BRNO UNIVERSITY OF TECHNOLOGY
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CONTROLLED PRODUCTION OF LIPIDS AND LIPIDIC SUBSTANCES BY SELECTED YEASTS AND MICROALGAE

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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 hydrolyzed 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

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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][4][5]:

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.

2.1.2 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][6]. 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][7][9].

2.1.3 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 [10]-[12].

2.1.4 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.

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 [14][15]:

- 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)[14]-[16].

2.1.5 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)[18]-[20].

2.1.6 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. 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 [1]-[3].

2.1.7 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 [1]-[3].

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* [20][22]-[25].

2.2.1.1 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* [22]-[25]..

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 [22]-[25].

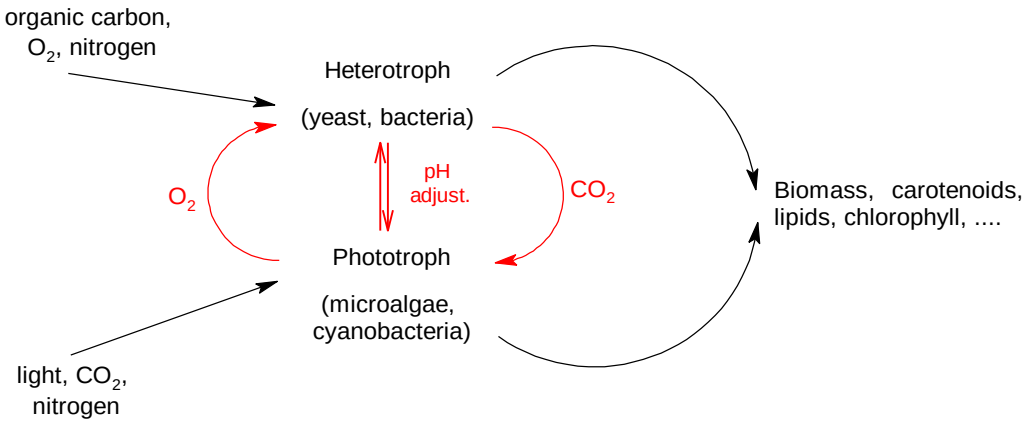
2.2.2 Algae

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. 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 [26]-[29].

2.3 Co-cultivation

The idea of a mixed cultivation (heterotroph – autotroph) 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 1). 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 [30]-[34].



Scheme 1. *Scheme 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 [30]-[34]. 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. 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 [30]-[34].

2.4 Methods for microorganism cells and biomass analysis

2.4.1 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. 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 [39]-[43].

2.4.2 HPLC/PDA

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 [39]-[43].

2.4.3 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. 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. [39]-[43].

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 1. *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. Strains were kept on agar plates at room temperature under constant illumination.

Table 2. *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 Cultivation techniques and media composition

4.2.1 Cultivation of carotenogenic yeasts and experimental design

All yeast strains were cultivated using double inoculation method. 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 was cultivated for 24 hours on a shaker and then transferred to Inoculum II with ratio 1:5 (V/V). After 24 hours, the inoculum was transferred to fresh sterile production media with the same ratio. Production media was prepared according to the procedure above. Composition of YPD media, basic production media, nitrogen sources, carbon sources, and others is listed in tables below (Table 3 and Table 4). All experiments in Erlenmeyer flasks were cultivated for 96 hours under constant irradiation at room temperature on a reciprocal shaker.

Table 3. *YPD media composition*

Media type		Components	Amount	Components	Amount
YPD solid media	YPD liquid media	Water	1000 mL	Tap water	1000 mL
		Pepton bacteriological	20 g	KH_2PO_4	4 g
		Yeast autolysate	10 g	$MgSO_4 \cdot 7H_2O$	0.696 g
		Glucose	20 g	$(NH_4)_2SO_4$	4 g
		Agar bacteriological	20 g	Glucose	23.63 g*

*C/N ratio 13

Table 4. *Nitrogen sources for yeast production media*

Nitrogen source	Amount	Carbon source	CN 13	CN 25	CN 50	CN 100
Urea	1.818 g	Glucose [g]	23.63	45.44	90.89	181.77
$(NH_4)_2SO_4$	4 g	Glycerol [g]	24.16	46.46	92.92	185.84
Coffee hydrolysate	*	Animal fat [g]	9.10	17.50	35.00	70.00
Whey hydrolysate	*	Coffe oil [g]	9.10	17.50	35.00	70.00
		Frying oil [g]	9.10	17.50	35.00	70.00

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

4.2.1.1 *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.

4.2.2 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.

4.2.2.1 *Microalgae cultivation in Brno*

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. These stock cultures were then used for inoculation of production media. All stock cultures were cultivated under constant illumination on orbital/reciprocal shakers and aerated with air.

4.2.2.1.1 Production media

Microalgae strains were cultivated in Erlenmeyer flasks and Pyrex flasks. Production media was prepared and sterilized according to the procedure mentioned above. Before inoculation, a sample of stock culture was taken under sterile conditions, checked for contamination and its absorbance at 680 nm measured. All microalgal cultivations were performed on the media listed in the table below (Table 5 and Table 6). 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.

Table 5. *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

4.2.3 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. For small scale tests, preparation of stock cultures and production media was done according to procedure above. All cultivations, except raceway pond experiment, were done in cultivation chamber under constant illumination 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photons and gently aerated with 2% CO_2 enriched air. All cultivations were performed in 500 mL Erlenmeyer's flasks, except for *B. braunii* diluted media experiment, where 1L Rouxe flasks were used.

The basic cultivation experiments were oriented to optimize media composition with the aim of enhanced carotenoid and lipid production. During culture growth, culture samples were taken regularly to determine culture growth, medium pH, and quantum fluorescence yield. Culture samples were then analysed for total carotenoid, chlorophyll, and lipid content. In experiments with different composition of one component, stock solution was prepared and added before inoculation into sterile media. All cultivations were performed in duplicate, and the results shown are the average of these duplicates.

Table 6. *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).

4.2.3.1.1 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. Stock culture of *Coccomyxa* was used to inoculate several plastic bags containing 25 L of NPK fertilizer media. The medium was prepared under non-sterile conditions with the tap water and common commercial fertilizers (Table 6). After sufficient growth, the stock culture from bags was used to inoculate open 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. Each tank was inoculated with 20 L of stock culture. Cultivation conditions are shown below (Table 7). 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 7. *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

4.2.4 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 and long 10-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 Pyrex flasks under illumination to confirm small scale experiments.
- Co-cultivation under controlled conditions in the 3L bioreactor

All cultivations and media preparations were done according to the methods described above. Standard BBM media (suitable for all chosen microalgal strains) with different nitrogen and carbon sources was used for the experiments (Table 10). In phases 1-3, cultivations were performed in Erlenmeyer flasks under constant illumination on a reciprocal shaker. Fourth phase cultivations were done in aerated Pyrex flasks. Final fifth phase was done in laboratory 3L bioreactor. Experiment scheme of each phase is briefly summarized in tables below (Table 8, Table 9 and Table 11).

Table 8. *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 9. *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	$(NH_4)_2SO_4$	$NaNO_3$	$NaNO_3$

Table 10. *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 11. *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

Table 12. *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 $\mu mol \cdot m^{-2} \cdot s^{-1}$

4.2.4.1 *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. Preparation of bioreactor vessel was done according to procedure above 4.2.1.1. A solution of 80% glycerol, which served as a carbon source, supplemented with a small antifoam addition. 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 12). 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.

4.3 Biomass and metabolite analysis

4.3.1 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.

4.3.2 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 13. 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 13. *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 %

4.3.3 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 14). All samples were measured twice and the results are reported as the average of their values.

Table 14. *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		

4.3.4 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.

4.3.5 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.

4.3.5.1 *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.

4.3.5.2 *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 [45], conference poster/abstract [44][46][51]. The summary of the main results is listed here.

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

5.1.1 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.

5.1.1.1 *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.

5.1.1.1.1 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. Hydrolyzed 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 [45]. The following paragraphs briefly summarize the results of this work.

5.1.1.1.1.1 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 [45]. 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 [45].

5.1.1.1.1.2 Biosurfactants

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 [45].

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 [45].

5.1.1.1.1.3 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 for 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 [45].

5.1.1.1.1.4 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 [45].

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 [45].

5.1.1.1.1.5 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. 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 [45].

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 [45]. 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 [45].

5.1.2 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 hydrolyzed. 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.

5.1.2.1 *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 hydrolysate.

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.

5.1.2.2 *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.

5.1.2.3 *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.

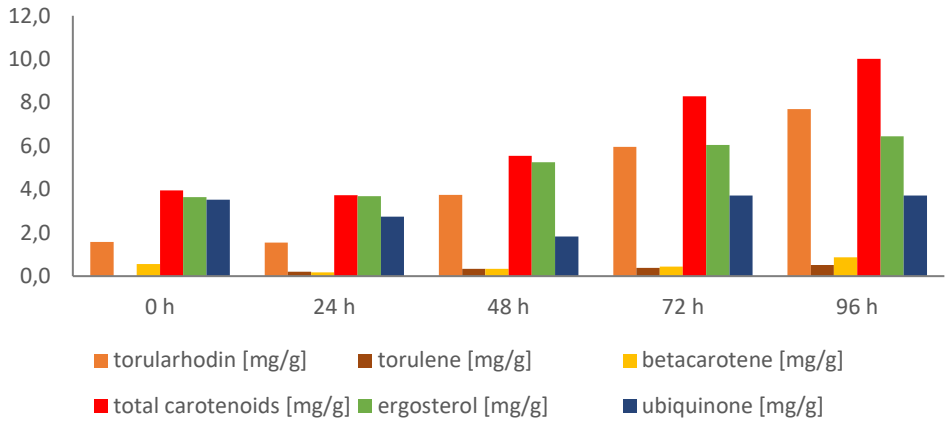
5.1.2.3.1 Rhodosporidium toruloides results:

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 15). 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.

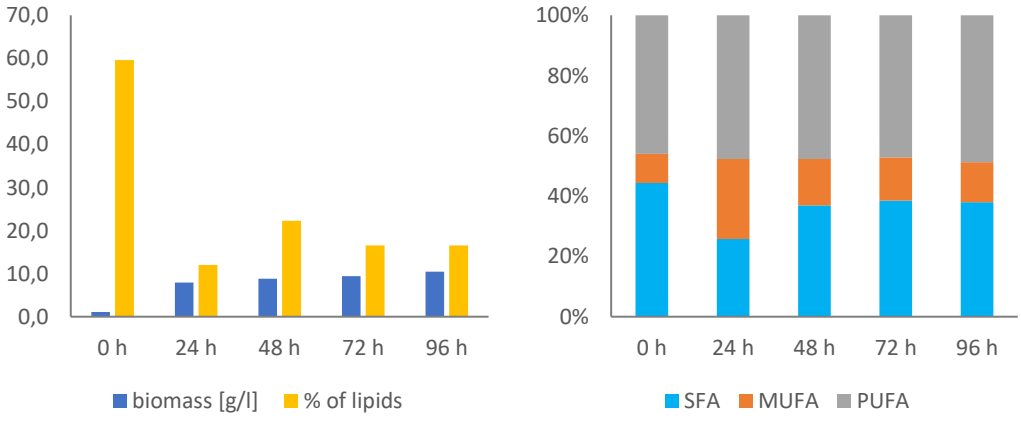
In the 96th hour of cultivation, carotenoids' production reaches 10.02 mg/g, ergosterol 6.44 mg/g and ubiquinone 3.71 mg/g dry biomass (Graph 1). From the overall trend of the obtained data, it can be assumed that by prolonging the cultivation in the case of this strain, an even higher production of all metabolites would be achieved. The profile of carotenoid pigments share the same features with *Rhodotorula* strains, 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 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 15. *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 1. *R. toruloides bioreactor cultivation on SGC hydrolysate and coffee oil: HPLC results*



Graph 2. *R. toruloides bioreactor cultivation on SGC hydrolysate and coffee oil: lipid production and fatty acid profile*

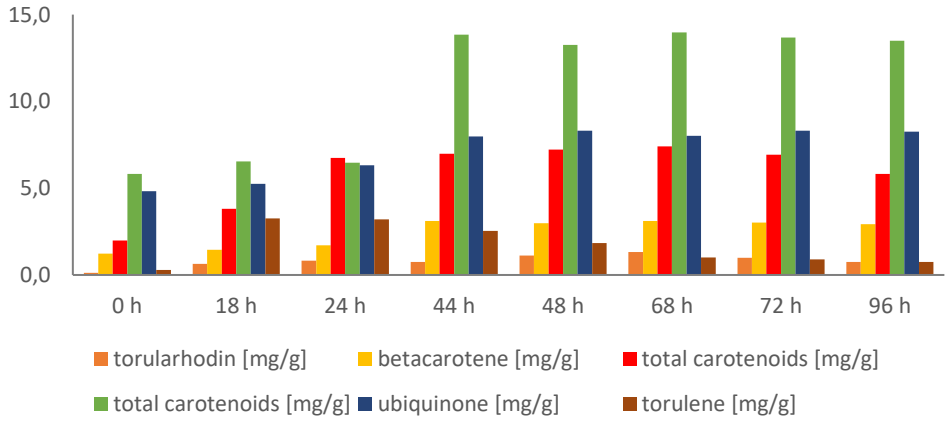
5.1.2.3.2 Sporidiobolus pararoseus results

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 16). 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 3). 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.

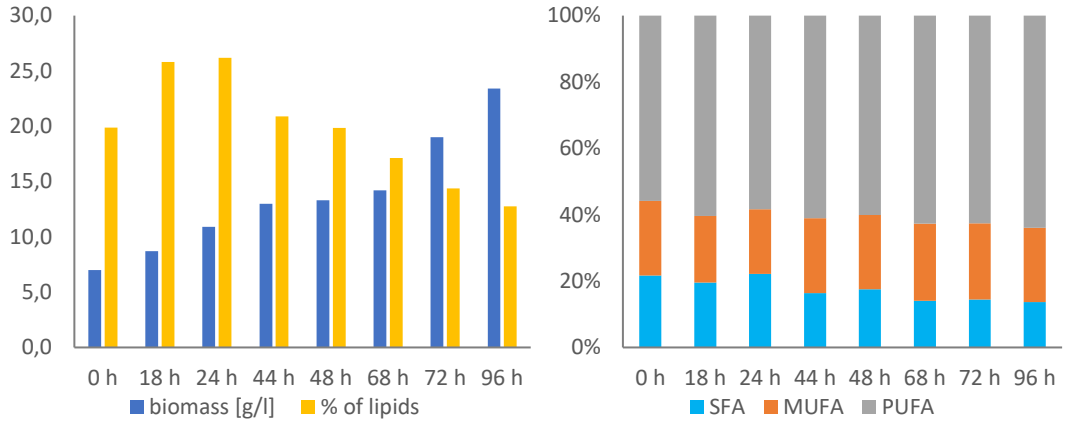
Lipid production data indicate differences from the previous four yeast strains. On the first day of cultivation we observe both the rapid growth of biomass and accumulation of lipids in the biomass with a maximum of 26.19% lipids at 24 hours (Graph 4). A linear decrease follows into 12.76% lipids in 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 16. *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 3. *S. pararoseus bioreactor cultivation on SGC hydrolysate and coffee oil: HPLC results*



Graph 4. *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).

5.1.2.4 *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.

5.1.2.4.1 Rhodotorula mucilaginosa results

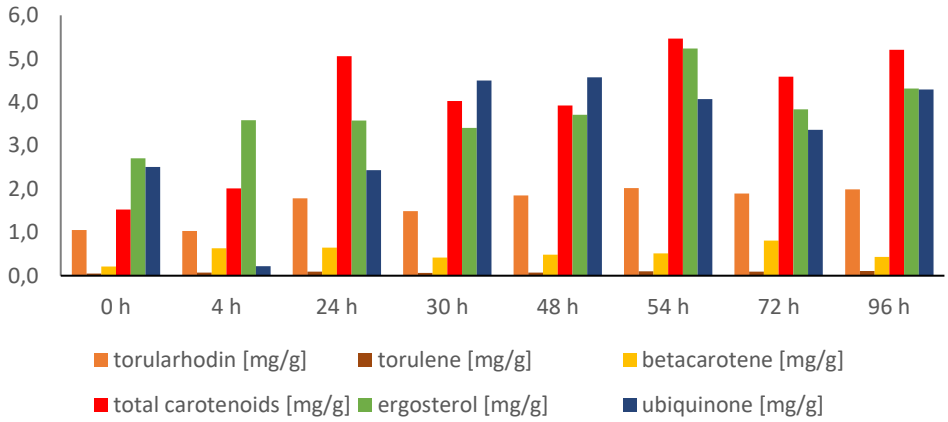
The results of cultivation with waste frying oil show much better conditions for cultivation of this yeast. Biomass production is more than 50% higher than that of coffee oil and reaches a maximum of 16.09 g/L at 96th hour of cultivation (Table 17). Here again, we see a rapid increase in biomass production 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 5). 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 6). The fatty acid profile of *R. mucilaginosa* copies the results of *R. kratochvilovae* yeast.

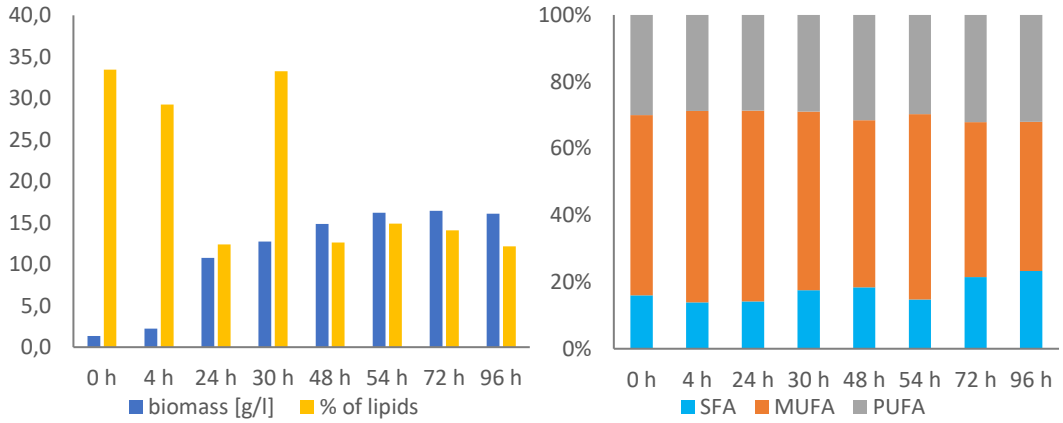
Here, too, we see a high content of monounsaturated fatty acids in waste oil, making up 45-54% of the lipid content. Again, we see a reduced SFA production, which is slightly lesser, but it increases to 23.27% in the end of cultivation. Thus, it can be said that in comparison with the results on coffee 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 17. *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 5. *R. mucilaginosa bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 6. *R. mucilaginosa bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production and fatty acid profile*

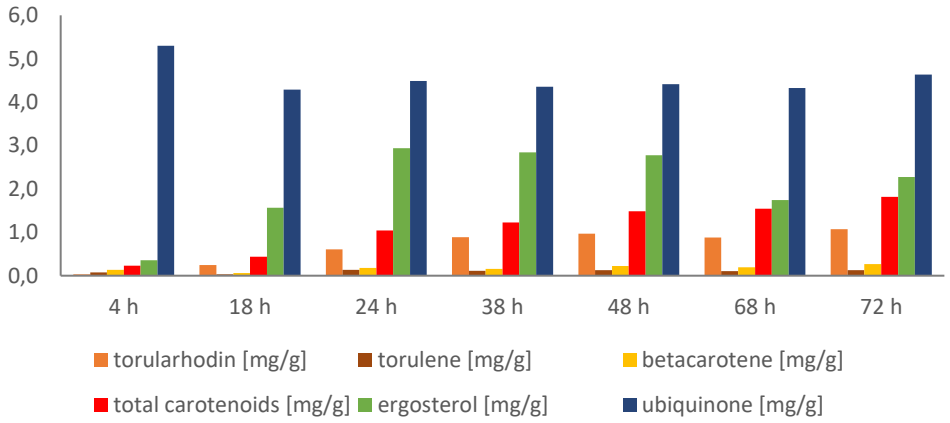
5.1.2.4.2 Cystofilobasidium macerans results

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 18). 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.

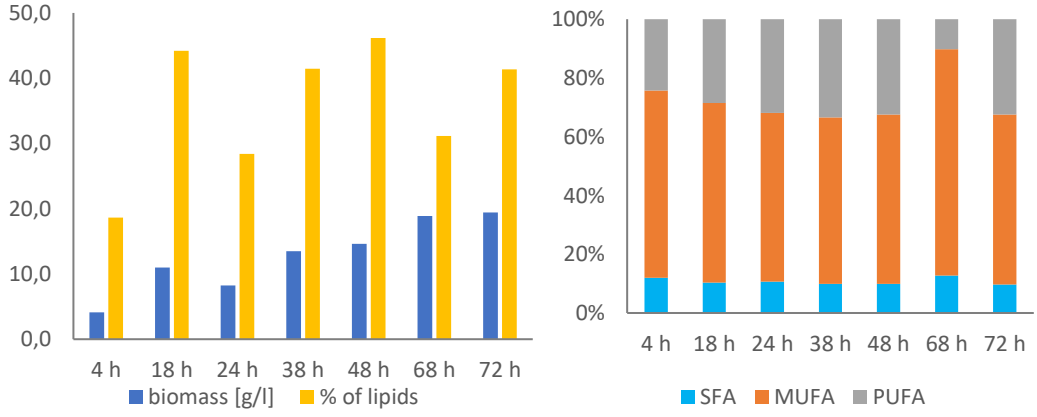
In this experiment, yeast's carotenoid content is very low and is maintained in the range of 1.5-1.7 mg/g of dry biomass, which is slightly lower than the medium with coffee oil. Likewise, ergosterol production has decreased and does not exceed 3.0 mg/g of dry biomass. (Graph 7). On the other hand, there 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. 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 8). 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 18. *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 7. *C. macerans bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 8. *C. macerans bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production and fatty acid profile*

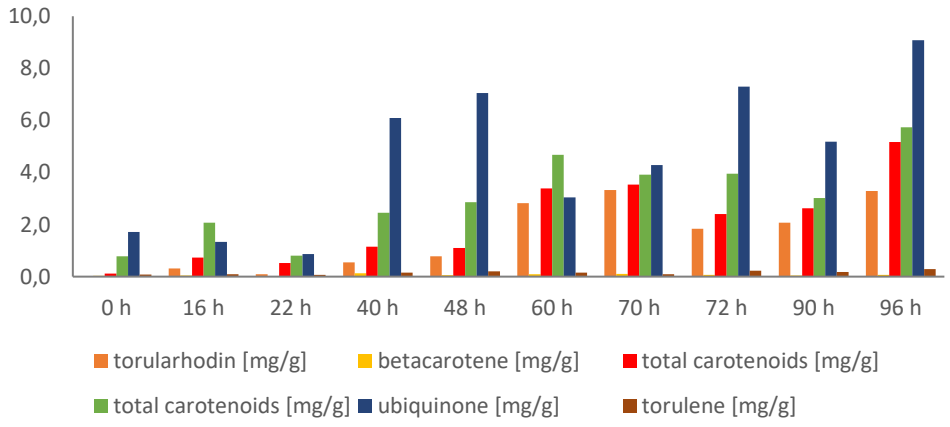
5.1.2.4.3 Sporidiobolus pararoseus results

In table 19, 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, 21.36 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 9). Otherwise, the production of ergosterol and especially ubiquinone shows a relatively large fluctuation.

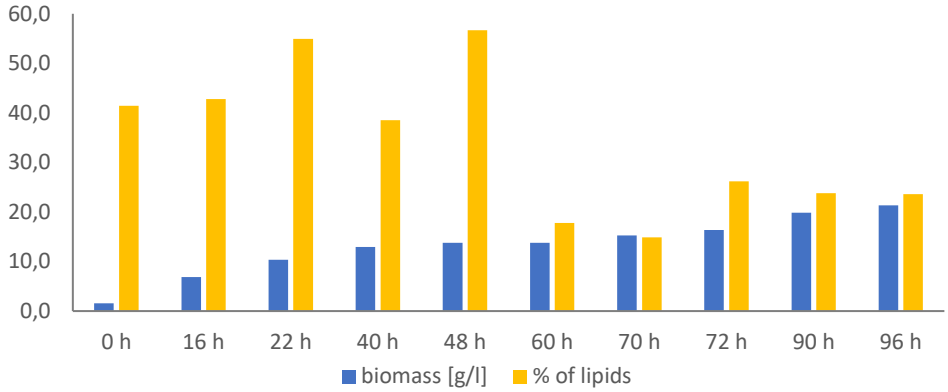
The lipid analysis results of the *S. pararoseus* strain showed a very high increase in the first 48 hours of cultivation, where the lipid content reached 56.65% of the lipids in the biomass (Graph 10). In the second half of the cultivation, there is a dramatic decrease of lipid content. At the end of 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 11). 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 19. *S. pararoseus bioreactor cultivation SCG hydrolysate + frying oil*

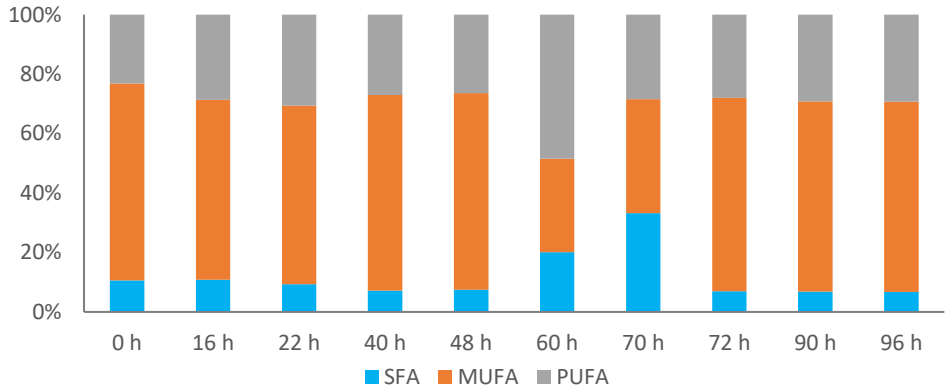
Biomass [g/L]									
0 h	16 h	22 h	40 h	48 h	60 h	70 h	72 h	90 h	96 h
1.59	6.87	10.33	12.93	13.80	13.75	15.25	16.34	19.84	21.36



Graph 9. *S. pararoseus bioreactor cultivation on SGC hydrolysate and waste frying oil: HPLC results*



Graph 10. *S. pararoseus bioreactor cultivation on SGC hydrolysate and waste frying oil: lipid production*



Graph 11. *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.

5.1.3 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.

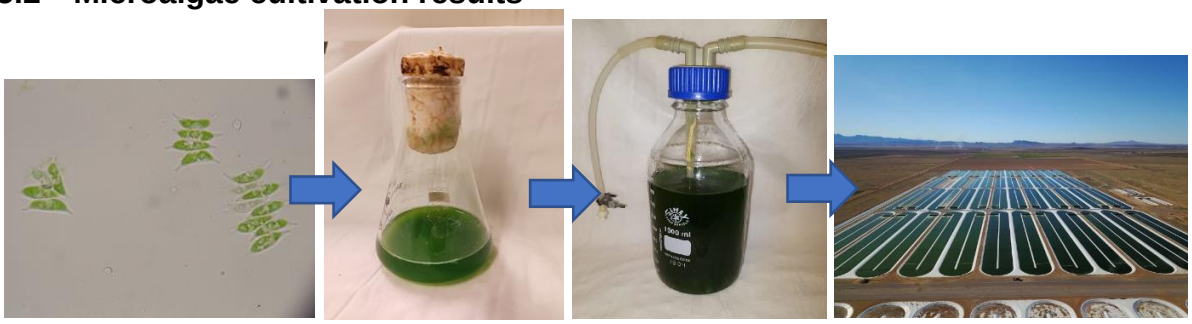
In these experiments, larger differences were observed between the tested strains. In general, the yeast strains were limited by the presence of phenolic substances and achieved lower production biomass, which was compensated by higher production of carotenoids, ergosterol and ubiquinone. Compared to 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 2. *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)

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 *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 20), 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.

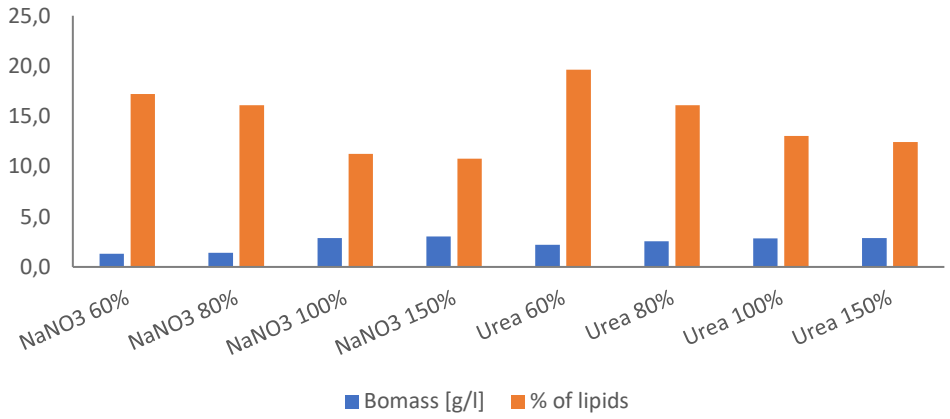
Table 20. *Multicultivator experiments: biomass production*

	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

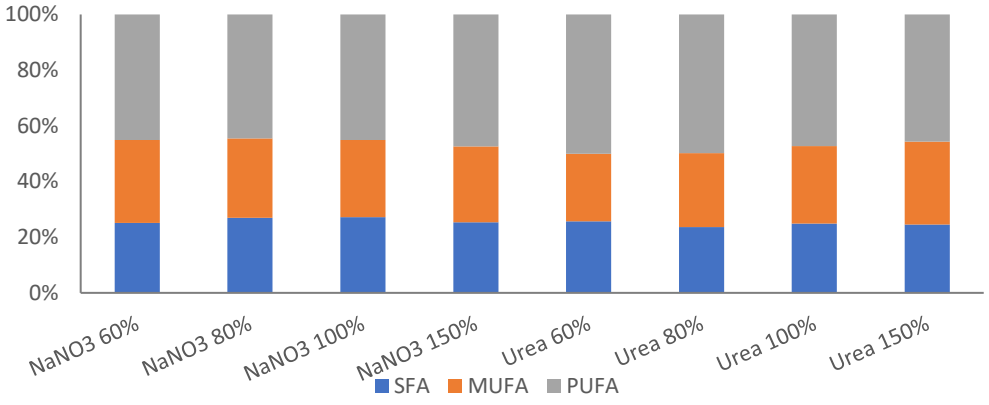
5.2.1.1.1 Desmodesmus velitaris cultivation results

The chromatographic analysis results show an apparent effect of the medium's reduced nitrogen concentration, which led to increased lipid production (Graph 12 and Graph 13). 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.

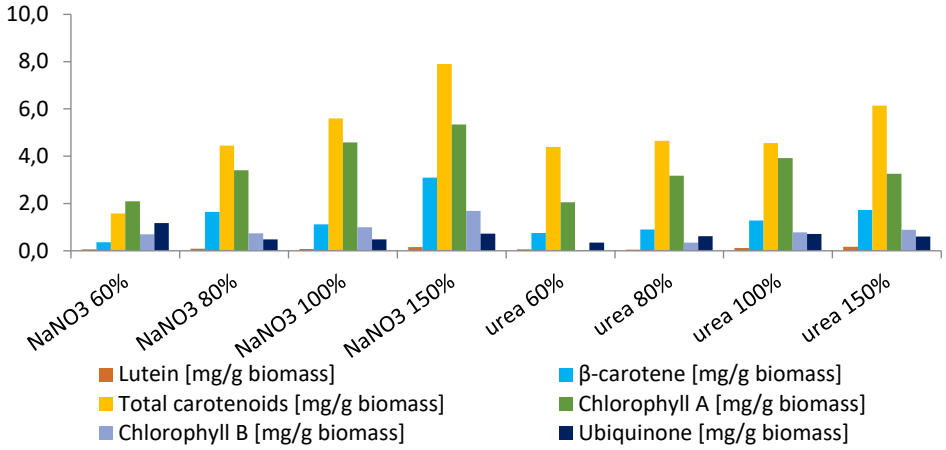
The results of the HPLC analysis of the pigments copy the results of biomass production. We see that with increasing nitrogen concentration in the medium, monitored pigments' level increases. Here, too, the highest production is achieved on a medium with 150% $NaNO_3$, namely 7.90 mg/g carotenoids and approximately 7 mg/g dry of chlorophylls (Graph 14). 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 analysed.



Graph 12. *Desmodesmus velitaris multicultivator cultivation on urea and sodium nitrate: lipid production results*



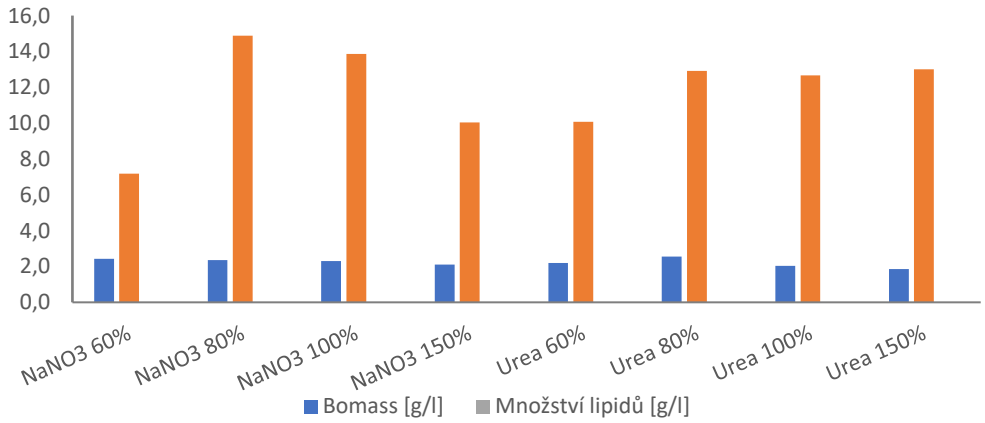
Graph 13. *Desmodesmus velitaris multicultivator cultivation on urea and sodium nitrate: fatty acid profile results*



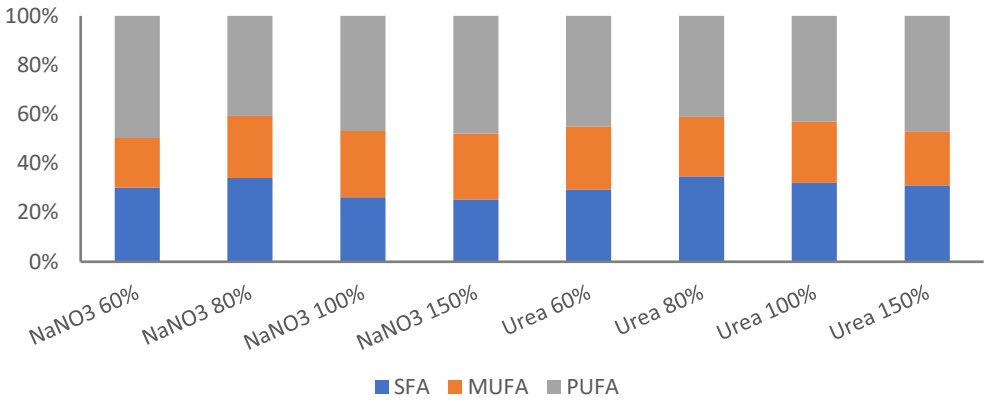
Graph 14. *Desmodesmus velitaris multicultivator cultivation: HPLC analysis results*

5.2.1.1.2 Desmodesmus armatus cultivation results

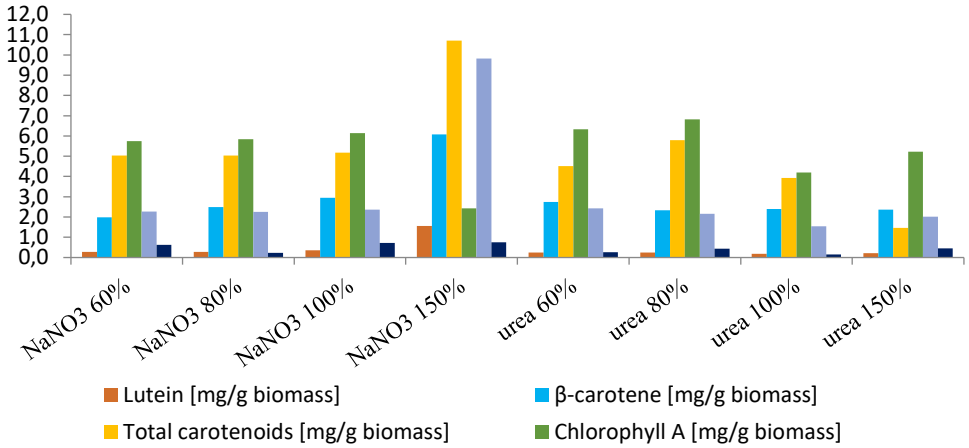
The chromatographic analysis results shown in the graphs below show a particular trend (Graph 15 and Graph 16). In media with 60% nitrogen, this algae produces the least amount of lipids, due to the unusually high growth rate. The maximum of lipid production is reached here only in media with 80% nitrate 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 (Graph 17). 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 15. *Desmodesmus armatus multicultivator cultivation on urea and sodium nitrate: lipid production results*



Graph 16. *Desmodesmus armatus multicultivator cultivation on urea and sodium nitrate: fatty acid profile results*



Graph 17. *Desmodesmus armatus multicultivator cultivation: HPLC analysis results*

5.2.1.2 *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 experiment sonclusions*

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. 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 obtained.

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 table 7. 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. Next chapters show the main results of biomass, pigment and lipid analysis.

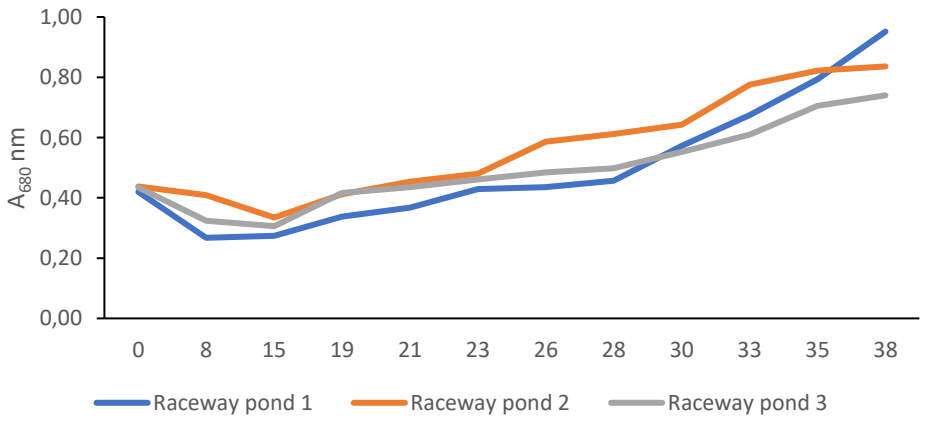
5.2.2.2.1 Biomass production and growth kinetics in different raceway ponds.

Table 21Table 21 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 21. *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

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 18). 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.



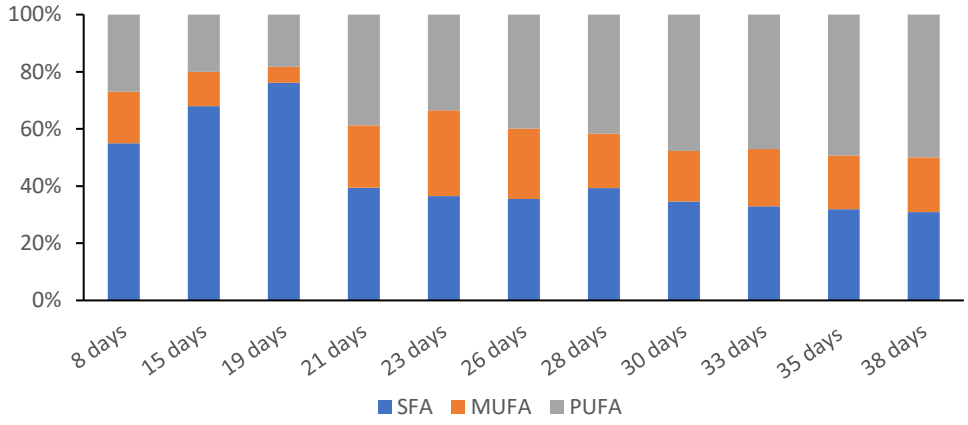
Graph 18. *The absorbance A_{680} of Coccomyxa cultures measured throughout the experiment*

5.2.2.2.2 Lipid production and fatty acid composition

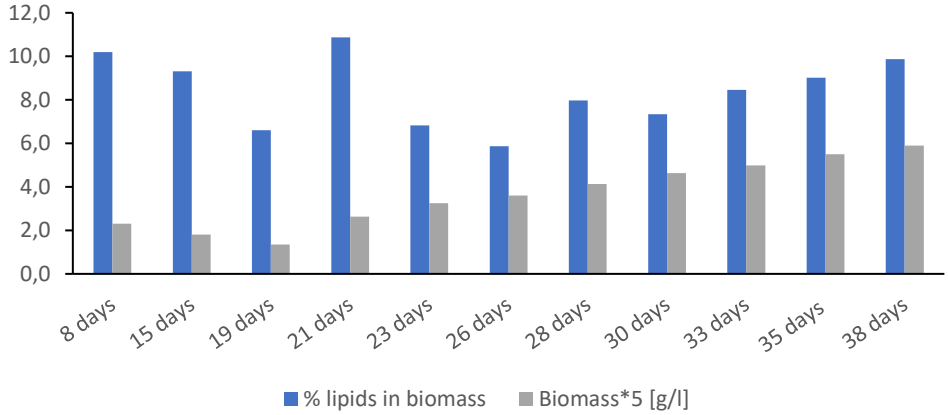
As mentioned in the introductory chapters, microalgae's lipids can best be used in the food and pharmaceutical industries. During the experiment, biomass samples were subjected to GC analysis to determine the fatty acid profile and total lipid production. Microorganisms maintain an optimal fatty acid 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.

5.2.2.2.2.1 Raceway pond 1

In the case of cultivation in the first pond (Graph 19), 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 20), 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.



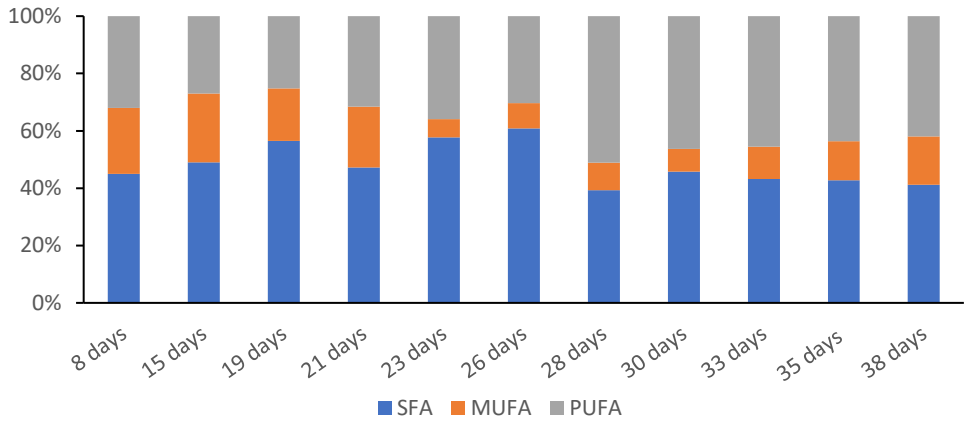
Graph 19. *Coccomyxa onubensis: Fatty acid profile of raceway pond 1 cultivation*



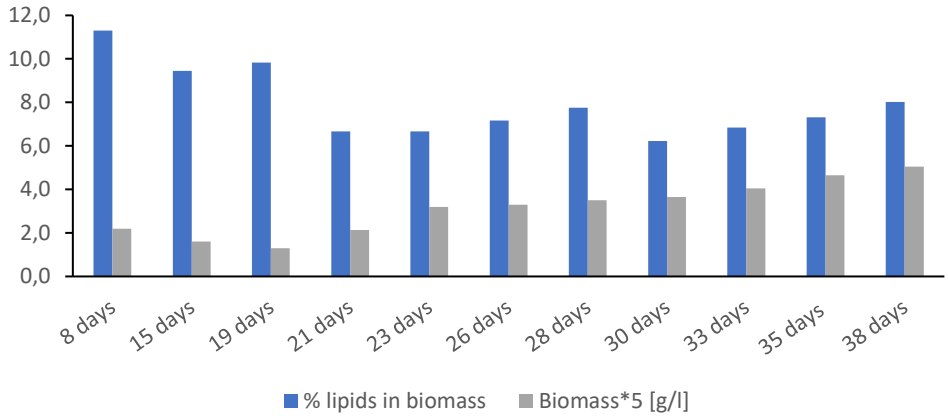
Graph 20. *Coccomyxa onubensis raceway pond 1 cultivation: % of lipids in biomass*

5.2.2.2.2.2 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 (Graph 21). 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 22). 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 21. *Coccomyxa onubensis: Fatty acid profile of raceway pond two cultivation*

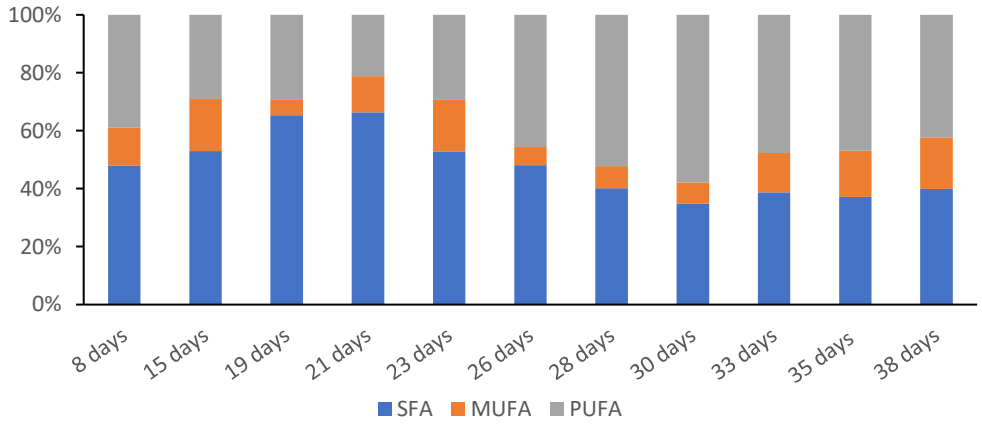


Graph 22. *Coccomyxa onubensis raceway pond two cultivation: % of lipids in biomass*

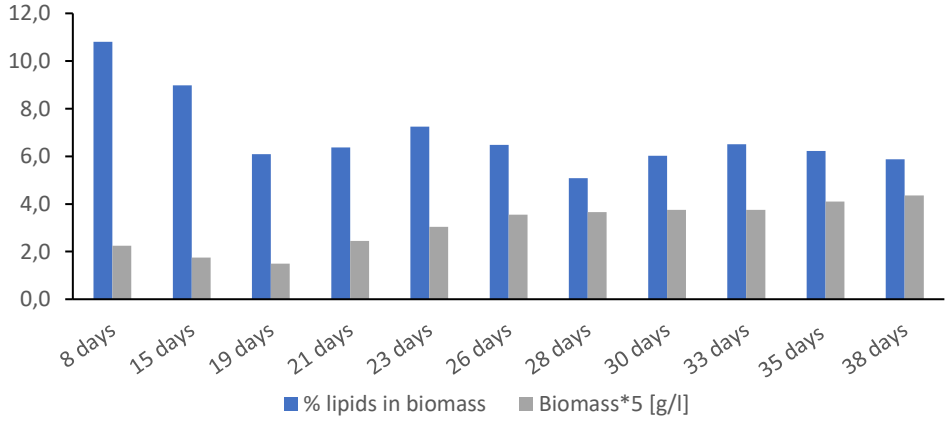
5.2.2.2.2.3 Raceway pond 3

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% (Graph 23). 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 24).



Graph 23. *Coccomyxa onubensis: Fatty acid profile of raceway pond three cultivation*



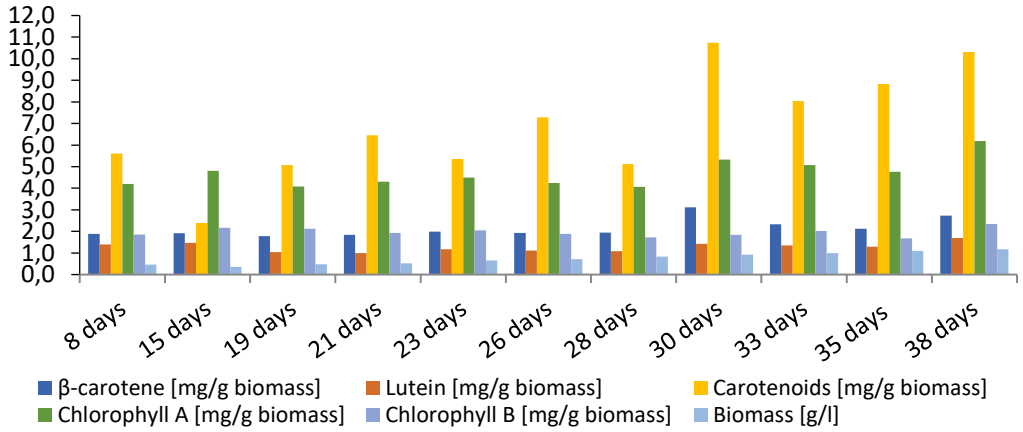
Graph 24. *Coccomyxa onubensis raceway pond three cultivation: % of lipids in biomass*

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.

5.2.2.2.3 Pigment production

5.2.2.2.3.1 Raceway pond 1

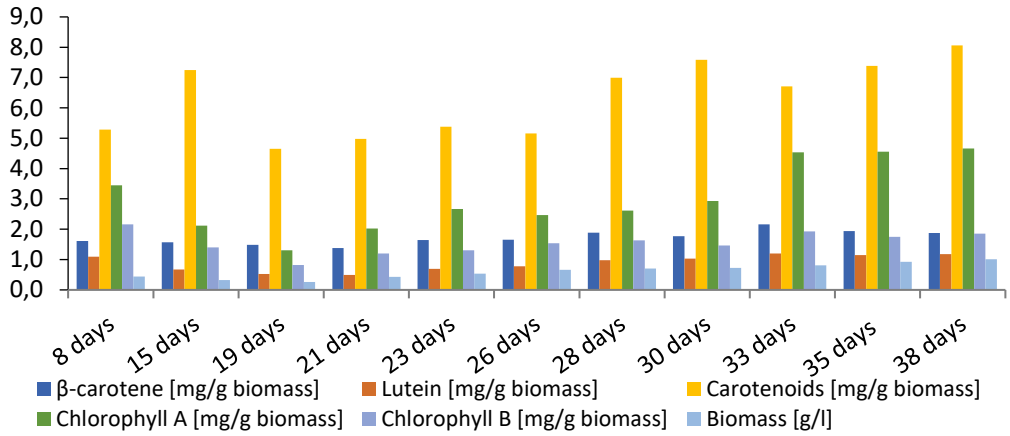
The following graph below shows the chromatographic analysis of pigments produced by the microalgae *Coccomyxa onubens*is cultivated in raceway pond one. We see a drop from 7.93 to 4.58 mg/g of dry biomass in the second week of cultivation in carotenoid production (Graph 25). Furthermore, during cultivation, 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 25. *Raceway pond 1: HPLC analysis results*

5.2.2.2.3.2 Raceway pond 2

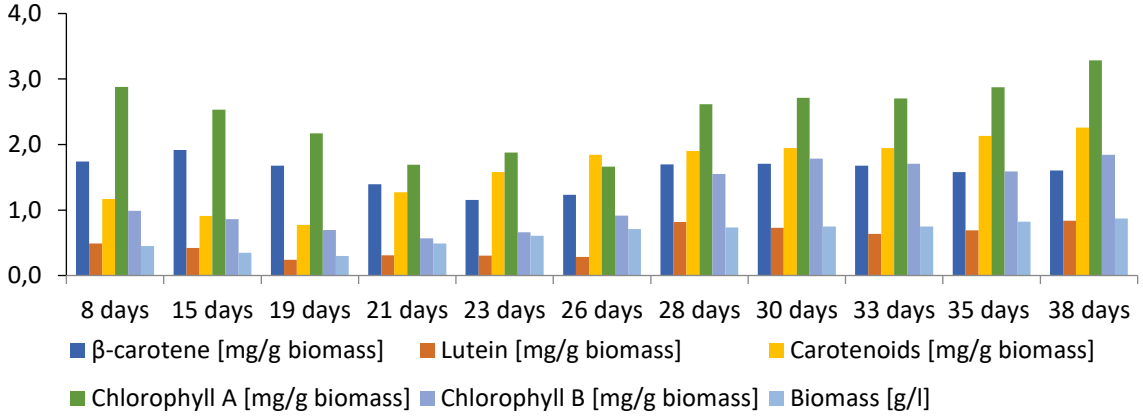
The analysis results from the second pond (Graph 26) show a direct effect of the concentration of nutrients in the medium. Half of the medium's nutrient content compared to the first culture had a negative effect on all observed pigments' production. The highest production of carotenoids 8.07 mg/g of 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 26. *Raceway pond 2: HPLC analysis results*

5.2.2.2.3.3 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 27) 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 27. *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.

5.2.2.2.4 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 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 GC and HPLC analysis are shown in graphs. The first graph describes the % composition of fatty acid groups (SFA, MUFA, PUFA).

Scheme 3. *Co-cultivation process graphical abstract*

5.3.1 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.

5.3.2 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 analysed. 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).

5.3.2.1 *Bioreactor co-cultivations of Rhodosporidium toruloides*

5.3.2.1.1 Bioreactor co-cultivations of Rhodotorula kratochvilovae + Desmodesmus quadricauda C/N = 100 – first trial

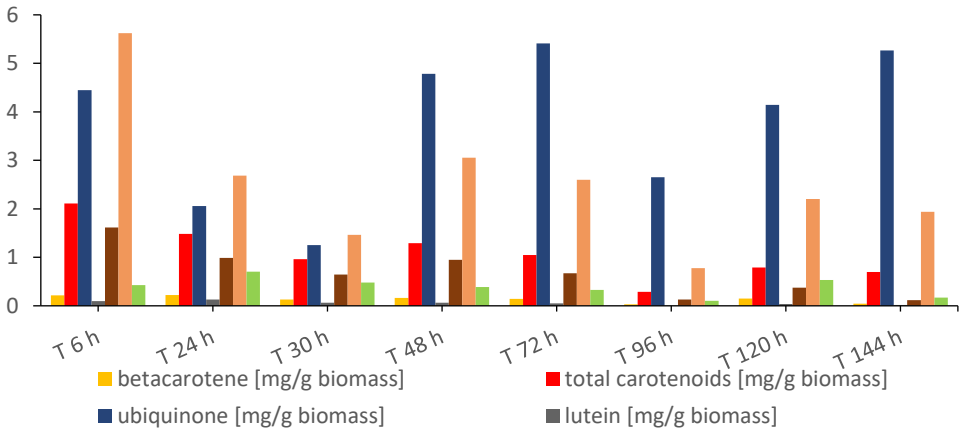
The first bioreactor experiment was co-cultivation of *R. kratochvilovae* and *D. quadricauda* in BBM media with glycerol + urea on C/N ratio 100. The table below (Table 22) shows the biomass production of a co-cultivation experiment.

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 28) 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 22. *Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. quadricauda – first trial*

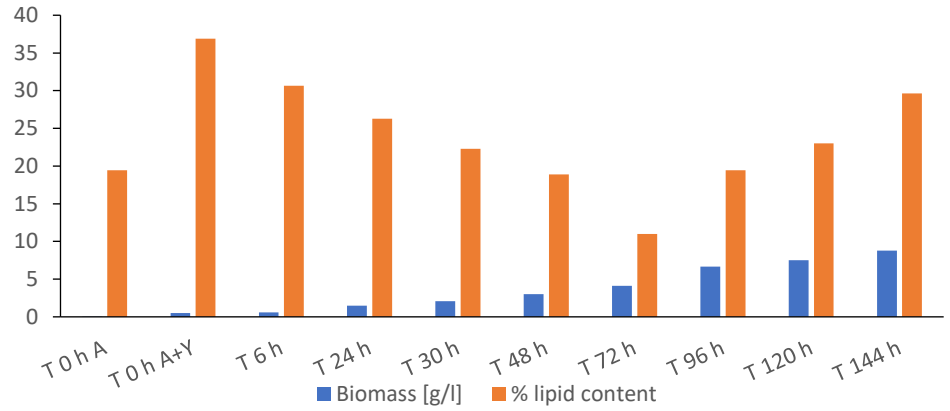
Cultivation time [hours]										
Sample name	T 0H A	T 0h A+Y	T 6H	T 24h	T 30h	T 48h	T 72h	T 96h	T 120h	T 144h
Biomass [g/L]	0.05	0.50	0.60	1.50	2.07	3.00	4.13	6.66	7.53	8.78



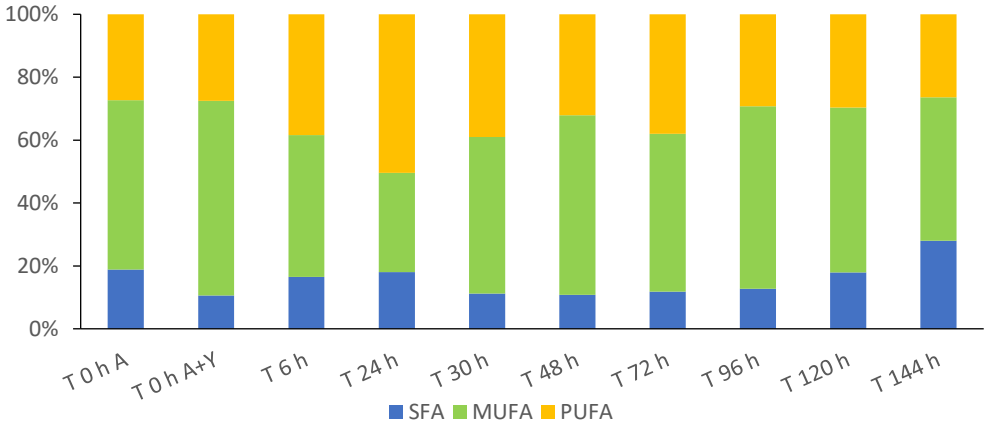
Graph 28. *Bioreactor co-cultivation R. kratochvilovae + D. quadricauda HPLC results – first trial*

From the graphs of lipid production (Graph 29 and Graph 30), 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 29. *Total lipid production in bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus quadricauda – first trial*



Graph 30. *Fatty acid profile of bioreactor co-cultivation of Rhodotorulak with Desmodesmus quadricauda - second trial*

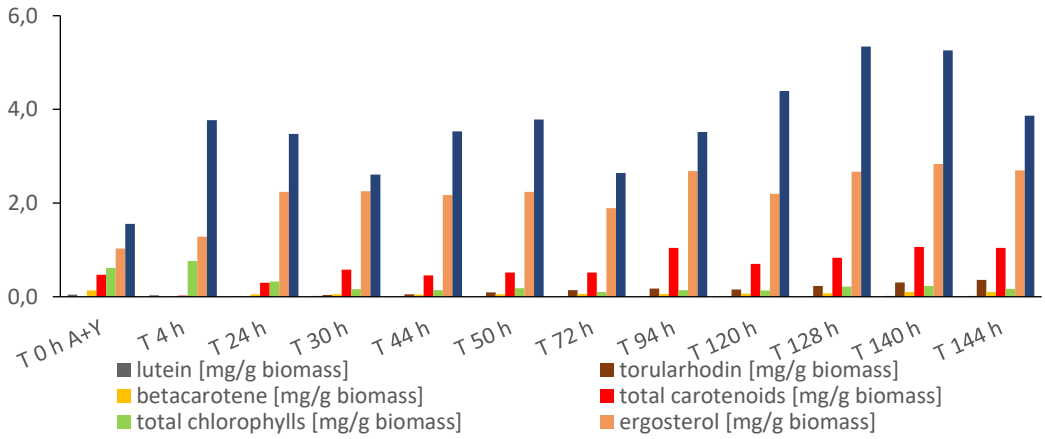
5.3.2.1.2 Bioreactor co-cultivation Rhodotorula kratochvilovae + Desmodesmus quadricauda C/N = 100 – second trial

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 (Table 23). 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 (Graph 31). 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.

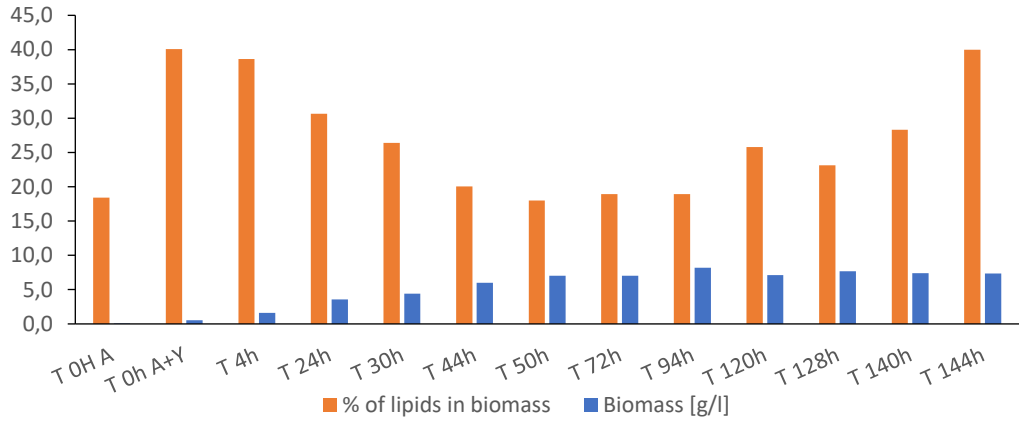
Table 23. *Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. quadricauda – second trial*

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

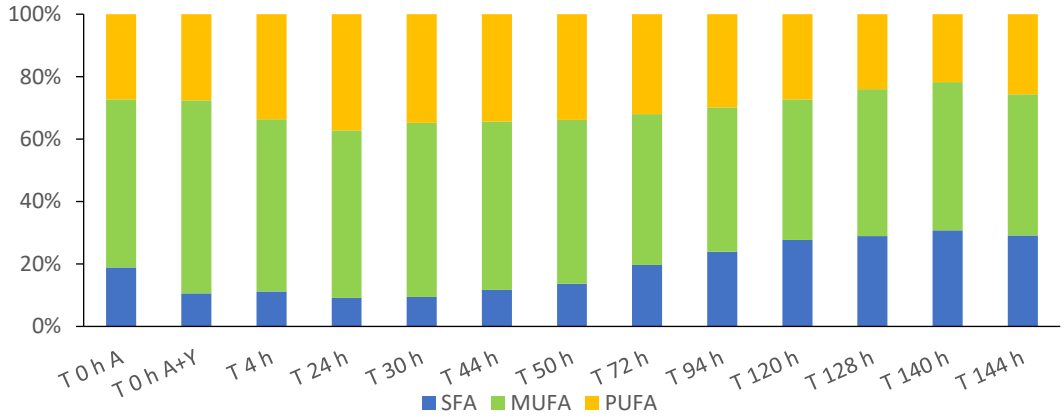


Graph 31. *Bioreactor co-cultivation R. kratochvilovae + D. quadricauda HPLC results – second trial*

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 32), 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 33), 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 32. *Total lipid production in bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus quadricauda – second trial*



Graph 33. *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.

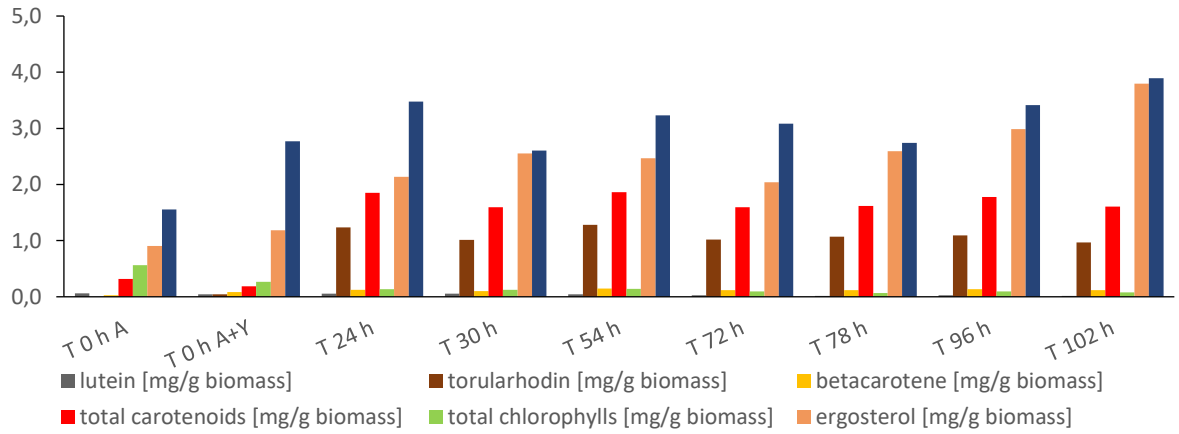
5.3.2.1.3 Bioreactor co-cultivation Rhodotorula kratochvilovae + Desmodesmus quadricauda C/N = 25

Table 24 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 dry biomass (Graph 34). 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 24. *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	3.77	4.85	6.03	6.08	6.78	7.35	6.63



Graph 34. *Bioreactor co-cultivation R. kratochvilovae + D. quadricauda C/N=25 HPLC results*

5.3.2.1.4 Bioreactor co-cultivation Rhodotorula kratochvilovae + Desmodesmus dimorphus C/N=100

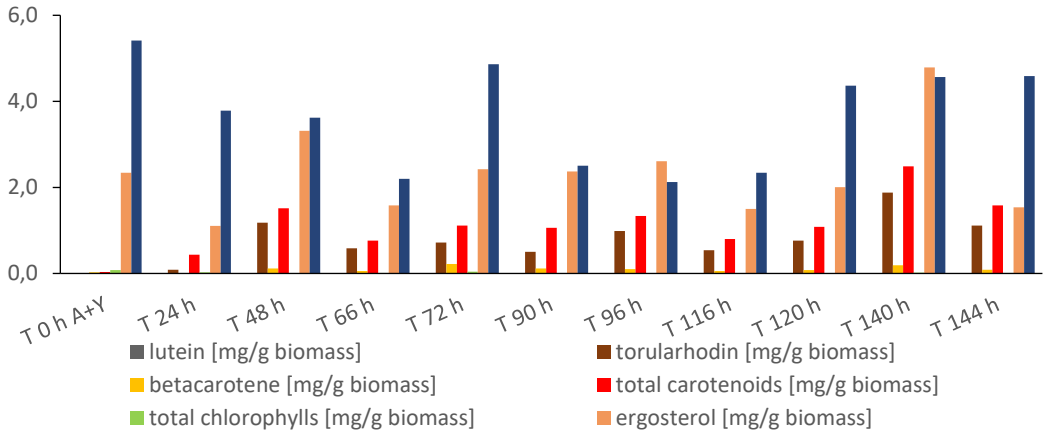
Biomass production in this co-cultivation reached the absolute highest values of all tests performed. At the beginning of cultivation, we see a rapid increase in biomass by the 66th hour of cultivation, slowing down slightly but still growing. In the last sample, the production reaches 11.28 g/L. It 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 (Graph 35). 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 36 and Graph 37) 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%.

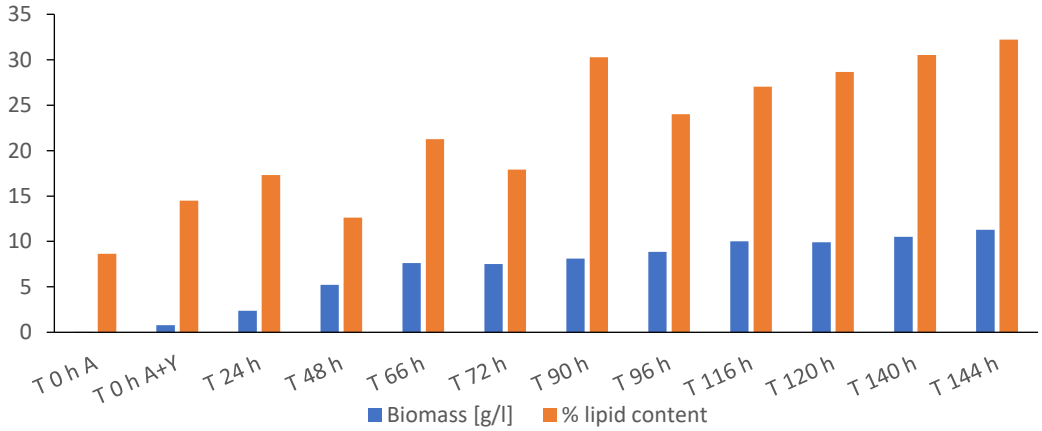
After the first day of cultivation, the fatty acid profile stabilised and remained virtually unchanged until the end of the cultivation. At the last sample, the SFA content jumped to 62% at the expense of PUFA production. The best harvest time for achieving maximum enrichment of biomass, both with lipids 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 25. *Biomass production: Bioreactor co-cultivation R. kratochvilovae + D. dimorphus C/N=100*

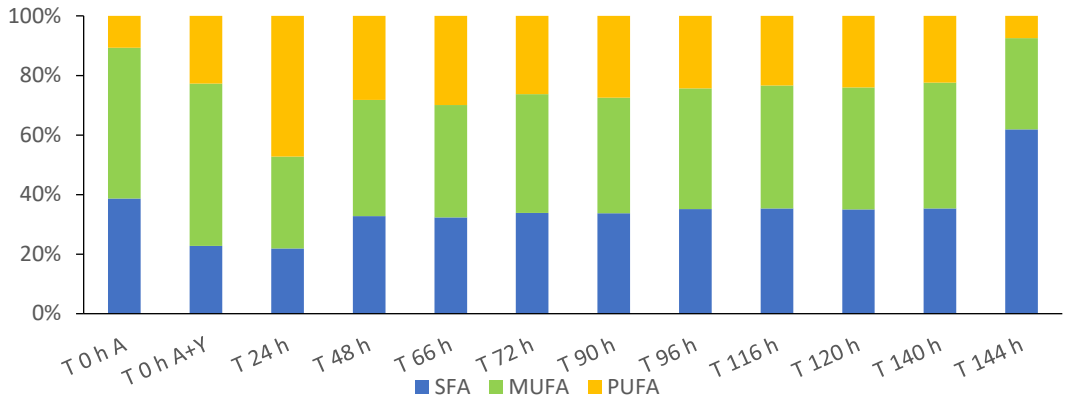
Cultivation time [hours]												
Sample name	T 0 h A	T 0 h A+Y	T 24 h	T 48 h	T 66 h	T 72 h	T 90 h	T 96 h	T 116 h	T 120 h	T 140 h	T 144 h
Biomass [g/L]	0.05	0.79	2.36	5.22	7.63	7.52	8.11	8.84	10.00	9.89	10.50	11.27



Graph 35. *Bioreactor co-cultivation R. kratochvilovae + D. dimorphus C/N=100 HPLC results*



Graph 36. *Total lipid production in bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus dimorphus*



Graph 37. *Fatty acid profile of bioreactor co-cultivation of Rhodotorula kratochvilovae with Desmodesmus dimorphus*

5.3.3 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 *Desmodesmus* 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 unhydrolyzed 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

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9 ŽIVOTOPIS – CURICILUM VITAE

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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.

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