KEALEY a Christopher D. REEVES. Microbial production of isoprenoids. *Process Biochemistry*. 2011, 46(9), 1703-1710. DOI: 10.1016/j.procbio.2011.05.012. ISSN 13595113.
[81] NIU, Fu-Xing, Qian LU, Yi-Fan BU a Jian-Zhong LIU. Metabolic engineering for the microbial production of isoprenoids: Carotenoids and isoprenoid-based biofuels. *Synthetic and Systems Biotechnology*. 2017, 2(3), 167-175. DOI: 10.1016/j.synbio.2017.08.001. ISSN 2405805X.
[82] LOHR, Martin, Jörg SCHWENDER a Jürgen E.W. POLLE. Isoprenoid biosynthesis in eukaryotic phototrophs: A spotlight on algae. *Plant Science*. 2012, 185-186, 9-22. ISSN 01689452. Dostupné z: doi:10.1016/j.plantsci.2011.07.018
[83] PANIAGUA-MICHEL, J., Willian CAPA-ROBLES, Jorge OLMOS-SOTO a Luis Enrique GUTIERREZ-MILLAN. The Carotenogenesis Pathway via the Isoprenoid-β-carotene Interference Approach in a New Strain of Dunaliella salina Isolated from Baja California Mexico. *Marine Drugs*. 2009, 7(1), 45-56. ISSN 1660-3397. Dostupné z: doi:10.3390/md7010045
[84] TAKAICHI, Shinichi. Carotenoids in Algae: Distributions, Biosyntheses and Functions. *Marine Drugs*. 2011, 9(6), 1101-1118. ISSN 1660-3397. Dostupné z: doi:10.3390/md9061101
[85] EISENREICH, W., A. BACHER, D. ARIGONI a F. ROHDICH. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cellular and Molecular Life Sciences*. 2004, 61(12). ISSN 1420-682X. Dostupné z: doi:10.1007/s00018-004-3381-z
[86] ZINGLÉ, Catherine, Lionel KUNTZ, Denis TRITSCH, Catherine GROSDEMANGE-BILLIARD a Michel ROHMER. Isoprenoid Biosynthesis via the Methylerythritol Phosphate Pathway: Structural Variations around Phosphonate Anchor and Spacer of Fosmidomycin, a Potent Inhibitor of Deoxyxylulose Phosphate Reductoisomerase. *The Journal of Organic Chemistry*. 2010, 75(10), 3203-3207. ISSN 0022-3263. Dostupné z: doi:10.1021/jo9024732
[87] KOPECKÝ, Jiří, Richard LHOTSKÝ a Jindřiška PAICHLOVÁ. Aktivní látky mikrořas ve výživě. Praha: Academia, 2017. Věda kolem nás.

[88] GÁLOVÁ, Eliška, Iveta ŠALGOVIČOVÁ, Viktor DEMKO, Katarína MIKULOVÁ, Andrea ŠEVČOVIČOVÁ, Ľudmila SLOVÁKOVÁ, Veronika KYSELÁ a Ján HUDÁK. A short overview of chlorophyll biosynthesis in algae. *Biologia*. 2008, 63(6). ISSN 1336-9563. Dostupné z: doi:10.2478/s11756-008-0147-3

[89] WILLOWS, Robert D. Biosynthesis of Chlorophyll and Bilins in Algae. *Photosynthesis in Algae: Biochemical and Physiological Mechanisms*. Cham: Springer International Publishing, 2020, 2020-06-04, , 83-103. Advances in Photosynthesis and Respiration. ISBN 978-3-030-33396-6. Dostupné z: doi:10.1007/978-3-030-33397-3_5

[90] YAHIA, E. M. Fruit and vegetable phytochemicals: chemistry and human health. 2nd edition. Hoboken, NJ, 2018. ISBN 978-111-9157-977.

[91] MIZOGUCHI, T., Y. KIMURA, T. YOSHITOMI a H. TAMIAKI. The stereochemistry of chlorophyll-c3 from the haptophyte Emiliania huxleyi: The (132R)-enantiomers of chlorophylls-c are exclusively selected as the photosynthetically active pigments in chromophyte algae. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2011, 1807(11), 1467-1473. DOI: 10.1016/j.bbabio.2011.07.008. ISSN 00052728. Dostupné také z: https://linkinghub.elsevier.com/retrieve/pii/S0005272811001745

[92] MICHAL, Gerhard a Dietmar SCHOMBURG, ed. Biochemical pathways: an atlas of biochemistry and molecular biology. 2nd ed. Hoboken: Wiley, c2012. ISBN 978-0-470- 14684-2

[93] BLANKENSHIP, Robert E. Molecular mechanisms of photosynthesis. 2nd ed. Chichester: Wiley Blackwell, 2014. ISBN 978-1-4051-8976-7..

[94] Photosynthesis. In: *Wikipedia: the free encyclopedia* [online]. San Francisco (CA): Wikimedia Foundation, 2001- [cit. 2021-01-23]. Dostupné z: https://en.wikipedia.org/wiki/Photosynthesis

[95] VOLKMAN, J. Sterols in microorganisms. *Applied Microbiology and Biotechnology*. 2003, 60(5), 495-506. ISSN 0175-7598. Dostupné z: doi:10.1007/s00253-002-1172-8

[96] BELT, Simon T., Thomas A. BROWN, Lukas SMIK, Philipp ASSMY a C.J. MUNDY. Sterol identification in floating Arctic sea ice algal aggregates and the Antarctic sea ice diatom Berkeleya adeliensis. *Organic Geochemistry*. 2018, 118, 1-3. DOI: 10.1016/j.orggeochem.2018.01.008. ISSN 01466380.

[97] DUPONT, Sebastien, Guillaume LEMETAIS, Thierry FERREIRA, Philippe CAYOT, Patrick GERVAIS a Laurent BENEY. ERGOSTEROL BIOSYNTHESIS: A FUNGAL PATHWAY FOR LIFE ON LAND?. *Evolution*. 2012, 66(9), 2961-2968. DOI: 10.1111/j.1558-5646.2012.01667.x. ISSN 00143820.

[98] ROBERTS, C.W., R. MCLEOD, D.W. RICE, M. GINGER, M.L. CHANCE a L.J. GOAD. Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. *Molecular and Biochemical Parasitology*. 2003, 126(2), 129-142. ISSN 01666851. Dostupné z: doi:10.1016/S0166-6851(02)00280-3

[99] WEETE, John D., Maritza ABRIL, Meredith BLACKWELL a Geraldine BUTLER. Phylogenetic Distribution of Fungal Sterols. *PLoS ONE*. 2010, 5(5). ISSN 1932-6203. Dostupné z: doi:10.1371/journal.pone.0010899

[100] AUSSEL, Laurent, Fabien PIERREL, Laurent LOISEAU, Murielle LOMBARD, Marc FONTECAVE a Frédéric BARRAS. Biosynthesis and physiology of coenzyme Q in bacteria. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 2014, 1837(7), 1004-1011. DOI: 10.1016/j.bbabio.2014.01.015. ISSN 00052728.

[101] BENTINGER, Magnus, Michael TEKLE a Gustav DALLNER. Coenzyme Q – Biosynthesis and functions. *Biochemical and Biophysical Research Communications*. 2010, 396(1), 74-79. DOI: 10.1016/j.bbrc.2010.02.147. ISSN 0006291X.

[102] KWON, Ohsuk a R. MEGANATHAN. Biosynthesis of Menaquinone (Vitamin K2) and Ubiquinone (Coenzyme Q). *EcoSal Plus*. 2009, 3(2), -. DOI: 10.1128/ecosalplus.3.6.3.3. ISSN 2324-6200.

[103] KAGAN, V. E. a P. J. QUINN. *Coenzyme Q: molecular mechanisms in health and disease*. Boca Raton: CRC Press, c2001

[104] LANGE, B. M., T. RUJAN, W. MARTIN a R. CROTEAU. *Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes.* Proceedings of the National Academy of Sciences. 2000, 97(24), 13172-13177.
[105] WEETE, J. D., M. ABRIL, M. BLACKWELL a G. BUTLER. *Phylogenetic Distribution of Fungal Sterols.* PLoS ONE. 2010-5-28, 5(5).
[106] NIGAM, Poonam. Microbial Enzymes with Special Characteristics for Biotechnological Applications. Biomolecules [online]. 2013, 3(4), 597-611 [cit. 2018-11-16]. DOI: 10.3390/biom3030597. ISSN 2218-273X. Dostupné z: http://www.mdpi.com/2218-273X/3/3/597
[107] ANBU, Periasamy, Subash C. B. GOPINATH, Bidur Prasad CHAULAGAIN a Thangavel LAKSHMIPRIYA. Microbial Enzymes and Their Applications in Industries and Medicine 2016. BioMed Research International [online]. 2017, 2017, 1- 3 [cit. 2018-11-16]. DOI: 10.1155/2017/2195808. ISSN 2314-6133. Dostupné z: https://www.hindawi.com/journals/bmri/2017/2195808/
[108] SINGH, Rajendra, Manoj KUMAR, Anshumali MITTAL a Praveen Kumar MEHTA. Microbial enzymes: industrial progress in 21st century. 3 Biotech [online]. 2016, 6(2) [cit. 2018-11-16]. DOI: 10.1007/s13205-016-0485-8. ISSN 2190-572X. Dostupné z: http://link.springer.com/10.1007/s13205-016-0485-8
[109] RAMNATH, Lucretia, Bruce SITHOLE a Roshini GOVINDEN. Classification of lipolytic enzymes and their biotechnological applications in the pulping industry. Canadian Journal of Microbiology [online]. 2017, 63(3), 179-192 [cit. 2018- 11-07]. DOI: 10.1139/cjm-2016-0447. ISSN 0008-4166. Dostupné z: http://www.nrcresearchpress.com/doi/10.1139/cjm-2016-0447
[110] BORNSCHEUER, Uwe. Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS Microbiology Reviews [online]. 2002, 26(1), 73-81 [cit. 2018-11-07]. DOI: 10.1111/j.1574-6976.2002.tb00599.x. ISSN 1574-6976. Dostupné z: https://academic.oup.com/femsre/article-lookup/doi/10.1111/j.1574-6976.2002.tb00599.x
[111] ANDUALEMA, Berhanu a Amare GESSESSE. Microbial Lipases and Their Industrial Applications: Review. Biotechnology[online]. 2012, 11(3), 100-118 [cit. 2018-11-12]. DOI: 10.3923/biotech.2012.100.118. ISSN 1682296X. Dostupné z: http://www.scialert.net/abstract/?doi=biotech.2012.100.118
[112] PRIJI, Prakasan, et al. MICROBIAL LIPASES - PROPERTIES AND APPLICATIONS. Journal of Microbiology, Biotechnology and Food Sciences [online]. 2016, 6(2), 799-807 [cit. 2019-02-27]. DOI: 10.15414/jmbfs.2016.6.2.799-807. ISSN 13385178. Dostupné z: https://www.researchgate.net/publication/311107937_Microbial_LipasesProperies_and_Applications
[113] HATZINIKOLAOU, Dimitris, Ekaterini KOURENTZI, Haralambos STAMATIS, Paul CHRISTAKOPOULOS, Fragiskos N. KOLISIS, Dimitris KEKOS a Basil J. MACRIS. A novel lipolytic activity of Rhodotorula glutinis cells: Production, partial characterization and application in the synthesis of esters. Journal of Bioscience and Bioengineering [online]. 1999, 88(1), 53-56 [cit. 2019-02-17]. DOI: 10.1016/S1389- 1723(99)80175-3. ISSN 13891723. Dostupné z: http://linkinghub.elsevier.com/retrieve/pii/S1389172399801753
[114] PAPAPARASKEVAS, Dimitris, Paul CHRISTAKOPOULOS, Dimitris KEKOS a Basil MACRIS. Optimizing production of extracellular lipase from Rhodotorula glutinis. Biotechnology Letters [online]. 1992, 14(5), 397-402 [cit. 2019-02-17]. DOI: 10.1007/BF01021254. ISSN 0141-5492. Dostupné z: http://link.springer.com/10.1007/BF01021254
[115] Biosurfactants from Yeasts: Characteristics, Production and Application. SEN, Ramkrishna, et al. Biosurfactants [online]. New York: Springer, c2010, s. 236-249 [cit. 2018-10-27]. Advances in experimental medicine and biology, v. 672. ISBN 978- 1-4419-5979-9. Dostupné z: https://link.springer.com/book/10.1007/978-1-4419- 5979-9#about

[116] CAMPOS, Jenyffer Medeiros, Tânia Lúcia MONTENEGRO STAMFORD, Leonie Asfora SARUBBO, Juliana Moura DE LUNA, Raquel Diniz RUFINO a Ibrahim M. BANAT. Microbial biosurfactants as additives for food industries. Biotechnology Progress [online]. 2013, 29(5), 1097-1108 [cit. 2019-02-19]. DOI: 10.1002/btpr.1796. ISSN 87567938. Dostupné z: http://doi.wiley.com/10.1002/btpr.1796
[117] BOGNOLO, Giulio. Biosurfactants as emulsifying agents for hydrocarbons. Colloids and Surfaces A: Physicochemical and Engineering Aspects [online]. 1999, 152(1-2), 41-52 [cit. 2019-02-19]. DOI: 10.1016/S0927-7757(98)00684-0. ISSN 09277757. Dostupné z: http://linkinghub.elsevier.com/retrieve/pii/S0927775798006840
[118] KATEMAI, Wichuda. Biosurfactants from Yeasts. Walailak Journal of Science and Technology [online]. 2012, 9(1), 8 [cit. 2018-10-27]. DOI: 10.2004/wjst.v9i1.24. Dostupné z: https://www.researchgate.net/publication/307652877_Biosurfactants_from_Yeasts
[119] SHEKHAR, Sudhanshu, Arumugam SUNDARAMANICKAM a Tangavel BALASUBRAMANIAN. Biosurfactant Producing Microbes and their Potential Applications: A Review. Critical Reviews in Environmental Science and Technology [online]. 2014, 45(14), 1522-1554 [cit. 2018-10-28]. DOI: 10.1080/10643389.2014.955631. ISSN 1064-3389. Dostupné z: http://www.tandfonline.com/doi/full/10.1080/10643389.2014.955631
[120] KURTZMAN, C., J. W. FELL a T. BOEKHOUT (eds.). *The yeasts: a taxonomic study*. 5th ed. Amsterdam: Elsevier Science Publishers, 2011.
[121] SHARMA, P. D., S. LIAAEN-JENSEN a H. PFANDER. *Microbiology: molecular aspects and health issues.* Meerut [India]: Rakesh Kumar Rastogi for Rastogi Publications, 2007.HOHMANN S. and MAGER W. H.: *Yeast Stress Responses*. 2003, Berlin: Springer-Verlag. 389 p. ISBN 3-540-43926-9
[122] WALKER, Graeme M.Yeast physiology and biotechnology. New York: J. Wiley, c1998. ISBN 0-471-96446-8
[123] SATYANARAYANA, T. a Gotthard. KUNZE.Yeast biotechnology: diversity and applications. New York: Springer, [2009]. ISBN 978-1-4020-8292-4.
[124] Molecular mechanisms in yeast carbon metabolism. New York: Springer, 2014. ISBN 978-3-642-55012-6.
[125] SAMPAIO, José Paulo. *The Yeasts*. Elsevier, 2011, 2011, , 1423-1432. DOI: 10.1016/B978-0-444-52149-1.00111-7. ISBN 9780444521491.
[126] ŠILHÁNKOVÁ, L. *Mikrobiologie pro potravináře a biotechnology*. Vyd. 3. [i.e. 4.], opr. a dopl., v nakl. Academia 1. vyd. Praha: Academia, 2008.
[127] MANNAZZU, Ilaria, Sara LANDOLFO, Teresa Lopes DA SILVA a Pietro BUZZINI. Red yeasts and carotenoid production: outlining a future for non-conventional yeasts of biotechnological interest. *World Journal of Microbiology and Biotechnology*. 2015, **31**(11), 1665-1673. ISSN 0959-3993. Dostupné z: doi:10.1007/s11274-015-1927-x
[128] BURKE, D., D. DAWSON a T. STEARNS. *Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual*. New York: Cold Spring Harbor Laboratory Press, c2000.
[129] WANG, Q a F BAI. Four new yeast species of the genus from plant leaves. *FEMS Yeast Research*. 2004, 4(6), 579-586. DOI: 10.1016/j.femsyr.2003.11.002. ISSN 15671356.
[130] VRANÁ, D. a A. KOCKOVÁ-KRATOCHVÍLOVÁ. *Kvasinky ve výzkumu a praxi*. Praha: Academia, 1986
[131] SEDLÁČEK, Ivo. *Taxonomie prokaryot*. Brno: Masarykova univerzita, 2007. ISBN 978-80-210-4207-0.
[132] OBERWINKLER, Franz, Robert BANDONI, Paul BLANZ a Ljuba KISIMOVA-HOROVITZ. Cystofilobasidium: a New Genus in the Filobasidiaceae. *Systematic and Applied Microbiology*. 1983, 4(1), 114-122. DOI: 10.1016/S0723-2020(83)80039-3. ISSN 07232020.

[133] HERZ, Stephanie, Roland W.S. WEBER, Heidrun ANKE, Adele MUCCI a Paolo DAVOLI. Intermediates in the oxidative pathway from torulene to torularhodin in the red yeasts Cystofilobasidium infirmominiatum and C. capitatum (Heterobasidiomycetes, Fungi).Phytochemistry[online]. 2007,68(20), 2503-2511 [cit. 2018-08-02]. DOI: 10.1016/j.phytochem.2007.05.019. ISSN 00319422. Dostupné z: http://linkinghub.elsevier.com/retrieve/pii/S0031942207003342

[134] SAMPAIO J. P., GADANHO M. BAUER R.: Taxonomic studies on the genus Cystofilobasidium: description of C. ferigula sp. nov.and clarification of the status of C. lari-marini, International Journal of Systematic and Evolutionary Microbiology, 2001, p. 221-229

[135] MUSSAGY, Cassamo Ussemane, Ana Alice Carizia GUIMARÃES, Lara Vicente Ferreira ROCHA, James WINTERBURN, Valéria de Carvalho SANTOS-EBINUMA a Jorge Fernando Brandão PEREIRA. Improvement of carotenoids production from Rhodotorula glutinis CCT-2186. *Biochemical Engineering Journal*. 2021, **165**. ISSN 1369703X. Dostupné z: doi:10.1016/j.bej.2020.107827

[136] CHENG, Yu-Ting a Chu-Fang YANG. Using strain Rhodotorula mucilaginosa to produce carotenoids using food wastes. *Journal of the Taiwan Institute of Chemical Engineers*. 2016, 61, 270-275. DOI: 10.1016/j.jtice.2015.12.027. ISSN 18761070.

[137] HERNÁNDEZ-ALMANZA, Ayerim, Julio CESAR MONTANEZ, Miguel A. AGUILAR-GONZÁLEZ, Cristian MARTÍNEZ-ÁVILA, Raúl RODRÍGUEZ-HERRERA a Cristóbal N. AGUILAR. Rhodotorula glutinis as source of pigments and metabolites for food industry. *Food Bioscience*. 2014, 5, 64-72. DOI: 10.1016/j.fbio.2013.11.007. ISSN 22124292.

[138] MAZA, D. Daniela, Silvana C. VIÑARTA, Ying SU, José Manuel GUILLAMÓN a Manuel J. AYBAR. Growth and lipid production of Rhodotorula glutinis R4, in comparison to other oleaginous yeasts. *Journal of Biotechnology*. 2020, **310**, 21-31. ISSN 01681656. Dostupné z: doi:10.1016/j.jbiotec.2020.01.012

[139] DA COSTA, Willyan Araújo, Carlos Eduardo DE ARAÚJO PADILHA, Júlia Maria DE MEDEIROS DANTAS, Maylla Maria Correia Leite SILVA, Hugo Alexandre DE OLIVEIRA ROCHA, Flávio Luiz Honorato DA SILVA, Márcio FERRARI a Everaldo Silvino DOS SANTOS. Fractional recovery of oleaginous bioactive produced by Rhodotorula mucilaginosa CCT3892 using deep eutectic solvents. *Bioresource Technology Reports*. 2020, **12**. ISSN 2589014X. Dostupné z: doi:10.1016/j.biteb.2020.100561

[140] AKSU, Z. a A. T. EREN. *Carotenoids production by the yeast Rhodotorula mucilaginosa: Use of agricultural wastes as a carbon source. Process Biochemistry*. 2005, 40(9), 2985-2991.

[141] TRAN, Tuyet Nhung, Dai-Hung NGO, Ngoc Tuan NGUYEN a Dai-Nghiep NGO. Draft genome sequence data of Rhodosporidium toruloides VN1, a strain capable of producing natural astaxanthin. *Data in Brief*. 2019, **26**. ISSN 23523409. Dostupné z: doi:10.1016/j.dib.2019.104443

[142] OSORIO-GONZÁLEZ, Carlos S., Krishnamoorthy HEGDE, Pedro FERREIRA, Satinder Kaur BRAR, Azadeh KERMANSHAHIPOUR, Carlos Ricardo SOCCOL a Antonio AVALOS-RAMÍREZ. Lipid production in Rhodosporidium toruloides using C-6 and C-5 wood hydrolysate: A comparative study. *Biomass and Bioenergy*. 2019, **130**. ISSN 09619534. Dostupné z: doi:10.1016/j.biombioe.2019.105355

[143] SARAN, Saurabh, Arushi MATHUR, Jyotsana DALAL a R.K. SAXENA. Process optimization for cultivation and oil accumulation in an oleaginous yeast Rhodosporidium toruloides A29. *Fuel*. 2017, **188**, 324-331. ISSN 00162361. Dostupné z: doi:10.1016/j.fuel.2016.09.051

[144] BAI, F. Y. Reclassification of the Sporobolomyces roseus and Sporidiobolus pararoseus complexes, with the description of Sporobolomyces phaffii sp. nov. *International journal of systematic and evolutionary microbiology*. 52(6), 2309-2314. DOI: 10.1099/ijs.0.02297-0. ISSN 14665026.

[145] ZENG, Jiaying, Shaoan LIAO, Ming QIU, Mingfeng CHEN, Jianmin YE, Jiayi ZENG a Anli WANG. Effects of carbon sources on the removal of ammonium, nitrite and nitrate nitrogen by the red yeast Sporidiobolus pararoseus Y1. *Bioresource Technology*. 2020, **312**. ISSN 09608524. Dostupné z: doi:10.1016/j.biortech.2020.123593.
[146] LI, Chunji, Ning ZHANG, Bingxue LI, Qiong XU, Jia SONG, Na WEI, Wenjing WANG a Hongtao ZOU. Increased torulene accumulation in red yeast Sporidiobolus pararoseus NGR as stress response to high salt conditions. *Food Chemistry*. 2017, **237**, 1041-1047. ISSN 03088146. Dostupné z: doi:10.1016/j.foodchem.2017.06.033
[147] COLET, Rosicler, Letícia URNAU, Jaine BAMPI, et al. Use of low-cost agro products as substrate in semi-continuous process to obtain carotenoids by Sporidiobolus salmonicolor. *Biocatalysis and Agricultural Biotechnology*. 2017, **11**, 268-274. ISSN 18788181. Dostupné z: doi:10.1016/j.bcab.2017.07.015
[148] MANOWATTANA, Atchara, Charin TECHAPUN, Masanori WATANABE a Thanongsak CHAIYASO. Bioconversion of biodiesel-derived crude glycerol into lipids and carotenoids by an oleaginous red yeast Sporidiobolus pararoseus KM281507 in an airlift bioreactor. *Journal of Bioscience and Bioengineering*. 2018, **125**(1), 59-66. ISSN 13891723. Dostupné z: doi:10.1016/j.jbiosc.2017.07.014
[149] BOROWITZKA, Michael.The physiology of microalgae. New York, NY: Springer Berlin Heidelberg, 2016. ISBN 978-3-319-24943-8.
[150] PEREIRA, Leonel a Joao MAGALHAES NETO. *Marine Algae: Biodiversity, Taxonomy, Environmental Assessment, and Biotechnology*. CRC Press, 2014. ISBN 9781466581678.
[151] BARSANTI, L. a Paolo. GUALTIERI.Algae: anatomy, biochemistry, and biotechnology. Second edition. Boca Raton, [Florida]: CRC Press/Taylor & Francis Group, [2014]. ISBN 978-1-4398-6732-7.
[152] RICHMOND, Amos. Handbook of microalgal culture: biotechnology and applied phycology. Ames, Iowa, USA: Blackwell Science, 2004. ISBN 06-320-5953-2
[153] MARKOU, Giorgos, Dries VANDAMME a Koenraad MUYLAERT. Microalgal and cyanobacterial cultivation: The supply of nutrients. *Water Research*. 2014, **65**, 186-202. ISSN 00431354. Dostupné z: doi:10.1016/j.watres.2014.07.025
[154] YOUSUF, Abu. Fundamentals of Microalgae Cultivation. Microalgae Cultivation for Biofuels Production. Elsevier, 2020, , 1-9. DOI: 10.1016/B978-0-12-817536-1.00001-1. ISBN 9780128175361. Dostupné z: https://linkinghub.elsevier.com/retrieve/pii/B978012817536100001
[155] ZUCCARO, Gaetano, Abu YOUSUF, Antonino POLLIO a Jean-Philippe STEYER. Microalgae Cultivation Systems. Microalgae Cultivation for Biofuels Production. Elsevier, 2020, , 11-29. DOI: 10.1016/B978-0-12-817536-1.00002-3. ISBN 9780128175361.Dostupné z: https://linkinghub.elsevier.com/retrieve/pii/B9780128175361000023
[156] CUARESMA, María, Marcel JANSSEN, Carlos VÍLCHEZ a René H. WIJFFELS. Horizontal or vertical photobioreactors? How to improve microalgae photosynthetic efficiency. *Bioresource Technology*. 2011, **102**(8), 5129-5137. ISSN 09608524. Dostupné z: doi:10.1016/j.biortech.2011.01.078
[157] SMALLEY, Tressa, Francis J. FIELDS, Anthony J.E. BERNDT, Joseph T. OSTRAND, Vanessa HEREDIA a Stephen P. MAYFIELD. Improving biomass and lipid yields of Desmodesmus armatus and Chlorella vulgaris through mutagenesis and high-throughput screening. *Biomass and Bioenergy*. 2020, **142**. ISSN 09619534. Dostupné z: doi:10.1016/j.biombioe.2020.105755
[158] POZZOBON, Victor, Wendie LEVASSEUR, Cédric GUERIN, Nathalie GAVEAU-VAILLANT, Marion POINTCHEVAL a Patrick PERRÉ. Desmodesmus sp. pigment and FAME profiles under different illuminations and nitrogen status. *Bioresource Technology Reports*. 2020, **10**. ISSN 2589014X. Dostupné z: doi:10.1016/j.biteb.2020.100409

[159] SIJIL, P.V., Vinaya R. ADKI, R. SARADA a V.S. CHAUHAN. Strategies for enhancement of alpha-linolenic acid rich lipids in Desmodesmus sp. without compromising the biomass production. *Bioresource Technology*. 2019, **294**. ISSN 09608524. Dostupné z: doi:10.1016/j.biortech.2019.122215
[160] RODAS-ZULUAGA, Laura Isabel, Lizbeth CASTAÑEDA-HERNÁNDEZ, Eduardo Israel CASTILLO-VACAS, et al. Bio-capture and influence of CO2 on the growth rate and biomass composition of the microalgae Botryococcus braunii and Scenedesmus sp. *Journal of CO2 Utilization*. 2021, **43**. ISSN 22129820. Dostupné z: doi:10.1016/j.jcou.2020.101371
[161] SAFI, Carl, Bachar ZEBIB, Othmane MERAH, Pierre-Yves PONTALIER a Carlos VACA-GARCIA. Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. *Renewable and Sustainable Energy Reviews*. 2014, **35**, 265-278. ISSN 13640321. Dostupné z: doi:10.1016/j.rser.2014.04.007
[162] AZAMAN, Siti Nor Ani, Norio NAGAO, Fatimah M. YUSOFF, Sheau Wei TAN a Swee Keong YEAP. A comparison of the morphological and biochemical characteristics of Chlorella sorokiniana and Chlorella zofingiensis cultured under photoautotrophic and mixotrophic conditions. *PeerJ*. 2017, **5**. ISSN 2167-8359. Dostupné z: doi:10.7717/peerj.3473
[163] JEON, Seon-Mi, Ji Hyung KIM, Taeho KIM, et al. Morphological, Molecular, and Biochemical Characterization of Monounsaturated Fatty Acids-Rich Chlamydomonas sp. KIOST-1 Isolated from Korea. *Journal of Microbiology and Biotechnology*. 2015, **25**(5), 723-731. ISSN 1017-7825. Dostupné z: doi:10.4014/jmb.1412.12056
[164] FUENTES, Juan L., Volker A. R. HUSS, Zaida MONTERO, Rafael TORRONTERAS, María CUARESMA, Inés GARBAYO a Carlos VÍLCHEZ. Phylogenetic characterization and morphological and physiological aspects of a novel acidotolerant and halotolerant microalga Coccomyxa onubensis sp. nov. (Chlorophyta, Trebouxiophyceae). *Journal of Applied Phycology*. 2016, **28**(6), 3269-3279. ISSN 0921-8971. Dostupné z: doi:10.1007/s10811-016-0887-3
[165] DARIENKO, Tatyana, Lydia GUSTAVS, Anja EGGERT, Wiebke WOLF, Thomas PRÖSCHOLD a Diego FONTANETO. Evaluating the Species Boundaries of Green Microalgae (Coccomyxa, Trebouxiophyceae, Chlorophyta) Using Integrative Taxonomy and DNA Barcoding with Further Implications for the Species Identification in Environmental Samples. *PLOS ONE*. 2015, **10**(6). ISSN 1932-6203. Dostupné z: doi:10.1371/journal.pone.0127838
[166] RUIZ-DOMÍNGUEZ, Mari Carmen, Isabel VAQUERO, Virginia OBREGÓN, Benito DE LA MORENA, Carlos VÍLCHEZ a José M. VEGA. Lipid accumulation and antioxidant activity in the eukaryotic acidophilic microalga Coccomyxa sp. (strain onubensis) under nutrient starvation. *Journal of Applied Phycology*. 2015, **27**(3), 1099-1108. ISSN 0921-8971. Dostupné z: doi:10.1007/s10811-014-0403-6
[167] TANOI, Takako, Masanobu KAWACHI a Makoto M. WATANABE. Effects of carbon source on growth and morphology of Botryococcus braunii. *Journal of Applied Phycology*. 2011, **23**(1), 25-33. ISSN 0921-8971. Dostupné z: doi:10.1007/s10811-010-9528-4
[168] TANOI, Takako, Masanobu KAWACHI a Makoto M. WATANABE. Iron and glucose effects on the morphology of Botryococcus braunii with assumption on the colony formation variability. *Journal of Applied Phycology*. 2014, **26**(1), 1-8. ISSN 0921-8971. Dostupné z: doi:10.1007/s10811-013-0026-3
[169] CHENG, Pengfei, Junfeng WANG a Tianzhong LIU. Effect of cobalt enrichment on growth and hydrocarbon accumulation of Botryococcus braunii with immobilized biofilm attached cultivation. *Bioresource Technology*. 2015, **177**, 204-208. ISSN 09608524. Dostupné z: doi:10.1016/j.biortech.2014.11.088
[170] UYEDA, Josef C., Luke J. HARMON, Carrine E. BLANK a Brett NEILAN. A Comprehensive Study of Cyanobacterial Morphological and Ecological Evolutionary Dynamics through Deep Geologic Time. *PLOS ONE*. 2016, **11**(9). ISSN 1932-6203. Dostupné z: doi:10.1371/journal.pone.0162539

[171] DVOŘÁK, Petr, Dale A. CASAMATTA, Petr HAŠLER, Eva JAHODÁŘOVÁ, Alyson R. NORWICH a Aloisie POULÍČKOVÁ. Diversity of the Cyanobacteria. *Modern Topics in the Phototrophic Prokaryotes*. Cham: Springer International Publishing, 2017, 2017-04-19, , 3-46. ISBN 978-3-319-46259-2. Dostupné z: doi:10.1007/978-3-319-46261-5_1

[172] PALINSKA, Katarzyna A. a Waldemar SUROSZ. Taxonomy of cyanobacteria: a contribution to consensus approach. *Hydrobiologia*. 2014, **740**(1), 1-11. ISSN 0018-8158. Dostupné z: doi:10.1007/s10750-014-1971-9

[173] STUART, Rhona K., Chris L. DUPONT, D. Aaron JOHNSON, Ian T. PAULSEN a Brian PALENIK. Coastal Strains of Marine Synechococcus Species Exhibit Increased Tolerance to Copper Shock and a Distinctive Transcriptional Response Relative to Those of Open-Ocean Strains. *Applied and Environmental Microbiology*. 2009, **75**(15), 5047-5057. ISSN 0099-2240. Dostupné z: doi:10.1128/AEM.00271-09

[174] CABELLO-YEVES, Pedro J., Jose M. HARO-MORENO, Ana-Belen MARTIN-CUADRADO, Rohit GHAI, Antonio PICAZO, Antonio CAMACHO a Francisco RODRIGUEZ-VALERA. Novel Synechococcus Genomes Reconstructed from Freshwater Reservoirs. *Frontiers in Microbiology*. 2017, **8**. ISSN 1664-302X. Dostupné z: doi:10.3389/fmicb.2017.01151

[175] KIM, Yihwan, Jehyun JEON, Min Seok KWAK, Gwang Hoon KIM, InSong KOH, Mina RHO a Qunfeng DONG. Photosynthetic functions of Synechococcus in the ocean microbiomes of diverse salinity and seasons. *PLOS ONE*. 2018, **13**(1). ISSN 1932-6203. Dostupné z: doi:10.1371/journal.pone.0190266

[176] STUNDA-ZUJEVA, Agnese. Review of Spirulina/Arthrospira Growth Medium Modifications. *Key Engineering Materials*. 2020, **850**, 153-158. ISSN 1662-9795. Dostupné z: doi:10.4028/www.scientific.net/KEM.850.153

[177] DE LA JARA, A., C. RUANO-RODRIGUEZ, M. POLIFRONE, P. ASSUNÇAO, Y. BRITO-CASILLAS, A. M. WÄGNER a L. SERRA-MAJEM. Impact of dietary Arthrospira (Spirulina) biomass consumption on human health: main health targets and systematic review. *Journal of Applied Phycology*. 2018, **30**(4), 2403-2423. ISSN 0921-8971. Dostupné z: doi:10.1007/s10811-018-1468-4

[178] NUHU, Abdulmumin A. Spirulina (Arthrospira): An Important Source of Nutritional and Medicinal Compounds. *Journal of Marine Biology*. 2013, **2013**, 1-8. ISSN 1687-9481. Dostupné z: doi:10.1155/2013/325636

[179] MATA, Teresa M., António A. MARTINS a Nidia. S. CAETANO. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*. 2010, **14**(1), 217-232. ISSN 13640321. Dostupné z: doi:10.1016/j.rser.2009.07.020

[180] RIZWAN, Muhammad, Ghulam MUJTABA, Sheraz Ahmed MEMON, Kisay LEE a Naim RASHID. Exploring the potential of microalgae for new biotechnology applications and beyond: A review. *Renewable and Sustainable Energy Reviews*. 2018, **92**, 394-404. ISSN 13640321. Dostupné z: doi:10.1016/j.rser.2018.04.034

[181] VIJAYAKUMAR, Subramaniyan. Potential Applications of Cyanobacteria in Industrial Effluents-A Review. *Journal of Bioremediation and Biodegradation*. 2012, **03**(06). ISSN 21556199. Dostupné z: doi:10.4172/2155-6199.1000154

[182] MILLEDGE, John J. Commercial application of microalgae other than as biofuels: a brief review. *Reviews in Environmental Science and Bio/Technology*. 2011, **10**(1), 31-41. ISSN 1569-1705. Dostupné z: doi:10.1007/s11157-010-9214-7

[183] CHU, Ruoyu, Shuangxi LI, Liandong ZHU, Zhihong YIN, Dan HU, Chenchen LIU a Fan MO. A review on co-cultivation of microalgae with filamentous fungi: Efficient harvesting, wastewater treatment and biofuel production. *Renewable and Sustainable Energy Reviews*. 2021, **139**. ISSN 13640321. Dostupné z: doi:10.1016/j.rser.2020.110689

[184] ISHIKA, Tasneema, Navid R. MOHEIMANI a Parisa A. BAHRI. Sustainable saline microalgae co-cultivation for biofuel production: A critical review. *Renewable and Sustainable Energy Reviews*. 2017, **78**, 356-368. ISSN 13640321. Dostupné z: doi:10.1016/j.rser.2017.04.110
[185] LIU, Lu, Junhui CHEN, Phaik-Eem LIM a Dong WEI. Dual-species cultivation of microalgae and yeast for enhanced biomass and microbial lipid production. *Journal of Applied Phycology*. 2018, **30**(6), 2997-3007. ISSN 0921-8971. Dostupné z: doi:10.1007/s10811-018-1526-y
[186] CHEIRSILP, Benjamas, Suleeporn KITCHA a Salwa TORPEE. Co-culture of an oleaginous yeast Rhodotorula glutinis and a microalga Chlorella vulgaris for biomass and lipid production using pure and crude glycerol as a sole carbon source. *Annals of Microbiology*. 2012, **62**(3), 987-993. ISSN 1590-4261. Dostupné z: doi:10.1007/s13213-011-0338-y
[187] WANG, Shikai, Yong WU a Xu WANG. Heterotrophic cultivation of Chlorella pyrenoidosa using sucrose as the sole carbon source by co-culture with Rhodotorula glutinis. *Bioresource Technology*. 2016, **220**, 615-620. ISSN 09608524. Dostupné z: doi:10.1016/j.biortech.2016.09.010
[188] PLEISSNER, Daniel a Carol Sze Ki LIN. Valorisation of food waste in biotechnological processes. *Sustainable Chemical Processes*. 2013, **1**(1). ISSN 2043-7129. Dostupné z: doi:10.1186/2043-7129-1-21
[189] MIRABELLA, Nadia, Valentina CASTELLANI a Serenella SALA. Current options for the valorization of food manufacturing waste: a review. *Journal of Cleaner Production*. 2014, **65**, 28-41. ISSN 09596526. Dostupné z: doi:10.1016/j.jclepro.2013.10.051
[190] M. Vysoká, M. Szotkowski, K. Bradáčová, J. Holub, P. Matoušková, S. Šimanský, M. Čertík, I. Márová. *BIOCONVERSION OF LOW-COST FAT MATERIALS INTO HIGH-VALUE PUFA-CAROTENOID-RICH BIOMASS.* 34th Annual Conference on Yeasts, Book of abstracts. 2018. s. 64-64. ISSN: 1336-4839.
[191] SZOTKOWSKI, Martin, Dana BYRTUSOVA, Andrea HARONIKOVA, Marie VYSOKA, Marek RAPTA, Volha SHAPAVAL a Ivana MAROVA. Study of Metabolic Adaptation of Red Yeasts to Waste Animal Fat Substrate. *Microorganisms*. 2019, 7(11). ISSN 2076-2607. Dostupné z: doi:10.3390/microorganisms7110578
[192] O'BRIEN, Richard D. Fats and Oils Formulating and Processing for Applications. 3. USA: Taylor & Francis Group, LLC, 2009. ISBN 978-1-4200-6166-6.
[193] PROŠKOVÁ, Alexandra, Jiří KUČERA a Zdenka KOPICOVÁ. POROVNÁNÍ KYSELE A BAZICKY KATALYZOVANÉ TRANSESTERIFIKACE KAFILERNÍHO TUKU METHANOLEM. Chemické listy. 2009, 103(12), 1034-1036.
[194] ROGINSKI, Hubert, Patrick F. FOX a John W. FUQUAY. Encyclopedia of Dairy Sciences. 2. Amsterdam: Academic Press, 2003. ISBN 01-222-7239-0.
[195] FRENGOVA, Ginka, Emilina SIMOVA a Dora BESHKOVA. Use of Whey Ultrafiltrate as a Substrate for Production of Carotenoids by the Yeast <I>Rhodotorula Rubra</I>. Applied Biochemistry and Biotechnology. 2004, 112(3), 133-142. DOI: 10.1385/ABAB:112:3:133. ISSN 0273-2289. Dostupné také z: http://link.springer.com/10.1385/ABAB:112:3:133
[196] ONWULATA, Charles I. a Peter J. HUTH. Whey Processing, Functionality and Health Benefits. 1. Singapore: Fabulos Printers, 2008. ISBN 978-0-8138-0903-8.
[197] Singh, Ram. (2014). Biotechnological Approaches for Valorization of Whey.
[198] MOULIN, G. a P. GALZY. Whey, a Potential Substrate for Biotechnology. *Biotechnology and Genetic Engineering Reviews*. 1984, **1**(1), 347-374. ISSN 0264-8725. Dostupné z: doi:10.1080/02648725.1984.10647790
[199] http://www.ico.org, 2019.
[200] Getachew, A. T., Cho, Y. J., and Chun, B. S. (2018). Effect of pretreatments on isolation of bioactive polysaccharides from spent coffee grounds using subcritical water. *Int. J. Biol. Macromol.*109, 711-719

[201] Wang, H.-M. D., Cheng, Y.-S., Huang, C.-H., and Huang, C.-W. (2016). Optimization of High Solids Dilute Acid Hydrolysis of Spent Coffee Ground at Mild Temperature for Enzymatic Saccharification and Microbial Oil Fermentation. *Appl. Biochem. Biotechnol.* 180, 753-765.
[202] Choi, I. S., Wi, S. G., Kim, S.-B., and Bae, H.-J. (2012). Conversion of coffee residue waste into bioethanol with using popping pretreatment. *Bioresource Technol.* 125, 132-137.
[203] KOVALCIK, Adriana, Stanislav OBRUCA a Ivana MAROVA. Valorization of spent coffee grounds: A review. *Food and Bioproducts Processing*. 2018, 110, 104-119. ISSN 09603085. Dostupné z: doi:10.1016/j.fbp.2018.05.002
[204] Kwon, E. E., Yi, H., and Jeon, Y. J. (2013). Sequential co-production of biodiesel and bioethanol with spent coffee grounds. *Bioresour. Technol.* 136, 475-480.
[205] Obruca, S., Benesova, P., Petrik, S., Oborna, J., Prikryl, R., and Marova, I. (2014). Production of polyhydroxyalkanoates using hydrolysate of spent coffee grounds. *Process Biochem.* 49, 1409-1414.
[206] Obruca, S., Benesova, P., Kucera, D., Petrik, S., and Marova, I. (2015). Biotechnological conversion of spent coffee grounds into polyhydroxyalkanoates and carotenoids. *New Biotechnol.* 32, 569-574.
[207] Hudeckova, H., Neureiter, M., Obruca, S., Fruehauf, S., and Marova, I. (2018). Biotechnological conversion of spent coffee grounds into lactic acid. *Lett. Appl. Microbiol.* 66, 306-312.
[208] B. Maiorella, H.W. Blanch, C.R. Wilke, By-product inhibition effects on ethanolic fermentation by Saccharomyces cerevisiae, Biotechnol. Bioeng. 25 (1) (1983) 103–121.
[209] I.S. Chung, Y.Y. Lee, Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula, Biotechnol. Bioeng. 27 (3) (1985) 308–315.
[210] J.C. Parajo, H. Dominguez, J.M. Dominguez, Improved xylitol production with Debaryomyces hansenii Y-7426 from raw or detoxified wood hydrolyzates, Enzyme Microb. Technol. 21 (1) (1997) 18–24.
[211] E. Palmqvist, J.S. Almeida, B. Hahn-Hagerdal, Influence of furfural on anaerobic glycolytic kinetics of Saccharomyces cerevisiae in batch culture, Biotechnol. Bioeng. 62 (4) (1999) 447–454.
[212] L.R. Jarboe, L.A. Royce, P. Liu, Understanding biocatalyst inhibition by carboxylic acids, Front. Microbiol. 4 (2013) 272
[213] GU, Hanqi, Yuyong ZHU, Yanfang PENG, et al. Physiological mechanism of improved tolerance of Saccharomyces cerevisiae to lignin-derived phenolic acids in lignocellulosic ethanol fermentation by short-term adaptation. *Biotechnology for Biofuels*. 2019, 12(1). ISSN 1754-6834.
[214] HOPPERT, Michael. *Microscopic Techniques in Biotechnology*. Wiley, MArch 2006. ISBN ISBN: 978-3-527-60523-1.
[215] LEE, Soo Youn, Jun Muk CHO, Yong Keun CHANG a You-Kwan OH. Cell disruption and lipid extraction for microalgal biorefineries: A review. Bioresource Technology. 2017, 244, 1317-1328. DOI: 10.1016/j.biortech.2017.06.038. ISSN 09608524.
[216] POOJARY, Mahesha, Francisco BARBA, Bahar ALIAKBARIAN, Francesco DONSÌ, Gianpiero PATARO, Daniel DIAS a Pablo JULIANO. Innovative Alternative Technologies to Extract Carotenoids from Microalgae and Seaweeds. Marine Drugs. 2016, 14(11), DOI: 10.3390/md14110214. ISSN 1660-3397.
[217] WAITES M.J., MORGAN N. L., ROCKEY J.S., and HIGTON G.: Industrial Microbiology: An Introduction. 2001, Oxford: Wiley-Blackwellp. ISBN 978-0-632-05307-0
[218] Walker, J. M. (2002). The protein protocols handbook. Totowa, N.J: Humana Press.
[219] LICHTENTHALER, H.K., Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. In: Douce, R. and Packer, L. (eds.), Methods Enzymol. 148, 350-382, Academic Press Inc., New York 1987.

[220] VAN BREEMEN R. B.: Electrospray liquid chromatography-mass spectrometry of carotenoids. Analytical Chemistry, 1995. 67(13): p. 2004-2009. ISSN 0003-2700
[221] VAN BREEMEN R. B.: Liquid chromatography/mass spectrometry of carotenoids. Pure and Applied Chemistry, 1997. 69(10): p. 2061-2066. ISSN 0033-4545 (print), 1365-3075 (electronic)
[222] RIVERA, S.M. a R. CANELA-GARAYOA. Analytical tools for the analysis of carotenoids in diverse materials. Journal of Chromatography A. 2012, 1224, 1-10. DOI: 10.1016/j.chroma.2011.12.025. ISSN 00219673.
[223] SOMMER, L.. Základy analytické chemie. Vyd. 1. Brno: VUTIUM, 2000, 347 s.
[224] Schreiber, U.: Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview, pp. 279e319, in: Papageorgiou, G. C. and Govindjee. (Eds.),
[225] PRAKASH, S Bisen. *Laboratory Protocols in Applied Life Sciences*. USA: CRC Press, 2014. ISBN 978-1-4665-5314-9.
[226] RAPLEY R.; WALKER J.M.: *Molecular Biomethods Handbook*. Humana Press. Totowa, New Yersey. 1998. s. 725. ISBN 0-89603-501-8.
[227] MÁROVÁ, I.; RAPTA, M.; SZOTKOWSKI, M.; VANĚK, M.; MIKHEICHYK, N. *Screening of red yeasts for animal fat utilization and extracellular lipase production.* 34th Annual Conference on Yeasts, Book of abstracts. Institute of Chemistry, Slovak Academy of Sciences, Bratislava: Yeast Commission, 2017. s. 60-61. ISSN: 1336-4839
[228] SZOTKOWSKI, M.; MIKHEICHYK, N.; RAPTA, M.; HÁRONIKOVÁ, A.; MÁROVÁ, I. *Metabolic changes of non- conventional red yeasts grown on various substrates under oxidation stress.* 43rd Annual Conference on Yeasts - Book of Abstracts. 34th Annual Conference on Yeasts, Book of abstracts. 1. Bratislava: Chemický ústav SAV Bratislava, 2016. s. 46-46. ISBN: 1336- 4839. ISSN: 1336-4839.
[229] KOSTOVOVÁ, I.; ROUBALOVÁ, M.; POKRÝVKOVÁ, Z.; BYRTUSOVÁ, D.; SZOTKOWSKI, M.; HLAVÁČEK, V.; MÁROVÁ, I. *Improvement of lipid compound prooduction by red yeasts though random mutagenesis.* 44th Annual Conference on Yeast. Bratislava: Slovak Academy of Sciences, Bratislava, 2017. s. 58-58.
[230] *Application of physical stress factors on production characteristics of red yeasts.* 34th Annual Conference on Yeasts, Book of abstracts. 2017. ISSN: 1336-4839.
[231] *Comparison of metabolic activity, morphology and potential use of carotenogenic yeasts for production of high value products using fluorescence techniques.* Studentská konference Chemie je život. Brno: Vysoké učení technické v Brně, Fakulta chemická, Purkyňova 464/118, 612 00 Brno, 2017. s. 72-72. ISBN: 987-80-214-5488- 0.
[232] RAPTA, M.; SHAPAVAL, V.; KOHLER, A.; TAFINTSEVA, V.; SZOTKOWSKI, M.; BYRTUSOVÁ, D.; MIKHEICHYK, N.; MÁROVÁ, I. OPTIMIZATION OF SIMULTANUOUS CAROTENOID AND LIPID PRODUCTION IN RED YEAST. BOOK OF ABSTRACTS. Mikulov: Czech Society of Industrial Chemistry, 2017.
[233] HÁRONIKOVÁ, A.; BYRTUSOVÁ, D.; SZOTKOWSKI, M.; RAPTA, M.; MIKHEICHYK, N.; MÁROVÁ, I. *POTENTIAL USE OF CAROTENOGENIC YEASTS AND MICROALGAE FOR PRODUCTION OF HIGH VALUE PRODUCTS.* BOOK OF ABSTRACTS 8th International Symposium on RECENT ADVANCES IN FOOD ANALYSIS. 1. Prague: University of Chemistry and Technology, Prague, 2017. s. 339-339. ISBN: 978-80-7080-999-0.

9 ŽIVOTOPIS – CURICILUM VITAE

Jméno a příjmení: Martin Szotkowski
Datum narození: 15.09.1992
Adresa: Mosty u Jablunkova č. 592, PSČ 739 98

Vzdělání:
1998 – 2005 Základní škola v Mostech u Jablunkova

2005 – 2011 Gymnázium Komenského Třinec, Všeobecné gymnázium 6-ti leté

2011 – 2014 Vysoké učení technické v Brně, Fakulta chemická
Bakalářské studium oboru Chemie pro medicínské aplikace zakončené státní zkouškou, titul Bc. Téma bakalářské práce: Syntéza a studium nových typů difuranonových heterocyklů využitelných pro optoelektronické aplikace.

2014 – 2016 Vysoké učení technické v Brně, Fakulta chemická
Magisterské studium oboru Chemie pro medicínské aplikace zakončené státní zkouškou, titul Ing. Téma diplomové práce: Srovnání metabolické aktivity a potenciálního využití mikrořas a karotenogenních kvasinek.

2016 – současnost Vysoké učení technické v Brně, Fakulta chemická
Doktorské studium oboru Chemie potravin a biotechnologií

Zahraniční stáže:
Erasmus stáž:
Místo pobytu: University of Huelva, Biotechnological and algal group (BITAL)
Datum: 05.03.2018 – 31.07.2018
Téma: Optimalizace kultivace acidofilní mikrořasy *Coccomyxa onubensis* adaptované na solný stress.

Erasmus stáž:
Místo pobytu: University of Huelva, Biotechnological and algal group (BITAL)
Datum: 01.04.2019 – 10.08.2019
Téma: Velkoobjemové kultivace acidofilní mikrořasy *Coccomyxa onubensis* v otevřených nádržích.

10 BIBLIOGRAPHY

Published papers:

SZOTKOWSKI, M.; BYRTUSOVÁ, D.; NĚMCOVÁ, A.; VYSOKÁ, M.; RAPTA, M.; SHAPAVAL, V.; MÁROVÁ, I. Study of Metabolic Adaptation of Red Yeasts to Waste Animal Fat Substrate. *Microorganisms,* 2019, roč. 7, č. 11, s. 1-28. ISSN: 2076-2607

BYRTUSOVÁ, D.; SHAPAVAL, V.; HOLUB, J.; ŠIMANSKÝ, S.; RAPTA, M.; SZOTKOWSKI, M.; KOHLER, A.; MÁROVÁ, I. Revealing the Potential of Lipid and beta-Glucans Coproduction in Basidiomycetes Yeast. *Microorganisms,* 2020, roč. 8, č. 7, s. 1-19. ISSN: 2076-2607.

KOVALČÍK, A.; PERNICOVÁ, I.; OBRUČA, S.; SZOTKOWSKI, M.; ENEV, V.; KALINA, M.; MÁROVÁ, I. Grape winery waste as a promising feedstock for the production of polyhydroxyalkanoates and other value-added products. *FOOD AND BIOPRODUCTS PROCESSING,* 2020, roč. 124, č. 1, s. 1-10. ISSN: 0960-3085.

VANĚK, M.; MRAVEC, F.; SZOTKOWSKI, M.; BYRTUSOVÁ, D.; HÁRONIKOVÁ, A.; MÁROVÁ, I. Fluorescence lifetime imaging of red yeast Cystofilobasidium capitatum during growth. *The EuroBiotech Journal,* 2018, roč. 2, č. 2, s. 114-120. ISSN: 2564-615X.

MÁROVÁ, I.; RAPTA, M.; VANĚK, M.; HÁRONIKOVÁ, A.; SZOTKOWSKI, M. USE OF HIGH-THROUGHPUT TECHNIQUES TO STUDY SIMULTANOUS PRODUCTION OF LIPID-SOLUBLE METABOLITES IN CAROTENOGENIC YEASTS. *Journal of Biotechnology,* 2017, roč. 256S, č. 1, s. 542-543. ISSN: 0168-1656.

Manuscripts:

DZURENDOVA, SIMONA; SHAPAVAL, VOLHA; TAFINTSEVA, VALERIA; KOHLER, ACHIM; SZOTKOWSKI, MARTIN; BYRTUSOVA, DANA; MAROVA, IVANA; ZIMMERMANN, BORIS. *Assessment of fungal biomass by Fourier transform Raman spectroscopy for application in biotechnology and bioprocessing*. Manuscript.

Conference papers with full text:

MÁROVÁ, I.; SZOTKOWSKI, M.; RAPTA, M.; BYRTUSOVÁ, D.; VANĚK, M.; HÁRONIKOVÁ, A.; MIKHEICHYK, N. *Simultaneous production of lipids and pigments by red yeasts grown on animal fat using the concept of biorefinery.* Madrid, Spain: Formatex Research Centre, 2017. s. 199-202.

MÁROVÁ, I.; RAPTA, M.; SZOTKOWSKI, M.; BYRTUSOVÁ, D. Lipofungi - report. NMBU As, Norsko: NMBU As, Norsko, 2017. s. 1-17.

MÁROVÁ, I.; SZOTKOWSKI, M.; VANĚK, M. *Závěrečná zpráva o řešení inovačního voucheru - neenergetické využití pyrolýzního oleje (EVECO)*. Brno: FCH VUT Brno, 2017. s. 1-27.

SZOTKOWSKI, M.; BYRTUSOVÁ, D.; NĚMCOVÁ, A.; VYSOKÁ, M.; RAPTA, M.; SHAPAVAL, V.; MÁROVÁ, I. *Study of metabolic adaptation of red yeasts to waste animal fat substrate.* The Eurobiotech Congress 2020, Prague, September 24-26, Biotechnology and Biotehnol. Equipment 35(1), 2021.

Abstracts:

SZOTKOWSKI, M.; BYRTUSOVÁ, D.; CHRÁSTOVÁ, N.; TĚŠÍKOVÁ, K.; MATOUŠKOVÁ, P.; RAPTA, M.; MÁROVÁ, I.; ČERTÍK, M., SHAPAVAL. V. *Extracellular lipase production by red yeasts cultivated on animal waste fat.* Smolenice, Slovensko: 2019. s. 1 (1 s.).

MÁROVÁ, I.; SZOTKOWSKI, M.; VANĚK, M.; BYRTUSOVÁ, D.; RAPTA, M.; HÁRONIKOVÁ, A. PIGMENTED YEASTS AS BIOTECHNOLOGICAL FACTORIES FOR BIOPRODUCTS AND BIOFUELS. In *45th Annual Meeting on Yeasts. 34th Annual Conference on Yeasts, Book of abstracts.* 1. Bratislava: Chemický ústav SAV Bratislava, SR, 2018. s. 33-33. ISSN: 1336-4839.

Byrtusová D., Szotkowski M., Holub J., Bradáčová K., Vysoká M., Rapta M., Márová I.: TRANSFORMATION OF WASTE FAT INTO HIGH VALUABLE METABOLITES BY RED YEASTS. 45th Annual Conference on Yeast. May 15-18, 2018, Smolenice. Selected to the session Poster highlights. Book of Abstracts, p. 42-42. ISSN: 1336-4839.

RAPTA, M.; SHAPAVAL, V.; ZIMMERMANN, B.; TAFINTSEVA, V.; SZOTKOWSKI, M.; BYRTUSOVÁ, D.; MIKHEICHYK, N.; MÁROVÁ, I. THE EFFECT OF NITROGEN SOURCE ON LIPID AND CAROTENOID ACCUMULATION IN RED YEAST. 44th ANNUAL CONFERENCE ON YEAST. 44th Annual Conference on Yeasts, Book of abstracts. Bratislava: Chemický ústav SAV Bratislava, 2017. s. 62-62. ISSN: 1336-4839.

VANĚK, M.; TOMALA, L.; SZOTKOWSKI, M.; NOVÁČKOVÁ, I.; OBRUČA, S. *On Mixing and Separation During Polyhydroxyalkanoates Extraction – Yield and Purity Optimization.* 7th Meeting on Chemistry and Life 2018. Book of abstracts. 2018. ISBN: 978-80-214-5488-0.

M. Vysoká, M. Szotkowski, K. Bradáčová, J. Holub, P. Matoušková, S. Šimanský, M. Čertík, I. Márová. *BIOCONVERSION OF LOW-COST FAT MATERIALS INTO HIGH-VALUE PUFA-CAROTENOID-RICH BIOMASS.* 34th Annual Conference on Yeasts, Book of abstracts. 2018. s. 64-64. ISSN: 1336-4839.

HÁRONIKOVÁ, A.; BYRTUSOVÁ, D.; SZOTKOWSKI, M.; RAPTA, M.; MIKHEICHYK, N.; MÁROVÁ, I. *POTENTIAL USE OF CAROTENOGENIC YEASTS AND MICROALGAE FOR PRODUCTION OF HIGH VALUE PRODUCTS.* BOOK OF ABSTRACTS 8th International Symposium on RECENT ADVANCES IN FOOD ANALYSIS. 1. Prague: University of Chemistry and Technology, Prague, 2017. s. 339-339. ISBN: 978-80-7080-999-0.

KOSTOVOVÁ, I.; ROUBALOVÁ, M.; POKRÝVKOVÁ, Z.; BYRTUSOVÁ, D.; SZOTKOWSKI, M.; HLAVÁČEK, V.; MÁROVÁ, I. *Improvement of lipid compound prooduction by red yeasts though random mutagenesis.* 44th Annual Conference on Yeast. Bratislava: Slovak Academy of Sciences, Bratislava, 2017. s. 58-58.

Application of physical stress factors on production characteristics of red yeasts. 34th Annual Conference on Yeasts, Book of abstracts. 2017. ISSN: 1336-4839.

Comparison of metabolic activity, morphology and potential use of carotenogenic yeasts for production of high value products using fluorescence techniques. Studentská konference Chemie je život. Brno: Vysoké učení technické v Brně, Fakulta chemická, Purkyňova 464/118, 612 00 Brno, 2017. s. 72-72. ISBN: 987-80-214-5488- 0.

RAPTA, M.; SHAPAVAL, V.; KOHLER, A.; TAFINTSEVA, V.; SZOTKOWSKI, M.; BYRTUSOVÁ, D.; MIKHEICHYK, N.; MÁROVÁ, I. OPTIMIZATION OF SIMULTANUOUS

CAROTENOID AND LIPID PRODUCTION IN RED YEAST. BOOK OF ABSTRACTS. Mikulov: Czech Society of Industrial Chemistry, 2017.

MÁROVÁ, I.; RAPTA, M.; SZOTKOWSKI, M.; VANĚK, M.; MIKHEICHYK, N. *Screening of red yeasts for animal fat utilization and extracellular lipase production.* 34th Annual Conference on Yeasts, Book of abstracts. Institute of Chemistry, Slovak Academy of Sciences, Bratislava: Yeast Commission, 2017. s. 60-61. ISSN: 1336-4839.

SZOTKOWSKI, M.; MIKHEICHYK, N.; RAPTA, M.; HÁRONIKOVÁ, A.; MÁROVÁ, I. *Metabolic changes of non- conventional red yeasts grown on various substrates under oxidation stress.* 43rd Annual Conference on Yeasts - Book of Abstracts. 34th Annual Conference on Yeasts, Book of abstracts. 1. Bratislava: Chemický ústav SAV Bratislava, 2016. s. 46-46. ISBN: 1336- 4839. ISSN: 1336-4839.

VANĚK, M.; SZOTKOWSKI, M.; MRAVEC, F.; HÁRONIKOVÁ, A.; MÁROVÁ, I. *Fluorescence lifetime imaging of red yeast Cystofilobasidium capitatum during growth.* 43rd Annual Conference on Yeasts - Book of Abstracts. 34th Annual Conference on Yeasts, Book of abstracts. 1. Bratislava: Institute of Chemistry, Slovak Academy of Sciences, Bratislava, 2016. s. 54-54. ISBN: 1336- 4839. ISSN: 1336-4839.

MÁROVÁ, I.; HLAVÁČEK, V.; HÁRONIKOVÁ, A.; SZOTKOWSKI, M.; MIKHEICHYK, N. *Use of biorefinery concept to complex processing of waste rapeseed cake fractions for production of high- value added microbial metabolites.* Venezia: IUPAC, 2016. s. 234-234.