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ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

USE OF SOME MOLECULAR TECHNIQUES TO METABOLIC CHARACTERIZATION OF INDUSTRIALLY SIGNIFICANT YEASTS

VYUŽITÍ VYBRANÝCH MOLEKULÁRNÍCH METOD K METABOLICKÉ CHARAKTERIZACI PRŮMYSLOVĚ VÝZNAMNÝCH KVASINEK

DOCTORAL THESIS
DIZERTAČNÍ PRÁCE

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Use of some molecular techniques to metabolic characterization of industrially significant yeasts

Doctoral Thesis:

The dissertation thesis is focused on the production of valuable lipidic compounds using carotenogenic yeasts. To gain enhanced amounts of these compounds, the impact of cultivation conditions, mutagens and yeast strain diversity were employed.

The main aims:

• The impact of phenotype to production properties of carotenogenic yeasts
• The impact of carbon/nitrogen ratio on lipid metabolism and lipid droplets formation
• The impact of inhibitory compounds on carotenoid metabolism
• Employment of genetic transformation methods and random mutagenesis for enhanced metabolite production
• Variability of metabolite production among various species of carotenogenic yeasts.

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ABSTRACT

Carotenoids, ergosterol and fatty acids are valuable compounds used in food, feed and cosmetic industries. Since their conventional sources depend on weather conditions, season, geographical location, and availability of agricultural land, it is necessary to increase its availability for constantly increasing demand. Microbial production of aforementioned compounds exploiting carotenogenic yeast is promising due to their extraordinary capability to simultaneously produce carotenoids, ergosterol and fatty acids.

Hereby Doctoral Thesis was aimed to provide the molecular and metabolic characterisation of various carotenogenic yeasts and their potential for industrial applications. For this purpose, the first experimental parts are focused in great detail on yeast species *R. mucilaginosa* and *R. toruloides,* their production properties and the influence of nutritional stress and different carbon sources, such as xylose and glycerol. Besides the detailed characterization of their production properties, were these strains also characterized by molecular methods, including sequence analysis of ITS1, ITS2 and D1/2 of the ribosomal operon and analysis of mini and microsatellite sequences M13 and GTG5.

R. toruloides is known as an excellent producer of fatty acids and because of it, it recently became a target carotenogenic yeast for the development of tools for its genetic manipulation. In this work were successfully prepared genetically modified clones of *R.toruloides* bearing over-expressed genes for DGA1 (diacylglycerol acyltransferase) and GPD1 (glycerol-3-phosphate dehydrogenase). Their production of fatty acids was not higher in comparison to the original strain. Therefore, the next part was focused on the preparation of overproducing mutant strains prepared by random mutagenesis. The combination of nitrogen limitation and the carotenoid production inhibitor diphenylamine led to the successful selection of carotenoid overproducing robust mutant strains with resistance to diphenylamine.

The last part of the thesis deals with the production properties of lesser known carotenogenic yeast species among order Sporidiobolales and Cystofilobasidales in comparison to relatively well studied carotenogeic yeast species represented by *R. toruloides* and *P. rhodozyma*. The best fatty acid-producing strains were *S. metaroseus* CCY19-6-20 and *C. macerans* CCY 10-01-02. Moreover, the best carotenoid producer, *R.mucilaginosa* CCY 19-04-06, naturally produced lycopene in amounts representing more than 80 % of total carotenoids.

KEYWORDS

Carotenogenic yeasts, carotenoids, ergosterol, fatty acids, genetic modification, random mutagenesis, Sporidiobolales, Cystofilobasidiales, *Rhodotorula*, *Sporobolomyces*, diphenylamine, EMS, MMS, UV-C

ABSTRAKT

Karotenoidy, ergosterol a mastné kyseliny jsou velmi žádané látky využívané v krmivářském, potravinářském a kosmetickém průmyslu. Konvenční zdroje mastných kyselin a karotenoidů jsou závislé na sezónních podmínkách, geografické poloze a na dostupnosti zemědělské půdy, což znesnadňuje pokrýt jejich neustále se zvyšující spotřebu. Velmi slibným řešením je mikrobiální produkce výše uvedených látek pomocí karotenogenních kvasinek, které jsou schopny simultánně produkovat karotenoidy, mastné kyseliny i ergosterol.

Předložená disertační práce je zaměřená na molekulární a metabolickou charakterizaci karotenogenních kvasinek a na jejich potenciál pro průmyslové aplikace. Proto první experimentální části práce jsou zaměřeny na kvasinky druhu *R. mucilaginosa* a *R. toruloides*, jejich produkční vlastnosti, vliv nutričního stresu a různých zdrojů uhlíku, jakými byly xylóza a glycerol. Kromě podrobné charakterizace jejich produkčních vlastností, byly tyto kmeny také charakterizovány molekulárními metodami, zahrnující sekvenční analýzu ITS1, ITS2 a D1/2 ribozomálního operonu a analýzu mini a mikrosatelitních sekvencí M13 a GTG5.

Druh *R. toruloides* je známý jako vynikající producent mastných kyselin, a proto se v poslední době stal cílovou karotenogenní kvasinkou pro vývoj nástrojů pro jeho genetickou manipulaci. V této práci byly úspěšně připraveny geneticky modifikované klony kmene *R. toruloides*, nesoucí nadměrně exprimované geny pro diacylglycerol acyltransferázu (DGA1) a glycerol-3-fosfát dehydrogenázu (GPD1). Produkce mastných kyselin u modifikovaných klonů nebyla ve srovnání s původním kmenem vyšší. Proto byla další část práce zaměřená na přípravu nadprodukčních mutantních kmenů připravených náhodnou mutagenezí. Kombinace limitace dusíkem a inhibice produkce karotenoidů vedla k úspěšné selekci robustních mutantních kmenů s nadprodukcí karotenoidů vykazující rezistenci vůči difenylaminu.

Poslední část práce se zabývá produkčními vlastnostmi méně známých druhů karotenogenních kvasinek náležící do řádů Sporidiobolales a Cystofilobasidales, ve srovnání s relativně dobře prostudovanými karotenogenními druhy *R. toruloides* a *P.rhodozyma*. V této studii byly nejlešími producenty mastných kyselin kmeny *S.metaroseus* CCY 19-6-20 a *C. macerans* CCY 10-01-02. Nejlepší producent karotenoidů, kmen *R. mucilaginosa* CCY 19-04-06, navíc produkoval lykopen, který představoval více než 80 % celkového množství karotenoidů produkovaných tímto kmenem.

KLÍČOVÁ SLOVA

Karotenogenní kvasinky, karotenoidy, ergosterol, mastné kyseliny, genetická modifikace, náhodná mutageneze, Sporidiobolales, Cystofilobasidiales, *Rhodotorula*, *Sporobolomyces*, difenylamin, EMS, MMS, UV-C

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DECLARATION

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

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student's signature

Poděkování:
Na tomto místě bych chtěla poděkovat vedoucí mé disertační práce, prof. RNDr. Ivaně Márové, CSc., za odborné vedení a cenné rady v průběhu doktorského studia. Také bych chtěla poděkovat Jean-Macovi Nicaudovi a Rémimu Dulermovi, za jejich velkou ochotu, pomoc a zkušenosti, které jsem získala při pobytu ve Francii v týmu BimLip (BimLip, INRA, France). Velmi ráda bych také chtěla poděkovat Klausovi Natterovi, Govindprasadu Bhutada a Martinu Kavščekovi, kteří mě toho spoustu naučili a byli se mnou velmi trpěliví.
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CONTENTS

1 INTRODUCTION

Yeasts are ubiquitous unicellular eukaryotic organisms defined as fungi. Traditional uses of yeasts are connected to their ability to ferment many food products such as beer, ciders, wines, distilled spirits, bakery products, cheese, sausages, and other fermented foods. With increasing knowledge in the fields of biotechnology, microbiology, and molecular biotechnology also increased the potential of yeast as a source of industrially important chemicals. Carotenogenic yeast differ from the traditional concept of yeasts as organisms serving for food and beverage fermentation, because generally they are not capable of fermentation. The inability to ferment is in carotenogenic yeasts compensated by the unique ability to accumulate lipidic compounds such as carotenoids, ergosterol, and fatty acids at great yields. The carotenogenic yeast potential can be exploited in the microbial fabrication of above-mentioned compounds, and potentially serve as a replacement of traditionally used natural sources of carotenoids and fatty acids. Traditionally used natural sources of fatty acids and carotenoids are generally plants or aquatic animal species, both dependent on seasonal conditions with the need for a large area. On the contrary, microbial fabrication has many advantages over traditional sources, because it is easily scalable, readily available, independent of seasonal conditions and usage of a large area.

Fatty acids, sterols, and carotenoids serve for the production of detergents, industrial lubricants, emulsifiers as well as nutritional additives and provitamins in the human diet, animal feed and aquaculture. The usage of carotenogenic yeasts has many advantages. Carotenoids and ergosterol are produced simultaneously with fatty acids (FAs), which can form almost 70 % of their cell dry weight (CDW). Furthermore, FAs composition of carotenogenic yeasts is similar to the composition of vegetable oils, and can therefore be potentially used as a replacement.

Generally, microbial production of any desired metabolite has to go through many selection steps including selection of the suitable yeast strain and definition of optimal cultivation conditions. Carotenogenic yeasts are still considered as non conventional organisms with a lack of efficient tools for genetic manipulation. Furthermore, the current state of knowledge regarding their regulation of metabolic pathways for carotenoids, fatty acids and ergosterol, are still not sufficiently known. Hereby Doctoral thesis aimed to use molecular methods and methods of analytical chemistry to characterize the chosen carotenogenic yeasts and their production properties. The production properties were evaluated based on yeast strain capability to produce increased amounts of fatty acids, ergosterol and carotenoids under variable cultivation conditions and/or with the usage of genetic manipulation or random mutagenesis.

2 THEORY

2.1 The yeasts

Yeasts are unicellular eukaryotic organisms that belong to the kingdom of fungi. These unicellular organisms are defined as fungi that are generally characterized by budding or fission as a primary means of vegetative reproduction and do not form sexual states which are enclosed in a fruiting body [1, 2]. Yeast species are a diverse group of organisms determined by their ability to utilize specific carbon sources and by their preferred habitats. Depending on their specialization, they can be isolated from terrestrial, aquatic, and aerial environments [1, 2].

2.1.1 Yeast morphology and cytology

Yeast cell size varies from 2 to 50 μm depending on the yeast species and age of the cell. Yeast cell contains all subcellular structures typical of eukaryotes. These structures include the cytoskeleton, a nucleus, endoplasmic reticulum (ER), the Golgi apparatus, mitochondria, microbodies, and vacuoles. These cellular contents are encased by an envelope comprising the plasma membrane, periplasm, and cell wall [1, 2].

2.1.2 The cell wall

Yeast cell wall is not a static entity. Its thickness, composition, and structure depend on environmental conditions, such as pH, temperature, oxygen level, carbon source, nutrient availability, and the presence of pheromones. Thus, the cell wall protects the cell against physical damage, degradation by foreign proteins, and dehydration. The other but crucial role of the cell wall is in maintaining turgor pressure, in preserving cell shape, and in morphogenesis. The cell wall is taking part in cell-cell recognition, biofilm formation, and adhesion, and virulence. In yeast, the composition can vary in different yeast species. Chemical analysis of yeast cell wall polysaccharides proved that they consist of various polysaccharides and a combination of polysaccharides. As an example can serve the skeletal elements of the cell wall of yeast *S.cerevisiae*. The major load-bearing polysaccharide in *S.cerevisiae* is beta-1,3-glucan and in addition some chitin can be present. This composition strongly varies among *Ascomycetous* and *Basidiomycetous* yeasts [1, 2].

2.1.3 Cytoplasm and cytoskeleton

The cytoplasm forms an aqueous acidic colloidal fluid containing low and intermediate molecular weight compounds, dissolved proteins, glycogen, and other soluble macromolecules. In the cytoplasm are suspended ribosomes, proteasomes, and lipid droplets.

The cytosolic non-organelle enzymes of yeasts include glycosidic enzymes, the fatty acid synthase complex, and enzymes of protein biosynthesis [1].

2.1.4 Nucleus

The cells generally contain one nucleus (approximately 1 μm in diameter). The nucleus harbors and expresses an organism's essential genetic blueprint and ensures the proper expression, duplication, repair, and segregation of chromosomes and at the same time ensures proper processing and export of messenger and ribosomal RNA. The chromosomes and the nucleosomal fibers within them can be thought of as basic structural elements of the nucleus. Besides chromosomes and nucleosomal fibers, there can be found also molecules like RNA and non-histone proteins [2]. Another major structural elements of the nucleus that have a key role in budding yeasts are the nuclear envelope (NE), the nuclear pore complex (NCP), and the nucleolus. The NE contains a different type of chromatin anchorage sites, including the spindle pole body (SPB), and the unique protein components of the inner nuclear membrane that bind heterochromatin, ribosomal DNA (rDNA), or different types of DNA damage [3].

The endoplasmic reticulum, golgi apparatus and ribosomes

The endoplasmic reticulum (ER) is the largest membrane-bound organelle in eukaryotic cell. The necessary functions of the endoplasmic reticulum in every eukaryotic cell include protein translocation and folding, lipid synthesis, and calcium homeostasis. Three major classes of membrane lipids are synthesized in ER: sphingolipids, phospholipids, and sterols. As an example, can serve major glycerol-3-phosphate acyltransferases (Gat1p and Gat2p) in yeasts, that catalyze the first step in the synthesis of almost all membrane phospholipids and neutral glycerolipids [4].

Many newly synthesized proteins within the ER are transported to the Golgi apparatus via a secretory pathway. These proteins are termed secretory cargoes. The Golgi apparatus consists of membrane sacs called cisternae. There are different types of cisternae, which differ biochemically [2]. Depending on cisternal maturation, there can be early (cis, medial) and late (trans) cisternae. After the favorable theory of cisternal maturation model is at trans Golgi cisternae formed vesicles that fuse within the cell membrane. Those secretory proteins are then released into the growth medium [5].

Ribosomes are cell organelles that can be found "freely suspended" in the cytoplasm or bound to the ER to form a rough ER. These cellular nanomachines consist of two ribonucleoprotein subunits, the small 40S subunit (SSU) and the large one 60S subunit (LSU). The SSU in yeasts contains one ribosomal rRNA (18S) and 33 different ribosomal proteins (r-proteins), while the LSU includes three rRNAs (5S, 5.8S, and 25S) plus 46 r-proteins. The function of SSU is to bring the messenger mRNA and aminoacylated

transfer tRNAs together and decode the information in mRNA. The subsequent peptidyl transferase reaction in LSU forms peptide bonds between the growing peptide chain and the newly admitted amino acid [6]. Ribosomes have only a temporary existence and are urgently needed, thus more than 2000 ribosomes are assembled each minute in a rapidly growing yeast cell. Ribosomes are made in non-membrane-bound sub-compartment of the cell nucleus, the nucleolus [1].

Mitochondria

Mitochondria are organelles responsible for adenosine triphosphate (ATP) production in aerobically growing eukaryotic cells. The mitochondria are originated from an endosymbiosis between an ancestral alpha-proteobacterium and a eukaryotic host. Thus, mitochondria have its own DNA (mtDNA), RNA and its own ribosomes [2]. These organelles continuously divide and fuse to regulate their numbers. They are composed of four subcompartments: the outer membrane, the inner membrane, the intermediate space, and the matrix. The outer membrane contains porins which make the membrane permeable to molecules with a mass of up to 10 kDA. The other proteins of this membrane include mitochondrial lipid synthesis enzymes and enzymes converting lipids to forms that can be metabolized in the matrix. The inner membrane has an enlarged surface by the formation of numerous invaginations so-called cristae. In cristae can be found proteins of the electron transport chain which form the membrane potential across the inner membrane, the ATP synthase proteins producing ATP to the matrix, and the transport proteins enabling the input and output of matrix metabolites. Mitochondrial matrix is a place of oxidation of carbon sources such as fatty acids and sugars via the Krebs cycle. The reducing equivalents produced during the metabolic reactions mentioned above (NADH and $FADH_2$) are responsible for the generation of membrane potential across the inner membrane, which is used to generate ATP via oxidative phosphorylation [7].

Microbodies and lipid droplets

The microbodies in yeast cells include glyoxysomes and peroxisomes, which consist of proteinaceous matrix surrounded by a single membrane [1]. They are involved in a primary metabolism of various carbon and organic nitrogen sources, such as n-alkanes, fatty acids, ethanol, methanol D-amino acids, primary amines, and ureate [2]. The peroxisomes contain H_2O_2 producing oxidase and catalase that degrade hydrogen peroxide. It is assumed that in yeast, the fatty acid beta-oxidation pathway and glyoxylic acid cycle resides in peroxisomes [8].

Lipid droplets (LD) occur in cytosol, where they serve as storage vesicles for neutral lipids, sterols and/or steryl esters, and hydrophobic pigments such as carotenoids [1]. Lipid droplets contribute to diverse cellular functions, which include signaling, temporal

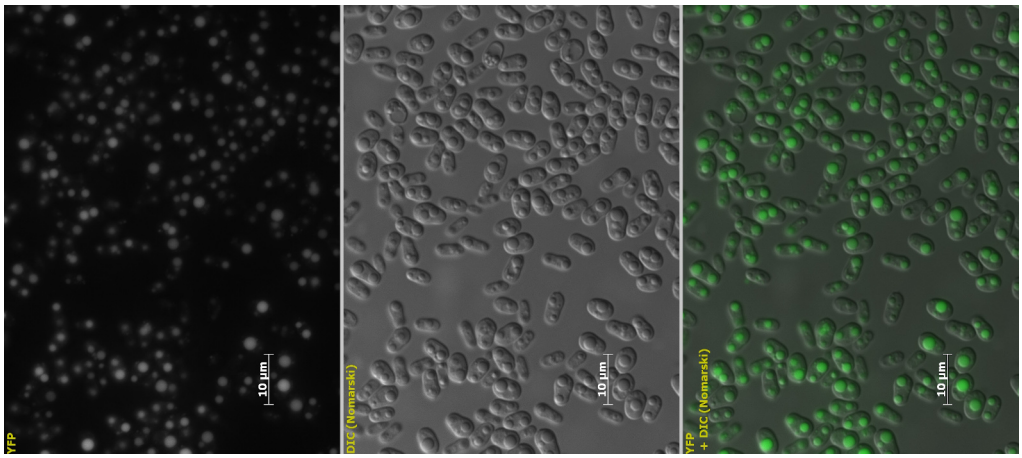

Fig. 2.1: Fluorescence microscopy of *R. toruloides* with BODIPY as lipid staining.

protein storage, and protein degradation. It was found out that yeast lipid droplets can serve as a depot for nonnatural and potentially harmful sterols that are taken up from the environment [9]. At the Fig. 2.1 can be seen lipid droplets of *R. toruloides*.

2.1.5 Yeast reproduction

The type of yeast reproduction is regarded as one of the morphological and physiological characterizations of the yeast strain besides temperature sensitivity or substrate utilization ability. The yeasts can be ascomycetous or basidiomycetous fungi that reproduce asexually or sexually and grow mainly in unicellular form [2, 1].

Asexual reproduction

The asexual reproduction of yeast can occur in a form of budding, by fission, or by the production of blastoconidia during conidiogenesis. The formation of pseudohyfae and septate (true) hyphae are other forms of asexual reproduction. The following chapter will deal only with some forms of asexual reproduction.

Budding may arise on yeast cells or on hyphal cells. At the beginning of bud formation, there is a small evagination or outgrowth at some point on the surface of the parent cell. The parent cell remains constant in shape and size during budding, while the bud (blastoconidium) increases in size and forms a new cell. The daughter cell (bud) can be after some time separated from the parent cell. Budding can be holoblastic or enteroblastic. During holoblastic budding is involved all layers of the cell wall of the parent cell [2]. The bud separates usually on a narrow base, leaving a remaining scar on the parent cell, through which no further budding occurs. During enteroblastic budding, the first bud arises through a rupture in the wall of the parent cell, through which the innermost layer evaginates and ultimately grows out to form the outermost layer of the bud.

Enteroblastic budding is characteristic of basidiomycetous yeasts. Budding can be also position-specific. The types of position-specific budding are monopolar, restricted to one pole of the cell, bipolar (on two poles of the cell), or it can occur on various sites of the cell, and then it is termed as a multilateral or multipolar budding [2].

The pseudohyphae are formed when the bud remains attached to the parent cell and continues budding. Pseudohyphae can consist of cells which may be of similar size and shape, and each of the cells may produce blastoconidia [1]. Some yeasts produce true septate branching hyphae, elongate by the continuous growth of the hyphal tip followed by the formation of septa [2].

The yeasts may also produce asexual spores like endospores (endoconidia), chlamydospores, or ballistoconidia. The ballistoconidia are forcibly discharged asexual spores known as ballistospores or ballistoconidia. The ballistoconida are a specialized mode of reproduction encountered in some basidiomycetous species such as *Sporobolomyces* [2].

Sexual reproduction

The final products of yeast sexual reproduction are spores, which are termed after the characteristic cell, in which reduction division takes place. If the site of meiosis is ascus, spores are called ascospores. In the case that the reduction division is restricted to either teliospore, or the basidium, on which the haploid basidiospores are formed externally, the spores are called basidiospores. Ascomycetous yeasts have usually asexual diploid or haploid phase, but sometimes both haploid and diploid cells are present in the same culture [2]. Basidiomycetous yeasts occur as a budding haplophase, a dikaryotic hyphal phase, or a self sporulating diplophase [2]. Among ascomycetous and basidiomycetous yeasts are homothallic and heterothallic yeast strains [1]. The homothallic yeast strains have only one mating type and during sexual reproduction is zygote often formed by the conjugation of two separate asexual cells or between the cell and its bud [2]. The heterothallic strains have two different mating types. The ascospores of the opposite mating types conjugate within the ascus. The conjugation may lead to diplophase or the ascospores germinate giving haploid cultures of the opposite mating type [2, 1]. The basidiospores of the opposite mating type conjugate and form dipolar, tetrapolar, or dikaryotic hyphae. The dikaryotic hyphae eventually form large, frequently lipid-rich, clamped cells, interpreted as probasidia. After karyogamy of haploid cells, the reduction division is going on and results in sporulation and formation of haploid ascospores or basidiospores [2].

2.2 Carotenogenic yeasts and their taxonomy

Carotenogenic yeasts grow as pigmented colonies and are for this reason known as "red yeasts"[10]. Furthermore, carotenogenic yeasts are also great producers of microbial

lipids, they can accumulate more than 70 % of lipids per its dry weight mass as triglycerides, with a similar composition to that of plants [11]. Additionally, these yeasts proved to utilize a wide range of carbon sources and transform them into a variety of primary and secondary metabolites. They also possess tolerance to inhibitory compounds which can occur as a by-product of the agro-food industry like plant biomass hydrolysates [10]. Altogether, the ability to naturally accumulate carotenoids and microbial lipids makes carotenogenic yeast one of the most promising producers of provitamins and oils, usable in staple food, animal feed and biodiesel industries [11]. The main characteristic of carotenogenic yeast is the production of carotenoids, strictly aerobic metabolism. The majority of carotenogenic yeasts are not able to ferment simple sugars into ethanol and carbon dioxide. The major form of their vegetative reproduction is budding, which can be multilateral or polar. Some carotenogenic strains form pseudomycelium or true mycelium [2].

2.2.1 Taxonomy of carotenogenic yeast

Yeast taxonomy falls under the authority of the International Code of Botanical Nomenclature. The yeast taxonomy in these days is not based on only their morphological properties. After the International Botanical Congress in Melbourne in 2011, there should be used the implementation of the "One Fungus = One Name" nomenclature principle [12]. This principle does not allow to treat anamorph and teleomorph of one yeast separately. Nowadays, taxonomy uses methods of molecular biology and molecular systematic. To reflect the phylogenetic relationships of yeasts by molecular methods, a multigene approach, including ribosomal genes, is used for the creation of monophyletic taxa [2, 12, 13].

Carotenogenic yeasts mainly belong to subdivision Basidiomycotina. A multigene approach based on ribosomal genes sequences proved a three subphylum within Basidiomycota. Previously, there was a three-class concept that needed to be elevated to subphyla after multigene analysis. The subphyla of Basidiomycota are Puccniomycotina (identical to class Urediniomycetes), Ustilaginomycotina (identical to class Ustilaginomycetes) and Agaricomycotina (identical to class Hymenomycetes) [2]. The most known carotenogenic yeasts are spread within subhylums Pucciniomycotina and Agaricomycotina [13, 14]. The subphylum Pucciniomycotina and Agaricomycotina were recently reclassified using multigene analysis including ITS regions (including 5.8S rRNA), the D1/D2 domains of the LSU (large subunit) rRNA, and genes for the largest *(RPB1)* and second largest *(RPB2)* subunits of RNA polymerase, translation elongation factor 1-alpha*(TEF1)*, and a cytochrome B coding gene *(CYTB)* [13, 14].

The Pucciniomycotina yeast species belong to four recognized classes, namely, Agaricostilbomycetes, Cystobasidiomycetes, Microbotriomycetes, and Mixiomycetess [13].

The yeasts of the genus *Rhodotorula* and *Sporobolomyces* are placed within these four classes to correspond with the closest phylogenetic relationships. Not in every case can be said that the relations are monophyletic. There is still *incertae sedis* in the genus *Buckleyzyma* (class *Cystobasidiomycetes*, family *Bukleyzymaceae*). In the genus *Buckleyzyma* was recently placed yeast *Rhodotorula auratiaca,* nowadays named *Buckleyzyma aurantiaca* [13]. The most known carotenoid forming yeasts within *Pucciniomycotina* are the yeast of genus *Rhodotorula* and *Sporobolomyces* [15]. The carotenogenic yeasts most commonly used in biotechnology belong to the genus *Rhodotorula, Rhodosporidiobolus* and *Sporobolomyces*, and are placed in the order of Sporidiobolales of the class Microbotriomycetes [13].

The most commonly used carotenogenic yeasts of the subphylum Agaricomycotina are mainly placed within the class Tremellomycetes. In the class Tremellomycetes are currently recognized five orders, namely, Cystofilobasidiales, Filobasidiales, Holtermanniales, Tremellales, and Trichosporonales [14]. Tremellomycetes comprehend yeasts, dimorphic taxa, and species that form hyphae and/or complex fruiting bodies [2]. The best-known examples of carotenogenic yeasts are yeasts of the genus *Cystofilobasidium* and *Phaffia,* which both belong to the order Cystofilobasidiales, and *Dioszegia* which belongs to the order Tremellales [14].

Sporobolomyces

The genus *Sporobolomyces* now includes *Sporobolomyces* species and their sexual counterparts *Sporidiobolus*. The first published name of this genus was *Sporobolomyces* in 1924 and thus it has taxonomic priority over *Sporidiobolus*, which was used later. In some species, sexual reproduction can be observed. The yeasts of genus *Sporobolomyces* form a clamp connection. Teliospores are formed and they germinate to produce transversely separate basidia. In some conditions, they form pseudohyphae or true hyphae. Interestingly, they can form balistoconidia [2]. A typical is a formation of salmon-ping or red-colored colonies. The microscopic picture of *S. roseus* culture can be seen at Fig. 2.2. The shape of the cells can be ellipsoid, spindle, or cylindrical. The asexual reproduction is mediated by budding. *Sporobolomyces* have strictly aerobic metabolism and they are unable to ferment sugars to alcohol [13]. The well-known members of this genus are: *Sporobolomyces roseus*, *Sporobolomyces pararoseus* (homotypic synonym *Sporodiobolus pararoseus*), *S. salmonicolor*, *S. metaroseus*, and *Sporobolomyces jonsonii* [13, 2].

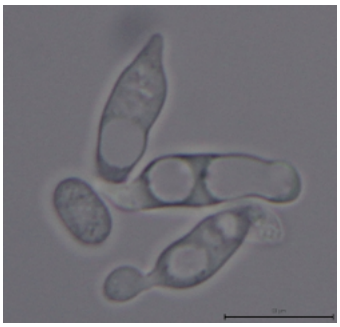

Fig. 2.2: Microscopic picture of *S. roseus*

Rhodotorula

Within the genus *Rhodotorula* are placed both anamorph and teleomorph so that there can be found both *Rhodotorula* species and their sexual counterpart *Rhodosporidium* [13]. In some species can be observed sexual reproduction. They also may form teliospores and produce transversely septate basidia. Pseudohyphae or true hyphae can be formed. *Rhodotorula* species are also able to form ballistoconidia. Asexual reproduction is performed by budding and their cells can be spherical, ellipsoidal, or elongated in shape. The majority of the genus is mesophilic with some exceptions. Every species in *Rhodotorula* genus has strictly aerobic metabolism with a lack of capacity to perform sugar fermentation [2, 13]. They can utilize many compounds as sources of carbon, including glucose, galactose, sucrose, maltose, trehalose, ethanol, glycerol, and hexadecane. Exceptionally, they can utilize also xylose as a carbon source [13, 16]. They can also produce carotenoids mainly β-carotene, γ-carotene, torulene, and torularhodin [15]. The production of carotenoids is responsible for colony color, which can change depending on cultivation conditions from pink, orange to dark red color. Some *Rhodotorula* species are exceptionally good at lipid accumulation, lipids can form almost 70 % of their biomass dry weight at certain conditions [17]. The biomass of these yeasts is a source of various enzymes like lipases, α-L-arabinofuranosidase, invertase, pectinase, and tannin acyl hydrolase. Biotechnologically very attractive is their ability to produce phenylalanine ammonia lyase, which can serve for the production of aspartame from L-phenylalanine [18]. The most well-known species of the genus are *Rhodotorula toruloides, R. glutinis R. kratochvilovae, R. babjevae, R. graminis*, and *R. diobovata* [13].

Rarely, some species are opportunistic pathogens which can cause dermatophytoses. The most common species causing these infections are strains of the species *Rhodotorula mucilaginosa* [18]. The culture of *R.mucilaginosa* can be seen at Fig. 2.3.

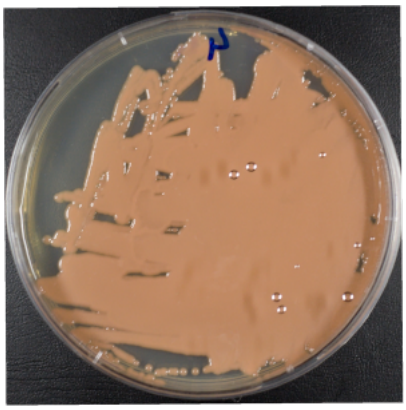

Fig. 2.3: Culture of *R. mucilaginosa* at YPD agar plate

Cystofilobasidium

The most well-known species within the genus *Cystofilobasidium* are *Cystofilobasidium capitatum, C. infirmominiatum, C. macerans, C. bisporidii* and *C. ferigula*. These species form pink to orange colonies. The reproduction can be asexual or sexual (in some species). During sexual reproduction, they may form true hyphae and pigmented teliospores, which are then budding by holobasidia [2]. The asexual reproduction can occur in a form of polar or bipolar budding. None of the species within the genus *Cystofilobasidium* can ferment sugars, in every case their metabolism is strictly aerobic. Mostly are *Cystofilobasidium* species mesophilic, exceptionally they can be psychrophilic. Among psychrophilic *Cystofilobasidium* species has been reported production of pectinolytic enzymes. They are able to produce β-carotene, γ-carotene, torulene, torularhodin, and unlike the *Rhodotorula* and *Sporobolomyces* species, they can also produce torularhodin aldehyde and 16-hydroxytrulene in small amounts. Typical is also the production of CoQ-9 (Coenzyme Q-9) and CoQ-10 [2, 19, 14].

Phaffia

The genus *Phaffia* currently contains only one species *Phaffia rhodozyma*. *Phaffia rhodozyma* was regarded as the anamorph of *Xanthophyllomyces dendrorhous*, because it has similar D1/D2 rDNA sequences. Despite of this fact, the next analyzes of ITS and IGS rDNA sequences and protein-coding gene sequences showed rather a complex population structure within the species *Phaffia rhodozyma* [14]. Interestingly, this yeast has an unusual sexual stage because it does not involve unicellular to filamentous stage transition. Unlike the other yeast species, *Phaffia rhodozyma* has homothallic mating behavior usually involving the conjugation between the mother cell and its bud in polyol-rich media [20]. The vegetative reproduction is done by budding [2].

The species *Phaffia rhodozyma* is unique among yeasts by the production of astaxanthin. As minor products, there can be produced also β-carotene and echinenone [20]. Due to the production of these carotenoids can be cultures colored from pink, orange to red color. They have the capacity to perform sugar fermentation. Typical is the production of CoQ-10 [14, 2]. *P.rhodozyma* lives in association with plant substrates in mountain environments, where are reactive oxygen species generated by high levels of UV radiation. In protection against UV radiation seems to be involved besides carotenoids also a compound named phaffiol and accumulation of mycosporine glutaminol-glucosides [20].

2.3 Yeast lipid metabolites

2.3.1 Carotenoids

Carotenoids represent a group of natural pigments with occurrence in plants, animals, and microorganisms. The animals are unable to synthesize carotenoids, thus their carotenoids are gained from diet, selectively or unselectively absorbed and then accumulated in modified or unchanged forms to fulfill their specific roles in animal metabolism. The carotenoids fulfill many remarkable functions which are possible due to their physical and chemical properties. Their overall molecular geometry is influenced by their size and by the presence of functional groups. Currently, there are over 700 specimens of carotenoids and they are the second most abundant pigments in nature [21, 22].

Carotenoid structure and nomenclature

A basic carotenoid compound which gives rise to many types of carotenoids, in every organism capable of carotenoid synthesis, is 15--*cis*-lycopene. There are three main ways of lycopene modification, which gave rise to numerous carotenoid specimens specific for a given organism. The first type of lycopene modification is modification by cyclization at one end or both ends of the molecule to give seven different end groups. The second type is modification by changes in lycopene hydrogenation levels and the last type of modification is the addition of oxygen-containing functional groups to the lycopene skeleton. Generally, carotenoids with one or more oxygen-containing functional groups are known as xanthophylls, while the parent hydrocarbons are known as carotenes [23]. In many biological studies, there is still a common practice to use well-known trivial names of carotenoids. The trivial names of carotenoids are based on the biological source from which the carotenoid was first isolated. However, trivial names, even well known, do not contain information about the structure of carotenoids. To describe the carotenoid structure, there is recommended to use semi-systematic names based on the stem name "carotene". Before the stem name, there are Greek letter prefixes that designate the two

end groups. As an example can be used β,β-carotene for β-carotene, and β,ϵ-carotene for α-carotene. Standard prefixes and suffixes used in organic chemistry are also used to describe changes in hydrogenation level and the presence of oxygen-containing substituents in carotenoids [21].

Physical and chemical properties

The characteristic trait of carotenoids is their conjugated double bond system. To stabilize this structure, the p orbitals are connected with delocalized electrons across an intervening σ bond in a molecule. The delocalization of π-electrons over the entire length of the carotenoid polyene chain influences their shape, chemical reactivity, and light-absorbing properties. In theory, there is a possible rotation about any C-C single bond in the polyene chain of carotenoids, so that they may form an enormous number of shapes and conformations. Possible conformations of carotenoids can be *cis* or *trans* and in carotenoid nomenclature can be equivalently used *Z* or *E*. Naturally occurring carotenoids are predominantly in thermodynamically more stable all-*trans* form. A *cis* form is not that stable because of the steric hindrance between nearby hydrogen atoms and/or methyl groups. Relatively stable *cis* isomers with small steric hindrance can be formed on those double bonds, which bear three substituents or also the disubstituted C-15,15' double bonds (e.g. 9-*cis*-, 13-*cis*-, and 15-*cis*-β,β-carotene. The overall shape of carotenoids is proved by X-Ray crystallography to be extended into linear molecules with only a slight S-shaped distortion [21]. Carotenoids are extremely hydrophobic molecules with little or no solubility in water. Due to their hydrophobicity, they are expected to occur mainly in the hydrophobic area of the cell-like inner core of membranes and lipid bodies. When associated with proteins, carotenoids can be as well in an aqueous environment. Their light absorption properties are based on their polyene chain with highly delocalized π-electrons. The energy needed for the excited state is of comparatively low energy and corresponds to the energy of the light in the visible region (400 - 500 nm wavelength) [21].

Most of the carotenoids have three wavelength maxims, which form a specific shape of their spectrum. Generally, the greater the number of conjugated double bonds, the higher the absorption maximum. As an example can be used lycopene, which possesses 11 conjugated double bond systems with the longest wavelengths of 444, 470, and 502 nm. This absorption spectrum corresponds to the red color. As the opposite example can be used ζ-carotene which is perceived as light yellow, its absorption maxima are 378, 400, and 425 nm [23]. The ζ-carotene possesses 7 conjugated double bonds and represents a limit of perceptible carotenoids (see Fig. 2.4). Carotenoids like phytoene and phytofluene with less than 7 conjugated double bonds are colorless [21]. The factors influencing the spectral characteristics of carotenoids are the cyclization on the ends (hypsochromic shift) and conjugated carbonyl group with the series of conjugated double bonds (bathochromic

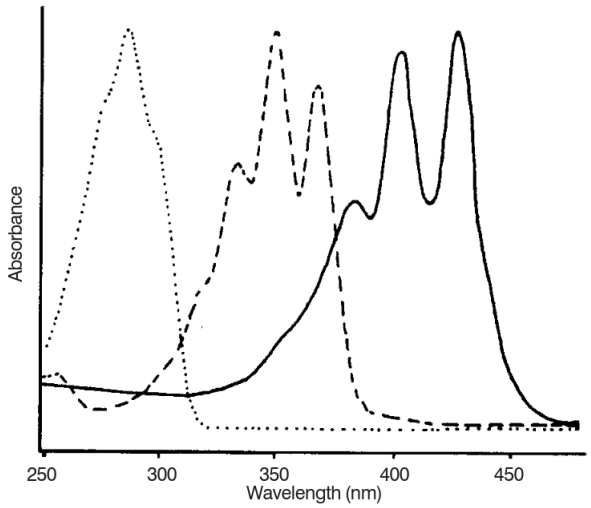

Fig. 2.4: UV-VIS spectrum of ζ-carotene (-), phytoene —, and phytofluene (...), solvent acetonitrile- ethyl acetate-methanol (85:10:5) [23]

shift) [21]. The conjugated double carboxyl group also causes a loss of spectral fine structure so that the three maxima spectrum is replaced by a single broad curve (echinenone, astaxanthin). The introduction of isolated carboxyl groups or hydroxy and methoxy substituents into the carotenoid molecule does not affect their absorption spectra [23].

Carotenoids in health and nutrition

The most common carotenoids that can be found in human blood are β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin [24]. The dietary carotenoids, which are considered as precursors of vitamin A are β-carotene, α-carotene, γ-carotene and β-cryptoxanthin [25]. Deficiency in vitamin A is connected with altered function of vision (night blindness and nyctalopia). In mammals is vitamin A in the form of *trans*-retinyl ester and provitamin A carotenoids. Retinyl esters are hydrolyzed to retinol in the small intestine and then absorbed by enterocytes. Provitamin A carotenoids like β-carotene must undergo oxidative cleavage to two molecules of *trans*-retinal. This reaction is catalyzed by β-carotene 15,15'-monooxygenase [26]. The other biological functions of vitamin A are connected to embryonic development, maturation of immune system, maintenance of epithelial integrity. In the adult brain is vitamin A important for learning and memory, and neurogenesis [27].

The other carotenoids with no vitamin A activity are lycopene, zeaxanthin, lutein, and other types of carotenoids. Zeaxanthin and lutein are macular carotenoids are their elevated level in human nutrition is investigated as a possible prevention to macular age degenerative diseases. The full range of biological effects of carotenoids is still unknown, but many studies focused on this problematic bring increasing evidence that carotenoids

are taking part in biological processes like oxidative defense, cell-to-cell communication, and that they are also affecting human health [28].

Yeast carotenoids

To date, the most known carotenogenic yeast are species of the genus *Rhodotorula*, *Sporobolomyces*, *Cystofilobasidium*, and *Phaffia*. The typical carotenoid species produced by *Rhodotorula*, *Sporobolomyces*, and *Cystofilobasidium* species are β-carotene, γ-carotene, torulene and torularhodin as the main carotenoids. For *Cystofilobasidium* species has been also reported the production of torularhodin aldehyde and 16-hydroxytrulene [15]. Interestingly, *Phaffia rhodozyma* produces as the main carotenoid astaxanthin, β-carotene and echinenone, production of torulene and torularhodin was also reported [29].

β-carotene is a carotenoid with 11 conjugated double bonds. The two of conjugated double bonds are located in β-rings on each end of the molecule [23]. The molecule of β-carotene is visualized at Fig. 2.5. The β-carotene is a principal carotenoid of carrot and red palm oil, and it is also predominant in orange-flashed sweet potatoes, squash, and pumpkins, etc. At certain conditions, it is a predominant carotenoid of some carotenogenic yeast [30]. A good natural source of β-carotene are also algae of *Dunaliella* species. There are many studies focused on possible health-promoting roles of β-carotene other than its provitamin A activity [31]. The most known studies are the study done by ATBC Study Group in 1994 and the CARET study. These studies were focused on the impact of β-carotene in the high-risk groups (smokers and workers exposed to asbestos and oxidative stress). The results of both studies were disappointing and had to be stopped earlier than was planned because of increased incidence of lung cancer [32, 33]. The problems of these studies were probably too high doses of β-carotene. It was estimated by other epidemiological studies that optimal daily intake of β-carotene is 4 mg (ATBC: 20mg of β-carotene daily, CARET 30 mg of β-carotene and 25,000 IU of vitamin A daily) moreover it is important to say that in epidemiological studies were subjects supplemented with diet with other carotenoids and constituents that could act synergistically with β-carotene . Needless to say, that the subjects of the two problematic studies were high-risk smokers and/or workers exposed to asbestos and oxidative stress. The cancer process might have reached a point where the carotenoids could no longer be effective [34]. The studies which used a combination of different antioxidants and micronutrients were more successful in decreasing the risk of cancer. The example study was done in the Linxian region in China, which is the region with the highest incidence of gastric and stomach cancer in the world. As a result of this study was a 21% reduction of deaths due to gastric cancer in the group supplemented with β-carotene, vitamin E, and selenium. Unfortunately, it is not known which component was responsible for the decrease, because the mixture was administrated [34].

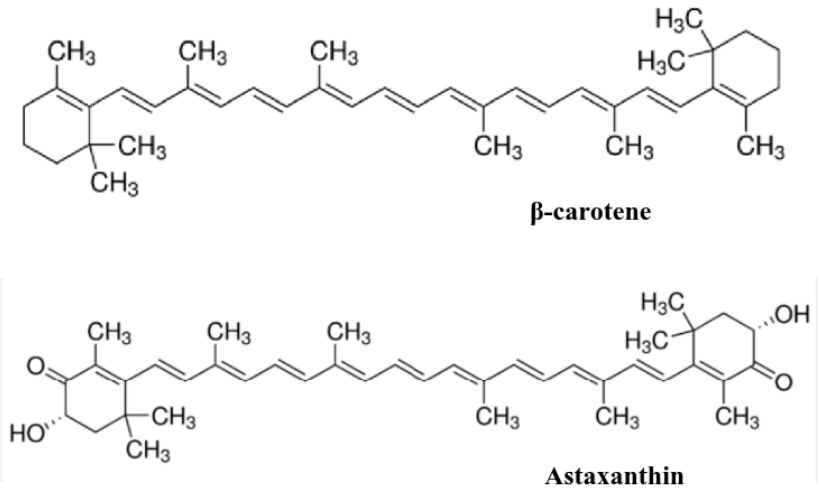

Fig. 2.5: Chemical formula of β-carotene and astaxanthin (source www.sigmaaldrich.com)

Astaxanthin is carotenoid which is responsible for the red color of lobsters, shrimps, crabs, and especially salmons. The molecular formula of astaxanthin is $C_{40}H_{52}O_4$ (see Fig. 2.5) [35]. It is used as a natural colorant because of its coloration capacity. It is essential in the aquaculture industry, where it serves as a nutritional component for adequate growth and reproduction of aquatic animals. Commercially synthesized astaxanthin is not esterified unlike the naturally occurring astaxanthin. The natural sources of astaxanthin are single-celled alga *Haematococcus pluvialis*, krill oil and meal, crayfish oil, and yeast *Phaffia rhodozyma*. The value and need for astaxanthin growths with the rapid growth of the aquaculture industry [31]. Astaxanthin is supposed to have some advantages compared to other carotenoids. It is more stable, it has higher antioxidant potential and it can easily cross the blood-brain barrier [24]. Astaxanthin is not a regular component of the diet and there is only a limited number of studies focused on this problematic. Until these days, there were reported studies focused on the impact of astaxanthin on Low-Density Lipoprotein (LDL) and apolipoprotein levels. Supplementation with astaxanthin may be responsible for lowering the levels of LDL cholesterol and apolipoprotein in overweight subjects. In moderately hyperlipidemic subjects, astaxanthin improved oxidative stress biomarkers (malondialdehyde, isoprostane, and superoxide dismutase), and total antioxidant capacity [24].

Torulene and **torularhodin** are not present in food and their impact on the human body has not yet been studied. To this date, there are studies focused on toxicity, anticancer activity, anti-microbial and antioxidative properties of torulene and torularhodin. The chemical formula of torulene and torularhodin is at Fig. 2.6. The majority of these studies used rats as a model system [15]. Latha and Jeevaratanm studied the toxicity of carotenoids produced by *R. glutinis* (β-carotene, torulene, and torularhodin) in rats. The

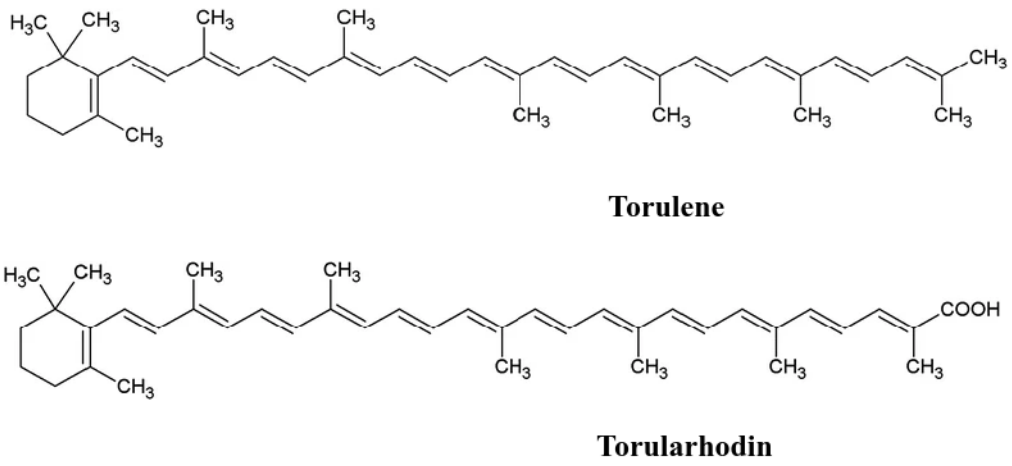

Fig. 2.6: Chemical formula of torulene and torularhodin [15]

results did not show any pathological changes in tested rat groups. This study concluded that carotenoids can be used as food additives [36]. The anticancer activity of torulene and torularhodin was tested in the study of Du. The anticancer activity was tested in male Wistar race rats. After daily supplementation by torulene and torularhodin were rats infected by PC-3 prostate cancer cells. After were rats supplemented with carotenoids in the same way for 7 weeks. Torulene proved to have anticancer properties in prostate cancer cells of the LNCaP line [37]. In the study of Wu torularhodin showed neuroprotective activity against hydrogen peroxide, which induces oxidative injury because of its strong antioxidative activity [38]. Additionally, several studies are dealing with torulene and torularhodin for possible antimicrobial activity. Ungureanu et al. recorded antimicrobial activity of torularhodin against both Gram-negative (*E. coli* ATCC 8738, *Pseudomonas aeruginosa* ATCC 9027) and Gram-positive bacteria (*S. aureus* ATTC 25923, *E. faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633). In this study, the titanium implants were dipped in torularhodin solution with an additional layer of titanium dioxide [15].

2.3.2 Yeast lipids

Lipids are a class of hydrophilic or amphiphilic molecules which are essential for diverse biological functions in every living cell. For a long time, they were seen only as storage molecules and components of cellular membranes. After a decade of lipid research, there is the finding that lipids can serve as regulators of energy metabolism, cell integrity, and membrane-based processes, such as endocytosis and vesicular trafficking [39].

Yeast lipids are also very interesting for biotechnological applications, such as a source of lipids for biofuel production or as a potential source of essential fatty acids for food, feed, and cosmetic industry [40]. Yeast species capable of relatively high lipid production are named oleaginous yeasts. The specificity of these strains is that they are able to produce more than 20 % of lipids per their dry weight. As examples of oleaginous yeast species can be mentioned *Yarrowia lypolitica*, *Lipomyces starkey*, *Cryptococcus curvatus*, *Cryptococcus albidus,* and some red yeast strains especially *Rhodotorula* species [17].

Biodiesel production

Biodiesel is composed of fatty acids esters, therefore, it is biodegradable, nontoxic and easily fabricated from various renewable lipid sources. Biodiesel has almost similar properties as fossil fuels. As sources for biodiesel production are now mainly worldwide used edible plant oils like rapeseed, sunflower, palm oil, soybean, and others. The advantage of these oils is primarily the high content of oleic acid which is necessary for superior ignition quality and ideal melting point, kinematic viscosity as well as improved oxidative stability [41].

Another advantage is that biodiesel decreases the life cycle of CO_2 emission by 50-75 %. Unfortunately, there is a competition with food resources, so these kinds of first-generation biofuels are no longer suitable. The European Parliament and Council provisionally agreed on a share of energy from renewable sources of at least 32 % of the Union's gross final consumption in 2030, with an upward revision clause by 2023. The European Union also agreed on phasing out palm oil-based biofuels (www.europarl.europa.eu). Biodiesel produced from palm oil has many negative impacts on the environment situation. The production of palm oil leads to deforestation, loss of natural habitat, and biodiversity, and it indirectly increases greenhouse emission.

Yeast lipids in the form of triacylglycerols (TG) have the same composition to that of plants. They produce TG with a high content of oleic (predominant), palmitoleic, palmitic, stearic, linoleic, and α-linolenic acids, and a small amount of myristic acid [11]. The main advantages of yeast lipid production are no dependency on climate conditions and seasonal conditions, no need for a large area, industrial-scale fermentation can be rapidly adapted to market needs, and sometimes a possibility to use waste substrates. The drawbacks of yeast lipid production are requirements of the controlled environment of aeration, temperature, and agitation, need for aseptic work, and recovery of the oil from the cells which leads to increased production prize [42]. The possibility of use waste substrates can reduce the production prize. As suitable substrates can be used glycerol, as a side product from biodiesel production or lignocellulosic biomass. For now, yeast biodiesel production is not economically suitable, because the production capacity is still relatively low. Economically suitable yeast biodiesel production should correspond to

lipid production over 80 % per dry weight of yeast biomass [39].

Polyunsaturated fatty acids

In animals, the Δ^9-desaturase is missing. Animals are not able to introduce a *cis* double bond into a saturated fatty acid between C-9 and C-10 carbons [43, 44]. Therefore, animals must gain these essential fatty acids from a diet. The essential polyunsaturated fatty acids (PUFA) are linoleic and α-linolenic acid. Arachidonic acid is conditionally essential in case that linoleic acid is missing. Linoleic acid is a precursor for the synthesis of arachidonic acid (AA). The α-linolenic is a precursor of eicosapentaenoic acid (EPA). Arachidonic acid and EPA are precursors for groups of eicosanoids like prostaglandins (PGs), thromboxanthines, and leucotrienes (LT). Eicosanoids influence blood pressure, contraction of smooth muscles and activity of the intestine. They are also responsible for the regulation of the immune system and the inflammatory effect in the human body. The ω-3 fatty acids are required for the normal composition of sperm, retina, and brain lipids. In preterm infants are necessary for optimal maturation of visual and cortical function. Dietary PUFA have also an important role in the prevention and treatment of cardiovascular disease [43].

The main sources of PUFA are aquatic animal species like fish, shrimps, prawns, crabs, shellfishes, and algae. Unfortunately, there can be contamination by heavy metals, polychlorinated biphenyls, or antibiotics. The other sources are plant oils like canola oil, olive oil, or plant seeds or nuts [45]. Promising sources of PUFA can be yeast oils. The main advantage of microbial sources is that they can be readily available, there is no contamination by heavy metals or antibiotics and other chemicals with negative effects on human health [42, 11].

2.4 Metabolism of yeast lipid compounds

The majority of studies dealing with regulation of lipid metabolism have been done in *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. For carotenogenic yeast species, there is still a limited number of studies dealing with lipid metabolism and regulation in brief detail. Most of these studies are based on knowledge gained by the study of lipid metabolism of *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. The most lately studied carotenogenic yeast with increased lipid production is *Rhodotorula toruloides*.

The branching point for lipid and central carbon metabolism is acetyl-CoA. The acetyl-CoA is the main precursor for all lipid biosynthetic pathways. The major consumers of acetyl-CoA are pathways for amino acid synthesis and pathways for membrane synthesis. Lipid metabolism is taking part in many different cellular compartments [39]. Lipid synthesis is taking part in the Endoplasmic reticulum and Golgi compartment. The lipid

droplets, mitochondria, and peroxisomes are necessary for other important roles in lipid metabolism like storage or fatty acids degradation. Scheme of fatty acids metabolism can be seen at Fig. 2.7 [8, 39].

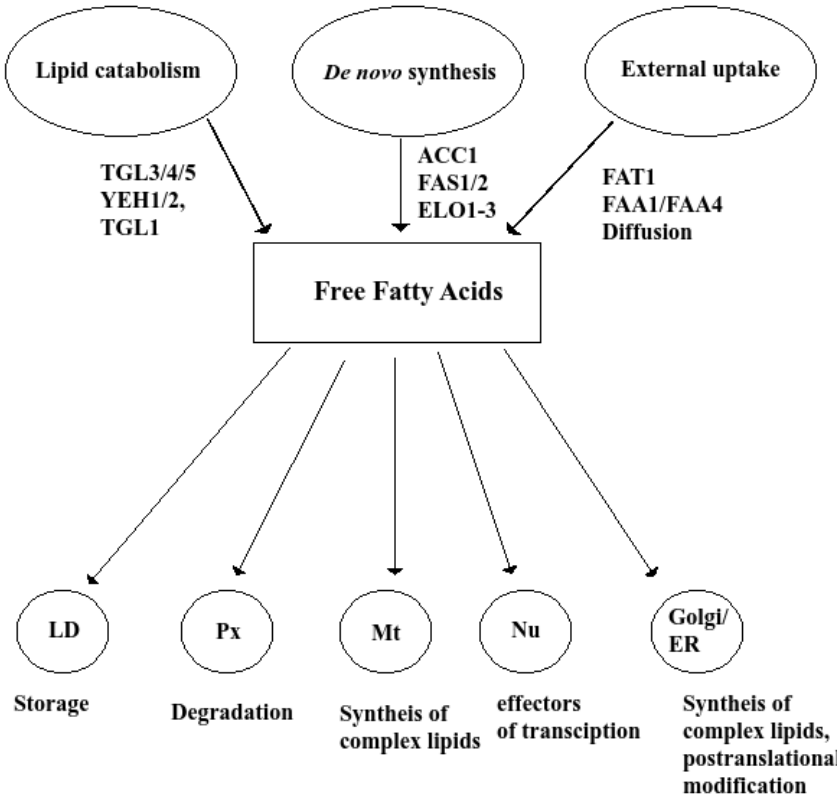

Fig. 2.7: Metabolism of Fatty acids in *S. cerevisiae*, LD -lipid droplets, Px - peroxisomes, Mt - mitochondria, Nu - nucleus, ER - endoplasmic reticulum [39].

Lipids are categorized based on their structure and function into eight diverse classes: fatty acids (FA), glycerolipids (GL), sterols and sterol derivatives, glycerolphospholipids (GFL), sphingolipids, prenols, glycolipids, and polyketides. Regarding the complexity of lipid metabolism, will be the following chapter focused merely on fatty acids, triacylglycerols, and sterols. The isoprenoid metabolism will be added to sterol synthesis because of shared biosynthetic pathways [39].

2.4.1 Fatty acids

Fatty acids (FAs) are carboxylic acids with long aliphatic chains, differing by length and saturation of the aliphatic chain [39]. The most frequent FAs produced by yeasts, including carotenogenic yeasts, are oleic acid (C18:1), palmitoleic acid (16:1), palmitic acid (16:0) and stearic acid (18:0), linoleic and α-linolenic acid, and small amount of myristic

acid (C14:0). Quantity of each fatty acid can differ depending on cultivation conditions and yeast strain [17, 46]. FAs are basic elements of complex and storage lipids. Additionally, FAs can serve as signal molecules, transcriptional regulators, and they have been involved in protein posttranslational modifications. They are frequently stored in lipid droplets (LD) in the form of triacylglycerols (TG) and steryl esters (SE). FA can be also incorporated into phospholipids and sphingolipids. FAs metabolism includes several variants: *De novo* synthesis of FAs, transport of FAs by specific transporters or complex lipid catabolism, and a small amount of FAs derivates is mediated by protein catabolism [39]. *De novo* synthesis of FAs takes place independently in cytosol and mitochondria. FAs biosynthesis in the cytosol includes enzymes coded by genes for acetyl-CoA carboxylase *ACCI* and fatty acid synthase, coded by two genes, *FAS1* and *FAS2* [39].

The fatty acid synthesis starts with the carboxylation of Acetyl-CoA to give malonyl-CoA. This step is mediated by the cytosolic acetyl-CoA carboxylase Acc1p. The Acc1p possesses activities as biotin carboxylase, as biotin carboxyl carrier protein, and as a transcarboxylase. Cytosolic acetyl-CoA carboxylase contains covalently bound biotin molecule. Deletion of the gene for acetyl-CoA carboxylase is lethal for cell even with FAs supplementation. Acetyl-CoA carboxylase Acc1p is located on the cytoplasmatic surface of the ER. The following reaction in fatty acid synthesis is the metabolization of malonyl-CoA by series of reactions catalyzed by FA synthases and elongases. The active FAS complex consists of six α-units and six β-units [39]. The genes *FAS1* and *FAS2* code for two distinct sub-units of fatty acid synthase. The *FAS1* codes beta-subunit, which has four different activities: acyltransferase activity, enoyl reductase activity, dehydratase, and malonyl-palmitoyl transferase activity. The *FAS2* decodes the alpha-subunit, which mediates 3-ketoreductase, 3-ketosynthase, and phosphopantenyl transferase activity [39, 47].

In oleaginous yeast, *Yarrowia lipolytica* is the main source of acetyl-CoA mediated through citrate lyase (encoded by *ACS1* and *ACS2* genes). The citrate lyase is found only in oleaginous yeasts and it is located in the cytosol where it cleaves citrate released from mitochondria to acetyl-CoA. This phenomenon is especially important in nitrogen limitation. When nitrogen levels in the cells drop, the intracellular concentration of AMP decreases. In oleaginous yeasts, the activity of isocitrate dehydrogenase depends absolutely on the concentration of AMP. In low AMP concentration, the activity of isocitrate dehydrogenase is downregulated and the enzyme can not effectively synthesize α-ketoglutarate from citrate. This leads to the accumulation of citrate in mitochondria to a critical concentration and then it is released to the cytoplasm to change for cytosolic malate [48]. *De novo* synthesis of fatty acids takes place mainly in the cytosol and the subsequent elongation and desaturation reactions are carried out in ER. The desaturation reactions are introduced by acyl-CoA Δ^9-desaturase encoded by *OLE1*. Elongation is carried out by Elo1p and elongation of very long fatty acids for sphingolipid synthesis is catalyzed

mainly by Elo2p and Elo3p [39, 47]. Fatty acids can be also transported into the cell by diffusion or by transporters. Transported fatty acids can fully compensate endogenously synthesized FA. The fatty acid must be activated before transport into the cell with coenzyme A, which is carried out by synthetases Faa1p, Faa2p, Faa3p, Daa4p, and Fat1p. The machinery for the utilization of extracellular complex lipids has been identified only in oleaginous yeasts, which can produce surfactants and/or extracellular lipases in medium containing lipids as substrates. In general, free FA can be very quickly metabolized or immediately incorporated into complex lipids. Elevated free FA content in the cell has harmful effects because it can perturb membrane properties due to changes in fluidity [39]. The catabolism of FA in yeast *Saccharomyces cerevisiae* takes place predominantly in peroxisomes by β-oxidation. In yeast *Rhodosporidium toruloides* are necessary both peroxisomal and mitochondrial β-oxidation for robust growth on fatty acids. A plausible model of β-oxidation is that large ensemble of peroxisomal enzymes shorten diverse long-chained FA in peroxisomes and a smaller ensemble of enzymes metabolize short-chained fatty acids in mitochondria [49].

2.4.2 Nonpolar lipids

TG and SE are a biologically inert form of free fatty acids and sterols. Because of these properties, they are labeled as neutral lipids. In TG, all three hydroxyl groups of glycerol are esterified with FAs. They are uncharged and have highly apolar structure. Steryl esters are esterified sterols. Nonpolar lipids serve as a source of energy and precursors for membrane lipid synthesis. TG and SE are typically stored in cytosolic lipid droplets, where they can be readily available for β-oxidation in a need of fatty acids or sterols. After recent evidence, lipid droplets are formed by budding of LD from the ER, when TG accumulate between the two leaflets of the ER membrane until it reaches its critical size. Mature LD have a highly hydrophobic core of TG, surrounded by the shell of SE and a phospholipid bilayer with a small amount of proteins. These proteins were identified as enzymes of lipid metabolism. The synthesis of TG is carried out by acyltransferases Dga1 and Lro1p. For SE synthesis are responsible for sterylestersynthases - Are1p and Are2p. TG and SE synthases are localized in Endpolasmic reticulum [9, 39]. A direct precursor for TG synthesis is diacylglycerol (DAG), which can be formed by three main ways: by dephosphorylation of *de novo* synthetized phosphatidic acid, by the degradation of phospholipids by phospholipases, and by deacylation of TG (see Fig. 2.8) [39].

In *Saccharomyces cerevisiae* are SE synthesized by two acyl-CoA-cholesterol acyltransferase (ACAT) related enzymes, Are1p and Are2p. Both proteins are localized in the ER. The storage of nonpolar lipids would be completely futile if there is no possibility to mobilize TG and SE when needed. FA and DAG are useful for the synthesis of membrane lipids and the production of energy when the nutrients are depleted. In this case,

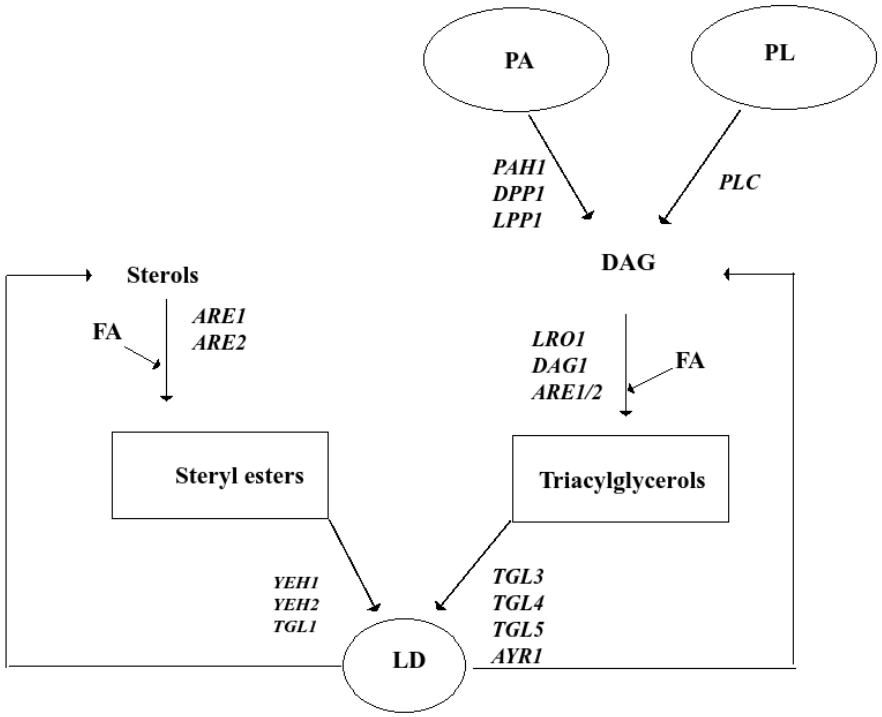

Fig. 2.8: Metabolism of nonpolar lipids in *S. cereveisiae* PA - phosphatidic acid, PL - phospholipids, DAG - diacylglycerol, FA - fatty acids, LD - lipid droplet [39].

the degradation of TG is achieved by TG lipases Tgl3p, Tgl4p, Tgl5p, which are located in the LD. When the LD is absent, the Tgl3p lipase is retained in ER [9, 39]. The SEs are hydrolyzed by three main SE hydrolyzes Yeh1p, Yeh2p, and Tg11p, where Yeh2p hydrolase possesses the highest activity. Yeh2p hydrolase is surprisingly located within the cytoplasmatic membrane [39].

2.4.3 Sterols

Sterols are important compounds in yeast cells that regulate membrane fluidity, plasma membrane biogenesis, and function. They serve as structural and signaling molecules. The yeast cells are not viable without sterols [39, 47]. The main yeast and fungal sterol is ergosterol. The biosynthesis of ergosterol is one of the most complex biosynthetic pathways, which consists of 30 different biochemical reactions. These reactions are catalyzed by so-called Erg proteins. The majority of Erg proteins are located in ER except for Erg1p, Erg6p, and Erg7p, which are localized in lipid droplets. Biosynthetic pathway of ergosterol is divided into pre-squalene and post-squalene pathway (see Fig 2.9)[39, 47]. The first steps of sterol synthesis are similar in fungi, plants, and animals. Firstly, Erg10p catalyzes the condensation of two acetyl-CoA molecules to yield acetoacetyl-CoA. Acetoacetyl-CoA then reacts with another acetoacetyl-CoA to form

(3S)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is in yeast subsequently reduced to mevalonate by HMG-CoA reductases 1 and 2 (*HMG1/2*). HMG-CoA reductase shows feedback inhibition by ergosterol and reduction of HMG-CoA to mevalonate is thus a rate-limiting step and one of the major control points in yeast sterol biosynthesis. Mevalonate is then phosphorylated and decarboxylated in a cascade of phosphorylation and decarboxylation reactions known as the mevalonate pathway. The mevalonate pathway results in isopentenyl pyrophosphate (IPP), the main precursor for squalene and other isoprenoids. Isomerization of IPP yields dimethylallyl pyrophosphate (DMAPP). DMAPP is a precursor for subsequent condensation reactions leading to squalene. These reactions are catalyzed by enzymes Idi1p, Erg20p, and Erg9. The post-squalene pathway starts with squalene epoxidation (Erg1p), then continues with a cascade of cyclization reactions which leads to lanosterol. These reactions are catalyzed by Erg7p enzyme [39, 47, 50].

The post-squalene pathway starts with squalene epoxidation (Erg1p) with several subsequent cyclization events that form from lanosterol. These cyclization reactions are catalyzed by the enzyme Erg7p. The lanosterol is the first intermediate with a typical sterol structure. Lanosterol is after complex demethylation, desaturation, and reduction reactions formed zymosterol. The reactions yielding zymosterol are conserved in all eukaryotic cells and are catalyzed by the genes *ERG24-ERG28*. The Erg6p enzyme catalyzes methylation of zymosterol at C-24 position and leads to fecosterol formation. Fecosterol is a unique intermediate of yeast and fungi. The next reactions, including shift, introducing and removing of double bonds lead to the formation of the final product, ergosterol [39, 47, 50].

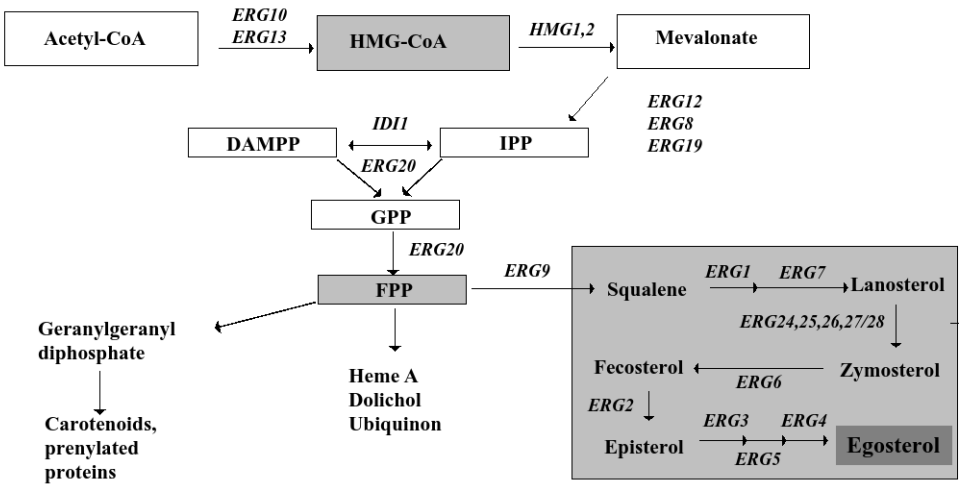

Fig. 2.9: Sterol biosynthesis in yeasts, DMAPP - dimethylallyl pyrophosphate, IPP -isopentenyl pyrophosphate, GGPP - geranylgeranyl pyrophosphate, FPP - farnesyl diphosphate.

In yeasts are sterols usually synthesized in excess and yeast cells are unable to degrade

them. If sterols are not degraded, they reach high levels which are toxic for yeast cells. Thus, yeast cells need to have detoxification mechanisms to avoid sterol harmful influence on membranes. The sterol homeostasis is maintained by the esterification of free sterols with FA, by downregulation of sterol biosynthesis, and by sterol acetylation which enables yeast cells to efficiently secrete excess sterols in the form of sterol acetate to medium. Acetylation of sterols is a potential quality control mechanism. Sterols that do not pass the quality control cycle are acetylated and secreted [39, 47, 50].

2.4.4 Carotenoid biosynthesis

Carotenoids belong to a group of isoprenoids which represent a large group of compounds produced mainly as secondary metabolites by plants and microorganisms. They are built from isoprene units, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Sterols and isoprenoids share a part of the pre-squalene biosynthetic pathway, the mevalonate pathway [39, 51, 15]. An important regulation step in the carotenoid synthesis is the formation of IPP and its subsequent isomerization which yields DMAPP. In the next step, an addition of three molecules of IPP to DMAPP molecule leads to the formation of geranylgeranyl pyrophosphate (GGPP). Condensation of two molecules of GGPP leads to the formation of phytofluene. The formation of phytofluene is catalyzed by bifunctional enzyme phytoene synthase/lycopene cyclase (CrtB) [51, 52]. The next reaction steps are carried out by enzyme phytoene desaturase CrtI. The first step leads to the formation of neurosporene, which may be transformed into lycopene or β-zeacarotene. Next metabolite γ-carotene can be formed from lycopene or β-zeacarotene. Lycopene cyclase activity results in the cyclization of lycopene to form γ-carotene (CrtY). Dehydrogenation of β-zeacarotene results in γ-carotene formation [53, 15]. γ-carotene is a precursor for β-carotene and torulene. Both reactions are catalyzed by the phytoene synthase/lycopene cyclase enzyme. Hydrogenation and oxygenation of torulene results in the formation of torularhodin [53, 15]. The proposed biosynthetic pathway can be seen at Fig. 2.10. The yeast *Phaffia rhodozyma* is capable of astaxanthin production as a major end product. Astaxanthin is formed from β-carotene through subsequent reactions carried out by ketolases and hydroxylase enzymes. The intermediate products are echinenone, hydroxyechinenone, and phoenicoxanthin [53, 54, 51].The proposed biosynthetic pathway can be seen at Fig. 2.11.

Another difference can be found in the carotenoid biosynthetic pathway of yeast *S. pararoseus*, where torulene can be synthesized from 3,4-didehydrolycopene. The transformation from 3,4-didehydrolycopene to torulene is supposed to be catalyzed by the enzyme phytoene synthase/lycopene cyclase AL-2. The 3,4-didehydrolycopene was also found to be a precursor of torulene in fungi *N. crassa* [15]. In recent years, there is a rapid increase in knowledge about carotenoid biosynthesis in carotenogenic yeast. It is con-

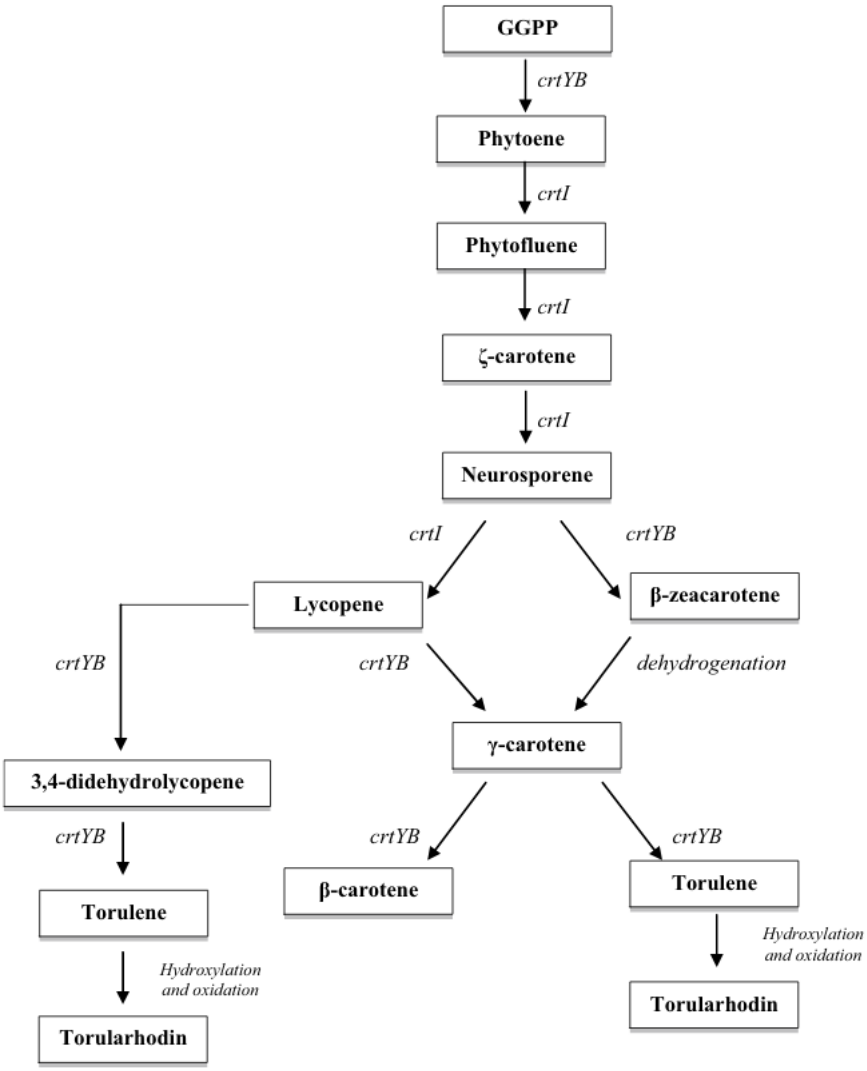

Fig. 2.10: Proposed biosynthesis of carotenoids from gernylgeranyl diphosphate to torulene and torularhodin in *Rhodotorula*, *Sporobolomyces*, and *Cystofilobasidium* sp. [15].

nected to the availability of molecular and omics tools. Recent genome sequencing of *R. toruloides* NP11 has led to the identification of two genes, phytoene synthase *(PSY1)* and phytoene dehydrogenase *(CRTI)* [55]. Lately, there were also identified genomic regions in *R. mucilaginosa* containing genes responsible for carotenoid production. It was found out that genes for phytoene synthase *(crtB)*, lycopene cyclase *(crtY)* and phytoene desaturase *(crtI)* were located in close proximity to one another. In numerous yeast species the *(crtB)* and *(crtY)* genes are fused, and thus there is a code for a bifunctional protein containing both lycopene cyclase and phytoene synthase activities. Surprisingly the gene coding for enzyme geranyl pyrophosphate synthase which is crucial for the production of early precursors of carotenoids is located on a separate contig. In *R. toruloides* genome is a gene for carotenoid oxygenase *(crtX)* which is responsible for cleavage of carotenoids

to retinal. The *(crtX)* gene is located in close proximity to *(crtY)*. These two genes are convergently transcribed, unlike in *R. mucilaginosa*, where are genes *(crtX)* and *(crtY)* transcribed divergently and are separated by a large gene coding for OPT (Oligopeptide transporters) family of small oligopeptide transporters. The *(crtX)* is presumably involved in the regulation of carotenoid synthesis by negative feedback mechanism [56].

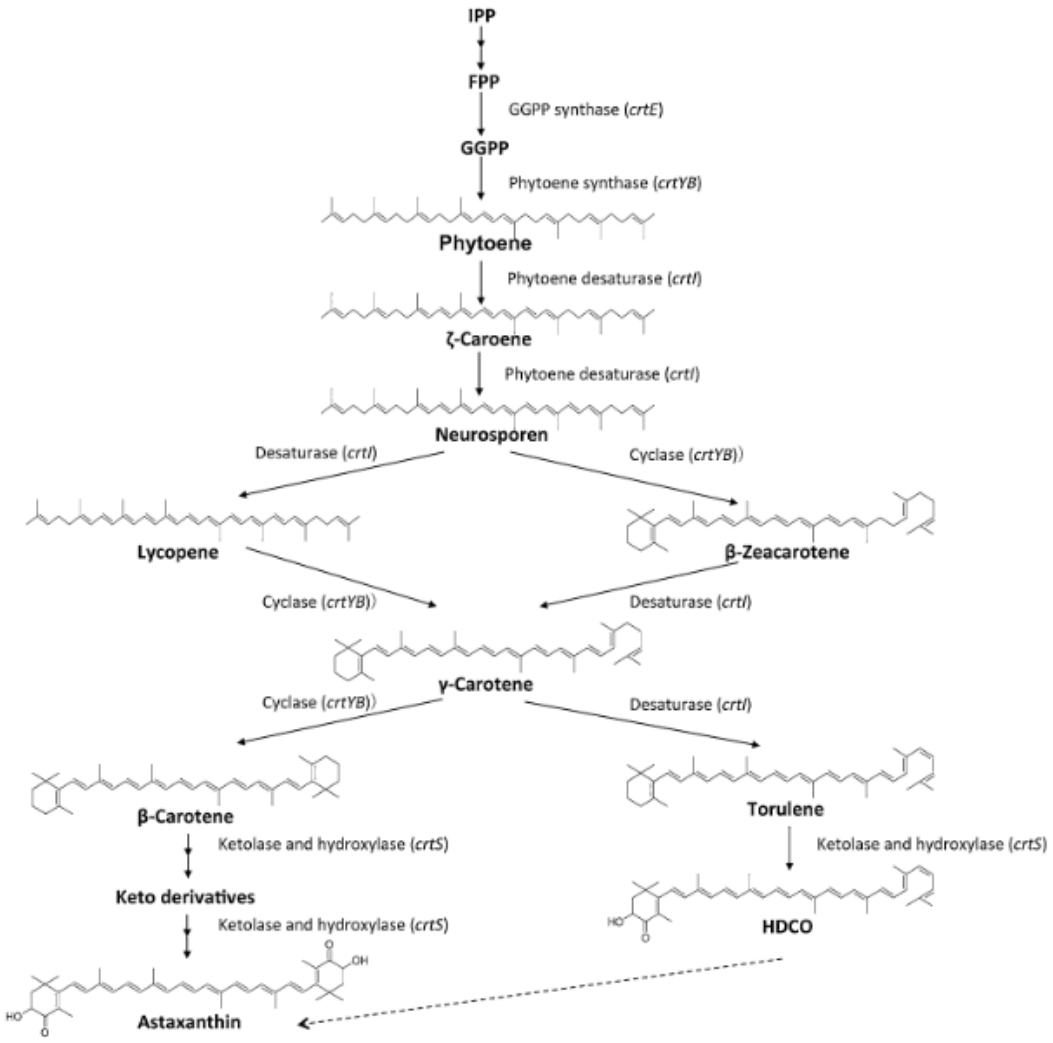

Fig. 2.11: Proposed carotenoid biosynthetic pathways by Chi et al. in *P. rodozyma* [54].

Biosynthesis of astaxanthin in yeast *Xanthophyllomyces dendrorhous* depends on a unique type of cytochrome P450 system, which consists of astaxanthin synthase (CrtS) encoded by *(crtS)* and its reductase encoded by *(crtR)* gene. Astaxanthin synthase catalyzes the ketolation and hydroxylation of β-carotene to produce astaxanthin. Biosynthesis of astaxanthin can be achieved only when *(crtS)* and its reductase encoded by *(crtR)* are expressed [57].

2.5 Factors influencing lipid metabolite production

Lipid and carotenoid metabolism regulation in carotenogenic yeasts are still not well known. Carotenoid and lipid production is known to be influenced by chemical and physical parameters or by the addition of a specific inhibitor which can result in accumulation of the desired metabolite. A very influential role has also oxidative stress, which influences mainly carotenoid production as a protection to reactive oxygen species.

2.5.1 Temperature and aeration

Temperature influence not only the general quantity but also the relative ratio of metabolites. In the case of carotenoids was reported increased production of torularhodin over torulene and β-carotene at a higher temperature [58, 15]. At low temperatures, yeast cells modulate their membrane fluidity. This adaptation is linked to the composition of fatty acids in membrane lipids, such as the length of fatty acid chains, structure, and saturation of fatty acids, and the rate of *cis* and *trans* fatty acids are closely related to membrane fluidity. In oleaginous yeast is generally the cultivation at low temperature responsible for increased amounts of PUFA including linoleic acid and α-linolenic acid [59]. Carotenogenic yeasts have mainly strictly aerobic metabolism. In studies done by Frengova and Beshkova, it was stated that the ideal aeration for the cultivation of the genus *Rhodotorula* should be in the range from 180 to 190 rpm, and the airflow should range from 0.5 to 1.9 l per min [58]. Other studies done by Aksu and Eren have reported that an increase in the aeration rate from 0 to 2.4 vvm significantly increased carotenoid synthesis by *Rhodotorula glutinis*. The total biosynthetic efficiency increased from 63.4 to 105.8 mg/l after 10 days of cultivation. The aeration rate has a negative influence on the biosynthesis of intracellular lipids. With increasing content of dissolved oxygen the total amount of intracellular lipids in *Rhodotorula glutinis* decreased [18].

2.5.2 Nutritional limitations

A significant role in lipid and carotenoid metabolism has nitrogen limitation. Nitrogen limitation is a well known effect with impact on lipid accumulation especially in oleaginous yeasts. Uniquely in oleaginous yeasts is present citrate lyase enzyme, which converts cytoplasmatic citrate to acetyl-CoA. This phenomenon is in detail described in chapter 2.4. Generally, with increasing C/N ratio, the lipid and carotenoid production is stimulated. However, with high C/N ratio is preferentially increased lipid content, the carotenoid production is increased at mild C/N rations. With increasing C/N ratio is reported increased production of torulene and torularhodin and decreased production of PUFA in carotenogenic yeasts [60]. There is still unexplained link between carotenoid and lipid metabolism in carotenogenic yeast. Some studies dealing with the impact of

nitrogen limitation to carotenoid and lipid production in yeast *P. rhodozyma* supposed the existence of two isoforms of ATP citrate lyase with different regulations. One type is supposed to be regulated by levels of dissolved oxygene (DO) and another which is regulated by nitrogen depletion. When DO is below 20 % saturation and there is a depletion of nitrogen, the citrate lyase activity is triggered to fatty acid biosynthesis. When the DO levels are above 20 % of saturation and sugar is shortly depleted, rapid carotenoid accumulation occurs [61, 62].

Influential role in nitrogen limitation has also the impact of carbon source and its concentration. As an example of concentration impact can serve glucose. It was proved that the initial high concentration of glucose has no positive economic effect on lipid production using batch fermentation and should thus be avoided. The price of carbon source is influential on the prize of the whole process [17]. Karanth and Sattur found, that the lipid production in batch fermentation was similar for initial glucose concentration of 60 and 80 g/l, while these treatments yielded around twice as much lipids as treatments with initial glucose concentration of 100 g/l. The lipid production with initial glucose concentration of 100 g/l had about the same level of lipid production compared to 40 g/l initial sugar glucose concentration at the end of cultivation time [60]. Papanikolaou et al. also pointed out that some key enzymes for lipid synthesis might be inactive at high glucose concentration. Other explanation is that the glucose is consumed in the same rate without considering glucose concentration and the difference of lipid accumulation can be observed once the cultivation time is prolonged adequately to initial glucose concentration [60].

Microbial lipid production is still economically demanding. The use of waste substrates as sewage juice and monosodium glutamate wastewater, or starch wastewater can reduce the cost of microbial lipid production. A rich nitrogen content does not allow to use a high C/N ratio which would increase the lipid production. Phosphorus limitation is reported to have an influential role to lipid accumulation in *R.toruloides*. In conditions of phosphorus limitation can be obtained lipid content over 60 % under conditions with a molar C/N ratio as low as 6. It is supposed that the regulation mechanism of phosphorus limitation is the same as in the case of nitrogen limitation. The levels of AMP are also decreased due to activation of AMP deaminase, which cleaves the AMP. Cleavage of AMP releases the inorganic phosphate and subsequently covers phosphate exhaustion. The detailed mechanism of phosphorus limitation in *R.toruloides* still needs to be proved by molecular methods [38].

2.5.3 Impact of physical and chemical mutagenes

The impact of physical and chemical mutagens may have a beneficial impact on lipid and carotenoid metabolism. To date, there are many studies focused on enhanced carotenoid production through random mutagenesis induced by physical or chemical mutagens in *P. rhodozyma*. Thanks to molecular methods and the impact of mutagens on lipid and carotenoid metabolism of *P. rhodozyma*, it was possible to reveal key enzymes in lipid and carotenoid biosynthesis. The most successful mutagenesis studies used mainly alkylation agents such as methyl methanesulphonate (MMS), ethyl methanesulphonate (EMS), N-methyl-N′-nitro-N-nitrosoguanidine (NTG) or ultraviolet radiation (UV) as a physical mutagen [63, 64, 65, 66]. The process of mutagenesis is time consuming due to the complicated selection of overproducing mutants. Great help can offer selection using flow cytometry but of course, there can be some drawbacks as false signal due to staining procedure or due to the presence of lipids and carotenoid inside of lipid droplets which differ in size and number inside of the cell. As a rapid way for selection can be used inhibitors of lipid or carotenoid synthesis or their combination. The most often used inhibitor for the selection of lipid overproducing yeast mutants is cerulenin which is generally used as a fungicide. Cerulenin binds covalently to a cysteine residue at the active site of the fatty acid synthase enzyme and irreversibly inhibits β-ketoacyl-ACP synthase activity of FAS [67]. Mutants that survive cerulenin treatment are expected to produce higher levels of lipids or PUFA [68]. Industrially used inhibitors that selectively block lycopene cyclase are tertiary amines, aminomethyl pyridine, imidazole, pyridine, quinolone, nicotine, and vitamin A acetate. The blocking of lycopene cyclase leads to the accumulation of lycopene [31]. Inhibitors that block lycopene cyclase activity cannot be used as a selection agens, because they only lead to production of lycopen. For the selection of carotenoid overproducing mutants is used diphenylamine (DPA). The DPA blocks the sequence of desaturation reactions by inhibiting phytoene synthase, leading to an accumulation of phytoene and other normally absent carotenoids. The mutant strain with overproduction of carotenoids can be then easily distinguished from others visually [69].

Alkylation agens

Alkylation agents such as ethylmethane sulfonate (EMS), methylmethane sulfonate (MMS) are mutagenes for most purposes, they are water soluble and thus easy to handle. These mutagenes cause high frequencies of base-pair substitutions with little lethality [70]. EMS and MMS transfer ethyl or methyl groups to a DNA base and cause changes in base pairing. EMS causes ethylation of guanin at 7-N and 6-O position. If 7-ethylguanin is formed, it is paired with thymin and thus there is a transition in G:C to A:T direction. EMS and MMS are agens which cause mutation during replication and in non-replicating DNA [70, 71].

Ultraviolet irradiation

Ultraviolet irradiation (UV) is a very strong mutagen for unicellular organisms. UV is absorbed by purins and pyrimidins in DNA. These bases are then excited to the state of higher reactivity or energy. The maximal absorption of DNA bases is at 254 nm, and doses of such radiation have the highest mutagenic effect [71]. The pyrimidins are after UV absorption highly reactive and product pyrimidine hydrates and pyrimidine dimers. The adjacent pyrimidines on the same strand of DNA are joined by the formation of a cyclobutane ring resulting from saturation of the double bonds between carbon 5 and 6. Pyrimidine, especially thymine dimers are causing mutation by breaching of the DNA structure and distract proper replication and reparation processes in DNA [70].

Reparation mechanisms

Many reparation mechanisms are conserved from bacteria and yeast through man. Reparation mechanisms involved in reparation of mutations caused by chemical mutagenes or UV irradiation are mainly photoreactivation and excision repair. The photoreactivation is a direct reparation process carried out by enzyme DNA photolyase. The activity of DNA photolyase is dependent on visible light, especially blue light. DNA photolyase recognizes and binds to thymine dimers in the dark, but without access of light it can not cleave covalent cross-bonds. This enzyme is also capable to cleave cytosine and cytosine-thymine dimers under the access of light. Whenever the UV mutagenesis is done for the gain of mutants, the yeast culture must be then incubated in dark without access to light to keep mutations unrepaired [72, 73]. Reparation of methylated lesions is carried out by an enzyme called O^6-methylguanine methyltransferase that transfers the methyl group from O^6-methylguanine to a cystein residue and its active site [72, 73].

Unlike photoreparation, the excision repair is not dependent on excess of light and it is fully functional in the dark. There are two types of excision repair, base excision repair and nucleotide excision repair. The base excision repair uses a set of DNA glycosilases which recognize abnormal bases and excise the base lesion to generate an abasic site - a sugar with no bases attached. Then AP-endonuclease together with phosphodiesterases cleaves sugar-phosphate groups. After these steps, the resulting single-base gap is filled by DNA polymerase. DNA ligase then completes the repair [74].

Unlike the DNA glycosylases which recognize only specific forms of damaged bases, the nucleotide excision repair system is able to recognize a wide variety of damaged bases that distort the DNA molecule. The repair is characterized by removing the damaged DNA in a form of an oligonucleotide fragment. The damaged DNA is firstly incised on both sides of the lesion by enzyme excinuclease and then the oligonucleotide fragment is removed. The reparation is then finalized with DNA polymerase which completes missing nucleotides and their subsequent ligation catalysed by DNA ligase [75, 71].

2.6 Molecular methods

Yeast molecular methods can be used for many purposes from yeast identification, gene expression studies to their genetic modification. Most of the molecular methods are based on polymerase chain reaction (PCR) and restriction endonuclease activity. PCR is a breakthrough method of molecular biology, which enables to amplify DNA molecules after a given template using two pairs of template-specific oligonucleotides, DNA polymerase, deoxyribonucleotides and suitable buffer. PCR reaction needs generally three different temperature conditions for denaturation of dsDNA, annealing, and elongation of DNA molecules after a given template. In laboratories are generally used thermal cycler machines, which are easily programmable to desired temperature programs for PCR.

2.6.1 Yeast identification methods

Traditionally were used morphological methods for yeast and fungal identification. However, morphological approaches used to describe the characteristics of species are important, these techniques may be misleading for low-level classification within an evolutionary framework. In some lineages of fungi, morphological character can be contentious or problematic even for trained mycologist [76]. It is given by the fact that morphology can be influenced by environmental conditions, and generally can be misleading due to hybridisation, cryptic speciation, and convergent evolution [2]. Nowadays are in mycology used short DNA sequence markers frequently based on ribosomal gene sequences for fungal identification [14, 13, 77, 76].

Ribosomal barcoding and single-copy protein-coding genes

The sequences of yeast ribosomal genes (rDNA) exhibit a sufficient level of variation to serve as short DNA sequence markers. Interestingly, the rDNA is composed of multiple repetitive transcription units and each of them is transcribed by RNA polymerase I. The rDNA genes are placed in subsequent order: 18S-rRNA, 5.8SrRNA, and 28S-rRNA, and they decode, respectively for small (SSU) and large (LSU) subunits. These genes are separated by two internal transcribed spacers called ITS1 and ITS2 (See Fig. 2.12), which are evolving the fastest and thus exhibit the highest variation. The SSU sequence is evolving the slowest and therefore possesses the lowest amount of variation among taxa. The SSU rDNA sequence serves for phylogenetic classification of a fungus on a higher level [78, 76]. If the classification needs to be done at the intermediate level, then can be used the LSU sequence which contains hypervariable domains D1 and D2. For species level identification, ITS sequence is the most useful, because it is the fastest evolving portion of rRNA cistron. Nowadays, the official barcodes that were chosen by the consortium

of mycologists for yeast and fungal identification are ITS sequences and D1/D2 hypervariable domains of LSU. The ITS sequence and sequence of D1/D2 domains are easily amplified, widely used, and they propose appropriately large differences between interspecific and itraspecific variation [76]. Another advantage of ITS and D1/D2 sequences as barcodes is that they were used in major studies in fungal systematics, such as Assembling the Fungal Tree of Life (AFTOL), and thus there is a large number of rDNA sequences of fungal rRNA in GenBank databases available for identification via barcoding. Identification procedure consists of next steps: extraction of genomic DNA from pure culture, amplification of barcode sequences by PCR, sequencing, sequence BLAST search (Basic Local Alignment Search Tool) International sequence Database, use Ref Seq Loci in GenBank, EMBL or other databases and search markers for further phylogenetic analysis. Ribosomal genes are also often used for rapid identification within 24 hours using methods as PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism), Real-Time PCR procedure or Denaturation Gradient Gel Electrophoresis (DGGE) in combination with PCR [2, 79, 77, 76].

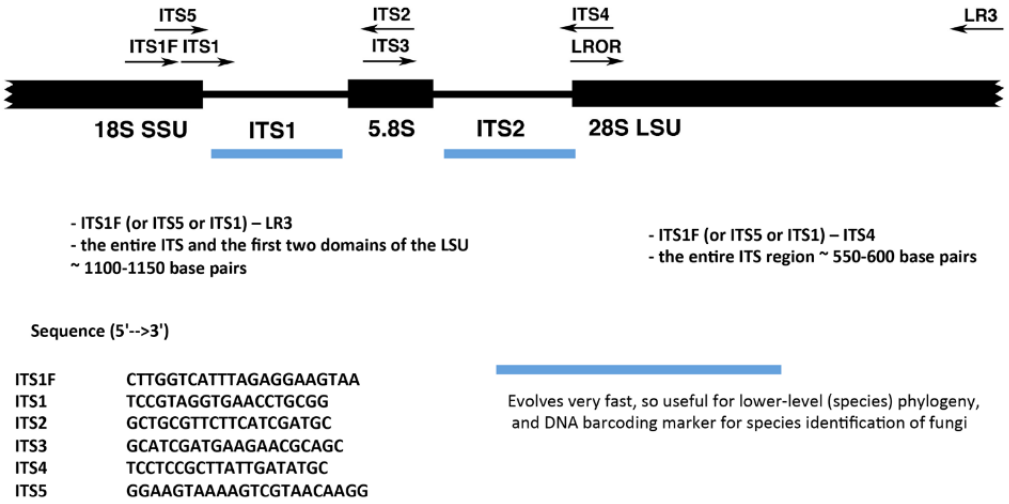

Fig. 2.12: Ribosomal operon and primers position and sequences for yeast identification [76].

If the ribosomal genes do not provide an accurate species identification, the sequences of protein-coding genes should provide better resolving power for a certain lineage of fungi. Protein-coding gene sequences contain intron regions which sometimes evolve at a faster rate compared to ITS sequences, and thus provide better resolution in phylogenetic analysis at higher taxonomic levels. Moreover, protein-coding genes are believed to occur as a single copy in fungi and they are less variable in their length since they accumulate fewer mutations in their exons [76]. Protein-coding genes are widely used in fungal systematics for reconstruction of molecular phylogenetics. The most widely used protein-coding markers are the largest *(RPB1)* and the second largest *(RPB2)* subunits of RNA polymerase, translation elongation factor 1-alpha *(TEF1)* and and beta-tubulin

(tub2/BenA). As promising markers for interfering phylogenetics, higher and lower-level phylogenetics relationships were more recently used genes for minichromosome maintenance protein *(MCM7)* and cytochrome B coding genes *(CYTB)* [12, 13, 14].

Denaturation gradient gel electrophoresis

DGGE - denaturation gradient gel electrophoresis is a suitable method for yeast and bacterial identification. It is used in combination with PCR, where firstly are the amplified regions of ribosomal genes (ITS and D1/D2 regions) and then are these fragments of DNA separated in polyacrylamide gel with increasing concentrations of denaturation agents. For PCR reaction are used special primers with so-called GC clamps which prevent complete denaturation of single-stranded DNA in DGGE gel and therefore prevent the production of smeared bands in the gel [80, 79]. As denaturation agens are mostly used acrylamide and urea or simple temperature gradient. The detection is based on successive denaturation of DNA fragments in acrylamide gel. DNA fragments rich in AT base pairs undergo denaturation faster than DNA fragments with high content of GC pairs. It is caused by the different number of hydrogen bonds holding double-stranded DNA together. DGGE is a very sensitive method used to detect changes even in one nucleotide between two or more sequences. Drawback of DGGE is that only DNA molecules of maximum size 500 bp can be effectively separated and a standard organism gained in culture collection is needed for further identification of the unknown organism. DGGE is also very demanding on the preparation and optimization of separation conditions [80, 79].

Restriction fragment length polymorphism

Restriction Fragment Lenth Polymorphism - RFLP is a very common method in microbial identification, because it is less demanding on preparation and optimization than DGGE and offers the possibility to compare results with restriction patterns utilizing specialized programs. First step is amplification of ribosomal gene regions by PCR using specific primers. Amplified DNA fragments are then cleaved with chosen commercial restriction endonucleases to generate fragments of different lengths. Restriction endonucleases recognize specific palindromic sequences. The results are interpreted through agarose gel electrophoresis. The restriction pattern on the gel is then analyzed by a specialized program and compared with *in silico* restriction digestion utilizing NEBcutter, MapDraw or BioEdit program [81]. RFLP is a method also used in methods of genetic engineering for creation of physical DNA maps or for gene localization in genetic diseases, or for vector preparation [71].

Sequencing

Sequencing is a method for determination of the primary sequence of DNA or RNA molecules. Knowledge of DNA sequences is indispensable in many fields of life sciences as phylogenetic studies, forensic studies, virology, biotechnology, and genetic engineering. Sequencing is a very precise method and thanks to technological expansion it has become a rapid, most precise, and economically available method [82].

At 1977, the first described methods used for sequencing were the Sanger enzymatic method and the Maxam and Gilbert chemical degradation method. Automated Sanger sequencing method helped to reveal the human genome sequence, and it is still commercially available till these days. The Sanger method is used for short fragments of DNA sequences like PCR products or plasmids. Sanger method has high precision which is typically more than 99%. The drawback of Sanger method is the limited length of reading frame (max 1100 bp), the relatively low number of samples which could be analyzed in parallel, and the difficulty of total automation of the sample preparation process [83].

A more sophisticated methods of sequencing are methods of Next generation sequencing including Second and Third generation sequencing. Their main goal is to decrease the prize and time of the whole process. The next generation sequencing technologies including Illumina sequencing, Roche 454 sequencing, SOLiD sequencing, etc., sequence millions or billions of DNA strands in parallel [83]. The most popular technique is Illumina sequencing for omic studies such as whole genome sequencing and RNA sequencing. Combination of whole genome sequencing and RNA sequencing can be very informative in the applications of microbial metabolic engineering [84, 85]. Sequencing of RNA is very helpful when working with non-conventional organisms and in combination with genome sequencing can help to reveal new metabolic pathways and regulation of gene expression generally [55]. RNA-seq is more useful than standard methods of gene expression like chip or probe technologies which are limited only to known genes [86, 87].

2.6.2 Yeast genetic modification

For yeast genetic modification are used numerous methods for ligase-dependent or independent cloning. The most notable methods are TOPO-TA cloning (Life Technologies), Gateway system (Life Technologies), and Gibson assembly. TOPO-TA uses Topoisomerase I to insert PCR-generated DNA fragments into a vector. Gateway system uses two vectors which can transfer already cloned piece of DNA from one vector to another. Gibson assembly is very useful in the construction of large DNA sequences, and it can be easily adapted even for smaller cloning experiments. Gibson assembly combines attributes of 5' exonuclease activity, thermostable DNA polymerase, and T4 DNA ligase. The drawbacks of these methods are the high price of a kit, maintain a steady supply of reagents, or they require time-consuming optimization and troubleshooting when being

extended [88]. Yeast cloning is still limited because of the need of compatible shuttle vectors and the demand of specific plasmids with specialized genetic elements. The shuttle vectors allow cloning steps in *Escherechia coli*, before introducing the construct into yeasts. The disadvantages of shuttle vectors are the difficulty and time-consuming construction of the plasmid, it does not allow to clone all open reading frames (ORF) into the expression plasmid. Furthermore, an introduction of extra DNA sequences other than the necessary selection marker, and promotors and ORF in yeast genome is difficult. The most common example is the presence of an antibiotic resistance marker gene for selection in *Escherechia coli* [89].

This problem has been solved by using a yeast cloning cassette (YCC) which can be prepared as a part of the chosen plasmid, which can be propagated in *E. coli* to the desired number of copies. These vectors are based on altered bacterial plasmids containing bacterial origin of replication, a marker for selection in bacteria, and YCC containing yeast selection marker, a gene of interest between promotor and terminator sequences and elements for integration into recipient genome. Transformed *E. coli* then serves as a ready-to-use deposit of the prepared vectors. When necessary, the parts of YCC cassette can be anytime amplified or excised by restriction endonucleases and used for subsequent applications. Prior to yeast transformation is prepared plasmid cleaved with restriction endonuclease and then the linear YCC is used for transformation. The advantage of transformation by linear DNA as YCC, is that integration into the recipient genome is prioritized by homologous recombination [90]. Linear YCC cassette is used in cases where lithium/acetate electroporation or biloistic transformation method is used. For transformation of carotenogenic yeast are frequently used *Agrobacterium tumefaciens* mediated transformation (ATM) or electroporation. Electroporation is preferable due to the high efficiency in comparison to other commonly used methods [91, 92, 93]

Except for yeast *Phaffia rhodozyma* are not yet developed genetic tools for the majority of carotenogenic yeasts. A great progress has been lately done in genetic modification methods for yeast *Rhodotorula toruloides*. Unfortunately, there is still limited efficiency in gene deletion, due to the preference of non-homolougous end-joining (NHEJ) pathway over homologous end-joining pathway in carotenogenic yeasts [11, 94]. NHEJ pathway uses the Ku protein that binds to the ends of introduced linear DNA, and together with DNA ligase they carry the DNA fragment at random sites in the host chromosome. For NHEJ integration system into the chromosome is not required homology between introduced DNA and the integration site. A target integration through homologous recombination can be improved when Ku protein or ligase is disrupted through mutation [95, 11].

Agrobacterium tumefaciens

Agrobacterium tumefaciens is a gram-negative soil bacterium that infects plants and causes crow gall tumor formation. Plant infection is mediated thanks to a special Ti (Tumor-inducing) plasmid consisting of a specific segment of bacterial plasmid DNA called T-DNA (transferred DNA) containing information for opine production, *vir* sequence, origin of replication and the genes for opine catabolism. Opines are condensation products of amino acids and and keto acid or amino acid and sugar, and serve as a carbon source for *Agrobacterium tumefaciens*. The infection process is induced by the presence of phenolic compounds or monosaccharides excreted by wounded plants [95]. Phenolic compounds are sensed via the *virA/virG* two-component regulatory system that induces *vir* genes. The *vir* genes are essential for the transfer and integration of T-DNA into plant chromosomes. Transferring process is supposed to be similar to plasmid transfer from donor to recipient cell during bacterial conjugation. T-DNA is transferred as a linear single-stranded DNA produced by a *virD1/D2*-encoded-site specific endonuclease that nicks within 24bp direct repeat sequences on the Ti plasmid. These repeats are called border sequences, and they are placed on the boundaries of the T-DNA. The T-DNA is then cleaved and excised by the single-stranded DNA-binding protein VirE2, transferred into the plant cell, and incorporated into genomic DNA [92].

In yeast ATM is necessary to prepare a suitable binary vector prior to transformation. The binary vector contains bacterial replication origin, genes for antibiotic resistance, desired ORF, and marker for selection in yeasts placed in between T-DNA border sequences. The *vir* genes are in other plasmid already present in *A. tumefaciens*. After transformation and selection of transformed *Agrobacterium tumefaciens*, the next step is co-cultivation with yeast culture in the presence of phenolic compounds such as acetosyringone or hydroxyacetosyringone. After cocultivation, yeast and bacterial cells are separated and yeast are inoculated on selection agar plates [92]. Successful metabolic engineering of *R. toruloides* thought ATM was reported by Zhang et al. In their experiment were used two *R. toruloides* strains for increased lipid production by over-expressing the native acetyl-CoA carboxylase and diacylglycerol acyltransferase genes. The best transformants were able to produce 16.4 g/l lipid from 70 g/l glucose and 9.5 g/l lipid from 70 g/l xylose in shake-flask experiments [66]. The first successful gene deletion was reported by Koh et al., where deletion was possible only in KU70 deficient mutants of *R. toruloides*. A KU70-deficient mutant was generated with the hygromycine selection cassette, which was subsequently removed by Cre--*lox*P recombination system. The targeted gene deletion frequency was improved to 90 % when used homology sequence length of at least 1kb [11]

Electroporation

Electroporation is a special method used generally in bacterial and yeast transformation. The main principle of DNA transfer into the cell is mediated through short electric pulses which help to create temporary pores in the cell wall. Vector is then transferred into the cell thought pores. Prior electroporation is necessary to prepare competent cells by addition of $CaCl_2$ or lithium/acetate to the cell culture. The salt solution causes adhesion of DNA molecules to the surface of the cell wall. The salts do not influence the transport itself, they just cause higher adhesion of DNA molecules to the cell wall, so that when the pores are created, there is a higher probability of DNA transport into the cell. Electroporation with linear YCC with hygromycin and bleomycin as selection markers, was used for successful transformation of *R. toruloides*. In this study reported by Liu et al., was also demonstrated the positive impact of dithiothreitol (DTT) and lithium acetate on competent yeast cells prior to electroporation [96]. Electroporation is also frequently used in genetic modification of yeast *P. rhodozyma* [97] or *R. gracilis* [98].

Biolistics

Biolistics transformation was first developed for plant cells, but it is used for genetic modification of many other organisms, yeast including. For transformation are used spherical gold or tungsten microparticles coated with DNA. As precipitants serve $CaCl_2$, spermidine, or polyethylene glycol. The prepared microparticles are then accelerated to a high speed with a special apparatus called a particle gun. The particle gun employs high pressure helium and the particles can be accelerated to speed in the range from 300 to 600 meters/second. Proper penetration of cells can be controlled by varying the intensity of the explosive burst, altering the distance of the target, or by using different size particles (the apparatus is visualized at Fig. 2.13)[99, 95, 100]. In case of yeast transformation are exponentially grown yeast cells in liquid medium transported to solid agar plates and "bombarded" with microparticles. For selection of transformed yeast cells are used selection plates depending on the used selection marker [99, 100].

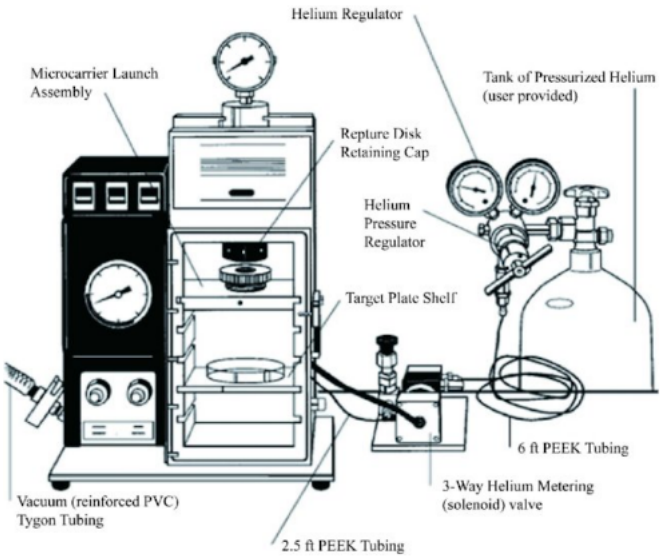

Fig. 2.13: The scheme of biolistic apparature [101]

2.7 Techniques to Yeast Metabolite Characterization

Usually, there are many necessary steps prior to any further analysis providing quantitative or qualitative properties of the metabolite. In the case of carotenogenic yeasts are the most investigated metabolites carotenoids and lipids which deserve special attention thanks to their nutritionally beneficial impact. Both lipids and carotenoids represent lipophilic compounds with little or no solubility in water, and it is always necessary to keep in mind during analysis their susceptibility to high temperature, oxidative agents and light sensitivity, especially in case of carotenoids. The extraction techniques are essential to the next steps of characterization [23]. In yeast extraction, there can be difficulties with sufficient disruption of the cells, so that the lipophilic compounds can freely pass into the desired solvents. Without sufficient disruption, there are problems with quantification of lipids and carotenoids, because some of them can still remain in the cell pellet and erroneously decrease final yeast productivity. The most effective extraction methods for lipidic compounds are Folch, Leese and Stanley method, Soxhlet extraction, and fatty acid transesterification method [102, 103]. After extraction, following separation techniques and qualitative and quantitative analysis methods. The most useful methods, providing sufficient qualitative and quantitative resolution power, are chromatographic methods, especially high performance liquid chromatography (HPLC), gas chromatography (GC) and thin layer chromatography (TLC). Chromatographic methods can be also combined with mass spectroscopy methods in tandem and provide even more sophisticated qualitative analysis. The mass detectors used in carotenoid analysis in tandem with HPLC are At-

mospheric Pressure Chemical Ionisation (APCI), MALDI-TOF (Matrix-Assisted Laser Ionisation Desorption - Time of Flight Detector [23, 104]. A very rapid and relatively precise is also UV-VIS spectrophotometry method [105]. Regarding the complexity and variability of analytical methods, will be the following chapter focused on chosen techniques of lipid compound analysis.

2.7.1 Lipid compound isolation methods

In carotenogenic yeasts, there are most of lipids and carotenoids stored together in lipid droplets. Most extraction methods of these nonpolar compounds are using a solvent system composed of non-miscible polar and nonpolar solvents. Excellent extraction powers provide the combination of methanol/hexane/water or methanol/chloroform. There are numerous effective extraction methods such as Soxhlet extraction used mainly for lipid extraction, Blight and Dyer method, or Folch, Leese, and Stanley methods providing effective extraction of carotenoids and lipids [102, 103]. Folch, Leese, and Stanley method, also known as Folch method, is the most effective for lipid extraction straightly from yeast biomass cell membranes or for extraction of lipids from cell compartments fractions. Combination of Folch solvent system with glass beads provides an effective cell wall disruption mechanism and effective lipid extraction. There exist also sophisticated methods for carotenoid extraction and purification including the step of lipid separation through saponification or enzymatic lipid degradation. These methods are time consuming with a high risk of carotenoid degradation. For carotenoids containing carboxyl groups like astaxanthine or torularhodin are saponification methods unsuitable, because besides lipid saponification it causes also saponification of these carotenoids [106].

2.7.2 High performance liquid chromatography

High performance liquid chromatography in combination with photodiode array detector (PDA) is a suitable method for carotenoid identification and quantification [23]. There are numerous methods for carotenoid separation employing HPLC and most of them are using reverse phase system with nonpolar stationary phase and polar mobile phase. A satisfactory separation of carotenoids can be achieved using C18 column containing chemically modified silica gel particles bearing octadecyl groups. As a mobile phase can be used isocratic elution with solvent mix acetone:water (9:1, v,v) or acetonitrile: 0.1% triethylamine methanol:ethyl acetate (75:15:10, v,v,v) [15]. Even if HPLC-PDA methods can provide information about retention time and UV-VIS spectrum and specific shape of absorption spectrum of each carotenoid, it is reasonable to compare these compound with standards. The process of carotenoid separation, especially in case of yeast carotenoids is not easy to optimize and not many methods provide satisfactory separation of all carotenoid species.

Excellent separation power has acetone as mobile phase in combination with water, but it is not suitable for screening in shorter wavelengths. The absorption spectrum of acetone covers the absorption spectrum of the less colored carotenoids, steryl esters, and coenzymes. Very effective separation of plant carotenoid pigments including their *cis* and *trans* isomers has been lately reported by Gupta et al. In this study a C30 column with isocratic elution solvent mixture methanol:water (95:5, v,v) was used. So far, there is no study reporting *cis* and *trans* isomers of yeast carotenoids mainly because of the lack of commercially available carotenoid standards [107].

2.7.3 Gas chromatography

Gas chromatography is the most common technology used for fatty acid analysis, which provides suitable separation and subsequent qualitative and quantitative analysis. In combination with the flame ionisation detector is GC standardized method for fatty acid esters analysis. Prior analysis, it is needed to derivatize yeast lipids into methyl esters. High boiling points of fatty acids are too high and GC is restricted only for analytes with boiling point under 400 °C. Samples for yeast fatty acids analysis can be prepared straightly from lyophilized yeast biomass or from yeast lipid extracts. The most known types of derivatisation are base-catalysed method and acid-catalysed transesterification method. Base-catalysed transesterification reaction is limited on samples with low concentration of free fatty acids. Free fatty acids are forming undesirable soaps in the form of stable emulsions that complicate phase separation, increase the consumption of catalysts, and decrease formation of methylesters. The most effective and widely used technique is acid-catalysed transesterification, where the transesterification mixture contains a solution of methanol with 0.2 M sulphuric acid or hydrochloric acid. To defined amount of yeast lyophilized biomass is added transesterification mixture and chosen internal standard. Internal standard should be represented by fatty acid which is not present in the analyzed sample and has very similar properties as analytes. In yeast, it is generally used heptadecanoic acid (C17) or tridecanoid acid (C13). Transesterification reaction takes place at relatively high temperature (80 -100°C). After transesterification are fatty acid methylesters (FAME) extracted with n-hexane and analyzed, or they can be dried under N_2 and frozen till next analysis [108, 109]. Subsequent separation of FAMEs using GC is based on compound distribution between stationary and mobile phase. As a stationary phase is used methylpolysiloxane in silica or glass capillary column as a carrier in diameter from 100 to 700 μm with length 15 - 100 m. As a mobile phase can be used helium, nitrogen, or hydrogen. Subsequent detection of FAME is taking part in FID detector, where ionisation current of a specific fatty acid ester gives different responses depending on the number of carbon atoms [109].

3 OBJECTIVES

The dissertation thesis is focused on the production of valuable lipidic compounds such as carotenoids, fatty acids, and ergosterol using carotenogenic yeasts. To gain enhanced amounts of these compounds, the impact of cultivation conditions, mutagens, yeast species, and even the impact of yeast strain was studied. To fulfill these aims, molecular methods for yeast identification and yeast strain diversity were employed. One part of this thesis was also focused on the genetic engineering of carotenogenic yeast *R. toruloides*.

The main aims:

- The impact of phenotype to production properties of carotenogenic yeasts
- The impact of carbon/nitrogen ratio on lipid metabolism and lipid droplets formation
- The impact of inhibitory compounds on carotenoid metabolism
- Employment of genetic transformation methods and random mutagenesis for enhanced metabolite production
- Variability of metabolite production among various species of carotenogenic yeasts

4 MATERIALS

4.1 Microorganisms

The used yeast strains were obtained from The Culture Collection of Yeasts (CCY, Slovak Academy of Sciences, www.ccy.sk). The designation used in experiment, yeast species and yeast collection numbers are listed in the Tab. 4.1.

Tab. 4.1: Used yeast strains

Designation	**Yeast strain**	**Collection number**
RK	*Rhodotorula kratochvilae*	CCY 20-2-26
RMa	*Rhodotorula mucilaginosa*	CCY 20-7-28
RMb	*Rhodotorula mucilaginosa*	CCY 20-9-7
RMc	*Rhodotorula mucilaginosa*	CCY 20-7-31
RMd	*Rhodotorula mucilaginosa*	CCY 19-4-6
RT	*Rhodotorula toruloides*	CCY 62-02-04
CM	*Cystofilobasidium macerans*	CCY 10-1-2
CI	*Cystofilobasidium infirmominiatum*	CCY 17-18-4
SR	*Sporobolomyces roseus*	CCY 19-6-4
SS	*Sporobolomyces salmonicolor*	CCY 19-4-25
SM	*Sporobolomyces metaroseus*	CCY 19-6-20
SP	*Sporobolomyces pararoseus*	CCY 19-9-6
PR	*Phaffia rhodozyma*	CCY 77-1-1
2402	*Rhodotorula mucilaginosa*	CBS 2402
2403	*Rhodotorula mucilaginosa*	CBS 2403
2404	*Rhodotorula mucilaginosa*	CBS 2404
2405	*Rhodotorula mucilaginosa*	CBS 2405
6016	*Rhodotorula toruloides*	CBS 6016

4.2 Materials and chemicals

Yeast autolysate (Himedia; India)
Peptone (Himeda; India)
Agar type I (Himedia; Indie)
D-mannosa (Sigma-Aldrich; DE)
D-xylosa (Serva; DE)
YNB base without AA and ammonium sulphate (Sigma-Aldrich; DE)
Arabinose (Sigma-Aldrich; DE)
DPA (Lachema, Cz)
Sodium carbonate, p.a. (Lach-Ner; Cz)
Sodium bicarbonate, p.a. (Penta; Cz)
Sodium-potassium tartrate tetrahydrate, p.a. (Lach-Ner; Cz)
Ammonium molybdate, p.a. (Lach-Ner; Cz)
Sulfuric acid, 96% (Penta; Cz)
Sodium hydrogen carbonate heptahydrate p.a. (Penta; Cz)
Copper sulfate pentahydrate, p.a. (Lach-Ner; Cz)]
Triton X-100 (Sigma-Aldrich, DE)
Tris-HCl + EDTA for molecular biology (Top-Bio;Cz)
Sodium Dodecyl Sulfate (SDS), p.a. (Penta;Cz)
75% ethanol for molecular biology (Top-Bio;Cz)
Sodium Chloride, p.a. (Lach-Ner;Cz)
Tris(hydroxymethyl) aminomethane, p.a. (Penta;Cz)
Q5 DNA Polymerase for molecular biology (BioLabs, UK)
Hydrochloric acid, 35% (Lach-Ner;Cz)
Sodium acetate for molecular biology (Top-Bio;Cz)
Ethylenediaminetetraacetic acid for molecular biology (Fischer scientific;Cz)
Ethanol for HPLC, (Lach-Ner;Cz)
DNA Ladder for molecular biology (Nipon Genetics, DE)
Glass beads 0.5 mm, (P-Lab; Cz)
Synthetic primers NL1, NL4, LS2, ITS1, ITS4, ITS2, ITS3, (E.Pharmacon, Cz)
Phenol-chloroform-isoamyl alcohol for molecular biology (Sigma-Aldrich, DE)
KAPA Long Range DNA Polymerase for molecular biology (KAPA BIOSYSTEMS; USA)
KAPA2G Robust HotStart for molecular biology (KAPA BIOSYSTEMS; USA)
Acetic acid, p.a., (Lach-Ner;Cz)
Agarose for molecular biology (Thermo Fischer Scientific; USA)
Ethidium bromide for molecular biology (Serva, DE)
Bromphenol Blue - Sodium Salt for molecular biology (Serva, DE)

MiliQ water for PCR for molecular biology (Top-Bio;Cz)
n-hexane for HPLC, (Lach-Ner;Cz)
Chloroform for HPLC, (Lach-Ner;Cz)
Acetone for HPLC, (Lach-Ner;Cz)
methanol for HPLC, (Lach-Ner;Cz)
Ethanol UV-VIS (Lach-Ner;Cz)
Heptadecanoic acid standard (Sigma-Aldrich, DE)
β-carotene standard (Sigma-Aldrich, DE)
Astaxanthin standard (Sigma-Aldrich, DE)
Torulene, HPLC standard (CaroteNature, Switzerland)
torularhodin, HPLC standard (CaroteNature, Switzerland)
Acrylamide for molecular biology (Serva, DE)
N, N'-methylene bisacrylamide for molecular biology (Serva, DE)
Formamide (Sigma-Aldrich, DE)
Urea, p.a., (Penta;Cz)
1.0 μm Gold Microcarriers (Biorad, Fr)
Dodecanoic acid standard (Sigma, Saint-Quentin Fallavier, France)
BodiPy® Lipid Probe (2.5 mg/ml) in ethanol (Invitrogen, SaintAubin, France)

4.2.1 Equipments

High-speed refrigerated centrifuge (Hermle, DE)
Centrifuge refrigerated (Eppendorf, DE)
Electrophoretic chamber (Bio-rad, USA)
ELISA Reader (BioTek; USA)
Filters for HPLC, PRE-CUT, (Alltech, UK)
Fluorescence Microscope for Biotechnology (Labomed, USA)
Incubator INCU-Line (VWR Collection, USA)
Laminar box (Esco, Singapore)
Microcentrifuge (Labnet International, USA)
Optical-mechanical adapter (Canon, Japan)
PCR cycler (Biorad, USA)
Dryer Room (Memert, DE)
UV/VIS Spectrophotometer (Boeco, DE)
UV transiluminator (Major Science, USA)
Vacuum evaporator RV 06 (IKA; DE)
Power source for electrophoresis (Major Science, USA)
The DCode™Universal Mutation Detection System (Biorad, USA)
Gene Pulser Xcell modular electroporation systems (Biorad, Fr)

Biolistic Particle Delivery Systems (Biorad, Fr)

Zeiss Axio Imager M2 microscope (Zeiss, Le Pecq, France)

100× objective lens and Zeiss filters 45 and 46 (Zeiss, Le Pecq, France)

Axiovision 4.8 software (Zeiss, Le Pecq, France)

HPLC UltiMate 3000 (Dionex Thermo Fisher Scientific, UK)

HyperREZXP carbohydrate H+ 8μm column (Thermo Fisher Scien-tific, Villebon sur Yvette, France)

HPLC/MS (Thermo Fischer scientific; USA)

Thermostat – LCO 10, Column Oven (ECOM;Cz)

Detector PDA – PDA Plus Detector (Thermo Fischer scientific; USA)

Pump – MS Pump Plus (Thermo Fischer scientific; USA)

Software Xcalibur

Column Kinetex C18, 5 mm, 4,6 x 150 mm (Phenomenex; USA)

Holder of the precolumn – KJ0 - 4282, (ECOM; Cz)

Precolumn – C18, AJ0 – 4287 (Phenomenex; USA)

Varian 3900 GC/FID (USA)

Varian FactorFour vf-23ms column (30 m, 0.25 mm, 0.25 µm) (USA)

TRACE GC/FID (ThermoQuest S.p.A., Italy)

Capillary column DB-WAX (30 m x 0,32 mm x 0,5 µm) (USA)

TRACE 1300 GC/FID (Trace GC Ultra, Thermo Scientific, USA)

Capillary column ZB-WAXplus (30 m x 0,25 mm x 0,25 µm) (USA)

5 METHODS

5.1 Media and cultivation conditions

5.1.1 Composition of used media

For yeast cultivation were used four types of media: YPD, YNB, mineral medium C/N 16 (MM) and mineral medium with only yeast extract C/N 80 (MMY). The YPD medium was used for pre-cultivation including incubation on agar plates for yeast proliferation prior inoculation to production media.

Tab. 5.1: Medium composition per 1l of medium

	YPD	**YNB**	**MM**	**MMY**
Glucose (g)	20	30		
Yeast Extract (g)	10	/	1.5	1.5
Peptone (g)	10	/	/	/
***YNB base (g)**	/	1.7	/	/
****$(NH_4)_2SO_4$ (g)**	/	/	2.8	/
NH_4Cl (g)	/	5	/	/
KH_2PO_4 (g)	/	/	4	4
$MgSO_4$ .7 H_2O (g)	/	/	0.696	0.696
Phosphate buffer (pH 6.8, mM)	/	50	/	/
*****Agar (g)**	20			
*without $(NH_4)_2SO_4$ and amino acids				
** depends on molar C/N ratio				
*** for solid media preparation				

Media were sterilized at 120 °C for 20 min. Except glucose, which was sterilized separately to avoid caramelization processes, and except YNB base which was sterilized using filter sterilization. The yeast biomass was taken during cultivation in chosen time intervals depending on experiment.

5.1.2 General preservation of yeast strains

Cryopreservation

Yeasts for cryopreservation were taken from precultivation steps on YPD media for 24 - 48 hours. The 750 μl of culture medium containing yeast culture in exponential phase was transferred into a sterile cryogenic tube with 750 μl of 50% glycerol solution (w,v). The

resulting solution in cryogenic tubes was gently mixed and stored in -80 °C. Before every cultivation was transferred one inoculation loop of cryo-preserved yeast culture to the agar plate. Yeast strains were then incubated for 24-72 hours, depending on experiment conditions and yeast strain. Cryopreservation is an efficient method to store yeast cultures with high reproducibility, because prior to every experiment can be easily influenced by yeast viability and speed of proliferation resulting in shortened lag phase.

5.1.3 Conditions of cultivation for diversity of *R. mucilaginosa* species

Media and cultivation

The cultivation was done with precultivation step in YPD media. After 24 hours were yeasts cells transferred into 500 ml Erlenmeyer flasks with production medium to reach optical density 1 (A_{600}). The production medium was composed of MMY medium (Minimal medium) containing yeast extract as an additional source of vitamins and nitrogen. Yeasts were then cultivated at 23°C, and 180 rpm for 72 hours. The samples for biomass, TAG, carotenoids, and ergosterol analysis were taken at 24 hours. At 8 hours, intervals were taken between samples to measure glucose consumption during cultivation and for measurement of cell optical density.

Vitamin requirements and carbon utilization tests

For substrate utilization tests were used solid media with/ without addition of YNB base as a source of vitamins. Media contained 1% (w,v) of individual carbon sources: Glucose, glycerol, lactose, arabinose, mannose, xylose, or starch. Yeast cultures were firstly inoculated on solid YPD media, after 48 hours of cultivation one small inoculation loop of yeast culture was transferred into 5 ml of YPD media. The cultivation was carried out in test tubes for 24 hours at 120 rpm. After this time were yeast cells two times washed by medium without carbon source and YNB base and yeast suspension was diluted/concentrated to optical density (OD) 1. Prepared yeast suspension was then diluted 1x, 10x, 100x, and 1,000x, and 3 μl of each cell concentration was inoculated into solid plates with different carbon source. As a reference a YNB solid medium without carbon source was used.

5.1.4 Cultivation conditions for nitrogen limitation and genetic modification experiments

Basic culture medium

Rich solid medium YPD was used for inoculation of yeast strains from cryopreserved stocks. Yeast strains were then cultivated at 28 °C and after 48 hours of incubation one

loop of yeast strain from the agar plate was transferred into liquid YPD media (same composition without addition of agar). Cultivation in liquid YPD was done at 28 °C for 24 hours and served as a precultivation step for better adaptation of yeast strains.

Nitrogen limiting and non limiting cultivation conditions

Lipid production among the used strains was examined by cultivation on YNB medium with xylose, glycerol, or glucose as carbon source with a precultivation step on every substrate for 24 hours at 160 rpm. For nitrogen limiting conditions was prepared YNB medium with decreased concentration of NH_4Cl to obtain C/N ratios 9, 30, 60, 180 on the beginning of cultivation. Yeasts were inoculated to reach optical density (OD, A_{600} nm) 0.2 at the beginning of cultivation. The yeast strains were cultivated for 120 hours, and at the 24 hour intervals were taken samples for fatty acid analysis, substrate consumption, biomass production, and OD. The cultivation experiments were done in biological duplicates.

Carbon utilisation tests

For substrate utilization tests were used solid YNB media with 0.2% (w,v) content of carbon source. Yeast biomass was taken from YPD precultivation step in test tubes (24 hours, at 120 rpm). After this time were yeast cells two times washed by YNB medium without carbon source and the yeast suspension was diluted/concentrated to optical density (OD, A_{600} nm) 1. The prepared yeast suspension was then diluted 1x, 10x, 100x, and 1,000x, and 3 μl of each yeast cell concentration was inoculated into solid plates with different carbon sources. As a reference, the YNB solid medium without carbon source was used. As carbon sources were used the following substrates for each plate: glucose, fructose, arabinose, xylose, glycerol, maltose, galactose, sorbitol, and starch.

5.1.5 Cultivation conditions for metabolite inhibition and mutagenesis experiments

Yeast strain, medium composition, and conditions of cultivation

For random mutagenesis experiments was chosen the haploid strain of *Rhodotorula toruloides* CCY 62-02-04 obtained from CCY collection in Bratislava. For every cultivation experiment was the strain inoculated on agar plates containing solid YPD medium from cryopreserved cultures. The plates were incubated for 48 hours, then one inoculation spoon was transferred to 10 ml of YPD medium and after 24 hours the production medium was inoculated to OD 1 (A_{600}). Two types of production media were used, one with C/N 16 - MM, and the second medium MMY with C/N 80.

The impact of DPA

The original strain of *R. toruloides* was exposed to different concentrations of diphenylamine (DPA). The used concentrations were 0, 10, 15, 25, 50, 100 and 150 μM of DPA using a stock solution of 10 mM DPA dissolved in UV/VIS grade ethanol. For these experiments were used two types of liquid media with C/N ratios 16 and 80. The amount of produced lipid metabolites and biomass was measured in comparison to control cultures without addition of DPA.

5.1.6 Cultivation conditions for variability within Sporidiobolales and Cystofilobasidiales

Conditions of cultivation

The yeasts were cultivated with precultivation step in liquid YPD media and then transferred into YNB media to reach an optical density (600 nm, OD) 0.5 at the beginning of cultivation. The yeast strains grew for four days at 23 °C, under constant shaking. The samples were taken every 24 hours for fatty acids, carotenoids, and biomass quantification.

5.2 Biomass quantification

5.2.1 Biomass quantification for diversity of *R. mucilaginosa* species

The biomass was isolated by centrifugation of 10 ml of culture medium at 4500 rpm for 8 min. The isolated biomass was washed twice with distilled water and evaporated at 80°C. The amount of biomass was measured gravimetrically.

5.2.2 General biomass quantification

The biomass was isolated by centrifugation (for 10 ml of culture medium 4,500 rpm for 8 min, for 4 ml of media at 10,000 rpm, for 3 min). The isolated biomass was washed twice with distilled water, frozen at -80 °C and lyophylized for 48 hours. The amount of biomass was measured gravimetrically and expressed as CDW. The fatty acid-free CDW (FF-DCW) was calculated as the difference between CDW and the measured amount of FAs in g/l.

5.3 Substrate consumption analysis

5.3.1 Glucose consumption analysis using Somogyi-Nelson method

A glucose was determined as the amount of reducing sugars by Somogyi-Nelson method. This method employs the reaction of cupric ions (Cu^{2+}) with reducing sugar in alkaline conditions and at high temperature (100 °C). The cupric ions Cu^{2+} are reduced by reduction sugar to cuprous ions Cu^{+} which are subsequently treated with arsenomolybdic acid to generate molybdenum blue. The concentration of molybdenum blue is directly proportional to the concentration of reducing sugar. This reaction allows determination of reducing sugars using UV-VIS spectrophotometry. The best wavelengths are 620 or 720 nm against appropriate reference sample with the addition of water instead of reducing sugar solution. The composition of all stock solutions is in the Tab. 5.2. To determine glucose concentration, the calibration solutions of glucose were prepared at concentrations: 35, 30, 25, 20, 15, 10, 5, 2.5, 1 g/l, and 100times diluted. For substrate utilization in liquid media was used 1 ml of yeast culture, where the yeast cells were separated by centrifugation (13,000 rpm for 1 min), and the supernatant was used for further analysis. Because Somoghyi-Nelson method is very sensitive, the samples were diluted 100times with water, in the way that the final absorbance does not exceed the value of 1. Then 100 μl of diluted sample or calibration solution was mixed with 900 μl of H_2O, with 500 μl of Solution I, and with 500 μl of Solution II. All components were mixed and heated for 10 min at 100 °C. When were samples cooled down to laboratory temperature, 500 μl of Nelson reagent and 7.5 ml of distilled water were added into the sample and all components were well mixed. After, were samples transferred into spectrophotometric kyvet and the absorbance was measured against the reference sample at 720 nm.

Tab. 5.2: Composition of Solutions

Solution	Chemical	Amounth (g/l)
Somogyi Solution I	Na_2CO_3	30
	$NaHCO_3$	20
	Na_2SO_4	180
	$KNaC_4H_4O_6$	15
Somogyi Solution II	$CuSO_4 \times 5H_2O$	20
	Na_2SO_4	120
Nelson Reagent	$(NH_4)_6Mo_7O_{24}$	50
	H_2SO_4 (concentrated)	42 ml/l
	$Na_2HASO_4 \times 7H_20$ /	6

All components were dissolved in distilled water to reach 1l of each solution

5.3.2 Substrate consumption analysis using HPLC

To quantify xylose, glucose, or glycerol, 1 ml of yeast culture was centrifuged for 1 min at 13,000 rpm, the supernatant was removed, filtered through 45μm pore-size membranes and analyzed with HPLC machine (UltiMate 3000;Dionex Thermo Fisher Scientific, UK) using a HyperREZXP carbohydrate H+ 8μm column (Thermo Fisher Scien-tific, Villebon sur Yvette, France). Mobile phase contained 0.1M H_2SO_4, every measurement was done at room temperature with a flow rate of 0.6 ml/min. For qualitative and quantitative analysis were used external standards of glucose, xylose, and glycerol.

5.4 Extraction of lipid compounds

5.4.1 Methanol-chloroform extraction

Hydrated yeast biomass was transferred into a 15 ml tube with 5 ml of chloroform:methanol mixture (2:1, v,v) and 1 ml of glass beads (0.25 – 0.5 mm). The tubes were then shaken for 20 min using a vortex shaker at maximum speed. After homogenization, a 1 ml of distilled water was added, and the sample was vortexed for 10 sec and then centrifuged at 4500 rpm for 7 min. The upper phase was discarded and 2 ml of MeOH/H_2O/$CHCl_3$ (48/47/3, v/v/v), the solution was vortexed and then centrifuged. The water phase was discarded and the organic lower phase was transferred into a glass test tube. Then 2 ml of chloroform:methanol mixture (2:1, v,v) and 1 ml of MeOH/H_2O/$CHCl_3$ (48/47/3, v/v/v) were added to the remaining biomass and glass beads, the suspension was vortexed, centrifuged, the upper phase was discarded, and the lower phase was transferred into a glass test tube containing previous organic phase. This step was repeated until the remaining biomass had a cream white color. The extract was evaporated and then dissolved in 1ml of chloroform for carotenoid and ergosterol analysis. For enzymatic quantification of total lipids, were the extracts evaporated and dissolved in 1 ml of n-hexane.

5.4.2 Methanol-hexane extraction

The lyophilyzed yeast biomass was resuspended in 1 ml of distilled water, then 3 ml of methanol were added, and the suspension was vortexed at maximum speed for 10 min. Then 2 ml of n-hexane and 0.5 ml of distilled water were added, vortexed for 3 min, and then centrifuged at 4,500 rpm for 7 min. Subsequently, the upper phase with pigments and lipids was separated and transferred into a glass test tube. To the remaining methanol suspension was added 1 ml of methanol, 2 ml of n-hexane, the sample was vortexed for 5 min, then centrifuged. The n-hexane phase was again removed and transferred into the test tube. To the methanol suspension the 0.25 ml of distilled water and 1 ml of n-hexane

were added, and the solution was vortexed for 3 min and then centrifuged. The process of extraction was repeated until the remaining biomass had a cream white color.

5.5 Analysis of yeast metabolites

5.5.1 Enzymatic quantification of total lipid content

For rapid analysis of TG content, the Serum Triglyceride Determination kit (STD-kit) was used (Sigma Aldrich). The STD-kit contains Triglyceride reagent and Free glycerol reagent and glycerol standard solution. The principle of TG determination is based on hydrolysing of TG by lipoprotein lipase to glycerol and free fatty acids. The glycerol is then oxidized in two-step reaction leading to the production of H_2O_2.The H_2O_2 then reacts with 4-aminopyridine and *N*-ethyl*N*-(3-sulphopropyl)*m*-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to the triglyceride concentration of the sample.

Lyophilized Free glycerol reagent and Triglyceride reagent were dissolved at 40 ml and 10 ml of miliq water. These two reagents were gently mixed and then mixed with each other. The method was optimized for ELISA reader, as a more rapid and less expensive variant to the usual UV-VIS spectrophotometry method. ELISA reader allows screening of 96 samples at the same time, another advantage is time measurement during the enzymatic reaction. For quantitative analysis of yeast TG, the chloroform lipid extracts prepared by Folch extraction method were dried at 60 °C for 20 min. Then were dried samples dissolved at 1 ml of n-hexane and 15 μl of each sample was transferred to microtitration plate and evaporated at 40 °C. As a reference sample was used pure n-hexane to simulate the same conditions for reference, standard solution and samples. After evaporation, 200 μl of mixed reagent solution and 10 μl of miliq water were added to each well. As a standard was used 10 μl of glycerol standard diluted 1×, 2×, 3×, 4×, 5×, and 6×. The microtitration plate was then placed into the ELISA reader. The samples were incubated at 37 °C and the enzymatic reaction was measured in time at 562 nm. The absorbance value was taken from the time point with the highest absorbance in time for each sample. The concentration of glycerol and TG, respectively, was calculated from the glycerol calibration curve, where the concentration of standard 2.5 mg/ml corresponds to the concentration 0.26 mg/ml of TG. As a simulation of yeast TG was used olive oil to optimize TG determination method for ELISA reader.

5.5.2 Carotenoid and ergosterol analysis for the diversity of *R. mucilaginosa* sp.

The samples were prepared using the extraction of lipidic compounds method, evaporated using a rotation vacuum evaporator, and dissolved in 1 ml of chloroform. Prior to HPLC analysis were samples filtrated through 0.2 μm filters. The 10 μl of sample was injected into RP-HPLC/PDA system and analyzed using column Kinetex 5 μm, C18, 100 A (Phenomenex, USA). Gradient elution of mobile phase A and B was used to separate carotenoid species. Mobile phase A contained acetonitrile, methanol, and 0.1 mM TRIS-HCL at pH 8 (84:2:14, v,v,v). Mobile phase B contained methanol and ethylacetate (6:4). The gradient elution program was following: 0-11min 100% of mobile phase A, 12-17 min 100% of mobile phase B, and 18-23 min 100% of phase A. The solvent flow rate was 1 ml/min at 23 °C, the wavelength scan of PDA detector was monitored at range 270 - 600 nm. Calibration curves for β-carotene were measured at channel with wavelength 450 nm and at a channel with 285 nm for ergosterol. To determine content of carotenoids and ergosterol, the RP-HPLC/PDA system was used (Thermo Fisher Scientific, USA), and the results were processed by the chromatographic software X-Calibur.

5.5.3 Carotenoid and ergosterol analysis

The lipidic extracts were evaporated using a rotation vacuum evaporator and dissolved in 1-3 ml of acetonitrile: ethylacetate mixture (3:1, v,v). Prior to HPLC analysis were samples filtrated through 0.2 μm filters. The 10 μl of sample was injected into RP-HPLC/PDA system and analyzed using column Kinetex 2.6 μm, XB-C18, 100 A (Phenomenex, USA). The gradient elution used two mobile phases A1 composed of acetonitrile:methanol: H_2O (11:8:1, v,v,v), and mobile phase B1 consisted of acetonitrile, methanol, and ethylacetate (3:3:4, v,v,v). The gradient elution program was following: 0-4 min 100% of mobile phase A1, 4-5 min 75% of mobile phase B1, 5-8 min 80% of B1, 8-11 min 100% of phase B1, 12-15 min 100% of phase A1. The solvent flow rate was 1 ml/min at 35 °C, the wavelength scan of PDA detector was monitored at range 280 - 600 nm. Calibration curves for β-carotene were measured at channel with wavelength 450 nm, for astaxanthine, torulene, and torularhodin, at channel with 450 nm, and for ergosterol at the channel with 290 nm.

To determine the content of carotenoids and ergosterol, the RP-HPLC/PDA system was used (Thermo Fisher Scientific, USA), and the results were processed by the chromatographic software X-Calibur. For qualitative and quantitative analysis were used the standards of torulene, torularhodin, lycopene, β-carotene, and ergosterol.

5.5.4 The UV-VIS multicomponent analysis

For rapid carotenoid analysis was used UV-VIS spectrophotometry with extension of the Beer-Lambert law adapted after [105]. The extension of Beer-Lambert law enables qualitative and quantitative analysis of the mixture of compounds whenever the absorption spectra of compounds differ significantly and measurements of absorbance are available as many different wavelengths as analytes to be analyzed. In theory, the absorbance of a mixture of i compounds at a given wavelength corresponds to the sum of absorbance of each compound in the mixture. The following expression in equation 5.5.4.1 describes the extension of the Beer-Lambert law:

$$A_{\lambda_j} = \sum_{i=1}^{m} E^i_{\lambda_j} c^i L \tag{5.5.4.1}$$

Equation 5.5.4.2 describes the extension of Beer-Lambert law, where A_{λ_j} is the absorbance of the mixture of *m* compounds at the *j*th wavelength. C^i is the concentration of *j*th component in g/100 ml of the solvent, and $E^i{}_{\lambda j}$ is the absorption coefficient of the *i*th component at *j*th wavelength. *L* is the cell path length (cm). Therefore, to determine the concentrations of *n* analytes in the mixture, it is necessary to have *n* equations and *n* readings at *n* wavelengths [105]. The optimization was done using commercial standards of β-carotene, torulene and torularhodin at a specified range of concentrations. Torulene and torularhodin were measured at the concentration range 0.5-5 μg/ml and β-carotene at 0.4-4 μg/ml. As a solvent was used pure n-hexane as well as blank for the measurement. The calculations were based on the extension of Lamber-Beer law. Firstly, the individual carotenoids were measured separately at a range of concentrations. The choice of the wavelengths influences the values of the absorption coefficients. Therefore, it is essential to choose the wavelengths that offer the best differences in absorption values between all three carotenoids. The wavelengths 451 nm, 481 nm, and 537 nm were chosen for the calculation of absorption coefficients as well as for sample measurements. The absorption spectra of carotenoid standards used are visualized in Fig. 5.1.

Firstly, the calibration curves for each carotenoid standard were measured at given wavelengths. The absorption coefficients were calculated from the slope of the linear regression of the concentration of each carotenoid standard against the absorbance measured at each selected wavelength. With the absorption coefficients known for each selected wavelength, the concentration equations were solved using the mathematical application WolframAlpha (www.wolframalpha.com) The application of the extended Beer-Lambert law describe the following equations (5.5.4.2- 5.5.4.7):

$$A_{451} = E_1 c_1 + E_2 c_2 + E_3 c_3 = 3549 c_1 + 1735 c_2 + 1710 c_3 \tag{5.5.4.2}$$

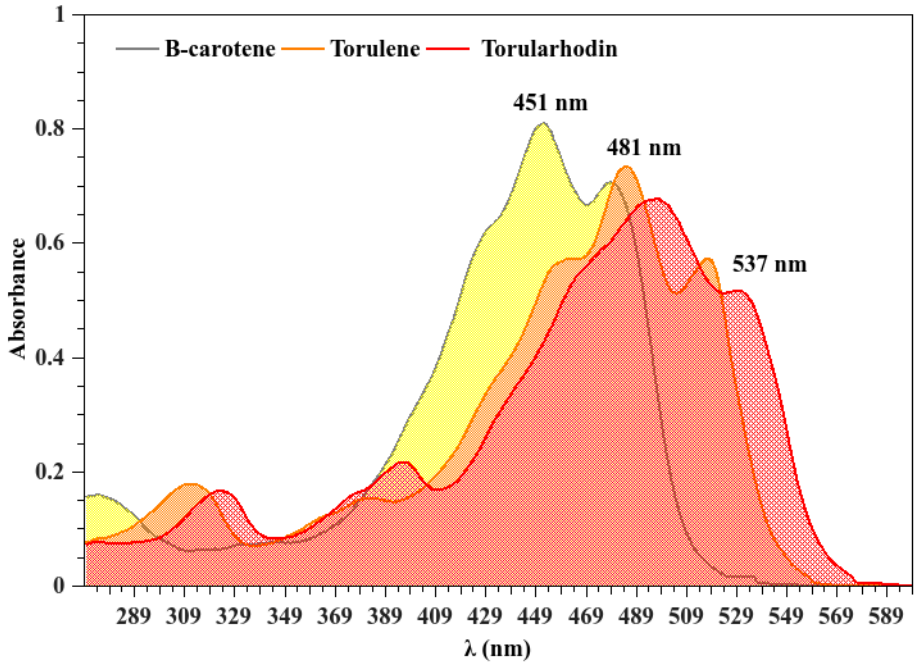

Fig. 5.1: UV-VIS spectrums of torulene, totularhodin, and β-carotene standards dissolved in in n-hexane

$$A_{481} = E_1c_1 + E_2c_2 + E_3c_3 = 3074c_1 + 2384c_2 + 2513c_3 \tag{5.5.4.3}$$

$$A_{537} = E_1c_1 + E_2c_2 + E_3c_3 = 0c_1 + 576c_2 + 1943c_3 \tag{5.5.4.4}$$

Solved equations based on the previous equations with calculated extinction coefficients are following:

$$c_1 = \frac{3184624A_{451} - 2386145A_{481} + 283415A_{537}}{3967220846} \tag{5.5.4.5}$$

$$c_2 = \frac{-5972782A_{451} + 6895707A_{481} - 3662097A_{537}}{3967220846} \tag{5.5.4.6}$$

$$c_3 = \frac{885312A_{451} - 1022112A_{481} + 1563713A_{537}}{1983610423} \tag{5.5.4.7}$$

Where c means concentration, E is an absorption coefficient at a given wavelength for a specific compound, A is an absorbance at a given wavelength, 1 is an index for β-carotene and γ-carotene, 2 is the index for torulene and 3 is an index for torularhodin.

The total concentration of carotenoids was calculated as a sum of the concentration of a specific carotenoid:

$$c_{Total} = c_1 + c_2 + c_3 \tag{5.5.4.8}$$

5.5.5 Fatty acid analysis for the nitrogen limitation and genetic modification studies

Fatty acids were extracted from 10-20 mg of lyophilized cells biomass and converted into their equivalent methyl esters. As transesterification mixture a 15% of H_2SO_4 and methanol (v,v) was used. Into a glass vial was added 10-20 mg of lyophilised biomass and 1.8 ml of transesterification mixture with internal standard (C12, 0,5 mg/ml). The mixture was incubated for 1.5 hours at 85°C. After incubation were samples let to cool down to laboratory temperature and mixed with 1 ml of n-hexane and 0.5 ml of water. The mixture was then centrifuged for 5 min at 1500 rpm. For analysis using GC was used 300 μl of the upper phase. The GC analysis was performed using a Varian 3900 gas chromatograph equipped with a flame ionization detector and a Varian Factor Four vf-23ms column, where the bleed specification at 260 °C was 3 pA (30 m, 0.25 mm, 0.25 µm). The fatty acids (FAs) were identified by comparing chromatography peaks produced to those generated by commercial FA methyl ester standards (FAME32; Supelco); FA levels were quantified by the internal standard method to the commercial C12:0 standard.

5.5.6 Fatty acid analysis

As transesterification mixture 15% of H_2SO_4 and methanol (v,v) was used. Into a glass vial was added 10-20 mg of lyophilised biomass and 1.8 ml of transesterification mixture with internal standard (C17, 0,5 mg/ml). The mixture was incubated for 3 hours at 85 °C. After incubation were the samples let to cool down to laboratory temperature. Then a whole sample after transesterification was transferred into a 5 ml glass vial where was then added 0.5 ml of 0.05 M NaOH and 1 ml of n-hexane. The mixture was shaken for 30 s to transfer FAME to n-hexane phase. When organic and water phases were separated, 100 μl of n-hexane phase was transferred into a 1 ml glass vial and diluted with 0.9 ml of pure n-hexane. Analyzes were done at TRACE GC/FID machine (ThermoQuest S.p.A., Italy) with capillary column ZB-WAXplus (30 m x 0,25 mm x 0,25 µm). The FAs were identified by comparing the chromatogram peaks produced to those generated by commercial FA methyl ester standards (FAME32; Supelco); FA levels were quantified by the internal standard method, which involved the addition of 50 µg of commercial C17:0 fatty acid standard.

5.5.7 Fluorescence microscopy analysis

The images were acquired using a Zeiss Axio Imager M2 microscope equipped with a 100× objective lens and Zeiss filters 45 and 46 for fluorescent microscopy. Axiovision 4.8 software was used for image acquisition. Lipid bodies were visualized after adding BodiPy® Lipid Probe (2.5 mg/ml) in ethanol to the cell suspension (A_{600} of 5) and after incubating the cells at room temperature for 10 min.

5.6 Molecular methods

5.6.1 Nucleic acid isolation

Genomic DNA isolation

To lyse yeast cells, a lytic solution was prepared after Tab. 5.3. The yeast biomass was isolated from agar plates (one inoculation spoon) or from liquid culture (1 ml of overnight culture, YPD). The yeast biomass was resuspended in 300 μl of lytic solution with addition of 0.3 g of glass beads. The 300 μl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v) was added and then were samples shaken with a vortex for 10 min at maximum speed. After cell desintegration were the samples centrifugated for 5 min at 13, 000 rpm. From two phase system, the upper phase was isolated and mixed with 500 μl of cold ethanol (100%, UV-VIS quality) and the mixture was incubated for 20 min at -20 °C. The DNA pellet was isolated by centrifugation process as in the previous step, the ethanol was removed, and the pelet was mixed with 500 μl of cold ethanol (70% UV-VIS quality). Then the centrifugation step was repeated and the ethanol solution was discarded and the samples were let dry at 37 °C. The isolated DNA was dissolved at 200 μl of sterile miliq water.

Tab. 5.3: Composition of Lytic Solution

Chemical	Concentration
Triton X-100	2%
Sodium dodecyl sulfate (SDS)	1%
NaCl	100 mM
Tris-HCl (pH 8)	10 mM

Plasmid isolation

Plasmids were isolated from *E.coli* cultures bearing the desired plasmid (taken from the local collection of institute Micalis in France). The isolation was done using a commercial Plasmid miniprep kit (ThermoFisher Scientific). The bacterial culture was harvested by centrifugation at 8,000 rpm in a microcentrifuge for 2 minutes at room temperature. The remaining medium was removed and 250 μl of Resuspension Solution was added to the bacterial pelet and vortexed. Then 250 μl of Lysis Solution was added to the resuspended cell solution, and the tube was inverted 4-6 times. 350 μl of Neutralization Solution was added into the suspension and subsequently the tube was mixed by inversion of the tube 4-6 times and centrifugated for 5 min at 13,000 rpm. The supernatant was then transferred into the spin column and the samples were centrifugated for 1 minute at 13,000 rpm. Subsequently, membrane bound DNA was washed 2$\times$ by 500 μl of Wash Solution and centrifugated for 30-60 s. The flow-through was discarded, and the empty columns were centrifugated for 1 minute 13,000 rpm. Purified DNA in the spin column was transferred into a new tube. To elute DNA from the membrane, the 50 μl of Elution Buffer was added to the filter of the column and incubated for 2 minutes. After this time were tubes centrifugated and the flow through liquid containing plasmid DNA was collected.

5.6.2 Agarose gel electrohoresis

For agarose gel electrophoresis was used TAE buffer and 18 g/l of agarose. Agarose was prepared by heating in microwave owen until was agarose totally dissolved. Dissolved agarose was then spilled into the form for electrophoretic gel and ethidium bromide was added. When the agarose solution formed the gel, 14 μl of DNA solution and 6 μl of staining solution (0.5 ml of 2% bromphenol blue and 7 ml of glycerol with 10 ml of miliq water) were placed into the well of the gel. The electrophoretic separation was done with 110V, 14 mA for approximately 1 hour. The DNA was then evaluated by comparison with adequate DNA molecular weight standards.

5.6.3 Yeast identification using PCR-DGGE analysis

Nested PCR

Ribosomal barcodes were amplified via nested PCR reaction with the following combination of primers modified for subsequent DGGE analysis with GC clamps. The species-specific ITS region was firstly amplified using universal primers ITS1-GC and NL4 . This sequence was used as a template for the following reactions: The internal sequences were amplified using primers ITS1-GC and ITS2, ITS3 and ITS4-GC after.The region D1/2 of LSU was amplified using primers NL1 and LS2. The combinations of primers and annealing temperatures were used after Raja et al. [76]. Sequence analysis of these regions is routinely used for yeast species identification and in this study, the PCR products were used for sequence analysis by the optimized DGGE method. The primer sequences are in the Tab. 5.4.

Tab. 5.4: Primers used for amplification of ribosomal barcode sequences

Primer Name	**Sequence**
ITS1	5'-TCCGTAGGTGAACCTGCGG-3‘
ITS2	5'-GCTGCGTTCTTCATCGATGC-3‘
ITS3	5'-GCATCGATGAAGAACGCAGC-3‘
ITS1-GC	5'CGCCCGCCGCGCGCGGCGGGCGGG-GCGGGGTCCGTAGGTGAACCTGCGG-3‘
ITS4-GC	5'CGCCCGCCGCGCGCGGCGGGCGGG-GCGGGGTCCTCCGCTTATTGATATGC-3'
NL1	5'-GCCATATCAATAAG-3‘
NL4	5'-GGTCCGTGTTTCAAGACGG-3‘
LS2	5'-ATTCCCAAACAACTCGACTC-3‘

For PCR was used EliZyme HS Robust kit. The reaction mixture (50 μl) was composed of EliZyme TM HS Robust 10 µl, forward primer (10 µM) 2 µl, reverse primer (10 µM) 2 µl, 1 μl of 10 mM dNTP, template DNA 2 μl EliZyme HS Robust polymerase (5 U/µl) 0,5 μl, PCR water up to 50 μl.

Denaturation gradient gel electrophoresis - DGGE

For DGGE polyacrylamide gels were prepared 8% solutions of AA/Bis (acrylamide *N,N'*-methylene-bisacrylamide, dissolved at 1×TAE buffer) with different concentrations of urea and formamide which served as denaturation agents. The used concentrations of denaturants were 50, 60, 70, and 80%. The solutions and gels were prepared after instruction manual (The DCode™Universal Mutation Detection System, Biorad). The used ranges of denaturant gradients in the gels were: 50-70 %, 60-80 % and 50-80 %. The separation was carried out at 60 °C, at 100 V and 140 mA in the time interval of 12 hours. As a DNA samples were used 10 μl of PCR products , stained with 5 μl of bromphenol blue solution. After separation was gel stained with ethidium bromide (50 μl of 10 mg/ml of stock solution per 250 ml of TAE buffer). Gels were visualized under UV in a transiluminator.

Amplification of mini and microsatelite sequences

Mini and microsatelite sequences were amplified using EliZyme HS Robust kit. Since for mini and microsatelite analysis only one primer is used, twice the amount of only one primer (M13/$(GTG)_5$) was used. See Tab. 5.5.

Tab. 5.5: Primer sequences for mini and microsatelite sequences

Primer Name	**Sequence**
M13	5´-GTAAAACGACGGCCAG-3´
$(GTG)_5$	5'-GTGGTGGTGGTGGTG-3‘

The parameters of amplification for ribosomal barcode sequences as well as for mini and microsatelite sequences are described in the Tab. 5.6:

Tab. 5.6: General Conditions of PCR

Step	**Temperature(°C)**	**Time(s)**	**Number of Cycles**
First Denaturation Step	98	30	1
Denaturation	98	60	
*Annealing	56, 58, 60	15	30
Elongation	72	30; 80**	
Final Elongation	72	2-5 min	1
*The values of annealing step can differ depending on used PCR primer pair			
Annealing temperature for (M13/$(GTG)_5$) was 55 °C			
**The elongation step for the longest PCR product (ITS1-NL4 primers)			

5.6.4 Yeast identification by sequence analysis of ribosomal barcodes

Amplification of ribosomal barcodes

PCR amplification of ITS1-2 and D1/D2 regions of rDNA was done using universal primers ITS1 and NL4 (Sequences of primers are listed in Tab. 5.4. Barcodes were amplified using Q5®High-Fidelity DNA Polymerase kit. The composition of the reaction mixture was following: Q5 reaction buffer 10 μl, 5$\times$ Q5 High GC enhancer 1 μl, 1 μl 10 mM dNTP, forward primer (10 µM) 2.5 μl, reverse primer (10 μM) 2.5 μl, template DNA 2 μl, and Q5 High-Fidelity DNA polymerase 0,2 μl, PCR water up to 50 μl. PCR was done after conditions in the Tab. 5.7.

Tab. 5.7: General Conditions of PCR

Step	Temperature(°C)	Time(s)	Number of Cycles
First Denaturation Step	98	30	1
Denaturation	98	60	
Annealing	56	30	30
Elongation	72	120	
Final Elongation	72	5 min	1

Sequencing and sequence analysis

The amplified sequences were sequenced as a paired-end read using commercial Sanger sequencing service (GATC BIOTECH) and processed by the program Staden Package into resulting consensus sequences. The resulting sequences were compared with CBS database using Pairwise sequence alignment tool (www.wi.knaw.nl).

5.6.5 Vector preparation and genetic modification

Amplification of genes from genomic DNA

The genes for diacylglycerol acyltransferase (DGA1), and glycerol-3-dehydrogenase 1 (GPD1) were amplified from isolated genomic DNA of *R. toruloides*. Sequences of used primers are in the Tab. 5.8. For gene amplification was used Q5® High-Fidelity DNA Polymerase from New England Biolabs.

The reaction mixture (50 μl) contained: Q5 reaction buffer 10 μl, 5$\times$ Q5 High GC enhancer 1 μl, 1 μl 10 mM dNTP, forward primer (10 μM) 2.5 μl, reverse primer (10 μM) 2.5 μl, template DNA 2 μl, and Q5 High-Fidelity DNA polymerase 0,2 μl, PCR water up to 50 μl.

Tab. 5.8: Primers for vector construction and verification of yeast transformants

Primer Name	**Sequence**
DGA1-For	TACGCAGGATCCCGAGGAGGTCAGGCGATAAT
DGA1-Rev	GATGGGCCAGCAGGCGACGGATCCTACTGC
GPD1-For	TACGCAGGATCCACACGCAACCATGGCACC
GPD1-Rev	ACTAGATGCCCTCGAAGATGGGGATCCTACTGC
DGA1-VerF	CCACCGACGACGATAGTGAG
DGA1-VerR	CTGACCTTCCGCCTTACTCC
GPD1-VerF	CTCATCAATTCTTGCCGCCG
GPD1-VerR	CGTGCACAAGTGTCAGCGAG
pTEF-VerF	CGACGCCATGCATGTAAAGG
tTEF-VerR	AATTCGGATGGAAGGTGAACGA

Subsequent verification of cloned genes after transformation was done using primers with suffix VerR/F, which correspond to forward or reverse primers. Scheme of verification reactions can be found in detail in chapter 6.3 in the Fig. 6.24 for better orientation in data. For PCR verification of *R. toruloides* was used GoTaq® DNA Polymerase from Promega. The composition of the reaction mixture was following: GoTaq buffer (5×) 10 μl, concentrated DMSO 1.5 μl, 1 μl 10 mM dNTP, forward primer (10 μM) 3 μl, reverse primer (10 μM) 3 μl, template DNA 1 μl, and GoTaq DNA polymerase 0,2 μl, PCR water up to 50 μl. The general conditions of PCR are in the Tab. 5.9.

Tab. 5.9: Conditions of PCR

Step	**Temperature(°C)**	**Time**	**Number of Cycles**
First Denaturation Step	95	3 min	1
Denaturation	95	30 s	
Annealing	60	30 s	30
Elongation	72	2 min	
Final Elongation	72	7 min	1

Excision and purification of amplified DNA from agarose gel

To isolate and purify DNA fragments excised from the agarose gel after electrophoresis, a commercial gel extraction kit from ThermoFisher Scientific was used. Firstly, a slice of the gel was excised using a clean scalpel and a slice of the gel was placed into the tube. Depending on the weight of the agarose slice, a volume of binding buffer was added (100 μl per 100 mg of agarose gel). The sample was incubated at 50-60 °C for 10 min, until the gel was completely dissolved.

Then the solution was transferred into the purification column, centrifuged for 1 min at 13,000 rpm, and the flow through liquid was discarded. Then was added 100 μl of binding buffer into the purification column and again centrifuged. Subsequently, 700 μl of wash buffer was placed into the same column. After centrifugation, the flow-through liquid was discarded and the empty purification column was centrifuged to dry a membrane. The purification column was placed into the new tube and 50 μl of Elution buffer was added into the Purification column, incubated for 2 min and centrifugated. The flow through liquid contained purified DNA, which was used in next experiments.

Vector preparation

The vectors were prepared for overexpresion of genes for diacylglycerol acyltransferase (DGA1), GPD1 (Glycerol-3-dehydrogenase 1) in *R. toruloides*. Firstly, the genes were amplified from genomic DNA of *R. toruloides* using primers with specific sequences for digestion using restriction endonucleases *Bam*HI and *Avr*II. Primer pair DAG1-For/Rev and GPD1-For/Rev (Tab. 5.8), were used to amplify, respectively, DAG1 and GPD1. Then after digestion, these fragments were ligated into plasmid P3612 (JMP62-pGPD rtNAT-tGPD-pTEF-rtRS-tTEF; plasmid collection of Institute Micalis, BimLip team in France), previously digested by *Bam*HI and *Avr*II using T4 ligase. As a result, two different plasmids bearing nourseothricin resistance (NAT) and gene DAG1 (JMP62-pGPD rtNAT tGPD-pTEF-DAG1-tTEF), or GPD1 (JMP62-pGPD rtNAT tGPD-pTEF-GPD1-tTEF) were obtained. The graphical scheme is visualized in the chapter 6.3, Fig. 6.21. Plasmids were linearized to expression cassettes using NotI restriction endonuclease, resulting in the release of kanamycine resistance and replication origin for *E.coli*. The prepared expression cassettes were used for biolistic transformation of *R. toruloides*. The conditions of digestion and ligation can be seen in the Tab. 5.10.

Tab. 5.10: Conditions of Ligation and Digestion

Component	**Ligation**	**Digestion**	
Used Enzymes	T4 Ligase	BamHI/AvrII	NotI*
Plasmid/PCR Product (μl) **	3/16	20	50
Reaction Buffer (μl)	4	8	7
BSA 10$\times$ (μl)	/	8	7
Enzyme 1 (μl)	2	2	3
Enzyme 2 (μl)	/	2	/
H_2O (μl)	/	40	3
Incubation Time (hours)	12	12	4
Temperature (°C)	16	37	laboratory
* after incubation inactivation of enzyme at 70 °C for 10 min			
** 1 μl of enzymes is used for 1 μg of DNA			

Transformation of *E.coli*

The *E.coli* competent cells were stored at -80 °C and prior to transformation were bacterial cells incubated on ice together with tubes containing 10 μl of the desired plasmid for 15 min. The 75 μl of the suspension of competent cells was added into the tube with plasmid, and the mixture was incubated for 30 min on ice. After incubation the mixture was placed into a water bath with 42 °C for 2 min to cause heat shock. After heat shock was the mixture was incubated for 5 min on ice, and after addition of 900 μl of LB medium (NaCl 10 g/l, tryptone 10 g/l, yeast extract 5 g/l) were cells incubated at 37 °C for 1 hour. The sorting of successful transformants was done on agar plates with LB medium and kanamycine as selection marker (50 μ/ml). To each plate was transferred 75 μl of cell suspension and the plates were incubated for 24 hours at 37 °C.

Transformation of *R. toruloides* by biolistic method

The biolistic method was done using customized Biolistic apparatus. Prior transformation the yeast culture grown overnight at 28 °C on YPD medium was used. Yeast cells were then adjusted to OD 14-16 and 200 μl of yeast culture was transferred to manitol-sorbitol agar plates (0.75 M sorbitol, 0.75 M manitol, 10 g/l glucose, 10 g/l peptone and 5 g/l yeast extract) and let dry.

To "shoot the gun", it was necessary to prepare golden beads which were firstly washed with ethanol. The suspension of golden beads had the concentration of 60 mg/ml, where 70 μl of this suspension can be used for 4-5 shoots. To wash the golden beads, the whole suspension was vortexed on horizontal vortex for 10 min. After vortexing, the beads were let to sediment and 20 μl of upper phase was removed, and 10 μl of DNA cassette, 50 μl of 2.5 M $CaCl_2$, and 20 μl of 0.1M spermidine solution were added. The mixture was then vortexed for 10 min at maximum speed, then centrifuged for 30s at 13,000 rpm, and redundant water was removed. The rest was twice washed with 140 μl of 70% ethanol and ones with 60 μl of 100% ethanol. The 15 μl resulting suspension of prepared golden beads was transferred to biolistic membranes.

The shoot gun was sterilized with 70% ethanol. The membrane was placed into the shotgun system and the uncovered agar plates were placed into the shooting chamber 6-9 cm distant from cannon. The shooting was done at the pressure of helium gas in the system 1,100-1,350 PSI.

After shoot gun were cells incubated on manitol sorbitol plates for 3-4 hours. Then were cells washed out from the plates with YNB medium without carbon source, concentrated by centrifugation (3 min, 4,000 rpm), resuspended in 1 ml of YNB without carbon source, and transferred to YNB agar plates with desired antibiotics for further selection. The used antibiotics were nourseothricin and geneticin depending on the expression resistance cassette (50 μ/ml)

5.7 Mutagenesis experiments

5.7.1 Chemical and physical mutagenesis

A three types of mutagens were used. As a chemical mutagens were used MMS and EMS and as a physical mutagen was used UV-C irradiation, 265 nm. For mutagenesis was used overnight culture

grown in 10 ml of YPD media. For EMS and MMS mutagenesis, the cell count was adjusted to 1.8×10^8. 1 ml of culture was transferred into a centrifugated, washed twice with sterile water, and resuspended at 1.5 ml of 0.1 M sodium phosphate buffer pH 6.8. To the cell solution was added 75 μl EMS or MMS solution, resuspended by vortexing, and then the cell suspension was incubated at 23 °C for 2 hours. To inactivate the effect of the mutagen, a 0.2 ml of yeast culture was transferred into 8 ml of sterile thiosulphate solution (5% w,w). The 100 μl of homogenized cell suspension was plated on agar plates containing solid production medium with C/N ratio 80. As a control was used culture suspension without addition of mutagens. For UV mutagenesis, the cell count of an overnight culture was diluted to the concentration of 100 cells per 100 μl. To the solid production medium C/N 80 was added 100 μl of the diluted cell suspension, the plates were dried in a sterile laminar box. The plates were then irradiated for 0, 1, 2, 3, and 4 min by UV-C irradiation, 265 nm in laminar box. After irradiation, the plates were covered by aluminium foil and then were the plates kept at dark for 24 hours. Most red-colored colonies were transferred to YPD plates and after 72 hours, most red-appearing strains were inoculated to agar plates with production medium C/N 80 with different concentrations of diphenylamine (0, 10, 15, 25, 50, and 100 μM). After 96 hours, the most colored strains on every DPA concentration were chosen for rapid selection of carotenoid overproducing strains.

For all types of mutagenesis, the impact on viability was measured as a colony forming unit on petri plates. As the control plates were used the plates, which were not treated with mutagen.

5.7.2 Hydrogen peroxide adaptation

The overproducing mutant strains were chosen for hydrogen peroxide adaptation. The adaptation was done in 50 ml of liquid media under nitrogen limitation with 10 mM concentration of hydrogen peroxide. The medium was inoculated to OD 1, after 24 hours was the next addition of hydrogene peroxide to 30 mM as the final concentration. After 72 hours of cultivation, the survival strains were plated on nitrogen-limited solid media and irradiated for 3 min with UV irradiation. After this procedure, the most colorful colonies were selected for the next experiments. Potentially overproducing mutant strains were selected by rapid selection method with UV-VIS quantification of carotenoids.

5.7.3 Rapid selection of mutants

Rapid selection of overproducing mutants was done on production medium with C/N 16 with decreased amount of glucose to 20 g/l for 48 hours, with 24 hours precultivation on liquid YPD medium. After cultivation, 4 ml of culture media and its biomass was isolated by centrifugation (10,000 rpm, for 3 min) and washed twice with distilled water. The biomass was then frozen to -30 °C and lyophylized for 48 hours. The biomass was measured by gravimetry. The carotenoids were extracted from lyophilized biomass using methanol-hexane extraction and carotenoids were quantified by UV-VIS spectrophotometry.

6 RESULTS AND DISCUSSION

6.1 Diversity of *Rhodotorula mucilaginosa* species

Rhodotorula mucilaginosa is one of the most widespread yeast species isolated from varied environmental sources including terrestrial, aquatic, and marine habitats. Their presence was also recorded in extreme environments in a range of deep-sea vents to arctic cold deserts to other extreme environments like chemical wastewater, evaporation ponds, and leachate of a uranium mineral heap [110, 111]. Moreover, *R. mucilaginosa* can be found in human samples from patients with compromised immune system [112]. This yeast species is characteristic by its ability to utilize a wide variety of substrates and exceptional ability to survive in extreme conditions. Generally, the carotenoids are known as antioxidants which can protect the cell against harmful influence of oxidative agents and oxidative stress [113], moreover the production of polysaccharides which are used for capsule production to help in protection of the cell [114] together with the ability to accumulate intracellular lipids even increases the survival rate of *R.mucilaginosa* species [115]. The strain variability within the same species can lead to different ability to produce metabolites or different ability to utilize some substrates. These properties can be influenced by different gene expression, which may be connected to adaptation of a particular strain to specific conditions. To distinguish different strains of the same species with different phenotype properties, the mini and microsatellite sequences with the high mutation rate were used.

6.1.1 Molecular characterization of yeast strains

In this study were chosen yeast strains with different phenotype properties which were in the previous study identified by sequencing of ITS and D1/2 rDNA regions of the ribosomal operon as *R. mucilaginosa* [116]. The identification was done by comparing obtained sequences with NCBI database. The chosen strains, their experimental numbers, and their identification results can be found in Tab. 6.1 and Tab. 6.2.

Tab. 6.1: Used yeast strains identified in previous studies as *R.mucilaginosa* species

Number	Old laboratory stock	Molecular Identification
2	*Rhodotorula glutinis*	*R. mucilaginosa*
5	*Rhodotorula mucilaginosa*	*R. mucilaginosa*
6	*Phaffia rhodozyma*	*R. mucilaginosa*
7	*Cystofilobasidium capitatum*	*R. mucilaginosa*
8	*Cystofilobasidium capitatum*	*R. mucilaginosa*
10	*Sporidiobolus salmonicolor*	*R. mucilaginosa*
11	*Sporidiobolus shibatanus*	*R. mucilaginosa*

Tab. 6.2: Reference yeast strains obtained from CCY and proved by molecular identification in previous studies

Number	CCY collection	Molecular Identification
3	*Rhodotorula glutinis* CCY 20-2-6	*Rhodotorula glutinis*
12	*Sporobolomyces roseus* CCY 19-6-4	*Sporobolomyces roseus*
18	*Rhodotorula toruloides* CCY 62-02-04	*Rhodotorula toruloides*

The species-specific regions ITS and D1/2 of rDNA were amplified via PCR. ITS region was amplified using universal primers ITS1-GC, ITS4, ITS4-GC, ITS2, and ITS3, region D1/2 of LSU was amplified using primers NL1, NL4, and LS2 via nested PCR reaction [76]. Sequence analysis of these regions is routinely used for yeast species identification and also in this study, the PCR products were used for sequence analysis by optimized DGGE method. Except *R.mucilaginosa* species were also analyzed other carotenogenic yeast strains (Tab. 6.2, number 18 -*R. toruloides* CCY 62-02-04, number 3 *R. glutinis* 20-2-26).The used range of denaturants in acrylamide gel was for ITS sequences 60-80%, for ITS2 sequences 50-70% and for D1/D2 sequences 50-80%.

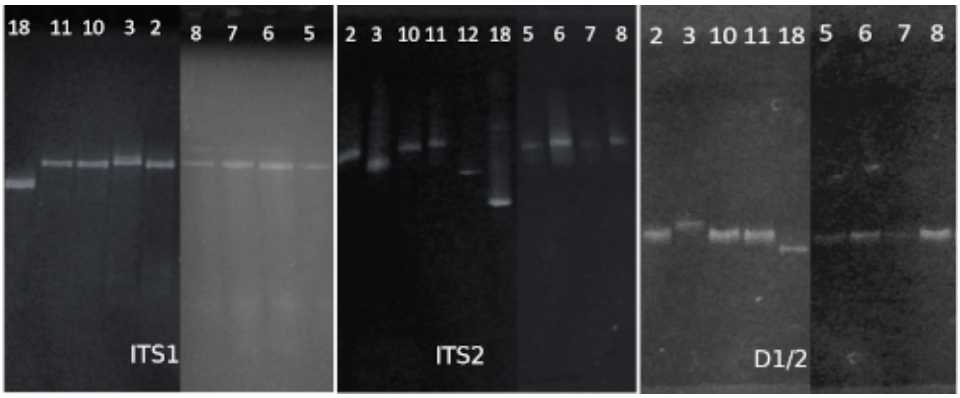

Fig. 6.1: DGGE gel profiles of ITS1 (range denaturants: 60-80%), ITS2 (range of denaturants: 50-70%) and D1/2 regions of rDNA (range of denaturants: 50-80%), 3 -*R. glutinis*, 18- *R. toruloides*, numbers 2,5,6,7,8,10, and 11 represents different *R.mucilaginosa* strains (see Tab. 6.1 and Tab. 6.2)

The analysis of these regions by the DGGE method proved that the chosen strains have very similar DGGE profiles for ITS1, and D1/2 regions of rDNA in comparison to other strains (Fig. 6.1). The results of DGGE analysis supported previous results of yeast identification by sequencing, where only strains *R. toruloides* and *R.glutinis* proved different DGGE profiles [116]. Strains *R. toruloides* and *R. glutinis* were recently obtained from CCY collection in Bratislava.

To analyze the intraspecific variability within the used strains of *R. mucilaginosa* species, the minisatellite/microsatellite-based PCR method was employed using primers for minisatellite M13 and minisatellite $(GTG)_5$. Minisatellites, also known as Simple Sequence Repeats (SSRs), are tandem repetitive DNA sequences ranging from 10 - 60 bp long sequences dispersed throughout

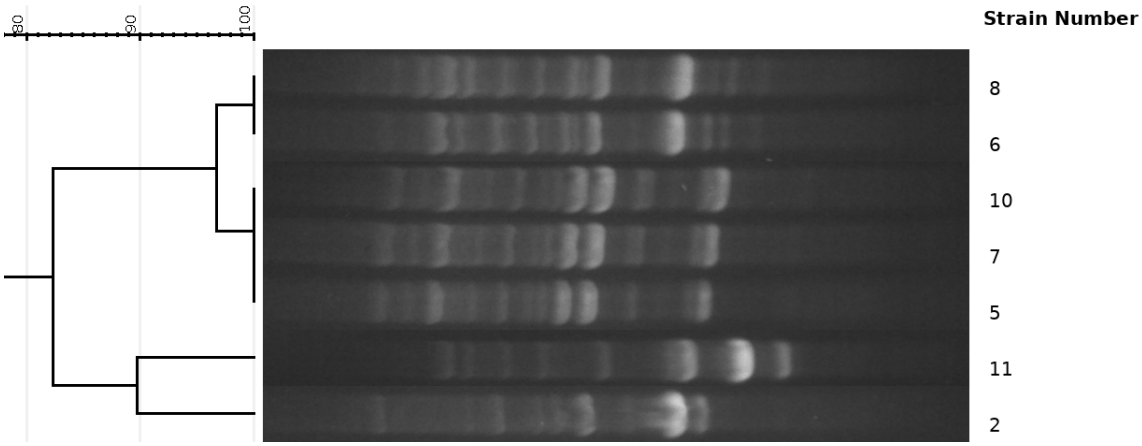

Fig. 6.2: The GTG_5 microsatellite length polymorphism based dendrogram of *R.mucilaginosa* strains, numbers correspond to Tab. 6.1

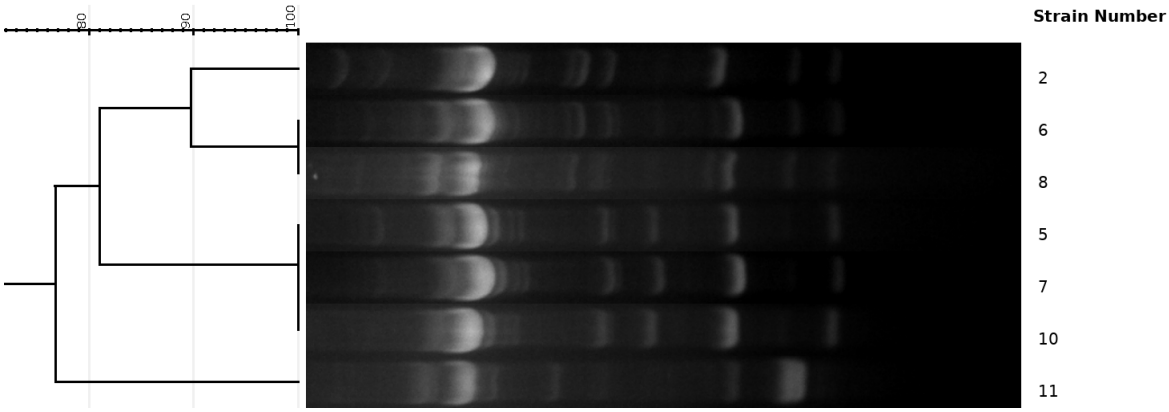

Fig. 6.3: The M13 minisatelite length polymorphism based dendrogram of *R.mucilaginosa* strains, numbers correspond to Tab. 6.1

the genome and dominantly inherited [117]. Minisatellites are larger repetitive sequences (several hundred base pairs to several kilobase pairs in length) also dispersed within the genome. Both mini and microsatelites exhibit size polymorphism with intraspecies variability in humans, animals, plants, and microorganisms [118]. Micro and minisatellite sequences are used for strain identification because they exhibit polymorphism among individuals of a population [119, 120, 121]. The resulting gel fingerprint profiles were analyzed using software GelJ, with Dice coefficients, and correlation coefficients, which were calculated by the unweighted pair group method algorithm (UPGMA).

Mini and microsatellite sequence polymorphisms for M13 and GTG_5, showed four different groups with specific band profiles (see Fig. 6.2 and 6.3). Into the first group can be placed strains number 5, 7, and 10. The specific band profile showed the second group, where are placed strains 6 and 8. Into the third group can be placed strain number 2. Forth group profile exhibited only strain 11.

6.1.2 Phenotype diversity

To specify phenotype diversity among *R.mucilaginosa* species, carbon utilization tests on solid medium were done. To distinguish growth dependency on vitamins, two types of solid media were prepared. The medium with the source of vitamins was done by the addition of the YNB base and media without the source of vitamins. The media without the source of vitamins had the same composition as vitamin medium but without the addition of the YNB base. The YNB base contains vitamins and trace mineral compounds. As carbon sources were chosen, glucose, mannose, D-xylose, lactose, D-arabinose, and starch. As a control for substrate utilization test were prepared the agar plates containing medium without carbon source. Foto-documentation of the plates is illustrated in Fig. 6.4, and the results of carbon utilization tests are summarized in Tab. 6.3. All used *R.mucilaginosa* strains were able to grow on glucose, mannose, and xylose, which were well utilized on media with and without the addition of YNB base except strain number 10 which had slight difficulties to grow without the presence of the YNB base. Glycerol and arabinose were utilized only in the presence of YNB base by all *R. mucilaginosa* species except strain 10. Lactose and starch were difficult to utilize and none of the *R. mucilaginosa* strains grew on these substrates. In the experiments was also included strain of *R. glutinis* species represented by number 3. The strain of *R. glutinis* was the only one with the ability to utilize lactose as a carbon source in the presence of the YNB base.

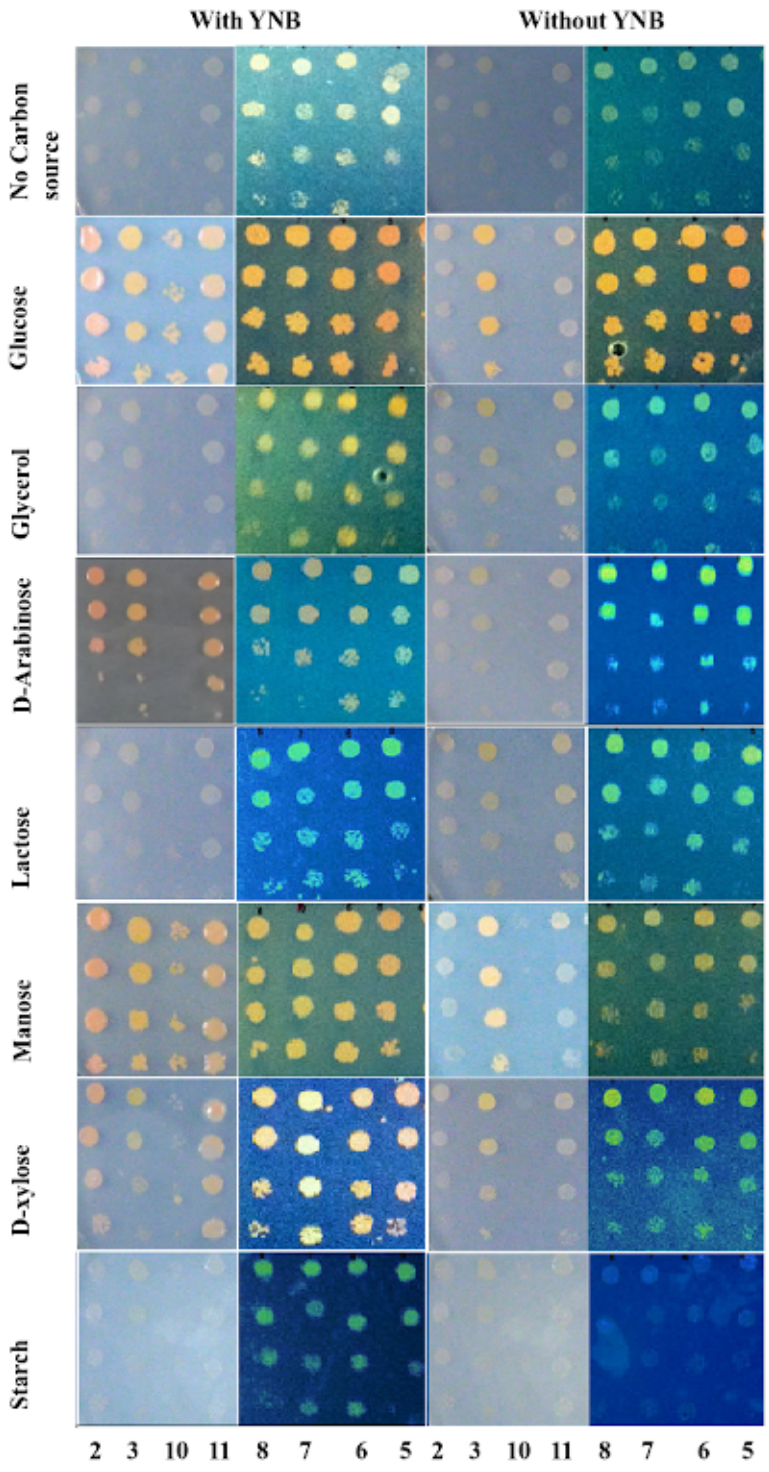

Fig. 6.4: Carbon utilization tests with and without addition of YNB, 3- *R. glutinis*,2, 5, 6, 7, 8, 10, and 11 - *R.mucilaginosa*, (see Tab. 6.1 and Tab. 6.2)

Tab. 6.3: Results of carbon utilization test, the numbers corresponds to yeast strain of *R. mucilaginosa* described in Tab. 6.1

No.	**Glucose**		**Mannose**		**Xylose**		**Lactose**		**Glycerol**		**Arabinose**	
	/	YNB	/	YNB	/	YNB	/	YNB	/	YNB	/	YNB
2	-	++	+	++	-	+	-	-	+	-	-	+
5	++	+	+	++	+	++	-	-	-	+	-	+
6	++	++	+	++	+	++	-	-	-	+	-	+
7	++	++	+	++	+	++	-	-	-	+	-	+
8	++	++	+	++	+	++	-	-	-	+	-	+
11	-	++	+	++	-	+	-	-	-	+	-	+
3*	++	++	++	++	+	+	-	+	-	+	-	+

* Reference strain *R.glutinis*

\- no growth, + growth, ++ good growth

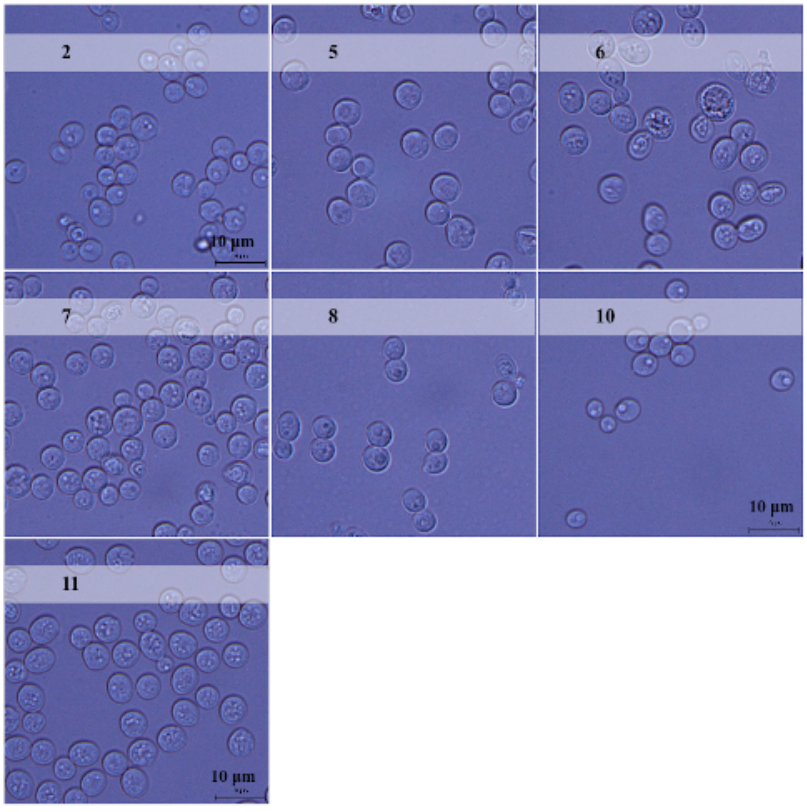

Fig. 6.5: Morphological properties of *R.mucilaginosa* strains after 72 hours of cultivation, numbers correspond to Tab. 6.1

The highest glucose utilization and biomass production measured as optical density (OD, A_{600} nm) were reached by strains number 7, 6, and 8 (Fig. 6.6). The stationary phase for the major-

ity of strains started at 32 hours of cultivation. Exceptional strain whose growth was strangely inhibited was strain number 10 (Fig. 6.7 and 6.6). Strain 10 also did not produce a satisfactory amount of lipids nor carotenoids due to poor growth. At the end of the cultivation, the microscopic observation of each yeast strain was done. The shape of all suspected strains was round. Inside of the cells were visible intracellular structures, probably lipid bodies. The more complicated intracellular structure had strains numbers 2, 5, 6, 7, and 11 (see Fig. 6.5)

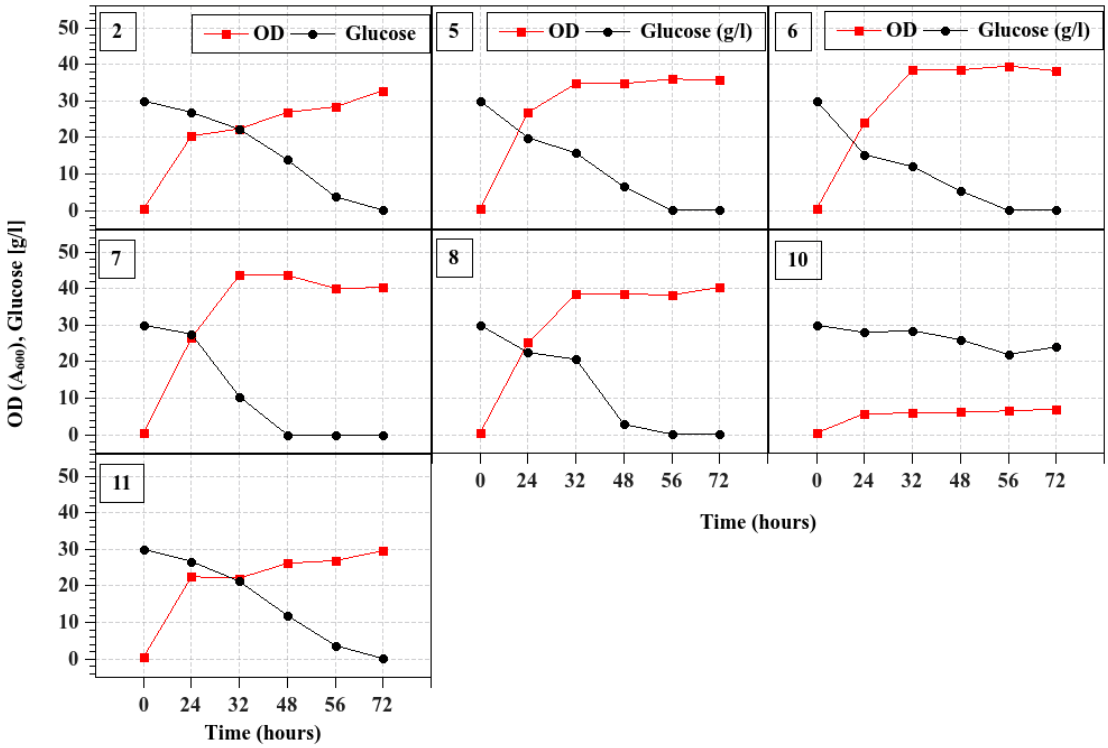

Fig. 6.6: Glucose consumption and biomass production expressed as optical density during cultivation time. Numbers 2, 5, 6, 7, 8, 10, and 11 belong to *R. mucilaginosa* strains described in Tab. 6.1, the results correspond to the average values calculated from the two independent experiments

6.1.3 Biomass, TG, carotenoids and ergosterol productions

The metabolite production was measured after 72 hours of cultivation when all strains were at the late stationary phase. The yeasts of *R. mucilagiosa* species produce torulene, torularhodin, γ-carotene, and β-carotene as the main carotenoids. Their representation within the total carotenoids produced can change depending on cultivation conditions [15]. In this study were torulene, torularhodin and γ-carotene quantified against β-carotene as a standard and their sum including β-carotene is represented as total carotenoids. The best carotenoid producer as for β-carotene and total carotenoid content per biomass were the strains 6, 8, and 2, which interestingly did not produce β-carotene. It has to be mentioned that carotenoid production by *Rhodotorula* species can

differ from 5-92 mg/l of production medium [122, 18]. The best carotenoid producing strain in this study was able to produce almost 2.19 mg/g of carotenoids per biomass, which is an excellent result for a small-scale experiment in Erlenmeyer flasks without strictly monitored and operated conditions (Tab. 6.4).

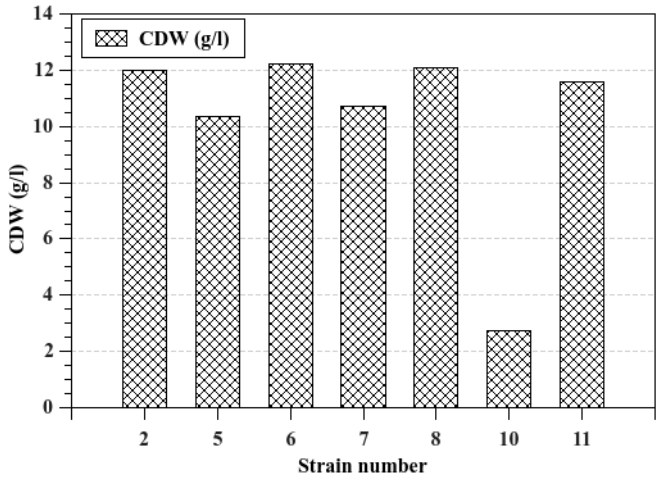

Fig. 6.7: Biomass production at 72 hours of cultivation, numbers 2, 5, 6, 7, 8, 10, and 11 belong to *R.mucilaginosa* strains (designation can be see in Tab. 6.1), the results correspond to the average values calculated from the two independent experiments

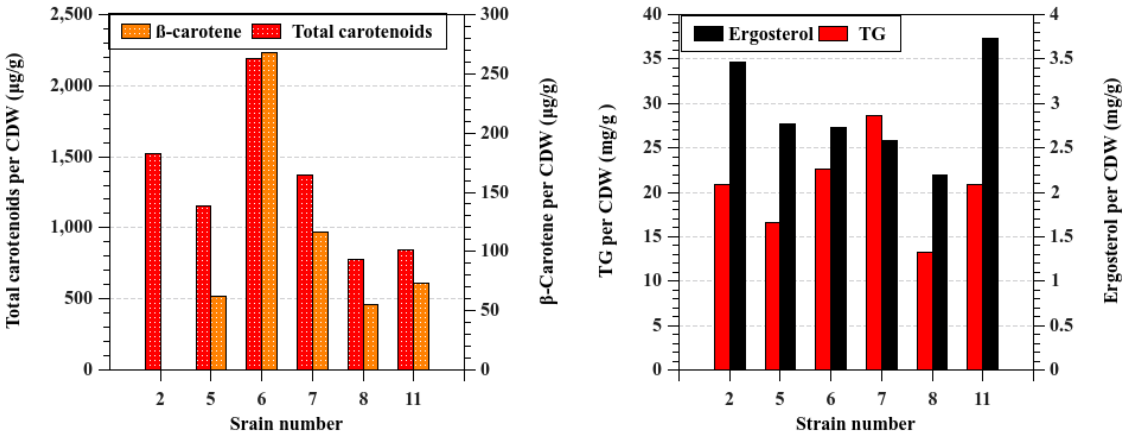

Fig. 6.8: Metabolite production at 72 hours of cultivation. Numbers 2, 5, 6, 7, 8, 10, and 11 belong to *R.mucilaginosa* strains (designation can be see in Tab. 6.1), the results correspond to the average values calculated from the two independent experiments

The TG production at given cultivation conditions did not outreach lipid content of 35 mg/g per biomass. The highest TG production per biomass proved to have strain number 7, which produced 28 mg/g. Strains 2, 6, and 11 outreach TG content of 20 mg/g. The lower production

Tab. 6.4: Production of total carotenoids, β-carotene, Ergosterol, TG and biomass by *R. mucilaginosa* strains (designation can be see in Tab. 6.1), the results correspond to the average values calculated from the two independent experiments

Num.	**Total carotenoids (μg/g)**	**β-carotene (μg/g)**	**Ergosterol (μg/g)**	**TG (mg/g)**	**Biomass (g/l)**
2	1519.53	-	3.46	20.83	11.95
5	1154.35	62.15	2.76	16.57	9.87
6	2190.62	268.01	2.73	22.66	12.23
7	1366.95	116.34	2.58	28.66	10.7
8	779.43	55.32	2.20	13.30	9.62
11	845.44	73.08	3.74	20.93	11.57

had strain number 8, which could not produce more than 15 mg/g of TG per biomass. TG production was generally not very high in every strain, which can be explained by the fact that the experiment was designed for low C/N ratio to specify natural production properties without any impact of nutritional limitation like nitrogen limitation which causes channeling of carbon source mainly into lipid production. The ergosterol is a yeast sterol connected to osmotic stress. It helps to equalize the osmotic stability of the cell in reaction to the environment. Yeasts are not able to degrade ergosterol and sterols generally [39]. At some stress conditions, for example in the presence of osmotic pressure it protects the membranes [123], but when ergosterol reaches a critical concentration which could harm the cell membranes, it needs to be acetylated and secreted out of the cell to maintain sterol homeostasis, or it can be acylated by fatty acids and stored in lipid bodies [47, 39]. The ergosterol accumulation could be thus connected to sterol homeostasis kept in balance. Production of these compounds subsequently increases the density of culture medium and the osmotic pressure or it could be also connected to different regulation of sterol metabolism [115]. The highest accumulated amount of ergosterol reached strains number 2 and 11 (more than 3 mg/g, see Fig. 6.8), these strains were also formed two separate genotype forms in mini and microsatellite analysis. Strain number 10 was excluded because of its poor growth and low level of biomass formation.

The strains of *R. mucilaginosa* proved to have different production properties regarding lipid and carotenoids production. These strains can provide high biomass content and especially their carotenoid production properties could be further optimized in controlled and operated conditions of bioreactors. Intraspecies variability among used strains brings an interesting proof of their different genotypes, which could be studied at the level of phenotype plasticity, which greatly differs among different strains of the same species. The analysis of these strains at the "omic" level could reveal in a greater detail the regulation of carotenoid, lipid, and sterol metabolism and its connection to carbon channeling.

6.2 Fatty acids production under nitrogen limitation

Following part of the thesis was done during ERASMUS+ internship in France at Institute Micalis, BimLip team (INRA). *Rhodotorula* sp. are frequently reported as oleaginous yeasts with the ability to accumulate more than 20 % of fatty acids in their cell dry weight (CDW) [17]. Moreover, *Rhodotorula* sp. can utilize a wide range of substrates, which can be subsequently transformed into valuable compounds as fatty acids, carotenoids or enzymes l-phenylalanine ammonia-lyase, and d-amino acid oxidase [18]. However, production of these compounds is still economically demanding regarding substrate price as well as fatty acid extraction process itself [39]. To decrease the price of cultivation processes, a usage of cheap substrates is needed. The hemicelluse is the second most abundant fraction of lignocellulose waste. Hemicellulose is composed mainly from xylose along with some others simple sugars, and acetic acid [124]. The hydrolysate of hemicellulose could be the cheap carbon source for microbiological fabrication of fatty acids, but its suitability is dependent on the ability of a particular yeast strain to utilize xylose. Another cheap waste substrate is glycerol, which is produced as a side product of biodiesel fabrication [125]. The essential parameters for fatty acid production are the selection of suitable yeast strains and optimized cultivation conditions [126]. The optimization of cultivation conditions is frequently done through optimization of suitable ratio of carbon to nitrogen (C/N ratio) leading to desired nitrogen limitation of yeast cells [126].

Nitrogen limitation is a stress condition leading to increased lipid accumulation in oleaginous yeasts. When nitrogen is depleted, the adenosine monophosphate (AMP) is broken down by monophosphate deaminase. Low levels of AMP cause the malfunction of isocitrate dehydrogenase (IDH), which can not efficiently convert isocitrate into α-ketoglutarate. Consequently, the concentration of isocitrate increases within mitochondria until it is released into the cytoplasm, where it is converted by citrate lyase into acetyl-CoA [55, 39, 48, 127].The acetyl-CoA is also precursor for fatty acid as well as polysaccharides synthesis as storage compounds, and its increased concentrations leads to increased synthesis of both types of metabolites [115]. Even if nitrogen limitation has a beneficial impact on fatty acids content within yeasts cells, it can lead to significant decrease of cell proliferation, and thus it leads to very low yields of produced biomass. Additionally, not every yeast strain reacts through increased fatty acid accumulation [17]. In this experiment, the impact of nitrogen limitation in combination with different carbon sources was studied. Yeast strains chosen for the experiment were *Rhodotorula mucilaginosa* and *Rhodotorula toruloides*. Used carbon sources were glucose, glycerol, and xylose in combination with C/N ratios 9, 30, 60, and 180. Carbon sources were chosen after the results of carbon utilization tests and after their frequent occurrence in the waste substrates.

6.2.1 Yeast strains identification and substrate utilization

Identification of yeast strains

Used yeasts strains were obtained from CBS yeast collection and were represented by yeast strains *R. toruloides* CBS 6016, and four strains of *R. mucilaginosa* - CBS 2402, CBS 2403, CBS 2404,

and CBS 2405. Their actual taxonomic status was verified by sequencing of ITS and D1/2 regions of rDNA. Used yeast strains were identified as *Rhodotorula mucilaginosa* (strains CBS 2402, 2403, 2404, 2405) and *Rhodotorula toruloides* - CBS 6016 (See Tab. 6.5).

Tab. 6.5: Results of sequence analysis and identification

CBS	Yeast strain	Identification	Score	Similarity	Ref. sequence
2402	*R. mucilaginosa*	*R. mucilaginosa*	1745	100.0 %	*KP223715.1
2403	*R. mucilaginosa*	*R. mucilaginosa*	1071	100.0 %	CBS 11292
2404	*R. mucilaginosa*	*R. mucilaginosa*	1145	100.0 %	CBS 12021
2405	*R. mucilaginosa*	*R. mucilaginosa*	1741	99.9 %	*KY218712.1
6016	*R. toruloides*	*R. toruloides*	902	100.0 %	CBS 350
*Source GenBank					

Substrate utilization tests

The carbon utilization tests were done using glucose, fructose, arabinose, xylose, glycerol, maltose, galactose, sorbitol, and starch. All used strains were able to grow in the presence of glucose, glycerol, fructose, and sucrose. Other substrates maltose, galactose and xylose were used differently depending on the yeast strain. The best utilization of these substrates seemed to have strain *R. mucilaginosa* 2402 since it was able to grow in all substrates except sorbitol and starch (see Tab. 6.6 and Fig. 6.9). *R. mucilaginosa* as well as *R. toruloides* species are known to grow on substrates such as xylose, arabinose, galactose, glycerol, and maltose [2, 13]. The different ability to grow on used substrates is probably connected to their different geographical origin. Despite the fact, that strains 2404 and 2405 were reported by CBS collection to grow on D-xylose, their ability to grow was somehow delayed. These strains were originally isolated form fermenting Kentucky tobacco from Italian city Salermo and surprisingly they both did not grow using xylose and galactose. Strain 2403 was isolated from a man with a case of emphysema in Netherlands and strain 2402 was isolated from boracic lotion in Indonesia. Their geographical source probably influence the ability of these strains to utilize some carbon sources (www.wi.knaw.nl). In *R. mucilaginosa* is frequently observed phenotype plasticity [2]. Sorbitol was utilized only by strain *R. toruloides* CBS 6016.

6.2.2 Yeast strain selection

The yeasts were selected after their ability to accumulate fatty acids and grow on glycerol, xylose, and glucose as carbon sources at low C/N ratios 9 and 30. The biomass production was expressed as cell dry weight (CDW).

The results of these first cultivation experiments are visualized in Fig. 6.10 (CDW production) and at Fig. 6.11 (FAs production per medium). *R. mucilaginosa* species 2402, 2403 and 2405 used more glucose as sources for biomass formation then for fatty acid accumulation when compared

Tab. 6.6: Results of carbon utilization tests

	Yeast CBS number				
Carbon source	**2402**	**2403**	**2404**	**2405**	**6016**
Glucose	+	+	+	+	+
D-xylose	+	+	-	-	+
Glycerol	+	+	+	+	+
Maltose	+	-	+	+	-
Galactose	+	+	-	-	+
Starch	-	-	-	-	-
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+/-
Sorbitol	-	-	-	-	+

to *R. toruloides* CBS 6016. When glucose was used as a carbon source, the majority of *R. mucilaginosa* species were able to grow to higher cellular density than *R. toruloides*. The only strain which did not grow well at C/9 and 30 even on glucose was strain 2404. Therefore this strain was excluded from subsequent experiments. The most amount of CDW produced strains *R. mucilaginosa* CBS 2402 and 2403 and even if they did not accumulate more than 13% of fatty acids within CDW produced a relatively high amount of fatty acids (FAs) reflecting FAs production per medium with no negative effect of C/N ratio 30. Glycerol was an excellent carbon source for *R. toruloides* in combination with both used C/N ratios 9 and 30. Production of FAs was in comparison to *R. mucilaginosa* strains increased, and the increase of C/N ratio to 30 even increased FAs production. *R. mucilaginosa* sp. did not react positively to the increased C/N ratio through FAs production and therefore were not used in the following cultivation experiments on glycerol with even higher C/N ratios. Xylose was well utilized and used for FAs production only by strains *R. toruloides* CBS 6016 and *R. mucilaginosa* CBS 2402. Strains *R. mucilaginosa* CBS 2403, and 2405 were not used in the next cultivation experiments with even higher C/N ratios and xylose as a substrate.

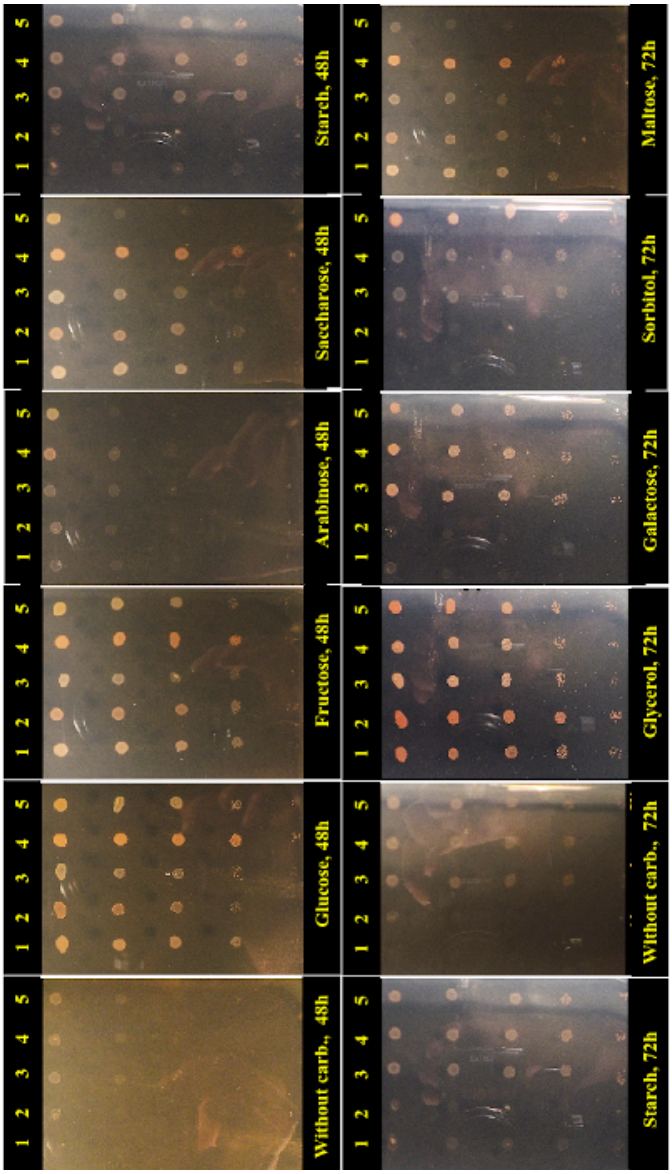

Fig. 6.9: Carbon source utilisation tests, 2402, 2403, 2404, and 2405 are CBS numbers of *R. mucilaginosa* strains, "Rhod" is the sign for *R. toruloides* CBS 6016

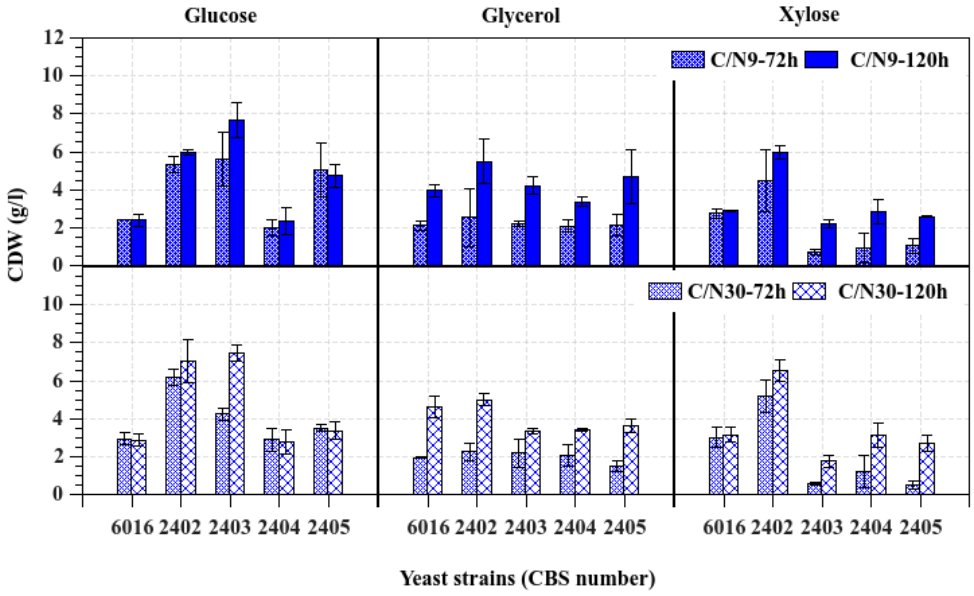

Fig. 6.10: CDW production on glucose, glycerol and xylose. Upper graph belongs to cultivation at C/N 9, graph below belongs to cultivation at C/N 30. The numbers of the x axis corresponds to CBS numbers of yeast strains: *R. toruloides* CBS 6016, *R. mucilaginosa* CBS 2402, 2403, 2404 and 2405

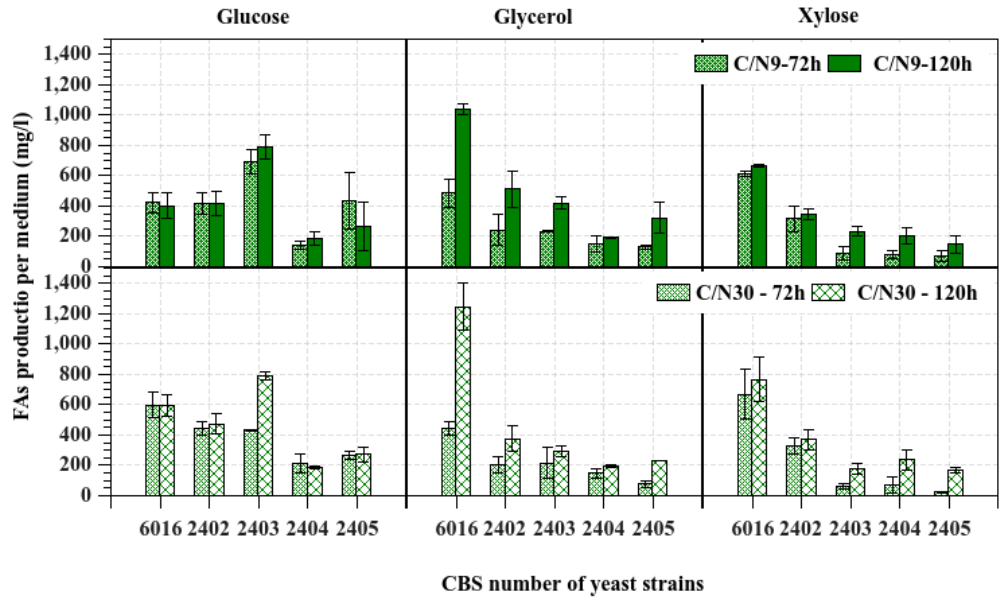

Fig. 6.11: FAs production on glucose, glycerol and xylose. Upper graph belongs to cultivation at C/N 9, graph below belongs to cultivation at C/N 30. The numbers of the x axis corresponds to CBS numbers of yeast strains: *R. toruloides* CBS 6016, *R. mucilaginosa* CBS 2402, 2403, 2404 ans 2405

6.2.3 Impact of nitrogen limitation and carbon source

For cultivation on glucose were selected almost all yeast strains: *R. toruloides* CBS 6016 and *R. mucilaginosa* sp. CBS 2402, CBS 2403, and CBS 2405. For cultivation with xylose as substrate and increased C/N ratio were chosen only two strains with the best growth and FAs accumulation, *R. toruloides* CBS 6016 and *R. mucilaginosa* CBS 2402. Glycerol was suitable only for *R. toruloides* CBS 6016. The chosen yeast strains were cultivated on glucose, xylose or glycerol medium with three different C/N ratios 30, 60, and 180. The concentration of carbon sources was kept to 30 g/l to avoid osmotic stress which would lead to decreased growth [128, 129]. As nitrogen source the ammonium chloride in concentrations corresponding to the desired C/N ratio was used. It is also necessary to point out that C/N ratios correspond to the initial concentrations at the beginning of the cultivation.

Biomass formation and carbon usage for FAs accumulation

Since FAs are intracellular metabolites [39] which form part of biomass, it is hard to capture the end of exponential phase, beginning of stationary phase and more importantly the time point when FAs start to be accumulated [130]. Therefore at Fig. 6.12, 6.14, and 6.13 (corresponding for cultivation on glucose, xylose and glycerol) are visualized the biomass productions expressed as CDW and FF-CDW (fatty acid free cell dry weight). The difference between CDW and FF-CDW captures the starting point for FAs accumulation within yeast cells.

The end of exponential phase and the beginning of the stationary phase was individual for each strain depending on the stress response to nitrogen limitation and used carbon source. The typical course of cultivation includes phase of balanced growth characterized by consumption of carbon and nitrogen leading to biomass production. If the nitrogen source is depleted, the biomass production is reduced and accumulation of storage metabolites begins. In oleaginous microorganisms, nitrogen limitation leads to the accumulation of fatty acids as a part of TG and SE [17].

Glucose as carbon source

In *R. mucilaginosa* strains CBS 2402 and 2403 with more limited conditions shortened the exponential phase, and the stationary phase started earlier with visible time point of FAs accumulation within CDW (see Fig. 6.12 (glucose), and 6.13 (xylose)). The best CDW productions were reached for C/N ratios 30 and 60 in strains *R. mucilaginosa* CBS 2402 and CBS 2403. Increasing C/N ratio, and therefore decreasing amount of nitrogen, dramatically decreased the amount of produced CDW in *R. mucilaginosa* sp.. The impact of nitrogen limitation is also well illustrated by glucose consumption (see Fig. 6.15). The glucose consumption was decreased with increasing C/N ratio in all *R. mucilaginosa* sp..

Strains *R. mucilaginosa* CBS 2402 and 2403 used substrate mostly for cell proliferation. The FAs accumulation in CDW did not reach more than 8 % in both strains at C/N 30. With increasing C/N ratio decreased the amount of produced CDW and increased the amount of accumulated FAs

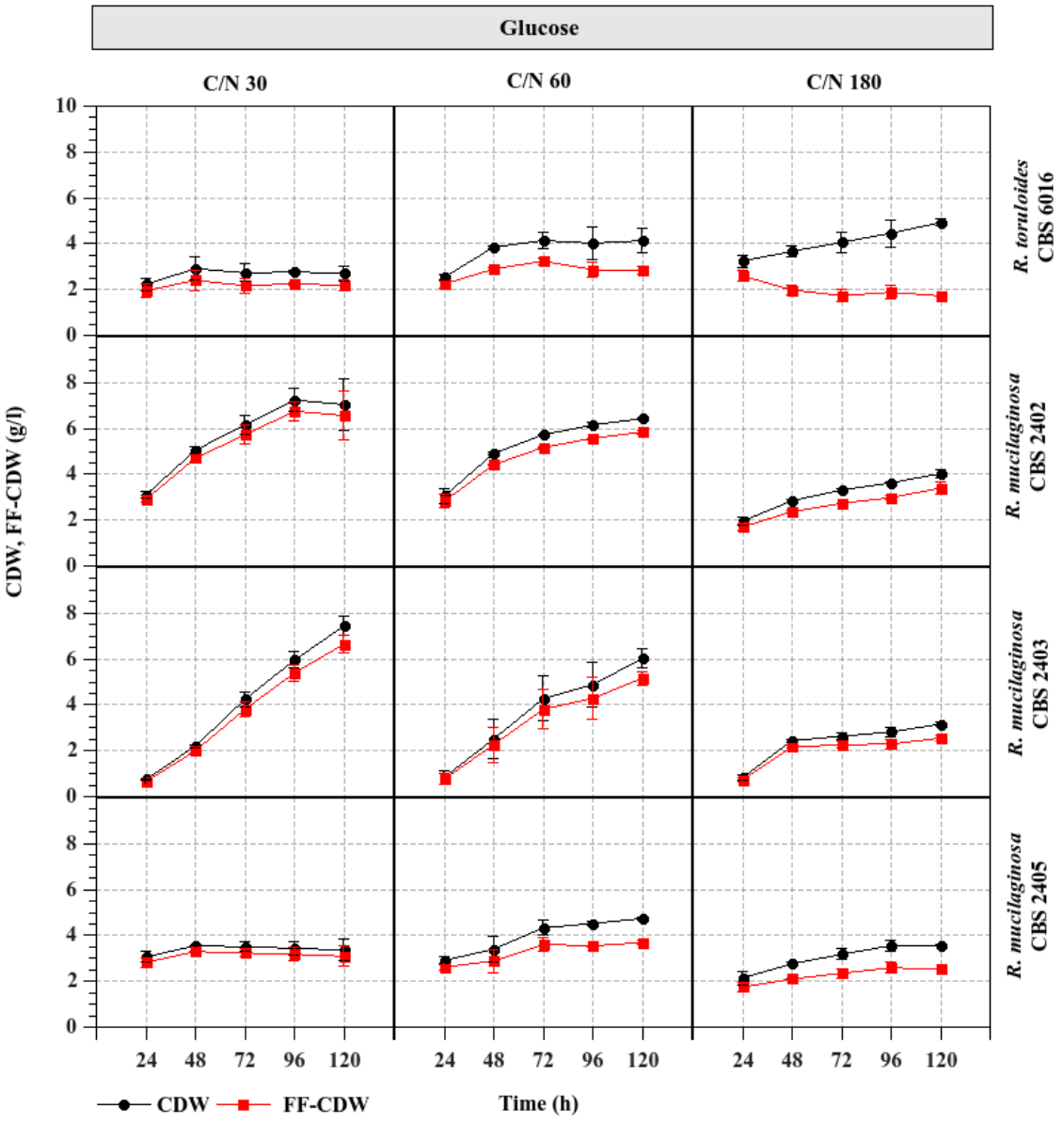

Fig. 6.12: Biomass production on glucose expressed as CDW and FF-CDW in time

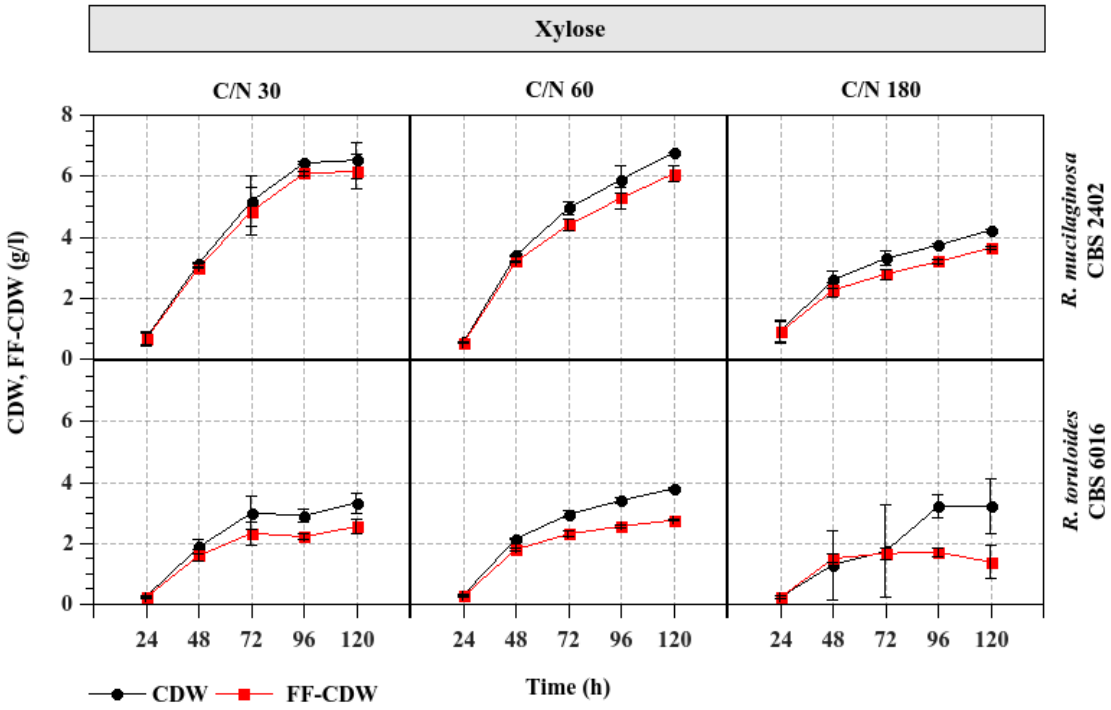

Fig. 6.13: Biomass production on xylose expressed as CDW and FF-CDW in time

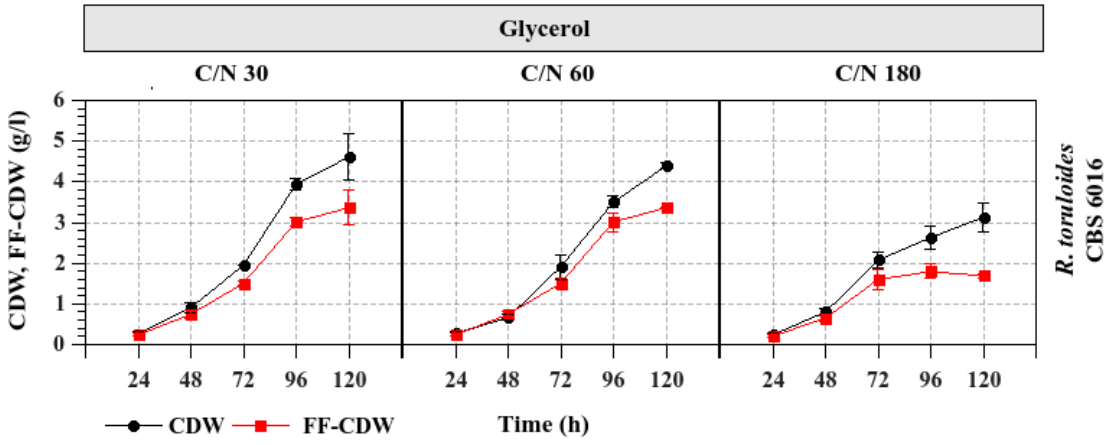

Fig. 6.14: Biomass production on glycerol expressed as CDW and FF-CDW in time

to 15% and 19 % respectively. Finally the FAs productions per medium were not much different at C/N 60 and C/N 180 for both strains.

With increasing C/N ratio *R. mucilaginosa* CBS 2405 increased the accumulation of FAs in CDW, but the productions of FAs per medium were comparable to strains CBS 2402 and CBS 2403. The strain CBS 2405 generally produced less CDW then these two strains. Carbon channeling to FAs was not very efficient in *R. mucilaginosa* sp. under nitrogen limitation. *R. mucilaginosa* sp. are reported to accumulate from 30-40 % of the fatty acids within CDW, and only strain *R. mucilaginosa* CBS 2405 on glucose medium (C/N 180) was able to accumulate almost 30 % of fatty acids [131]. The difference between CDW and FF-CDW in *R. mucilaginosa* strains CBS 2402 and CBS 2403 was not increased in all C/N rations. Their response to nitrogen limitation more corresponded to the behavior of non-oleaginous organisms, which in nitrogen limiting conditions stop cell proliferation or accumulate polysaccharides as glycogen and various glucans and mannans [132].

Nitrogen limitation in *R. toruloides* CBS 6016 and *R. mucilaginosa* CBS 2405 did not influence the beginning of the stationary phase in glucose medium, but influenced FAs accumulation in CDW. With increasing C/N ratio increased the difference between CDW and FF-CDW (see Fig. 6.12). Also, *R. toruloides* effectively utilized almost all glucose in all C/N ratios. With increasing C/N ratio increased the amount of FAs accumulated within the cells. The accumulation of FAs started generally at 48 hours of cultivation. Unlike *R. mucilaginosa* sp., *R. toruloides* CBS 6016 did not channeled carbon into biomass formation but preferred the accumulation of FAs within the cells. The difference between CDW and FF-CDW is therefore the most visible at C/N 180 (see Fig. 6.12). *R. toruloides* has a very effective metabolic response to nitrogen limitation through FAs accumulation. The best FAs productions were gained at C/N 180 and reached 3.2 g/l of medium, representing 65 % of CDW.

Xylose and glycerol as carbon source

In xylose cultivation, the strain *R. mucilaginosa* CBS 2402 entered into stationary phase at the same time points as glucose cultivation in all used C/N ratios. Nitrogen limitation influences the cell proliferation and with decreasing concentration of nitrogen dramatically decreases CDW productions of strain CBS 2402. This behaviour is not dependent on carbon sources used.

Only two of the yeast strains used, *R. mucilaginosa* CBS 2402 and *R. toruloides* CBS 6016, were capable to effectively utilize xylose. For strain *R. mucilaginosa* CBS 2402 as well as for *R. toruloides* CBS 6016, was xylose more easily utilizable substrate leading to higher CDW productions than xylose. The similar effect of xylose on biomass yield were observed in the study o Tiukova et al. [16]. The main characteristic of strain *R. mucilaginosa* CBS 2402 regarding the response to increased C/N ratio, was the capability to grow to high cell densities at C/N ratios 30 and 60 with high consumption of xylose. At C/N 180, the CDW production decreased 1.6x and substrate consumption was also minimal after 48 hours till 120 hours of cultivation. With increasing C/N ratio increased FAs accumulation within the cells at 72 hours of cultivation in every C/N. After 72 hours of cultivation, the FAs content slightly decreased. At this point, were the fatty acids probably used for biomass growth because there was not recorded that the cells would reach

the stationary phase. The highest FAs production per medium was reached at C/N 60 because of increased accumulation of FAs in CDW and without decreased CDW production (see Fig. 6.16). *R. mucilaginosa* sp. are reported to accumulate from 20-40 % of FAs within CDW, but it needs to be mentioned that this ability have only strains specially selected for increased content of FAs [131].

The impact of increased C/N ratio to *R. toruloides* cultivated on xylose was significant at C/N 180. The C/N ratios C/N 30 and 60 did not influence substrate consumption, production of CDW, or FAs production per medium. At both ratios was FAs accumulation comparable. A significant impact on fatty acid accumulation had C/N 180, wherein the end of cultivation content of FAs in CDW reached more than 50 %. Even if the highest nitrogen limitation slowed down cellular growth, significantly influenced the accumulation of fatty acids leading to FAs of 1.8 g/l of medium. At such limited conditions *R. toruloides* CBS 6016 used substrate mainly for the accumulation of fatty acids instead of cellular growth.

Glycerol was as well as xylose more difficult substrate than glucose for *R. toruloides* CBS 6016, which could be seen in delayed beginning of stationary phase. Furthermore, the increased C/N ratio and glycerol caused that the stationary phase started later than in glucose and with delayed FAs accumulation within CDW (see Fig. 6.13, and 6.14). Possibly, was nitrogen depleted later in medium with glycerol or xylose, than on medium containing glucose [16]. The capability of *R. toruloides* CBS 6016 to accumulate high yields of FAs in nitrogen limiting conditions corresponds to the behavior of oleaginous microorganism [133]. The formation of biomass as well as FAs were delayed in comparison to glucose or xylose. Glycerol surprisingly lead to increased DCW production at C/N ratios 30 and 60. At C/N 180 dramatically increased FAs content within CDW to 45 %. However, decreased production of CDW at C/N 180 caused comparable production of FAs per medium with C/N 30 and 60 (see Fig. 6.17).

R. toruloides is an excellent microbial producer of FAs using, glucose, xylose as well as glycerol as carbon source. The results of this study are in correlation with the results obtained by the studies of Wiebe et al. [134], Li [135], and Singh et al. [136].

6.2.4 Fatty acid composition

The majority of fatty acids in all used strains was predominantly formed by palmitic, stearic, oleic, and linoleic acids. Yeasts strains also produced myrictic (C14:0) acid and α-linoleic (C18:3) acid in small amounts. The α-linoleic was preferred metabolite to myristic acid and thus its amounts were monitored. Fatty acid composition was very similar in strains *R. mucilaginosa* CBS 2402 and CBS 2403 cultivated on glucose. The majority of fatty acids formed oliec acid (42 - 55 % of total FAs), palmitic acid (13-20 %), then linoleic acid (10-15%), stearic acid formed 6-18 % and α-linoleic was accumulated in the range from 1.3 -4 %. Composition of fatty acids produced by strains *R. mucilaginosa* CBS 2402 and 2403 is listed in Tab. 6.7, and 6.8

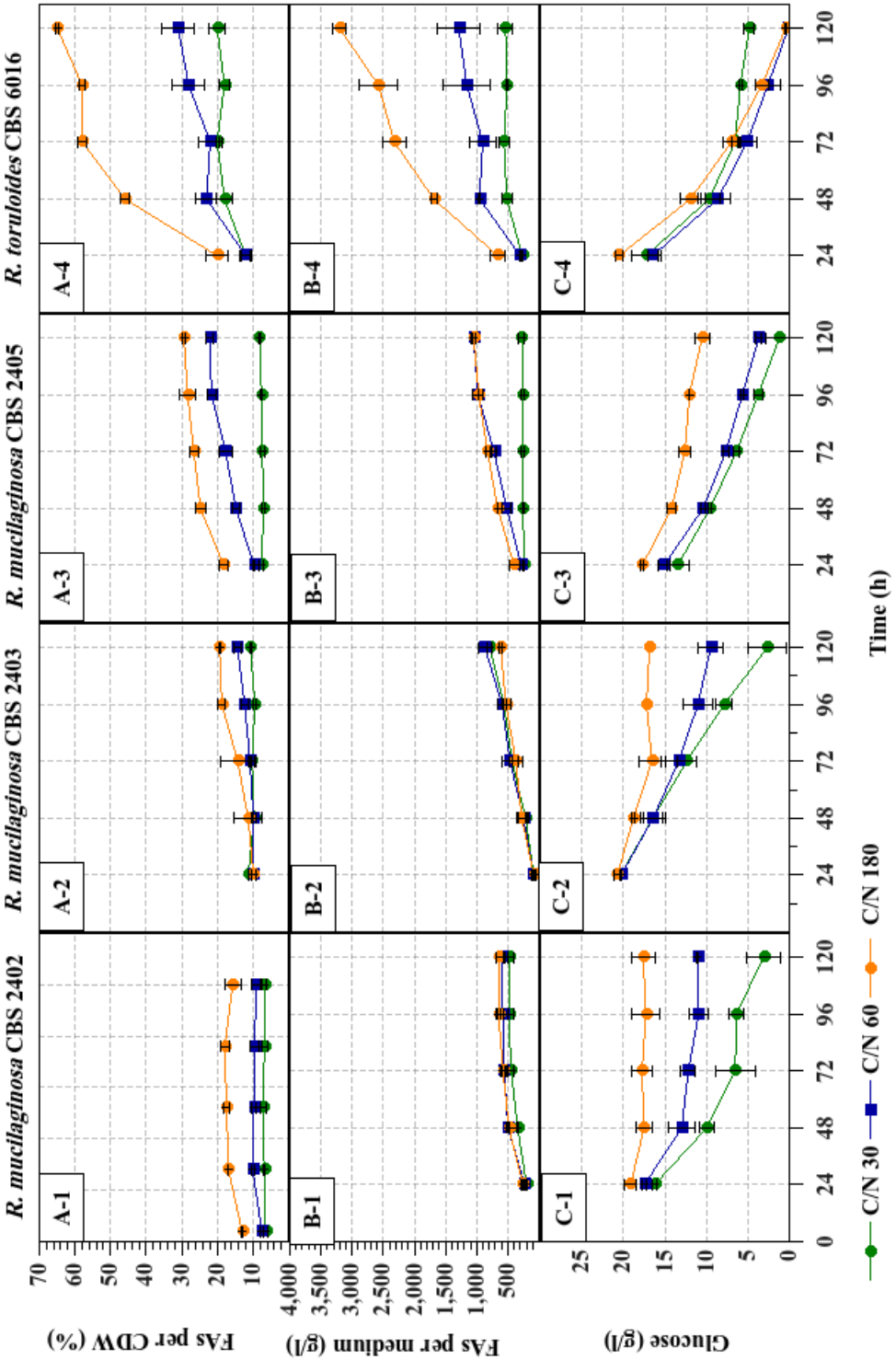

Fig. 6.15: Productions of FAs and glucose consumption in time. A(1-4) - FAs production per CDW, B(1-4)-FAs production per medium, C(1-4) - glucose consumption. (A-C)- 1-3 corresponds to strain *R. mucilaginosa* CBS 2402, 2403 and 2405,(A-C)-4 corresponds to strain*R. toruloides* CBS 6016

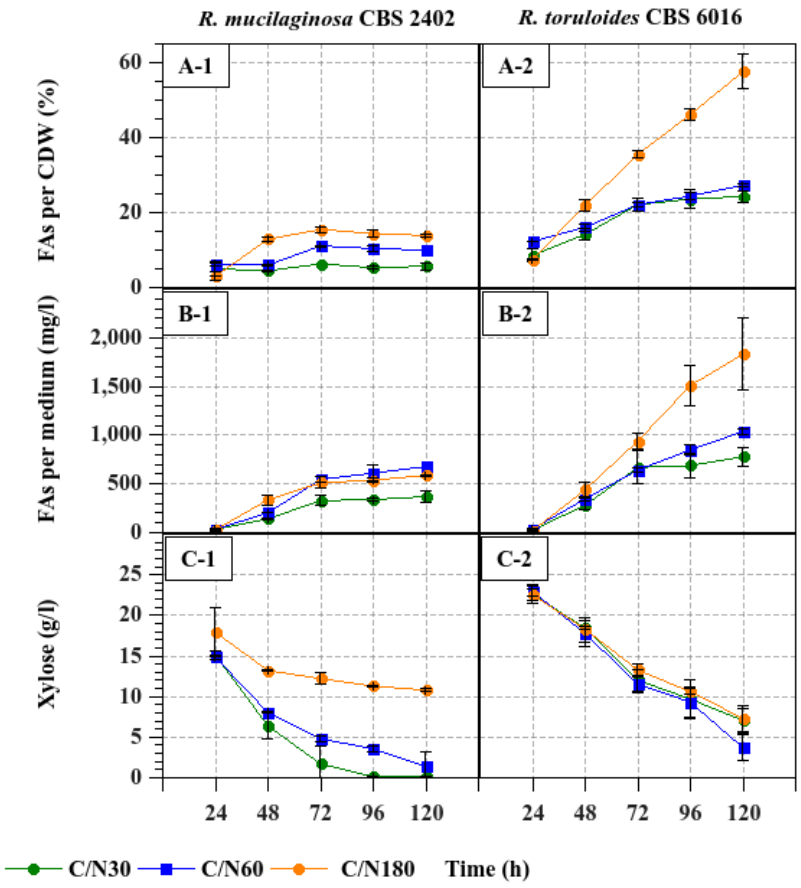

Fig. 6.16: Productions of FAs and xylose consumption in time. A(1-2) - FAs production per CDW, B(1-2)-FAs production per medium, C(1-2) - xylose consumption. (A)-1-2 corresponds to strain *R. mucilaginosa* CBS 2402, (A-C)-2 corresponds to strain*R. toruloides* CBS 6016

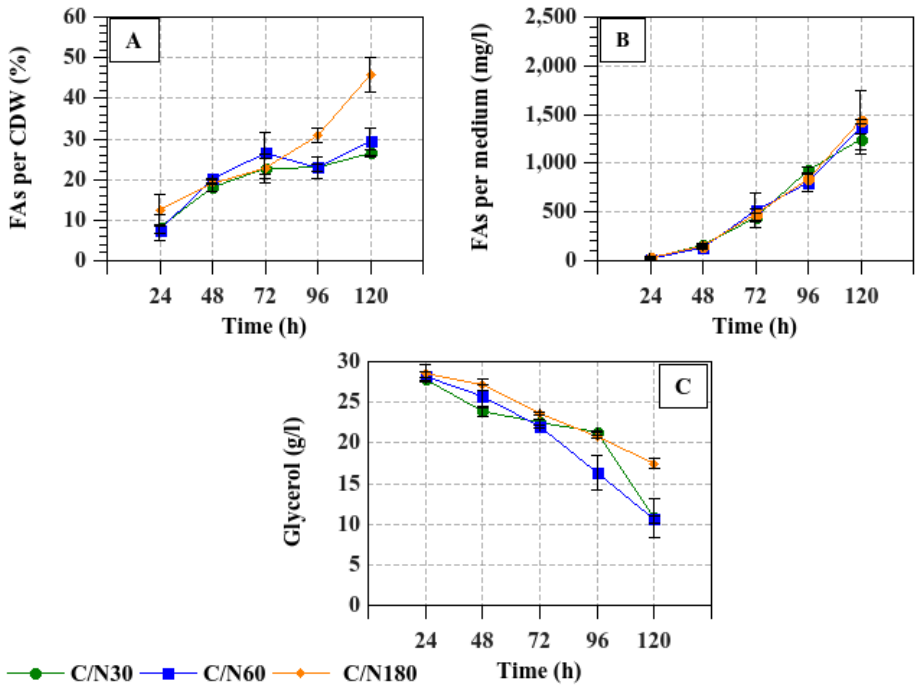

Fig. 6.17: Productions of FAs and glycerol consumption in time by strain *R. toruloides* CBS 6016

Tab. 6.7: Composition of FAs produced on glucose and xylose by strain *R. mucilaginosa* CBS 2402

R. mucilaginosa* CBS 2402 -composition FAs (%)						
Glucose cultivation						
C/N ratio	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
30	24	15.16	15.43	48.34	10.46	2.06
	48	14.70	14.53	51.24	10.32	1.48
	72	14.15	13.22	53.17	10.60	1.62
	96	14.17	9.79	54.88	12.33	1.39
	120	14.09	9.46	55.67	11.65	1.65
60	24	15.97	18.28	46.26	11.57	1.83
	48	15.07	16.17	51.85	10.60	1.21
	72	14.82	13.90	53.35	11.52	1.28
	96	14.70	12.70	53.89	12.09	1.36
	120	14.62	11.73	54.05	12.82	1.37
180	24	16.68	14.42	46.52	14.34	2.86
	48	15.00	12.58	50.42	13.93	2.87
	72	14.21	11.66	52.29	14.57	2.98
	96	14.10	11.60	51.61	14.71	2.93
	120	13.88	10.75	51.94	15.65	3.10
Xylose cultivation						
C/N ratio	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
30	24	18.44	11.56	22.76	25.38	8.14
	48	16.54	11.51	33.95	25.34	5.20
	72	14.41	17.97	44.90	13.01	2.26
	96	14.24	12.85	48.06	14.68	2.01
	120	14.43	10.11	53.23	12.40	1.84
60	24	22.34	17.89	22.56	21.26	6.06
	48	16.10	19.46	36.84	17.38	3.56
	72	15.49	15.55	45.57	15.22	2.00
	96	15.10	13.17	47.25	16.46	2.15
	120	14.84	11.48	46.75	18.29	2.44
180	24	17.16	6.33	26.96	33.39	10.38
	48	19.25	11.71	39.63	19.89	4.48
	72	17.35	11.46	42.75	19.13	4.43
	96	16.58	11.27	43.05	19.57	4.62
	120	16.14	10.09	43.25	20.68	4.83

The nitrogen limitation influenced only slightly the accumulation of C18:2 and C18:3, while the accumulation C18:1 slightly decreased. The FAs composition of *R. mucilaginosa* CBS 2405 was formed more by C18:1 and C18:3 acids at all C/N ratios than in other *R. mucilaginosa* strains. With increasing C/N ratio, increased production of C18:2 and C18:3 and at the same time the amount of C18:1 was decreased. The composition of fatty acids produced by strain *R. mucilaginosa* CBS 2405 is listed in the Tab. 6.8.

R. toruloides CBS 6016 produced in majority the same fatty acids as *R. mucilaginosa* strains. The most abundant fatty acid was also oleic acid, but it formed only 37-48 % of the total FAs. The palmitic acid formed 19-27 % and production of C18:2 and C18:3 was comparable to strains *R. mucilaginosa* CBS 2402 and 2403. The composition of fatty acids produced by strain *R. toruloides* CBS 6016 is listed in the Tab. 6.9

Tab. 6.8: Composition of FAs produced on glucose by strains *R. mucilaginosa* CBS 2403 and 2405

R. mucilaginosa* CBS 2403 -composition FAs (%)						
Glucose cultivation						
C/N ratio	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
30	24	19.53	11.34	42.77	13.78	3.38
	48	19.30	10.50	48.64	13.57	2.49
	72	17.70	10.61	51.68	12.10	1.87
	96	16.64	9.10	53.28	13.86	1.68
	120	15.90	6.72	54.63	15.37	1.74
60	24	19.83	10.07	45.83	15.03	4.29
	48	18.85	8.69	50.77	14.90	2.51
	72	19.57	9.34	49.82	14.17	1.91
	96	19.79	8.04	48.90	16.08	2.11
	120	19.99	8.06	48.41	15.93	2.31
180	24	19.64	11.16	43.39	14.28	3.99
	48	21.51	9.85	46.12	14.93	2.74
	72	26.74	9.83	56.87	14.94	3.04
	96	20.74	10.12	45.91	14.84	2.98
	120	20.49	9.39	46.89	15.16	3.29
R. mucilaginosa* CBS 2405 -composition FAs (%)						
Glucose cultivation						
C/N ratio	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
30	24	12.97	5.30	58.80	14.54	4.72
	48	9.85	1.54	68.01	13.94	4.42
	72	8.67	1.40	67.73	13.98	4.36
	96	8.89	2.64	65.72	14.36	4.42
	120	7.59	1.11	71.43	12.43	3.72
60	24	14.39	7.20	54.77	15.49	4.19
	48	14.24	4.87	56.76	16.91	2.63
	72	13.33	4.51	57.41	17.53	2.41
	96	12.20	4.47	57.32	17.75	2.33
	120	12.99	4.82	54.86	19.20	2.66
180	24	16.82	6.54	47.87	18.01	6.00
	48	14.99	6.00	52.77	16.99	4.84
	72	13.92	5.25	53.49	18.13	5.01
	96	13.47	5.22	52.47	18.74	5.11
	120	13.06	4.86	52.20	19.41	5.17

Tab. 6.9: Composition of FAs produced on glucose, xylose and glycerol by strain *R. toruloides* CBS 6016

R. toruloides* CBS 6016 -composition FAs (%)						
Glucose cultivation						
C/N ratio	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
30	24	21.64	10.92	37.92	18.45	4.78
	48	20.12	11.21	44.14	12.67	2.46
	72	19.13	9.02	48.00	13.27	2.63
	96	18.10	7.90	48.23	14.86	2.75
	120	19.42	9.03	46.37	13.77	2.69
60	24	21.43	10.13	38.46	17.77	4.40
	48	22.42	12.23	42.95	12.18	1.94
	72	22.47	10.10	41.28	15.07	1.94
	96	22.35	8.85	42.56	16.62	1.93
	120	21.63	7.56	42.43	18.79	2.11
180	24	22.81	12.37	34.85	14.42	4.14
	48	27.61	11.84	42.79	10.67	2.58
	72	27.15	11.12	44.10	10.24	2.43
	96	26.16	10.63	46.18	10.45	2.55
	120	25.32	9.86	46.70	11.46	2.63
Xylose cultivation						
C/N ratio	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
30	24	26.55	14.87	35.15	15.79	3.84
	48	22.95	13.80	39.34	14.45	2.18
	72	21.94	10.01	45.51	13.48	1.44
	96	20.28	8.76	43.13	16.06	1.57
	120	20.11	7.70	44.54	17.09	1.42
60	24	24.32	17.22	33.72	15.54	3.93
	48	21.27	12.84	41.79	13.93	2.06
	72	21.44	10.40	43.85	14.07	1.50
	96	20.99	8.49	42.77	16.12	1.50
	120	20.48	7.76	43.11	17.91	1.69
180	24	16.82	6.54	47.87	18.01	6.00
	48	14.99	6.00	52.77	16.99	4.84
	72	13.92	5.25	53.49	18.13	5.01
	96	13.47	5.22	52.47	18.74	5.11
	120	13.06	4.86	52.20	19.41	5.17
Glycerol cultivation						
C/N ratio	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
30	24	23.37	10.52	39.61	18.70	3.83
	48	26.16	11.21	40.53	13.84	2.05
	72	27.58	8.84	43.71	13.14	1.62
	96	27.61	8.81	44.12	12.86	1.49
	120	27.06	8.92	44.55	13.02	1.63
60	24	19.73	20.25	31.43	8.52	1.97
	48	16.90	21.17	40.03	11.59	3.43
	72	16.05	22.27	42.75	11.03	2.15
	96	18.52	21.25	42.02	9.63	1.34
	120	20.57	18.15	40.21	11.85	1.42
180	24	20.50	21.68	23.61	7.89	2.39
	48	18.29	22.09	38.85	9.89	2.24
	72	16.54	19.26	42.38	12.11	2.76
	96	22.21	16.73	40.76	11.64	2.10
	120	26.89	14.70	40.24	11.42	1.63

The combination of nitrogen limitation and xylose as a substrate caused a predominant accumulation of unsaturated fatty acids to saturated fatty acids in *R. toruloides*. The most significant was increase in accumulation of oleic, linoleic, and α-linoleic acids content. Xylose in combination with nitrogen limitation also changed preferences in fatty acid accumulation by *R. mucilaginosa* CBS 2402. The content of oleic acid was decreased in comparison to glucose medium and the content of linoleic and α-linoleic acids was slightly increased. The impact of increasing C/N ratio and glycerol as a substrate did not have any significant impact on fatty acid composition. It seems more that glycerol itself, when used as the only carbon source, leads to decreased accumulation of unsaturated fatty acids and to increased accumulation of saturated fatty acids in comparison to glucose as a carbon source. These data are in accordance with the study of Wiebe [134].

6.2.5 Lipid body formation in the cells of *R. toruloides*

R. toruloides strain CBS 6016 proved to have extraordinary potential to accumulate fatty acids. Therefore, fluorescent microscopy was used to visualize the accumulation of fatty acids within its lipid bodies (lipid droplets). The samples of the cells were taken for analysis in 24-hour intervals for all substrates and C/N ratios 30, 60 and 180. The impact of increasing C/N ratio on every substrate led to fatty acid accumulation within the lipid body as a main storage cellular compartment.

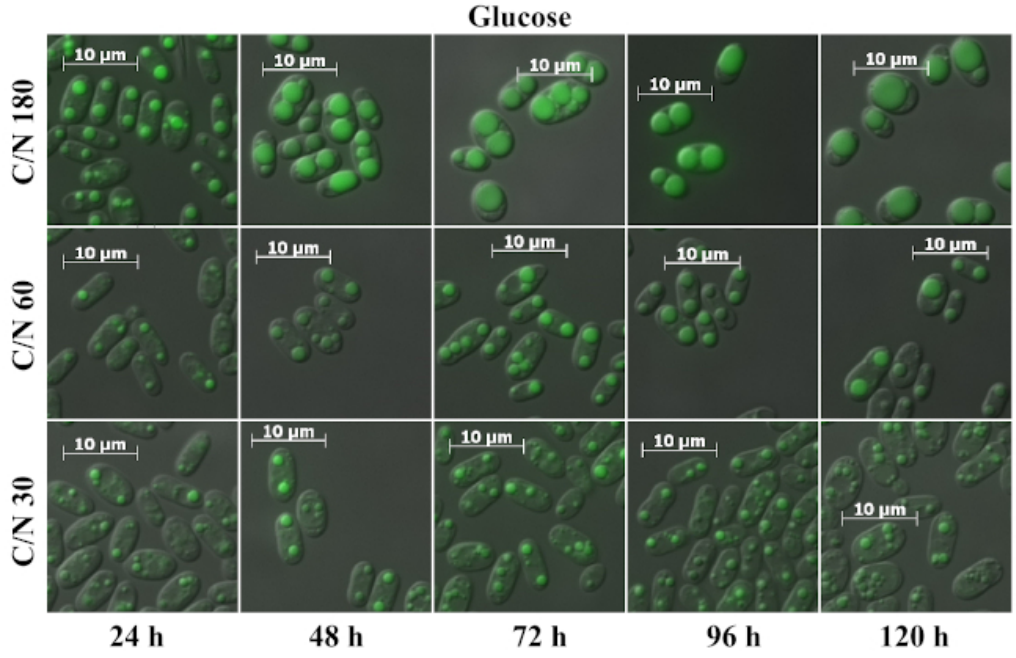

Fig. 6.18: Fatty acid accumulation within lipid bodies of *R. toruloides* CBS 6016 over cultivation time at C/N ratios 30, 60 and 180 using glucose as a substrate

Generally, at C/N 30 with glucose as a substrate there were visible in average 2-3 smaller lipid bodies, which grew over the cultivation time and at the end the cultivation cells contained numerous smaller lipid bodies. At C/N 60 was noticed the difference at the end of the cultivation

where yeast cells kept the same number of lipid bodies (see Fig. 6.18). This behaviour is typical for low-lipid cells [16]. The C/N 180 was completely different from the beginning of cultivation where yeast cells contained at average 2-3 lipid bodies slightly bigger than in previous C/N ratios at 24 hours of cultivation.

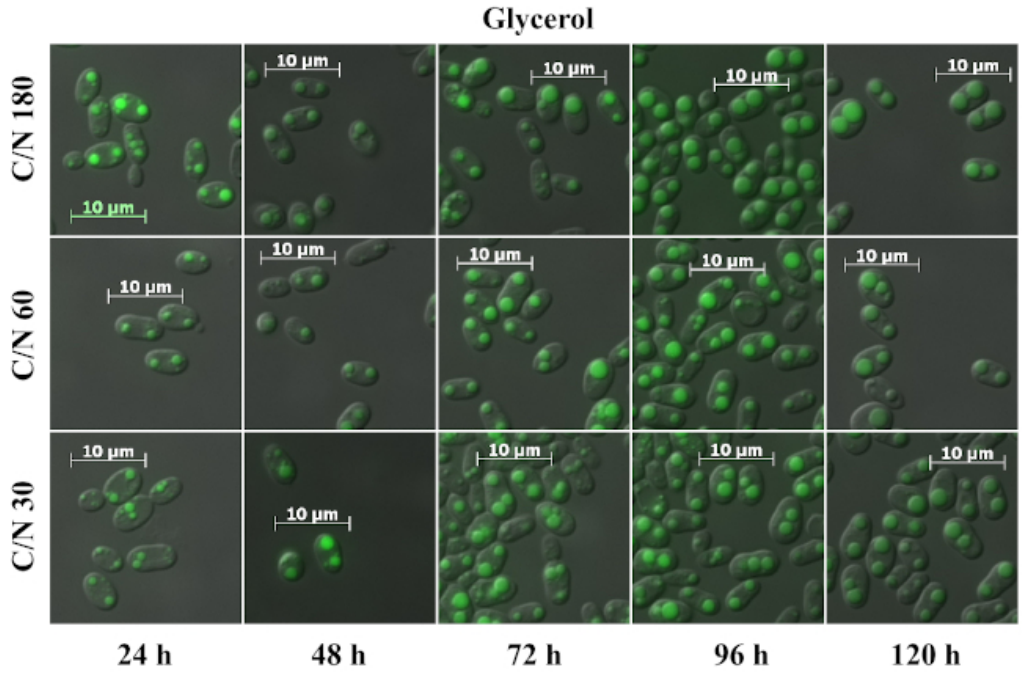

Fig. 6.19: Fatty acid accumulation within lipid bodies of *R. toruloides* CBS 6016 over cultivation time at C/N ratios 30, 60 and 180 using glycerol as a substrate

At 48 hours, lipid bodies started to increase their volume and at the end of cultivation split in one lipid body, filling almost all space of the cell. Therefore, the lipid bodies formation is consistent with accumulation of fatty acids within yeast cells [16]. With the increased accumulation of fatty acids changed also the shape of the cell from oval to the rounded shape of the cells (see Fig. 6.18). In glycerol cultivation, were images from fluorescent microscopy very similar to those obtained at all C/N ratios with glucose cultivation at C/N 60 (see Fig. 6.19). When xylose was used as a substrate there was a similar trend in fatty acids accumulation within the cells as those in glycerol for C/N 30 and 60. At C/N 180 at 48 and 72 hours of cultivation there were visible other compartments, probably vacuoles, which are connected to cellular starvation, when some components of the cell are decomposed and used as a source of missing nutrition for cell surviving [9]. At the end of cultivation, there were on average slightly increased lipid bodies (see Fig. 6.20).

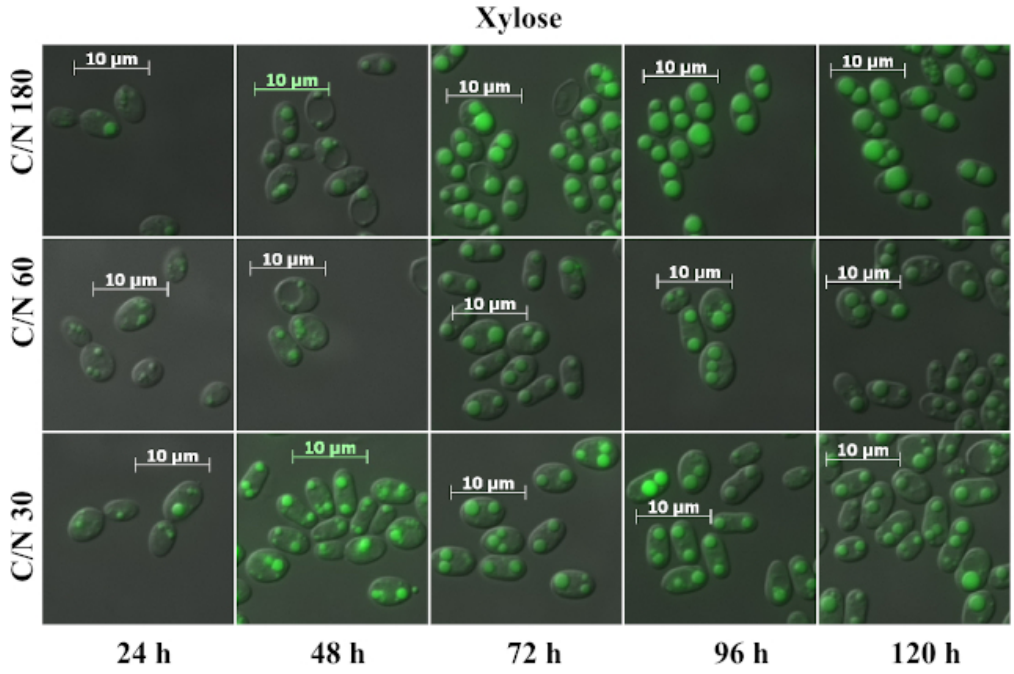

Fig. 6.20: Fatty acid accumulation within lipid bodies of *R. toruloides* CBS 6016 over cultivation time at C/N ratios 30, 60 and 180 using xylose as a substrate

6.3 Genetic modification of *R. toruloides* CBS 6016 using biolistic method

Following part dealing with yeast genetic engineering was done during ERASMUS+ internship in France at Institute Micalis, BimLip team (INRA). The genetic tools in *R. toruloides* are far less developed than in other oleaginous yeasts such as *Y. lipolytica*. So far, only a few research groups succeeded to genetically modify *R. toruloides* and in most cases it was done by ATM (Agrobacterium tumefaciens mediated transformation) method [94].

In this experiment was used biolistic method, which is not very common among oleaginous yeasts. In BimLip laboratory this method was specifically developed for *R. toruloides* transformation and this experiment is one of the early experiments leading to successful transformation. Since the deletion of genes is still inefficient the strategy of genetic modification was designed for gene over-expression. For the over-expression were chosen genes for GPD1 (glycerol-3-phosphate dehydrogenase) and DGA1 diacylglycerol acyltransferase. These genes are reported to lead to increased lipid accumulation in many studies dealing with oleaginous yeasts [137, 138]. DGA1 and GPD1 are genes involved in early steps of lipid metabolism. The GPD1 codes for NAD^+ dependent glycerol-3-phosphate dehydrogenase, which converts dihydroxyacetone phosphate into glycerol-3-phosphate. Glycerol-3-phosphate dehydrogenase is an enzyme localized mainly in cytosol and peroxisomes and it provides glycerol backbone for TG assembly [139]. The enzyme diacylglycerol-O-acyltransferase, product of gene DGA1 catalyze acyl-CoA-dependent acylation of sn-1,2-diacylglycerol and it is providing protection against toxicity of free fatty acids. Diacylglycerol-O-acyltransferase is localized on the lipid body membrane, where it is assumed to accumulate TG within lipid body and increase its volume. It is also localized in endoplasmic reticulum [140]. The DGA1 gene is used in combination with acetyl-CoA carboxylase as a "push and pull" strategy to increase lipid accumulation, and thus DGA1 was also used in this experiment [141, 138].

6.3.1 Vector construction and genetic modification

After gene amplification, genes were inserted into plasmid backbone and their correct insertion was verified by digestion after ligation of plasmid and PCR products. The verification is visualized on Fig. 6.22 where is also visible the site of gene GPD1 corresponding to 1774 bp and DGA1 corresponding to 1889 bp. The bigger fragment represents the rest of the plasmid P3612.

From the verified plasmids were prepared expression cassettes by NotI digestion and subsequently used for biolistic transformation. The map of plasmids is visualized in Fig. 6.21. The expression cassette represents bands on gels exceeding the size of 5 Kbp (see Fig. 6.23) .

After transformation by biolistic method were transformed yeasts grown on YNB plates with nourseotricine. Then the colonies which appeared to be the fastest growing were selected and inoculated to YNB plates with nourseotricine for 24 hours. Transformed yeasts were verified by two PCR reactions, where first of them included primers for pTEF promoter and a part of the

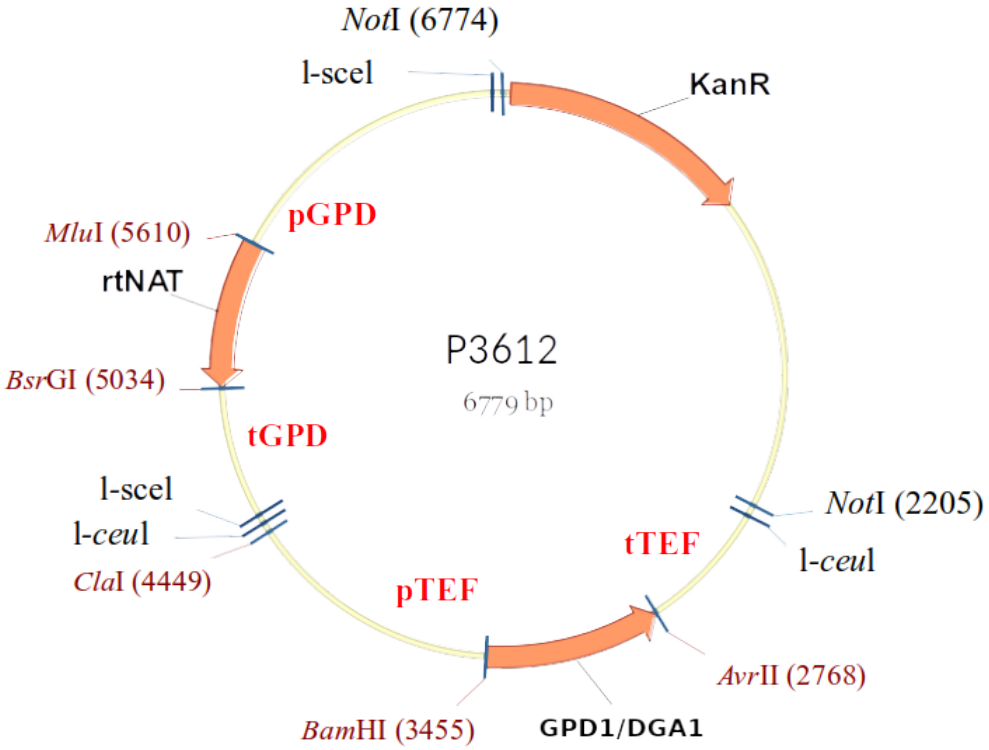

Fig. 6.21: Map of modified plasmid used for vector preparation

gene sequence (DAG1 or GPD1), while the second included tTEF terminator with a part of gene sequence (DAG1 or GPD1). The schemes of verification PCR reactions are visualized in Fig. 6.24.

Verification PCR was done for four selected clones for each overexpressed gene. As a positive control was used plasmid bearing the corresponding expression cassette, WT strain and negative control to exclude false positive results. The results of verification PCR proved only one positive clone for DGA1 and four clones positive for GPD1 (see Fig. 6.25). To examine impact of genetic manipulation on lipid production DGA1 clone 1 and GPD1 clone 4 were used for subsequent cultivation experiments.

6.3.2 Fatty acids production by genetically modified strains

Chosen clones were cultivated on YNB medium with glucose (30 g/l) at C/N ratio 180. Cultivation was done in Erlenmeyer flasks for 96 hours and samples were taken during cultivation in 24 hours time intervals. The experiments were done in triplicate in comparison with wild type (WT). The total lipid production was interpreted as FAs production, which was analyzed using gas chromatography. The chosen clones proved significantly better biomass production then the control WT strain, the results can be seen in Fig. 6.26. The biomass production was recorded every 24 hours for each strain. In control wild type strain WT was biomass production increased till 48 hour of cultivation in comparison to GPD1 and DGA1 clones. The GPD1 and DGA1 clones

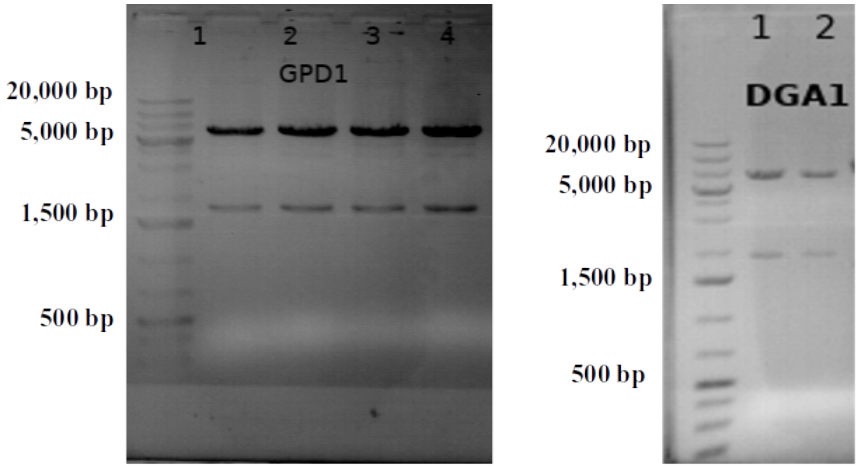

Fig. 6.22: Verification of inserted PCR products - genes DGA1 (1889 bp), and GPD1 (1774 bp) into plasmid backbone, the larger fragment in the upper side of the gel is plasmid backbone, numbers indicates successful *E.coli* clones

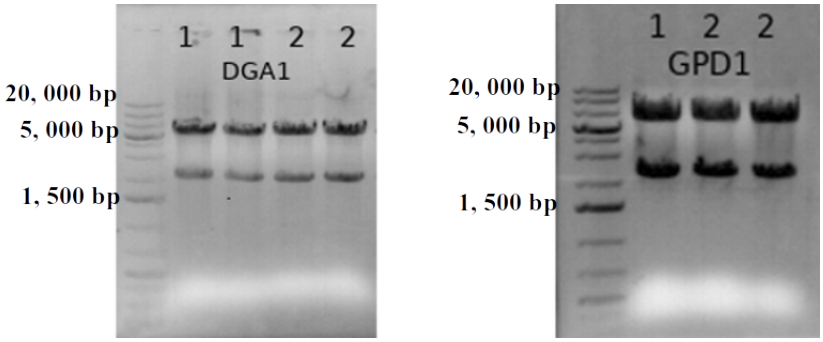

Fig. 6.23: Expression cassettes bearing genes DGA1 (size around 2kb) and GPD1 (size around 2kb) fragment over 5kb is the rest of plasmid backbone, numbers indicates successful *E.coli* clones

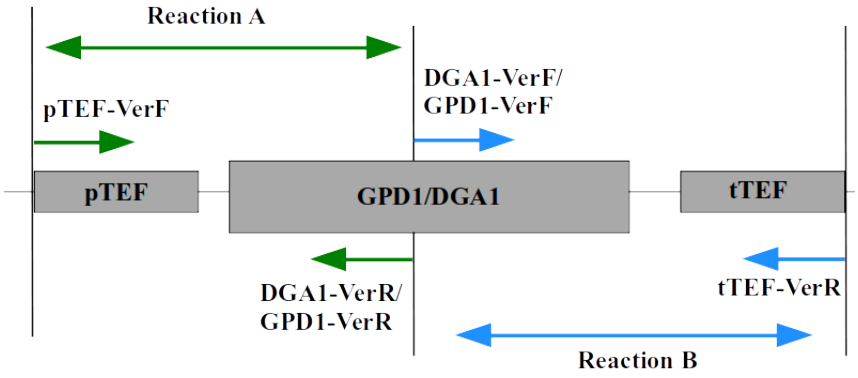

Fig. 6.24: Scheme of verification PCR reaction, pTEF - TEF promotor sequence, tTEF - TEF terminator sequence, GPD1/DGA1 - inserted genes, small arrows represents locations of verification primers and big arrows visualize amplified regions (reaction A/B)

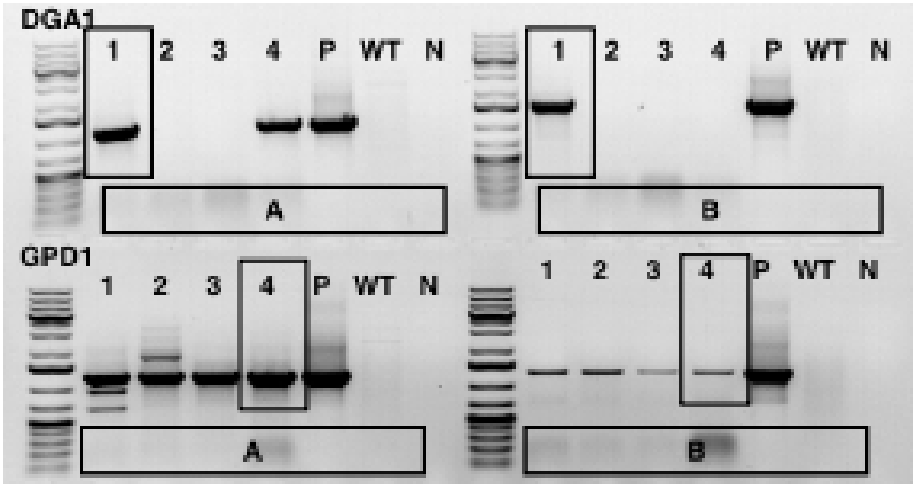

Fig. 6.25: Results of verification PCR, numbers 1- 4 represents chosen clones, P – plasmid bearing expression cassettes (DGA1 or GPD1), WT – wild type strain of *R. toruloides*, N – negative control

had proved to have slower growth during 48 hours of cultivation, but after 72 hours the biomass production of both clones was higher than in WT strain. Higher amount of biomass was recorded also after 96 hours of cultivation, where genetically manipulated clones produced 19.5 % (DGA1) and 24 % (GPD1) of biomass more than WT.

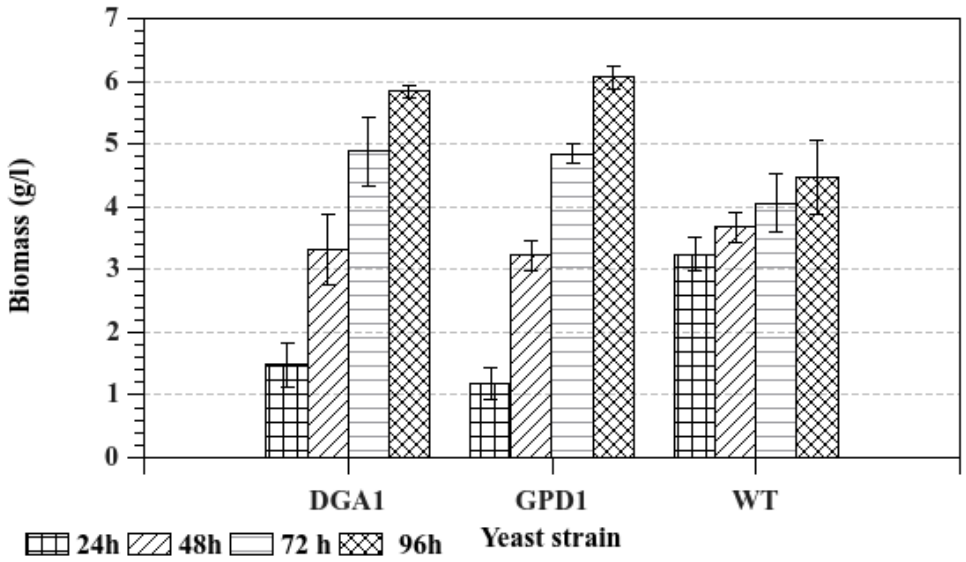

Fig. 6.26: Biomass growth during cultivation time by wild type and transformants

However, the final biomass production of genetically manipulated clones was higher than in WT, the impact of over-expressed genes was not reflected into FAs production which was decreased in comparison to WT (see Fig. 6.27). The production of FAs in both clones did not reach more than 50 % of FAs per CDW. The other study reporting increased lipids accumulation caused

by overexpression of GPD1 gene were only detectable in ß-oxidation deficient strains affecting the expression of genes involved in TG homeostasis [137].

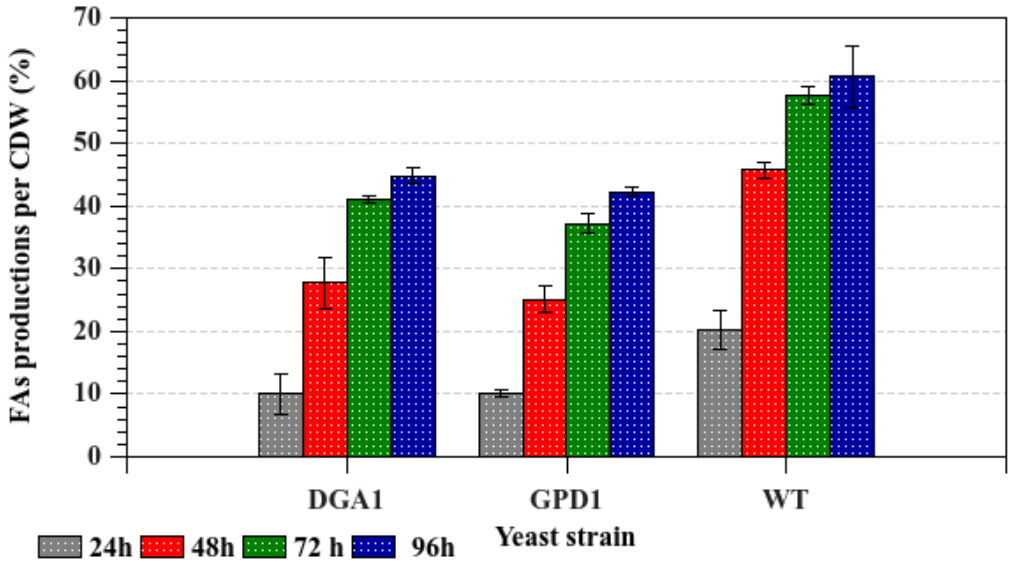

Fig. 6.27: Productions of FAs during cultivation time by wild type and transformants

The diacylglycerol acyltransferase (DAG1) is taking part in the terminal step in the formation of TG. Diacylglycerol acyltrasferase in yeasts is localized in lipid bodies and in the Endoplasmic reticulum. Generally, it is reported that overexpression of DGA1 in yeast *S. cerevisiae* and *Y. lipolytica* led to increased lipid production. On the contrary, it was also reported in the studies of Polburee et al. and Zhang et al., that increased lipid production was achieved only in some transformants. The study of Polburee et al. assumed that increased lipid production in *Rhodotorula fluvialis* depends on how many vector copies were simultaneously integrated into the genome and on specified conditions including relatively high concentrations of glycerol in combination with C/N ratio 80 [142]. In other study done by Zhang and Skerker dealing with *R. toruloides* NP11, was also reported diversity in lipid production among transformants. This phenomena is probably caused by random insertion of the genes into genome and possible inactivation of unknown genes [138]. Also in this experiment was not recorded significantly increased fatty acids productions in transformants bearing overexpressed DGA1 gene (2.615 g/l of FAs, 96 hour) in comparison to control strain WT (3.230 g/l of FAs, 96 hour). The results are summarized in the Tab. 6.10.

These experiments were mainly focused on the possibility to obtain genetically modified strains of *Rhodosporidium toruloides* by biolistic method and to prove the integration of the gene into the genome by PCR. This experiment resulted in two successfully genetically modified clones of *R. toruloides*, which could be used in subsequent experiments aiming at overexpression of other genes responsible for lipid overproduction in the same clone. The main candidate genes should be Citrate lyase (ACL) as a supplier of acetyl-CoA gene for Acetyl-CoA carboxylase (ACC), which is responsible for carboxylation of Acetyl-CoA to malonyl-CoA – main precursor for cytosolic

Tab. 6.10: Productions of FAs and biomass during cultivation time

Yeast strain	**Time (h)**	**FAs per CDW (%)**	**FAs per medium (g/l)**	**Biomass (g/l)**
DGA1	24	9.992 ± 3.238	0.146 ± 0.058	1.480 ± 0.361
	48	27.728 ± 4.042	0.932 ± 0.304	3.307 ± 0.566
	72	41.053 ± 0.446	2.005 ± 0.215	4.887 ± 0.549
	96	44.824 ± 1.325	2.615 ± 0.103	5.833 ± 0.099
GPD1	24	10.102 ± 0.547	0.119 ± 0.028	1.180 ± 0.262
	48	25.093 ± 2.090	0.811 ± 0.122	3.220 ± 0.231
	72	37.215 ± 1.636	1.805 ± 0.134	4.847 ± 0.150
	96	42.248 ± 0.692	2.560 ± 0.084	6.060 ± 0.183
WT	24	20.239 ± 3.143	0.601 ± 0.115	3.560 ± 0.275
	48	45.730 ± 1.336	1.815 ± 0.047	3.840 ± 0.238
	72	57.593 ± 1.400	2.567 ± 0.185	4.380 ± 0.454
	96	60.636 ± 4.846	3.230 ± 0.309	4.880 ± 0.593

synthesis of TG. GPD1 and DGA1 in combination with above-mentioned genes overexpressed together could create carbon flux invested into lipid accumulation. The GPD1 would supply a sufficient source of glycerol, which would be acylated in a faster rate by overexpressed DGA1 and the corresponding supply of fatty acids for the assembly TG. It would be also necessary to examine higher number of genetically modified clones and optimized conditions leading to increased lipid accumulation.

6.4 Impact of inhibitors, physical and chemical mutagenes on metabolite production

Rhodotorula toruloides is a yeast strain with a great capacity to accumulate fatty acids as well as carotenoids. Since there are not yet very efficient tools for genetic manipulation of *R. toruloides* the methods of random mutagenesis can be suitable for obtaining strains with overproduction properties [143, 94]. The random mutagenesis was already successfully used with *Phaffia rhodozyma* resulting in mutants with two times higher productions of astaxanthin then in the original wild type strain. Also, *P. rhodozyma* is so far one of the few carotenogenic yeast strains with functional tools for genetic manipulation. *P. rhodozyma* mutants helped to better understand the regulation of genes participating in carotenoid synthesis. Random mutagenesis is therefore a handy tool for metabolic regulation studies as well as in genetic manipulation studies aiming for preparation of yeast strains with exceptional properties [144, 29]. Generally, exploitation of random mutagenesis targeting increased carotenoid productions have frequently difficulties in selection of overproducing mutants. Carotenoid overproducing strains are generally not distinguishable by the naked eye. Therefore, the usage of appropriate selection tool is needed. In yeasts or algae are frequently used inhibitors of carotenoid synthesis as cinnamyl alcohol, thymol, dimethyl phthalate, veratrol, or diphenylamine (DPA) [144].

The majority of these inhibitors result in decreased cell proliferation leading to decreased biomass production [69]. Inhibitors of carotenoid biosynthesis frequently affect activity of phytoene desaturase (PDS) which is responsible for synthesis of neurosporene. Neurosporene can be cyclized into β-zeacarotene or it can be desatureted to produce lycopene. Phytoene is a colorless carotenoid synthetized through condensation of two molecules of geranylgeranyl pyrophosphate. When PDS enzyme is blocked, only the phytoene is accumulated with no subsequent biosynthesis of neurosporene nor lycopene or β-zeacarotene. Therefore, carotenogenic yeasts cultures exposed to the effect of carotenoid biosynthesis inhibitor have white to cream-white color. The resistance of yeast mutant strain to carotenoid synthesis inhibitors is a crucial factor for the selection of carotenoid overproducing mutant strains. The resistant mutants form yellow, pink or red colonies, while the nonresistant wild strain has a white to cream-white color [144, 69].

DPA is an inhibitor with sufficient carotenoid biosynthesis inhibition and minimal impact on biomass production. Moreover, there are some studies which successfully used DPA for inhibition of carotenogenesis in carotenogenic yeast [144, 69]. This study aimed to prepare overproducing mutant strains of *R. toruloides* using chemical, physical mutagenesis and evolutionary mutagenesis using hydrogen peroxide with subsequent UV-C mutagenesis. The selection of overproducing mutants was done using DPA.

6.4.1 Metabolite production under DPA inhibition and nitrogen limitation

The DPA inhibition lead in *Rhodotorula* sp. to decreased activity of phytoene desaturase [69]. But the general resistance to DPA is species specific and therefore prior usage, the experimentally

defined DPA concentration is needed [144, 69]. Also, the impact of the C/N ratio in comination with DPA is not reported. Firstly, the wild type of *R. toruloides* CCY 62-02-04 was exposed to different concentrations of DPA and two C/N ratios 16 and 80. Productions of carotenoids, fatty acids, and ergosterol were monitored after 96 hours of cultivation.

Impact of C/N ratio

Increased C/N ratio leads to enhanced accumulation of citrate, which then serves as a source of acetyl-CoA for fatty acid biosynthesis as well as for carotenoid biosynthesis. Therefore, amounts of fatty acids and carotenoids were increased taking in count production per medium (see Fig. 6.28). On the contrary, the accumulation of ergosterol was increased in C/N 16 ratio and decreased at C/N ratio 80 (see Fig. 6.28). Since ergosterol can not be metabolized by yeast cells there are mechanisms to maintain ergosterol homeostasis. Ergosterol homeostasis is maintained through ergosterol esterification with fatty acids, by down-regulation of sterol biosynthesis or by sterol acetylation with its subsequent secretion into production media [39, 47]. Therefore, there are numerous possibilities explaining decreased accumulation of ergosterol at a higher C/N ratio 80. The most probable are that acetyl-CoA is more triggered into carotenoids and fatty acids synthesis. The second explanation can be that ergosterol was released into the production media, or it was esterified by fatty acids and stored in lipid bodies.

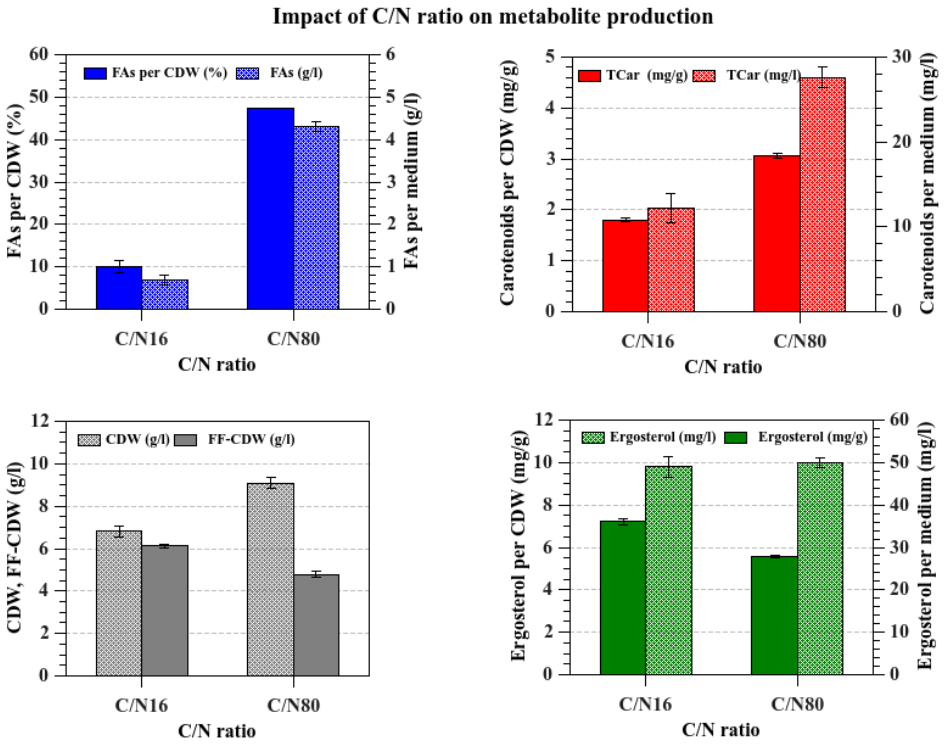

Fig. 6.28: Impact of C/N ratio on metabolite production in *R. toruloides*

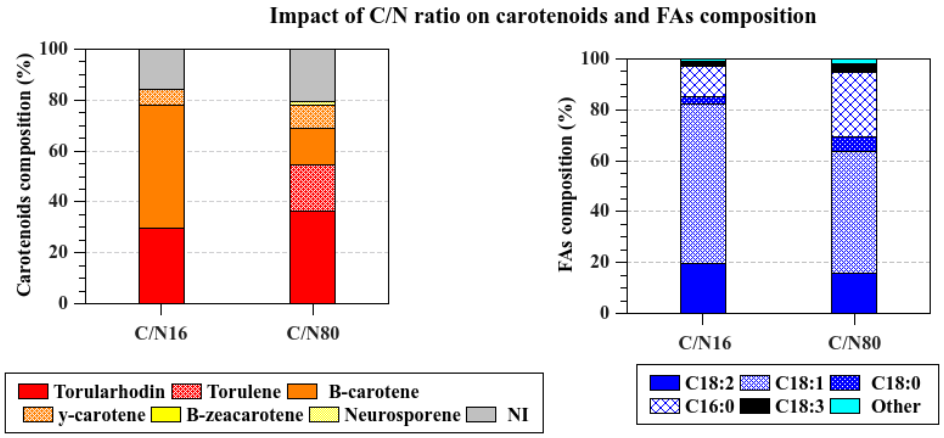

Fig. 6.29: Impact of C/N ratio to carotenoids and fatty acids composition in *R. toruloides*

Since fatty acids are intracellular components, they are part of the cell dry weight. To reflect cell proliferation, the fatty acids free biomass (FF-CDW) was calculated as a difference between CDW and fatty acids produced per medium (g/l). At high C/N ratio was significantly increased accumulation of fatty acids in comparison to low C/N ratio 16, it is well illustrated by the difference between the production of CDW and production of FF-CDW (see Fig. 6.28). In Fig. 6.29 is illustrated the impact of C/N ratio to carotenoid and fatty acids composition produced by *R. toruloides* in medium without addition of DPA (control). The majority of fatty acids produced by *R. toruloides* formed oleic acid at both C/N ratios. The C/N ratio 16 is favored even higher accumulation of oleic acid than in C/N 80. At C/N 80 increased production of saturated fatty acids as stearic and palmitic acids. Carotenoid composition was also influenced by C/N ratio. At C/N 16 formed majority of carotenoids β-carotene and torularhodin while at C/N 80 was as the main carotenoid produced torularhodin and in smaller amounts torulene and β-carotene with slightly increased production of γ-carotene in comparison to C/N 16.

Impact of DPA in combination with different C/N ratios

The impact of DPA on yeast cultures in liquid production media was visible by naked eye (see Fig. 6.30). In figures Fig. 6.31, and Fig. 6.32 are also visualized metabolite accumulation under impact of DPA, which correspond to the color change of yeast cultures. Addition of DPA into production media influenced metabolite production at both C/N ratios, but it had no lethal impact on cell proliferation. After the results of the study of Squina and Mercadante *R. toruloides* CCY 62-02-04 is probably more resistant to DPA than *R. mucilaginosa* (former *R. rubra*) [2] or *R. glutinis* species [69]. In the above mentioned study the concentrations of DPA above 10 μM caused severe inhibition of the cell growth of *R. mucilaginosa* and *R. glutinis*. Furthermore, in the study of Sanpietro and Kula was as well observed complete inhibition of growth of *P. rhodozyma* at 60 μM of DPA [145].

R. toruloides CCY 62-02-04 was able to grow without severe inhibition of growth even in DPA concentrations 100 and 150 μM. The most severe effects were noticed in carotenoid produc-

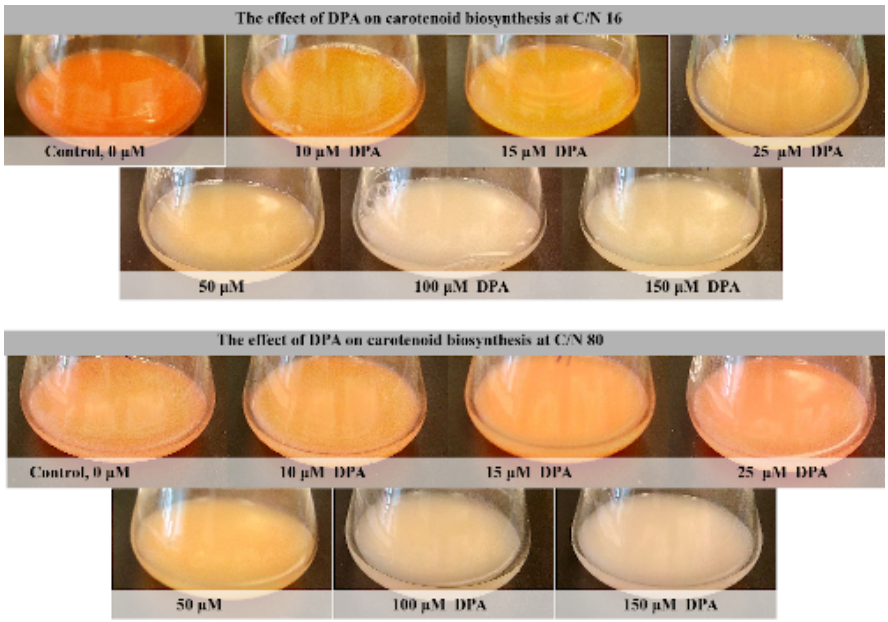

Fig. 6.30: The impact of C/N ration and concentration of DPA to coloration of yeast cultures in liquid production media

tion. With increasing concentration of DPA decreased the production of carotenoids. Surprisingly, the low C/N ratio 16 caused increased production of carotenoids in comparison to control, when concentrations of DPA 10 µM and 15 µM were used. The same effect observed also Squina and Mercadante in *R.mucilaginosa* at DPA concentrations 5 μM and Sanpietro and Kula, who observed this effect in *P.rhodozyma* with 10 μM of DPA [145, 69].

Concentrations 10 and 15 µM of DPA have a positive effect on carotenoid accumulation at C/N ratio 16 in *R. toruloides*. At DPA concentration 25µM and 50µM at C/N 16 greatly decreased production of carotenoids. Concentrations above 50 µM DPA caused almost complete inhibition of carotenoid accumulation.

Increased C/N ratio influenced the production of carotenoids. Even at concentration 25 µM of DPA was not carotenoid synthesis more inhibited in comparison to DPA concentrations 10 µM and 15 µM. The inhibitory effect was observed only in DPA 50 µM and in higher concentrations of DPA.

In DPA concentrations 10-50 µM at C/N 16 was slightly increased intracellular accumulation of FAs in comparison to control without addition of DPA (0). At concentrations over 50 µM, an increased accumulation of FAs per CDW was observed, and even if the CDW production slightly decreased, the FAs production per medium dramatically increased. At C/N 80 was accumulation of FAs per CDW slightly increased, but due to the decreased production of CDW decreased also the production of FAs per medium. Generally, production of FAs was triggered by a high C/N ratio resulting in production of more than 50 % of FAs per CDW (3-5 g/l of FAs per medium). Accumulation of ergosterol seemed to be connected to carotenoid inhibition. Generally with increasing DPA limitation decreased carotenoid production but increased accumulation of ergosterol at both C/N ratios. Exceptional were concentrations 10 and 15 µM at C/N 16. Under these con-

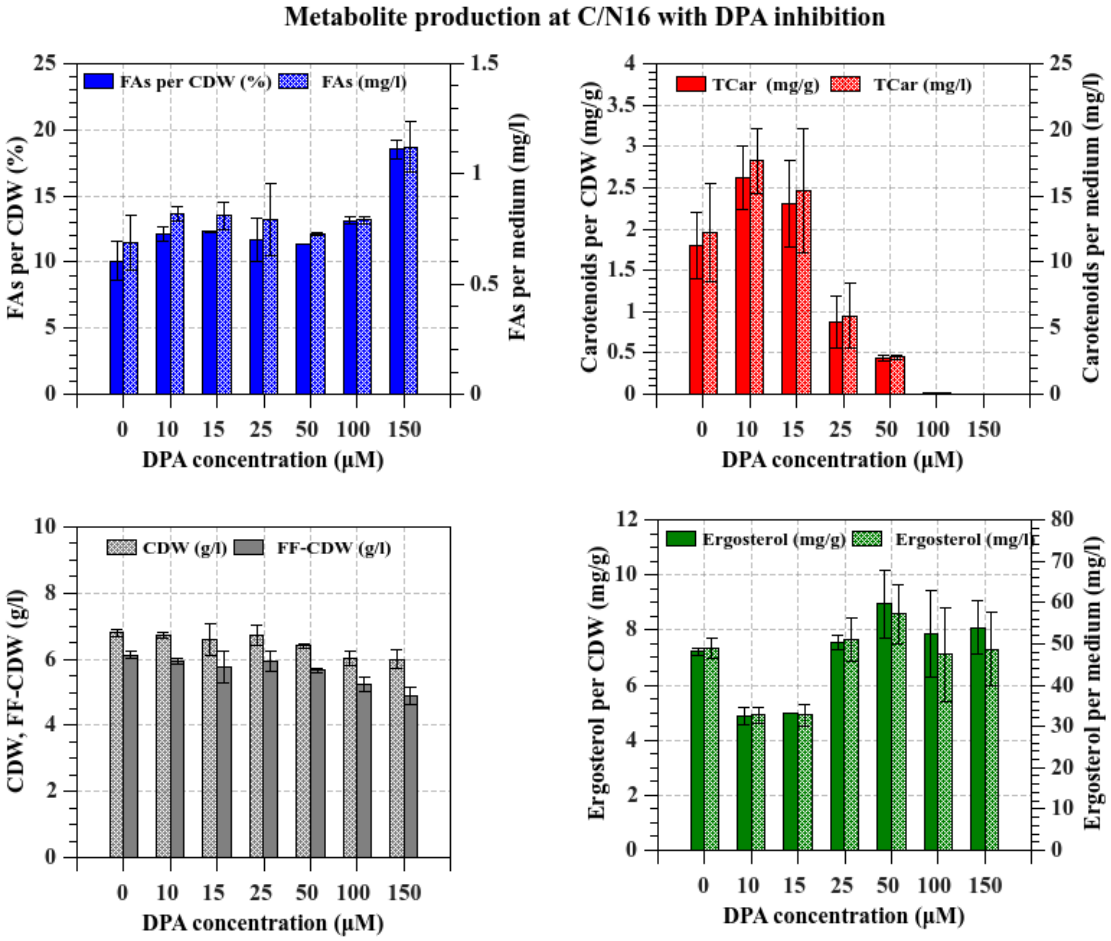

Fig. 6.31: Metabolite production by *R. toruloides* at C/N 16 under DPA inhibition

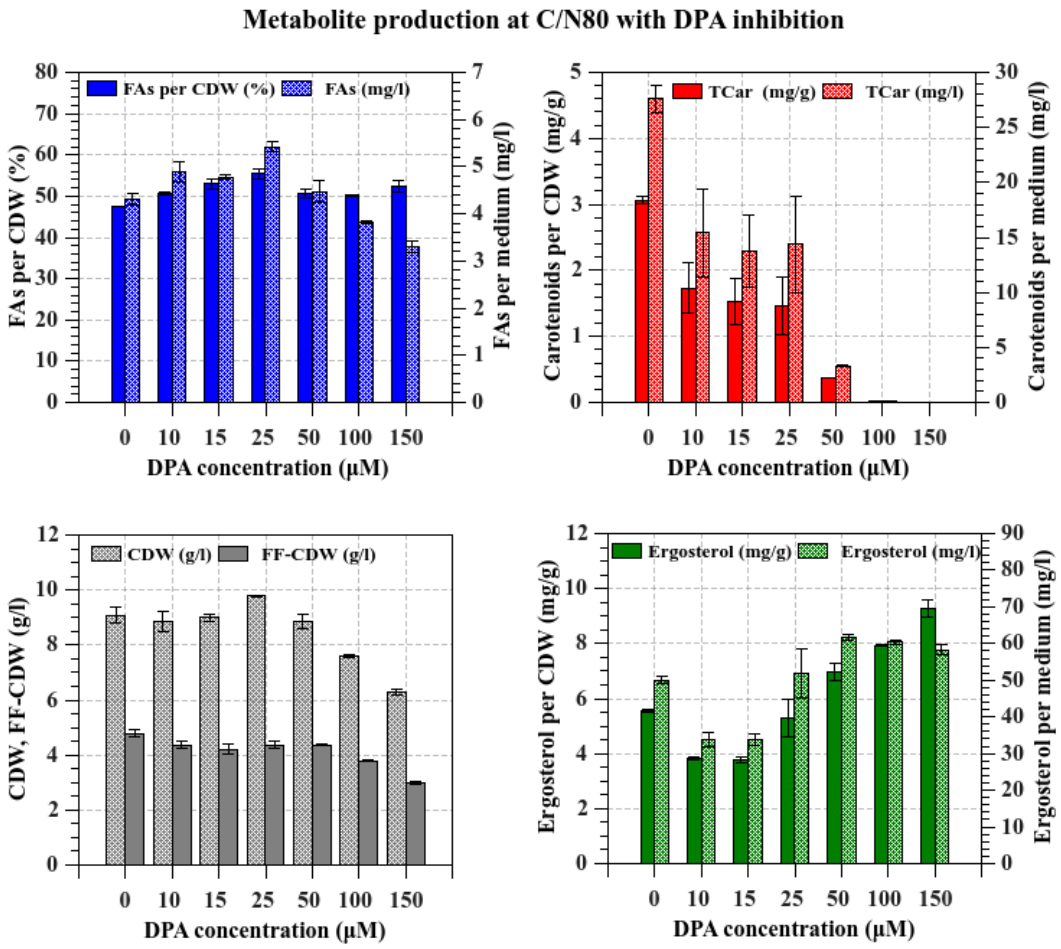

Fig. 6.32: Metabolite production by *R. toruloides* at C/N 80 under DPA inhibition

ditions were detected increased production of carotenoids in comparison to control. Furthermore, ergosterol content in CDW dramatically decreased. The same effect was observed also at C/N 80, but the carotenoid content against control was decreased. At C/N 16, the ergosterol content was increased at DPA concentration 50 μM. At C/N 80, there were the highest yield of ergosterol measured at DPA concentration 150 μM.

Carotenoids are important antioxidants incorporated also in the cell membranes where they protect the cells against the harmful impact of free radical species [15]. Some carotenoids can probably stabilize cell membrane fluidity and in their absence are needed increased amounts of ergosterol. The other explanation can also be that the downregulation of the carotenoid biosynthesis leads to upregulation of ergosterol biosynthesis.

Fatty acids and carotenoid composition

The composition of fatty acids and carotenoids is visualized in Fig. 6.33. The composition of FAs was not very influenced by addition of DPA into the production media and the major impact on FAs composition had C/N ratio with the same results as discussed in chapter above (Impact of C/N ratio). The carotenoids were identified using standards of torulene, torularhodin, β-carotene, lycopene, and using UV-VIS spectra obtained from HPLC-PDA analysis in comparison to study of Squina and Mercadante [69].

At C/N 16 was produced torularhodin in control and at 10 μM DPA. In concentrations above 10 μM was produced mainly β-carotene. Carotenoids produced at DPA concentration 50 μM, were formed predominantly by β-zeacarotene and neurosporene. At the DPA concentration 100 μM were detected only trace amounts of β-zeacarotene.

The increasing concentration of DPA inhibition in combination with nitrogen limitation (C/N 80) caused a gradual decrease in synthesis of torularhodin. At DPA concentrations 15 and 25 μM was recorded increase in torulene, γ-carotene and neurosporene synthesis. At the concentration 50 μM increased accumulation of γ-carotene and only at this concentration was accumulated β-zaecarotene as well.

On the contrary to C/N 16, at the C/N 80 and concentration 100 μM of DPA, was at the trace amounts recorded only neurosporene instead of β-zaecarotene. Interestingly, at DPA concentration 50 μM was at both C/N ratio was detected together with β-carotene, phytofluene as well (see supplementary materials, Fig. 9.1, and 9.1 and 9.12). Therefore, the enzyme phytoene desaturase *crtI* is probably the most inhibited enzyme under DPA inhibition. Surprisingly, lycopene was not detected at all. This effect can be caused by the immediate use of lycopene for the formation of γ-carotene or torulene, or there is a preference for formation of β-zeacarotene. The preference for formation of β-zeacarotene is more adequate according to present data, which report formation of β-zeacarotene and neurosporene at increased concentrations of DPA. These data are supported also by results obtained in the study of Squina and Mercadante [69]. In the presence of DPA the cyclisation reactions were favored to dehydrogenation at low C/N ratio.

At C/N 80 were cyclization, hydroxylation and oxidation reactions less inhibited. This can be caused by action and regulation of different enzymes which are probably up-regulated at higher

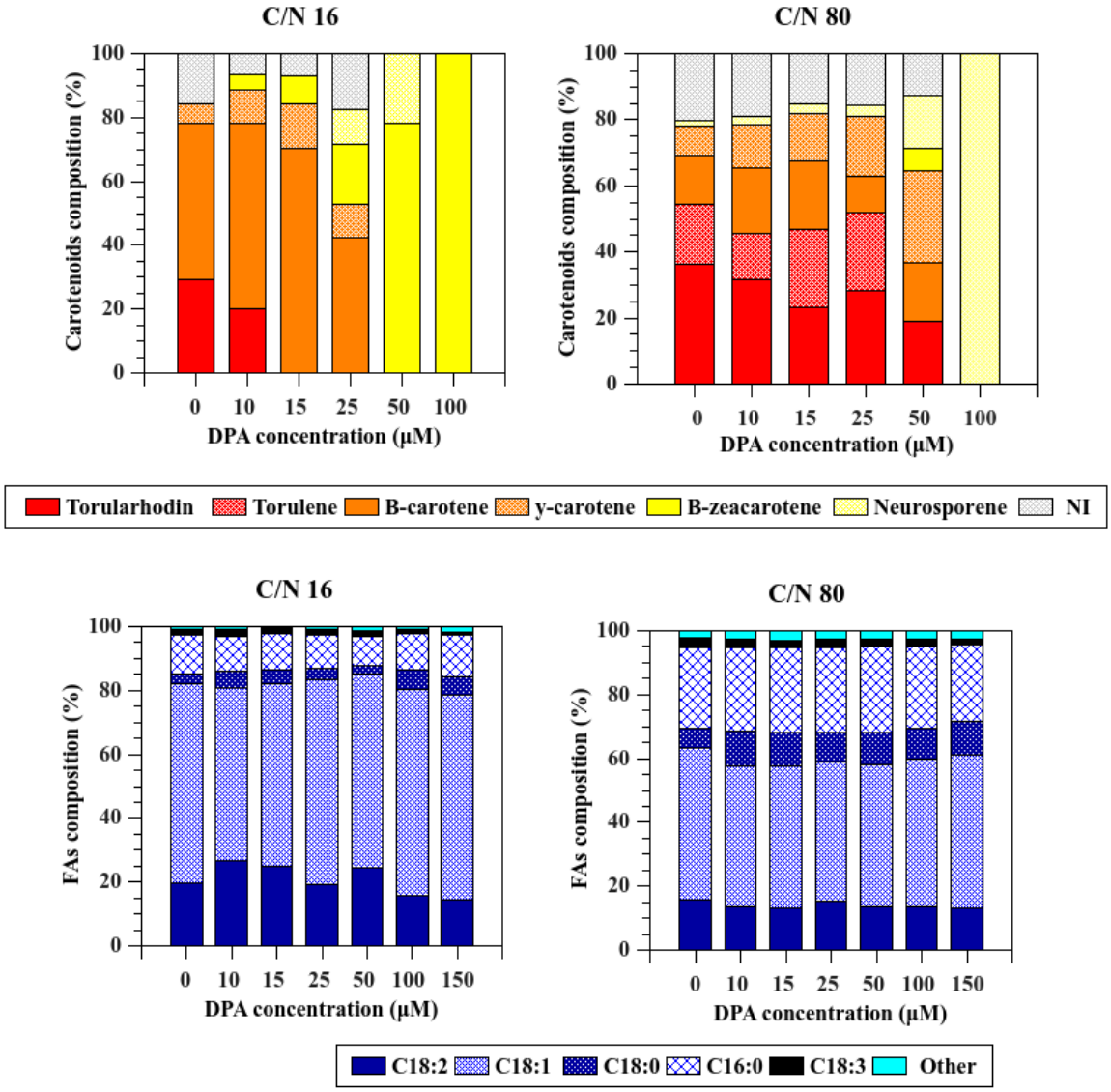

Fig. 6.33: Impact of nitrogen limitation and DPA inhibition to fatty acids and carotenoids composition

C/N ratios with connection to increased FAs production. In the omic study study of Zhu, focused on genome analysis of *R. toruloides* NP 11, were identified two genes that code carotenoid synthesis-related enzymes, phytoene synthase (*PSY1*) and phytoene dehydrogenase (*CRTI*) [55]. Phytoene synthase is reported as bifunctional enzyme which forms phytofluene as well as cyclizes lycopene into γ-carotene and catalyzes the formation of β-zeacarotene [15]. The torularhodin is reported to be a more efficient antioxidant than β-carotene, and therefore can be probably more efficient in protection of fatty acids against peroxidation [15].

The results obtained prove, that synthesis of carotenoids is regulated by availability of nitrogen. At the DPA concentration 150 M were not detected any carotenoids, which means that the carotenoid biosynthesis was stopped at the point of phytoen-phytofluene formation due to inhibition of phytoene desaturase. In the chromatograms at the supplementary materials can be seen relative amounts of phytoene (see Fig. 9.14, 9.15, and 9.16). Phytoene was identified after UV-VIS spectrum and after increased amounts in increased concentrations of DPA, which was reported in similar studies [69].

The phytoene is collorless carotenoid, and therefore it was detected at wavelenght 280 nm [21, 23]. Because of lack of the standard of phytoene, the amounts are just relative, but from the chromatograms is clear that the accumulation of phytoene increased with increased concentration of DPA. Also a non-identified (NI) sterol was detected and with increasing concentration of DPA used to treat yeasts cultures, dramatically increased the relative amount of this compound (see Supplementary materials Fig.(see Fig. 9.14, 9.15, and 9.16). After UV-VIS spectrum and relatively high polarity, it can be assumed that this compound is diphenylamine or geranylgeranyl diphosphate. Since there is no other study with the same observation, the identity of this metabolite remains unknown.

6.4.2 Mutagenesis and mutant selection

For the mutagenesis was specifically chosen strain *R. toruloides* CCY 62-02-04, because it is declared by the yeast collection to be haploid. The selection of a haploid yeast strain for random mutagenesis is essential, because it has only one set of chromosomes, where potentially mutated genes can not be compensated by the duplicate set of genes contained at the second set of chromosomes [146, 147]. Mutagenesis was done using ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and using UV-C irradiation. With increasing time of UV-irradiation decreased percentage of survivals (see Tab. 6.11). The greatest decrease in survivals was after 3 min of irradiation. After 3 and 4 min of irradiation survived only 0.9 % and 0.2 % of yeast cells.

The dosage of chemical mutagens was selected after [146]. EMS mutagen was used in two amounts 50 and 75 μl and after incubation of cell cultures with mutagens. Then the culture was transferred into sodium thiosulphate to inactivate harmful impact of MMS or EMS. The survival rate (see Tab. 6.11) was measured as colony forming units in comparison to control culture which was treated by the same approach except addition of mutagen. The survival rate was expressed in percents, where 100 % is represented by the control culture. The EMS as well as MMS mutagens had very harmful effect on viability of yeast cells. Addition of 50 μl or 75 μl to the yeast culture

survived only 1.4 and 1.7 %. The methods for mutagenesis were done after [146] and the results were also in accordance with this protocol.

Tab. 6.11: Impact of mutagens to yeast viability

Mutagen and its dose	**Survivals %**
UVC 1 min	74
UV 2 min	22
UV 3 min	0.9
UV 4 min	0.2
EMS 50 µl	1.4
EMS 75 µl	0.7
MMS 25µl	0
MMS 50µl	0
MMS 75µl	0.012

The MMS mutagen had a more drastic effect on yeast viability than EMS, the dosages of 25 µl and 50 µl of MMS killed all yeasts cells. Surprisingly, the highest doses of MMS had survived 0.012 % of the yeast cells. This effect is probably connected with induction of defensive mechanisms in the yeasts cells. The lower doses do not induce these mechanisms and then the cells are killed by mutagen action. Mutant yeast cells were selected from yeast cultures treated with 75 µl of EMS and 75 µl of MMS. The UV-C mutants were selected from yeasts cultures treated with 2 and 3 min of UV-C irradiation.

Mutant selection

The mutant strains were chosen after their ability to produce carotenoids under DPA inhibition. For the selection were mutants cultivated on agar plates with cultivation medium C/N 16 with concentrations of DPA 0µM (control), 10, 15, 25, 50, and 100 µM (See Fig. 6.34 as an example). After this selection, there were promising candidates selected for rapid cultivation screening and their production of carotenoids was evaluated using optimized UV-VIS multicomponent analysis against the control wild type strain. The results of this selection are visualized in Fig. 6.35. The successful mutants are highlighted in green color.

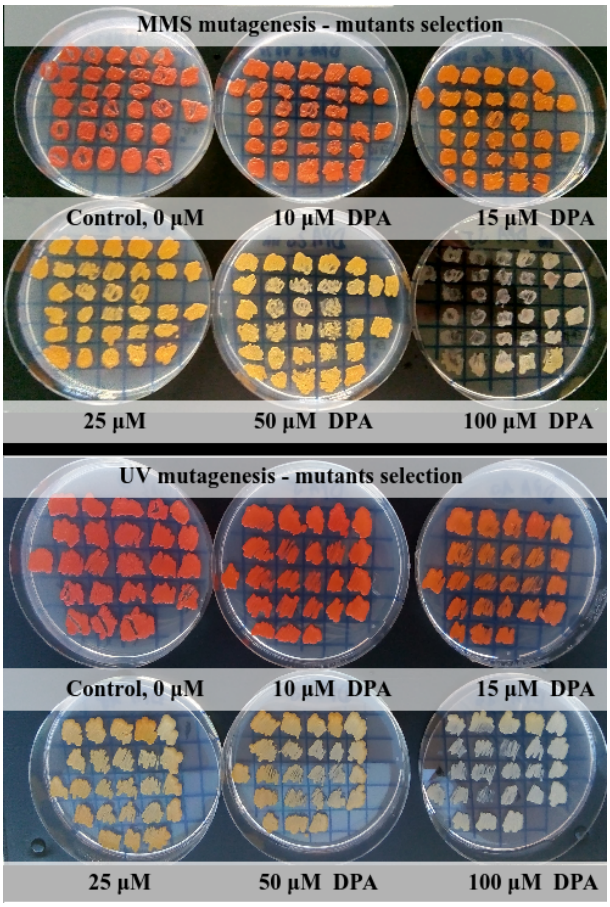

Fig. 6.34: The impact of DPA to mutant strain culture coloration (carotenoid production)

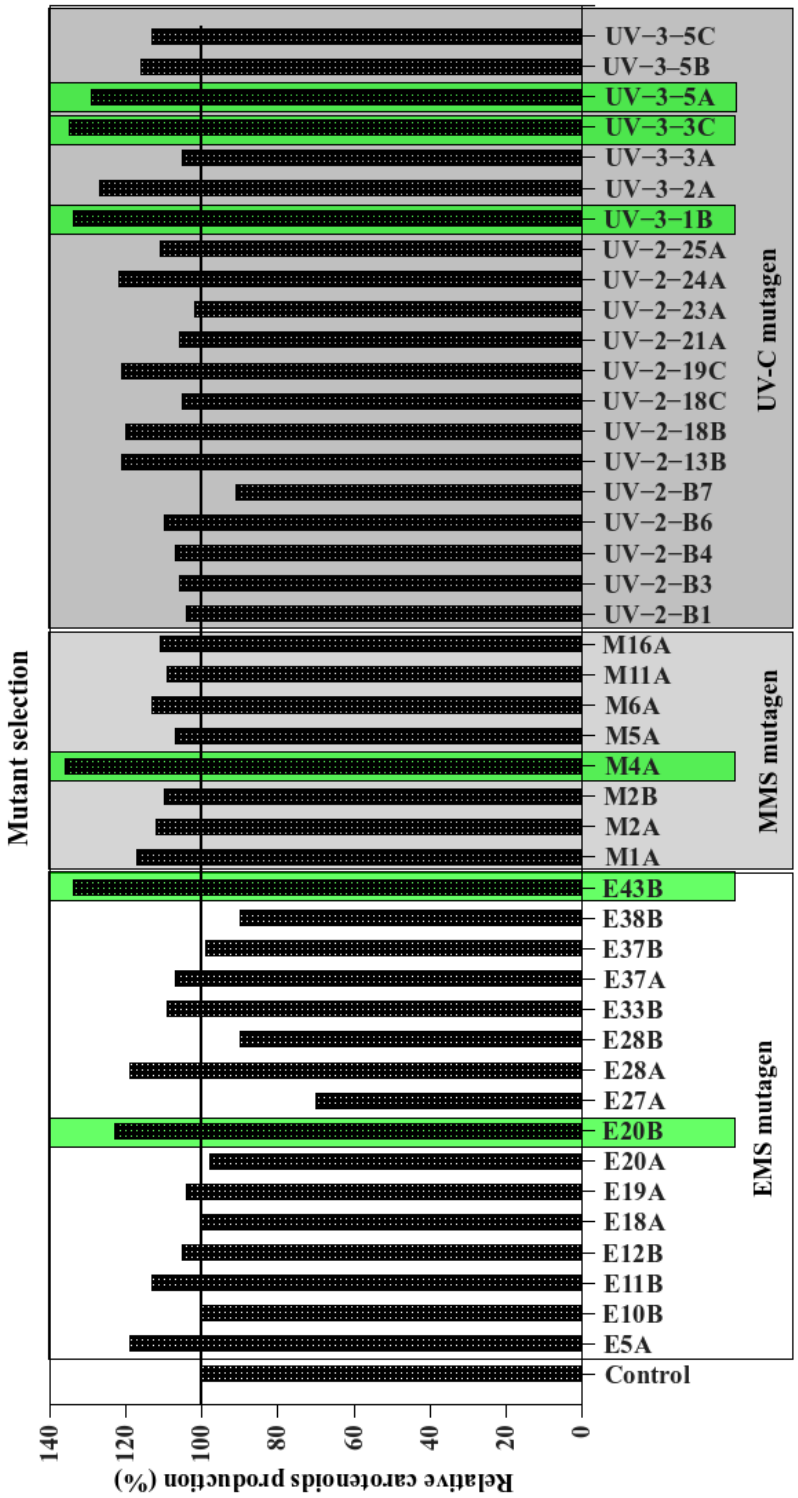

Fig. 6.35: Relative carotenoids productions of promising mutants strains in comparison to WT. The data were calculated as an average value of the two independent experiments. Selected over-producing mutants strains are highlighted in green color

Adaptation mutagenesis using hydrogen peroxide

The overproducing mutants were cultivated in the presence of hydrogen peroxide. The concentration of hydrogen peroxide was daily increased to increase carotenoid production as a protection against oxidative stress. Unfortunately, the only mutant strain which survived this treatment was strain UV-3-3C (See Fig. 6.36).

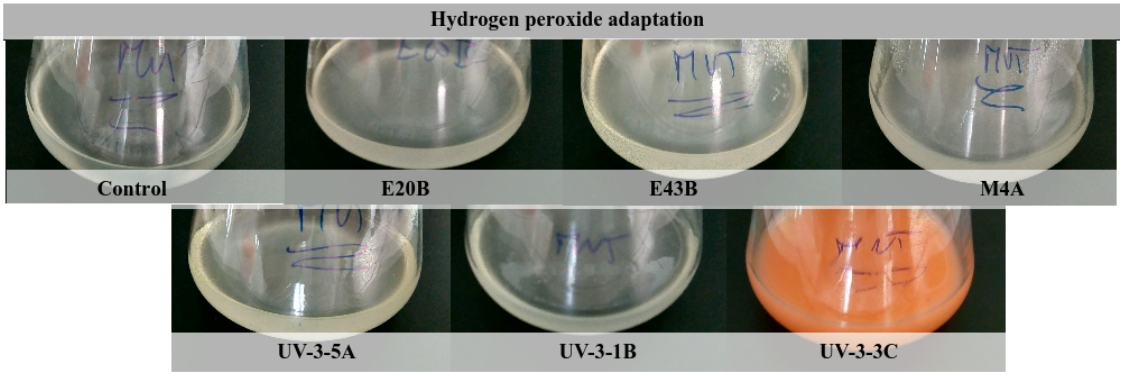

Fig. 6.36: The adaptation of selected mutants strains to hydrogen peroxide in liquid media.

The strain UV-3-3C was subsequently exposed to random mutagenesis using UV-C irradiation. The survival colonies were selected for carotenoid production under DPA limitation. Selected mutants were cultivated using rapid cultivation screening, and their production of carotenoids was evaluated using optimized UV-VIS multicomponent analysis against control - wild type strain. The best producing mutant strains produced almost 80 % more carotenoids then the control strain (see Fig. 6.37). These strains have extraordinary capability to survive under oxidative stress. The best producing strains, UV-H2O2-12, and UV-H2O2-13 were selected for next cultivation experiments. These strains are highlighted with green color in Fig. 6.37.

6.4.3 Production properties of mutant strains

The cultivation conditions leading to increased carotenoid production can differ among mutant strains. To fully describe the production properties of each selected mutant strain, all strains were cultivated in conditions with low C/N (16) and increased C/N ratio (80), and in conditions under DPA inhibition at both C/N ratios. For the DPA inhibition was chosen the concentration 50 μM, because it caused a rapid decrease in carotenoid production in comparison to control conditions, but not complete inhibition of carotenoid synthesis.

Biomass production and fatty acids accumulation

The mutagenesis influences the production of carotenoids, but frequently negatively influences the biomass production [66]. Biomass production was expressed as CDW (g/l) and its production was especially at C/N 80 influenced by intracellular accumulation of fatty acids. The majority of mutants reached comparable CDW production as wild type (WT) with no negative effect on

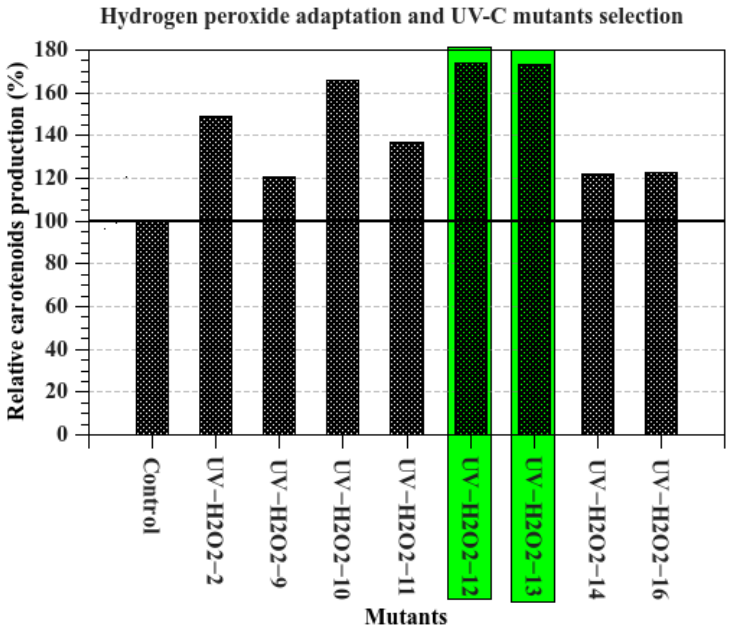

Fig. 6.37: Relative carotenoids productions of mutant strains adapted to hydrogen peroxide with subsequent UV-C mutagenesis. The data were calculated as an average value of the two independent experiments. The selected overproducing mutants are highlighted in green color.

cell proliferation, except mutant strains prepared by adaptation mutagenesis with subsequent UV-C mutagenesis (H_2O_2-UV^2) UV-H2O2-12 and UV-H2O2-13. These strains had decreased CDW production at C/N ratio 80 without and with addition of 50 µM DPA in comparison to WT (see Fig. 6.38).

In Fig. 6.39 can be seen the fatty acid production of WT and mutant strains. Increased fatty acid production at C/N 16 without addition of DPA had mutant strains UV-3-5A and UV-H2O2-13. However, only strain UV-3-5A accumulated increased amounts of FAs within the biomass (16.8 %) than WT at C/N 16 and under DPA inhibition (15.3 %). Other mutant strains produced at C/N 16 and with DPA comparable amounts of FAs to WT.

At C/N 80 without DPA was FAs production only slightly decreased in mutant strains UV-3-5A and UV-3-1B. Under C/N 80 and DPA inhibition, was FAs production only slightly decreased in mutant strains UV-3-5A, UV-3-1B and E20B in comparison to WT. The other mutant strains produced less FAs than WT at C/N 80 without and with DPA inhibition. The mutant strains UV-H2O2-12 and UV-H2O2-13 produced the lowest amounts of FAs in comparison to WT. Interestingly the low FAs production in g/l was caused in these strains by lower biomass production, because the content of FAs within the biomass was comparable to WT.

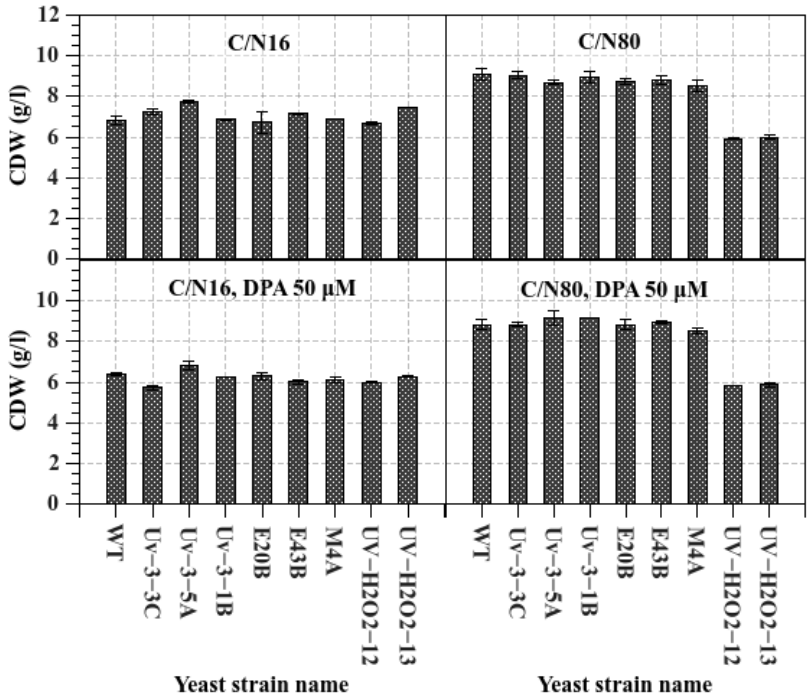

Fig. 6.38: Biomass production expressed as CDW under C/N 16, C/N 80 and with addition of DPA at both C/N ratios

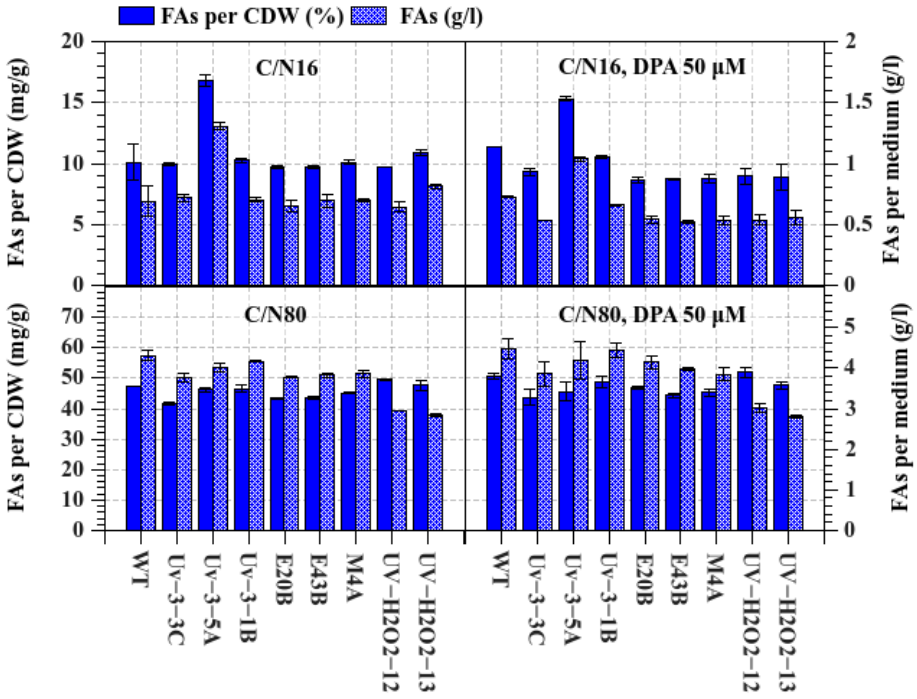

Fig. 6.39: The fatty acids production of WT and mutant strains at C/N 16, C/N 80 and with addition of DPA to both C/N ratios

Ergosterol accumulation and carotenoid production

Ergosterol accumulation serves mainly for cell wall stabilization, when the yeast cells are exposed to osmotic pressure. Since ergosterol can not be metabolized by yeast cells, its homeostasis is maintained through fatty acid esterification, by down regulation of ergosterol synthesis, or it is simply released into the production media [47, 39]. The ergosterol accumulation is mainly influenced by cultivation conditions. In this study, was the ergosterol accumulation influenced by C/N ratio as well as by DPA inhibition of carotenoid synthesis as was discussed on the beginning of this chapter.

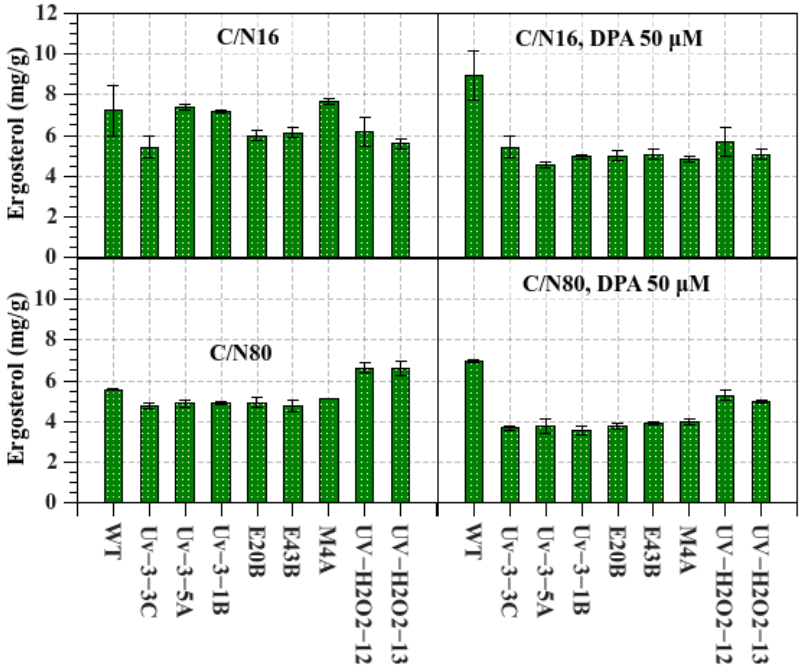

Fig. 6.40: Ergosterol accumulation of WT and mutant strains at C/N 16, C/N 80 and with addition of DPA at both C/N ratios

In Fig. 6.40 is visualized the accumulation of ergosterol by WT as well as by mutant strains. The accumulation of ergosterol within the majority of mutant strains was decreased in comparison to WT. Only three mutant strains UV-3-5A, UV-3-1B, and M4A, performed almost the same accumulation of ergosterol as WT. At C/N 16 and C/N 80 under inhibition by DPA was the accumulation of ergosterol significantly decreased in comparison to WT. Only strains UV-H2O2-12 and UV-H2O2-13 had slightly increased ergosterol accumulation in comparison the the other mutant strains at C/N 80 under DPA inhibition. At C/N 80 was ergosterol accumulation in mutant strains slightly decreased in comparison to WT, except mutant strains UV-H2O2-12 and UV-H2O2-13 which accumulated even higher amounts of ergosterol than WT.

To better compare the carotenoid productions of mutant strains with WT the relative carotenoid productions were calculated. The relative carotenoid productions were calculated as a percents of production of the WT carotenoid production, which represented 100 %. The best carotenoid

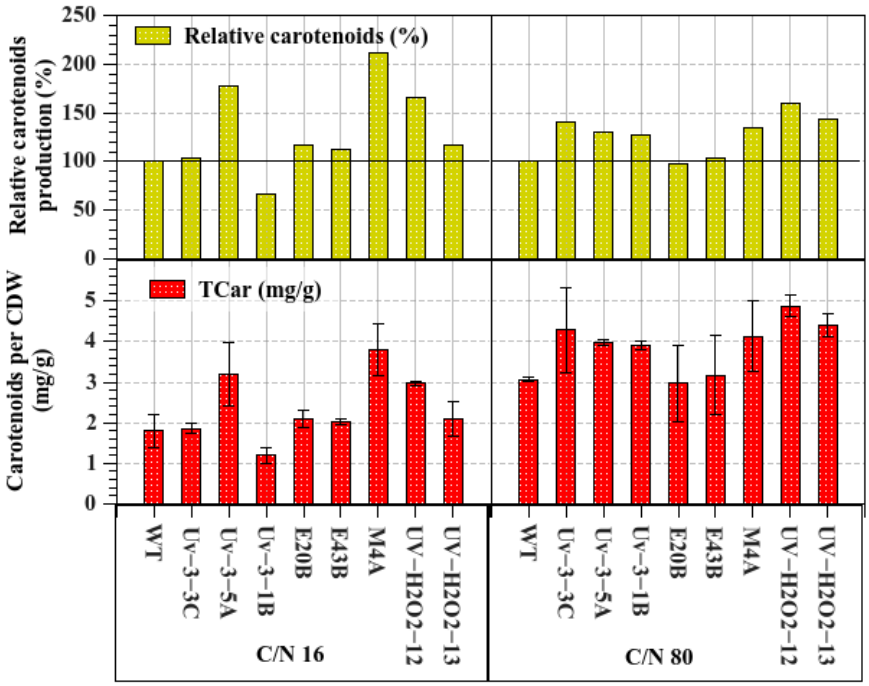

Fig. 6.41: Relative carotenoids production and carotenoid productions at C/N 16 and C/N 80.

producers in comparison to WT as a control were strains M4A, UV-3-5A, UV-H2O2-12, and UV-H2O2-13. These mutant strains produced dramatically increased amounts at C/N 16 as well as at C/N 80 (see Fig. 6.41). Furthermore, strains UV-3-5A, UV-H2O2-12, and UV-H2O2-13 had high resistance to DPA at C/N 16 (see Fig. 6.42). At C/N 80 all mutant strains had significantly higher resistance to DPA resulting in higher carotenoid productions (see Fig. 6.42).

The composition of carotenoids produced by mutant strains exposed to C/N16, C/N80, and to the effect of DPA is visualized in Fig. 6.43. Carotenoid composition was altered in some mutant trains at C/N 16. The mutants strains UV-3-3C, E20B, E43B, and M4A produced also torulene which was not found at these condition in control WT. Mutant strains prepared by chemical mutagenesis E20B, E43B and M4A had almost comparable percentual carotenoid composition as WT. On the other hand, the majority of UV-C mutants produced as the main carotenoid β-carotene, smaller amount of torularhodin and other unidentified carotenoids. The mutants strains UV-3-5A, UV-H2O2-12, and UV-H2O2-13 produced significantly more unknown catotenoids than other mutant strains (see chromatograms at the Fig. 9.3- 9.6 at the Supplementary materials). These strains also proved to have high resistance against inhibition of carotenoid synthesis by DPA in comparison to WT and to other mutant strains. At C/N 80 was carotenoid composition almost unchanged in comparison to the WT, except for strains UV-H2O2-12, and UV-H2O2-13, which produced even more unidentified carotenoids than other strains.

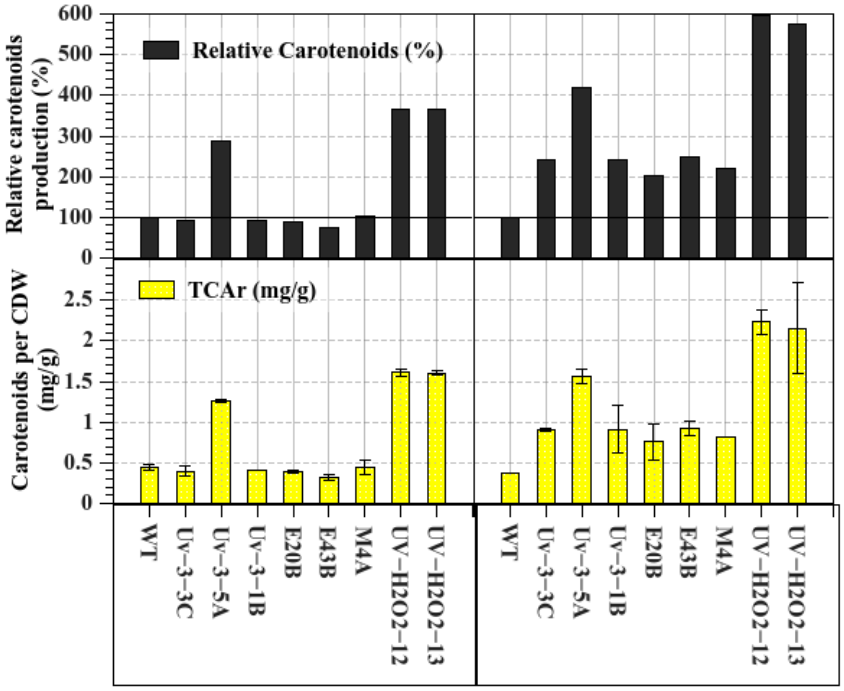

Fig. 6.42: Relative carotenoids production and carotenoid productions at C/N 16 and C/N 80 with addition of DPA.

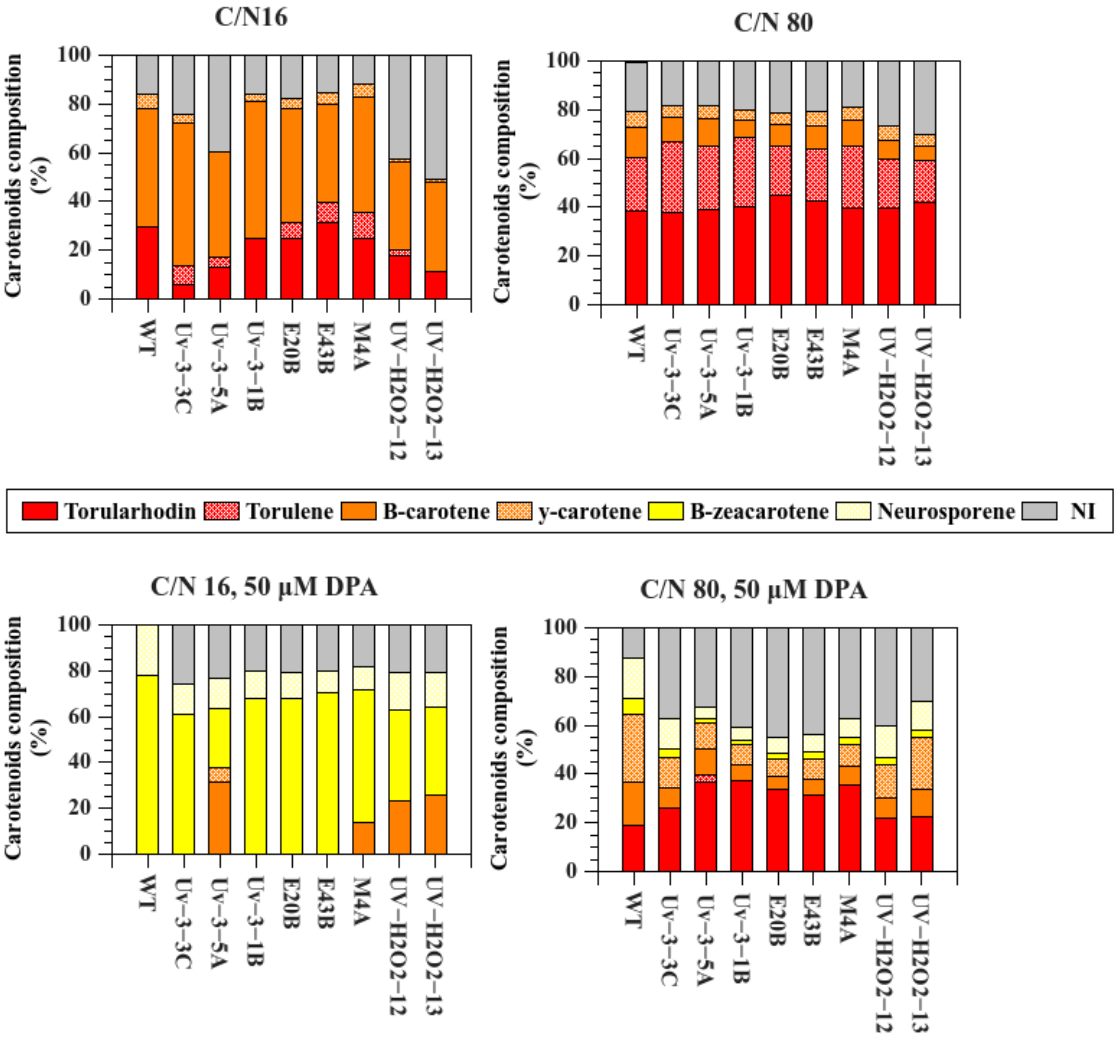

Fig. 6.43: Carotenoid composition produced by WT and mutant strains at C/N 16, C/N 80 and with addition of DPA to both C/N ratios.

When mutants strains were exposed to the carotenoid synthesis inhibitor DPA at C/N 16, the main produced carotenoids were β-zeacarotene and neurosporene. Interestingly, all mutant strains produced unidentified carotenoids (NI-carotenoids), probably as a response to DPA inhibition. Furthermore, the best carotenoid producing mutant strains were capable to produce, besides β-zeacarotene, neurosporene, and NI-carotenoids also β-carotene. Exceptionally, the strain UV-3-5A managed to produce γ-carotene. At C/N 80 under DPA inhibition composition of the produced carotenoids was altered in comparison to WT. The mutant strains produced mainly torularhodin and in smaller amounts β-carotene, γ-carotene, β-zeacarotene, and neurosporene in comparison to WT. At these conditions, mutants produced mainly NI-carotenoids as a response to DPA inhibition.

In this study were prepared carotenoid overproducing mutant strains. Mutants strains with the best carotenoid productions at both C/N ratios were M4A, UV-3-5A, and UV-H2O2-12. At C/N 16, strain M4A produced 3.8 mg/g of CDW, strain UV-3-5A 3.2 mg/g of CDW, and strain UV-H2O2-12 2.9 mg/g of CDW respectively. At C/N 80, the production of carotenoids even increased. The following yields of carotenoids per CWD were produced: 4.1 mg/g (strain M4A), 3.9 mg/g (UV-3-5A) and 4.9 mg/g (strain UV-H2O2-12). Very good productions proved to have as well mutant strains UV-3-3C (4.3 mg/g per CDW) and UV-H2O2-13 (4.4 mg/g per CDW). But in these strains was performed overproduction of carotenoids only at C/N 80.

Tab. 6.12: Carotenoid productions of mutant strains per medium.

Yeast strain	**Carotenoid production (mg/l)**	
	C/N 16	**C/N 80**
WT	12.225	27.609
UV-3-3C	13.424	38.627
UV-3-5A	24.783	34.487
UV-3-1B	8.159	34.953
M4A	26.321	35.467
E43B	14.409	27.851
E20B	14.098	25.823
UV-H2O2-12	19.797	28.827
UV-H2O2-13	15.631	26.324

To evaluate the carotenoid production properties of the obtained mutant strains, the sorting proposed in the study of El-Banna et al. was used [122]. The ability to produce carotenoids was categorized as follows: low amount < 0.100 mg/g of CDW, the average for 0.101-0.500 mg/g of CDW and high amount > 0.500 mg/g of CDW. Accordingly, mutant yeast strains and even WT in this study belong to the category with high carotenoid production, because the carotenoid productions were higher than 0.5 mg/g of CDW. Furthermore, the best mutant strain produced more than two times higher amounts of carotenoids than in original strain at C/N 16. When are carotenoid productions of prepared mutant strains compared to other studies dealing with random mutagenesis in carotenogenic yeasts, the results of this study are extraordinary. In the studies of

Cong et al. [148], Zhang [66] and Chumpolkulwong [144] is documented successful preparation of carotenoid overproducing yeast strains. However, only in some studies were achieved similarly high yields of produced carotenoids as in this study. Productions of carotenoids per medium are in Tab. 6.12. Very good productions of carotenoids in the mutant strain of *R. glutinis* were achieved in the study of Bhosale and Gadre. In this study, their mutant strain produced 14 mg/l of carotenoids at the basic medium containing 40 g/l of reducing sugars. After optimization of the cultivation conditions was achieved 185 mg/l of medium [149].

6.5 Variability of metabolites produced by some yeasts within Sporidiobolales and Cystofilobasidiales

The coproduction of ergosterol, fatty acids and carotenoids among carotenogenic yeasts is not frequently compared. In this study is present a comparison of production properties of relatively well-studied species, for which genetic manipulation tools are available, *Rhodotorula toruloides* and *Phaffia rhodozyma* to closely related but lesser-known carotenogenic yeasts. As the representatives of lesser known Cystofilobasidiales we have chosen strains *Cystofilobasidium macerans* and *C. infirmominiatum*. Lesser known Sporidiobolales were represented by *Sporobolomyces metarosues*, *S. salmonicolor*, *S. pararoseus*, *R. kratochvilovae* and *R. mucilaginosa*.

Aforementioned lesser known carotenogenic yeast strains were chosen due to their high fatty acid productions proved in the study of Byrtusová [150]. Based on the results, we decided to study simultaneous production of fatty acids, carotenoids and ergosterol in Sporidiobolales and Cystofilobasidiales in the greater detail. This study provides insights into variability of metabolite quality and quantity of lesser-known carotenogenic yeasts in comparison to relatively well studied species. Furthermore, this study reports to our best knowledge for the first time, production of lycopene by the strain *R. mucilaginosa* CCY 19-04-06 at great yields.

6.5.1 Biomass production and fatty acids accumulation

Fatty acids (FAs) are basic molecules for biosynthesis of membrane lipids as well as neutral lipids. They are incorporated into phospholipids, sphingolipids, triacylglycerols (TAGs) and steryl esters which are accumulated intracellularly in so-called lipid droplets (LDs) [39, 47]. The lipid droplets are generally seen as storage compartments composed mainly from nonpolar lipids, including triacylglycerols, steryl esters and ether lipids. The LD is formed by nonpolar lipids where triacylglycerols (TAG) form a core surrounded by a steryl ester shell covered by phospholipid monolayer and associated proteins [151]. Since the accumulated fatty acids form part of the biomass, the beginning of the stationary phase and the point of fatty acid accumulation can be less visible. Therefore, the production of biomass was interpreted as cell dry weight (CDW), and as fatty acid-free cell dry weight (FF-CDW). Fig.6.44 shows the plots of CDW and FF-CDW production during the cultivation time. The production of FAs during cultivation time can be seen in the Tab. 6.13.

The beginning of stationary phase was evaluated base on FF-CDW data (ANOVA, Tukey, $P < 0.05$). Stationary phase was not observed in some strains and their FF-CDW continuously increased. The stationary phase was observed after 72 hours of cultivation in strains *C. infirmominiatum* CCY 17-18-04 (CI), *R. mucilaginosa* CCY 20-09-07 (Rma) and CCY 20-07-31 (Rmb), *R.toruloides* CCY 62-02-04 (RT), *R. kratochvilae* CCY 20-02-26 (RK) and *S. pararoseus* CCY 19-09-06 (SP). Exceptional were strains *C. macerans* CCY 10-01-02 (CM) and *S. metaroseus* CCY 19-6-20 (SM) which entered stationary phase after 48 hours of cultivation. Accumulation of FAs generally started after 48 hours of cultivation and, depending on a strain, continued till the end of the cultivation. The most dramatic accumulation of FAs, as a difference between CDW and FF-CDW values, was observed in strains *C. macerans* CCY 10-01-02 (CM) and *S. metaroseus*

CCY 19-6-20 (SM). Fig. 6.45 shows productions of FAs at 96 hours of cultivation. *R. mucilaginosa* strains had the lowest FA production. The FAs productions of *R. mucilaginosa* strains in this study were comparable to FAs productions of *R. glutinis* in the study of Tkáčová and to the productions of *R. mucilaginosa* in the study of Byrtusová, considering amount of glucose as a substrate [128, 150]. The highest yields of FAs were accumulated by strains *S. metaroseus* CCY 19-6-20 (SM) and *C. macerans* CCY 10-01-02 (CM).

These yeast species accumulated almost double the amount of FAs, forming 4.9 g/l (42 % of CDW) and 4 g/l (36 % of CDW) per medium in comparison to other strains. Species *S. metaroseus* CCY 19-6-20 (SM) and *C. macerans* CCY 10-01-02 (CM) are not frequently cited as strains with exceptional FAs production. This is mainly due to the fact that there is a limited number of studies dealing with the production of FAs by *Cystofilobasidium* and *Sporobolomyces* yeast species. Production of these strains is comparable to production obtained by *R. toruloides* and *R. glutinis* in the study of Lian and even better when compared to FAs production of *R. babjevae* Y-SL7 in the study of Guerfali et al., using glucose substrate [152, 153].

Furthermore, it should be emphasized that strains *S. metaroseus* CCY 19-6-20 (SM) and *C. macerans* CCY 10-01-02 (CM) were able to accumulate high amounts of FAs without the use of initial nitrogen limitation (used C/N 16), which is frequently reported to increase FAs accumulation in oleaginous yeasts. Generally, when the amount of FAs forms more than 20 % of CDW the organism is considered to be oleaginous [17, 103, 153]. The parameter of oleaginous yeast was met also by the following yeast strains with an excellent FAs production ranging from 2-2.6 g/l with content of FAs in CDW 20 – 29 %: *P. rhodozyma* CCY 77-01-01 (PR), *C. infirmominiatum* CCY 17-18-04 (CI), *R. kratochvilae* CCY 20-02-26 (RK), and *S. pararoseus* CCY 19-09-06 (SP). Yeast strains *C. infirmominiatum* CCY 17-18-04 (CI), *R. kratochvilae* CCY 20-02-26 (RK), *S. pararoseus* CCY 19-09-06 (SP), and especially strains *S.metarosues* CCY 19-6-20 (SM) and *C. macerans* CCY 10-01-02 (CM), are promising non-conventional lesser-known producers of fatty acids.

Fatty acid production can be influenced by various factors including impact of temperature, aeration, different carbon source, nitrogen source and mainly by ratio of carbon to nitrogen (C/N ratio). The optimization of cultivation conditions can lead to even better and economically less demanding production of FAs [103]. Tkáčová et al. studied FAs production in *R. glutinis* CCY 20-2-26 and reached the high FAs production at C/N 70 forming 47.6 % of CDW (8.9 g/l, glucose concentration 70 g/l). However, higher ratio C/N 100 caused growth inhibition due to increased osmotic pressure, causing prolonged lag phase and low production of FAs [128]. Byrtusová et al. reported an increase in FAs accumulation in CDW reaching to more than 45 % of CDW at C/N 100 in *C. infirmominiatum*, *R. toruloides* and *S. macerans* [150]. Also in both of these studies were saturated FAs accumulated in larger relative quantities. Carbon concentration is the key factor in FAs production because, as too high concentrations can lead to decreased FAs and biomass production per carbon, substrate consequently increasing the prize of the whole process. Therefore its concentration has to be optimized. Very economically suitable solution is combination of waste substrates such as glycerol, xylose and acetic acid in combination with nitrogen limitation, as shown in the study of Lopes et al.. In their study *R. toruloides* CCT 0783 proved to have very

good lipid production rates when grown on glycerol, xylose or acetic acid between C/N ratios 60 and 100 [154]. The beneficial uses of nitrogen limitation and different carbon sources, leading to increased FAs accumulation depend mainly on the properties of a particular yeasts strain. Strains used in this study are promising candidates with high FAs production even in the lower C/N ratio.

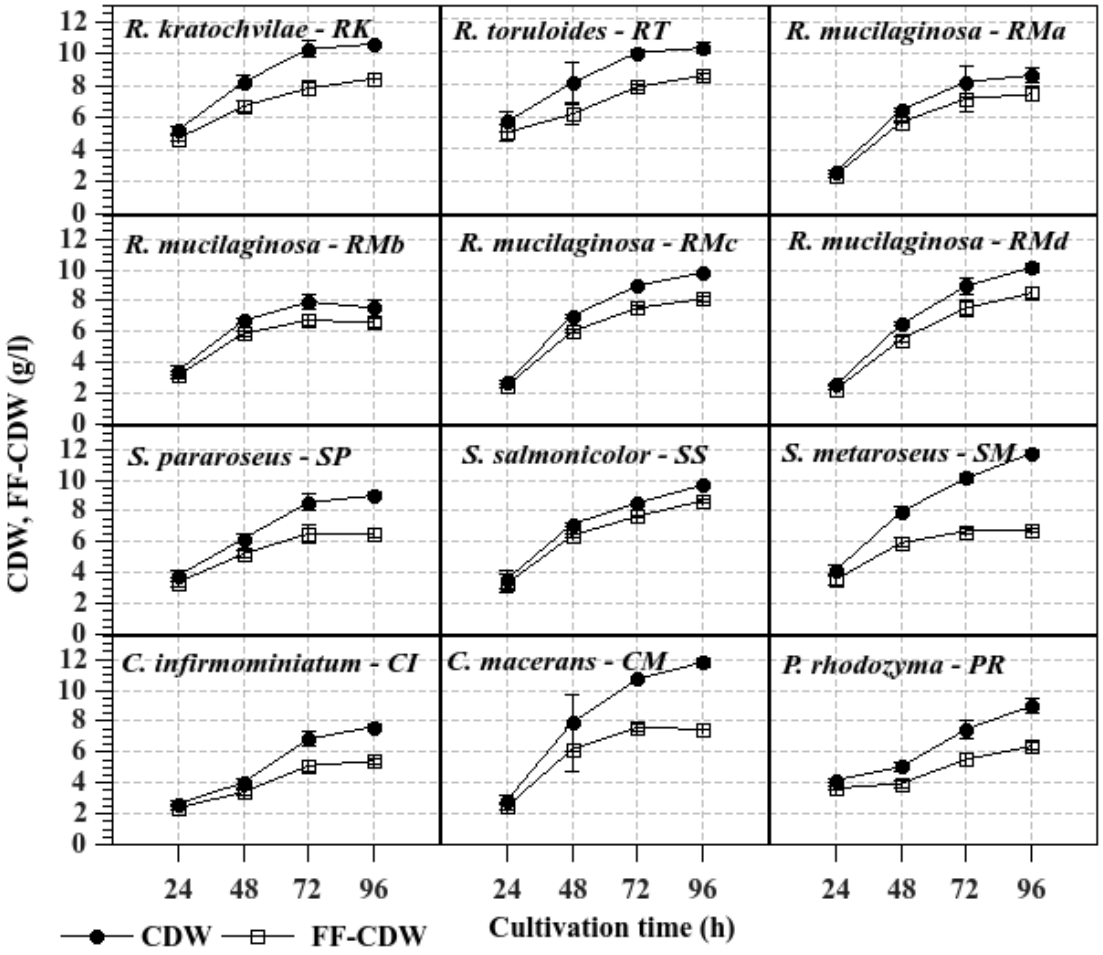

Fig. 6.44: Biomass production in time expressed as dry cell weight (CDW) and as fatty acid-free dry cell weight (FF-CDW)

Tab. 6.13: Productions of FAs during cultivation time

Yeast strain	Time (h)	FAs per CDW (%)	FAs per Medium (g/l)	FAs per CDW (mg/g)
RK	24	9.887± 0.494	0.519 ± 0.045	98.874 ± 4.936
R. kratochvilae	48	18.199 ± 0.784	1.498 ± 0.093	181.991 ± 7.835
CCY 20-2-26	72	23.706 ± 1.016	2.440 ± 0.113	237.057 ± 10.165
	96	20.395 ± 1.034	2.166 ± 0.134	203.947 ± 10.336
RT	24	11.591 ± 2.942	0.663 ± 0.215	115.909 ± 29.417
R. toruloides	48	23.449 ± 4.742	1.922 ± 0.681	234.491 ± 47.425
CCY 62-02-04	72	21.299 ± 0.543	2.115 ± 0.024	212.988 ± 5.425
	96	16.509 ± 1.142	1.686 ± 0.171	165.089 ± 11.419
RMa	24	8.083 ± 0.411	0.213 ± 0.018	80.826 ± 4.107
R.mucilaginosa	48	11.593 ± 1.312	0.753 ± 0.097	115.932 ± 13.120
CCY 20-7-28	72	12.797 ± 0.640	1.055 ± 0.103	127.966 ± 6.402
	96	13.652 ± 0.875	1.179 ± 0.022	136.518 ± 8.746
RMb	24	9.459 ± 0.262	0.330 ± 0.019	94.589 ± 2.619
R. mucilaginosa	48	12.291 ± 0.152	0.825 ± 0.026	122.912 ± 1.519
CCY 20-9-7	72	14.793 ± 0.607	1.173 ± 0.101	147.926 ± 6.069
	96	12.635 ± 2.308	0.961 ± 0.236	126.351 ± 23.083
RMc	24	8.072 ± 0.525	0.220 ± 0.015	80.718 ± 5.254
R. mucilaginosa	48	14.365 ± 0.363	1.008 ± 0.038	143.646 ± 3.627
CCY 20-7-31	72	16.040 ± 0.182	1.441 ± 0.029	160.398 ± 1.824
	96	17.517 ± 0.526	1.724 ± 0.039	175.173 ± 5.257
RMd	24	11.280 ± 0.787	0.287 ± 0.023	112.798 ± 7.873
R. mucilaginosa	48	14.969 ± 0.612	0.969 ± 0.021	149.695 ± 6.116
CCY 19-4-6	72	15.963 ± 0.760	1.430 ± 0.063	159.630 ± 7.602
	96	16.328 ± 1.262	1.657 ± 0.100	163.282 ± 12.621
SP	24	11.526 ± 0.344	0.440 ± 0.049	115.265 ± 3.437
S. pararoseus	48	15.812 ± 1.206	0.980 ± 0.087	158.116 ± 12.062
CCY 19-9-6	72	23.688 ± 2.465	2.020 ± 0.151	236.883 ± 24.650
	96	27.220 ± 1.774	2.449 ± 0.172	272.197 ± 17.744
SS	24	7.720 ± 0.202	0.274 ± 0.040	77.199 ± 2.016
S. samonicolor	48	10.387 ± 0.167	0.743 ± 0.016	103.873 ± 1.670
CCY 19-4-25	72	10.230 ± 0.619	0.873 ± 0.059	102.304 ± 6.185
	96	10.518 ± 0.710	1.018 ± 0.068	105.185 ± 7.100
SM	24	14.440 ± 0.484	0.599 ± 0.063	144.395 ± 4.843
S. metaroseus	48	25.221 ± 0.623	2.003 ± 0.090	252.207 ± 6.230
CCY 19-6-20	72	33.960 ± 0.892	3.440 ± 0.128	339.596 ± 8.918
	96	42.258 ± 0.313	4.964 ± 0.046	422.582 ± 3.132
CI	24	10.334 ± 0.858	0.275 ± 0.046	103.344 ± 8.585
C.infirmominiatum	48	15.074 ± 1.783	0.608 ± 0.112	150.741 ± 17.832
CCY 17-18-4	72	25.282 ± 1.300	1.733 ± 0.163	252.815 ± 13.004
	96	29.161 ± 0.763	2.226 ± 0.016	291.607 ± 7.630
CM	24	13.576 ± 0.602	0.388 ± 0.044	135.763 ± 6.018
C. macerans	48	21.659 ± 0.561	1.709 ± 0.391	216.592 ± 5.612
CCY 10-1-2	72	29.506 ± 1.422	3.169 ± 0.116	295.061 ± 14.215
	96	36.676 ± 1.479	4.337 ± 0.282	366.757 ± 14.787
PR	24	12.455 ± 0.139	0.520 ± 0.010	124.553 ± 1.395
P. rhodozyma	48	21.392 ± 0.721	1.077 ± 0.084	213.924 ± 7.206
CCY 77-1-1	72	26.035 ± 0.727	1.948 ± 0.195	260.354 ± 7.267
	96	29.170 ± 2.208	2.631 ± 0.337	291.703 ± 22.083

6.5.2 Fatty acids composition

The fatty acid composition of carotenogenic yeasts was strain-specific with a predominant accumulation of oleic acid regarding the percent composition of total fatty acids. The results summarised in Fig. 6.46 correspond to the highest accumulated amounts of FAs, obtained after 96 hours of cultivation. The FAs composition during cultivation can be seen in the Tab. 6.14. The FAs produced by *Rhodotorula* species were predominantly composed of oleic acid (C18:1, 59 – 69 %) and palmitic acid (C16:0, 16 – 18 %). The composition of FAs was slightly different for *R. kratochvilae* which accumulated 39.5 % of oleic acid, 27.6 % of palmitic acid and 19.7 % of linoleic acid. These results are in agreement with other studies dealing with *R. kratochvilae*, *R. mucilaginosa* and *R. toruloides* strains [155, 129].

Sporobolomyces species (previously known as *Sporidiobolus*) [156] accumulated mainly oleic acid (42-56 %), with linoleic acid being the second most accumulated fatty acid (22-30 %). Gientka et al reported linoleic acid as the second most abundant FA (20.2 %) in the biomass of *S. salmonicolor* LOCK53. However,*S. salmonicolor* LOCK53 accumulated lesser amounts of oleic acid (39.4 %) and higher share of stearic acid (14.5 %) and arachidic acid (C20:0, 16 %) [157] than *S. salmonicolor* CCY 19-04-25 (SS) in this study. Also, *Sporobolomyces* strains in this study produced no arachidic acid (C20:0). Share of palmitic acid differed among examined *Sporobolomyces* strains. While strains *S. pararoseus* CCY 19-09-06 (SP) and *S. metaroseus* CCY 19-6-20 (SM) accumulated 18 % of palmitic acid, strain *S. salmonicolor* CCY 19-04-25 (SS) accumulated only 8 %.

Fatty acid composition of *C. macerans* CCY 10-01-02 (CM) was very similar to *Sporobolomyces* species except for strain *C. infirmominiatum* CCY 17-18-04 (CI), which produced mainly unsaturated fatty acids like oleic acid (42 % of total FAs), linoleic acid (25 % of total FAs), and a relatively high percentage of α-linolenic acid (13 % of total FAs) in comparison to other strains. The share of oleic, linoleic and especially α-linolenic acid within accumulated TAG was higher than in the study of Řezanka et al. In his study, Increased share of above mentioned unsaturated FAs, were achieved only when the yeasts were cultivated at 4 °C [158]. In general, the amounts of stearic acid stayed relatively constant during cultivation time and, depending on the strain, represented 1.6 -14 % of total fatty acids. During the cultivation time, oleic acid predominantly accumulated while linoleic and α-linolenic acids decreased. With the exception of the strain *C. infirmominiatum* CCY 17-18-04 (CI) which started to accumulate α-linolenic acid at 72 hours of cultivation. Yeasts of genera *Sporobolomyces* and *Cystofilobasidium* proved to be promising producers of linoleic acid and strain *C. infirmominiatum* CCY 17-18-04 (CI) has especially great potential as a natural source of both dietary essential fatty acids (Benatti et al. 2004).

Tab. 6.14: Composition of FAs produced during cultivation time

Composition FAs (%)*						
Yeast strain	**Time (h)**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
RK	24	18.4	3.8	40.6	26.6	9.1
R. kratochvilae	48	29.0	6.7	40.6	21.3	nd.
CCY 20-2-26	72	28.6	7.5	38.0	18.7	4.8
	96	27.6	6.0	39.5	19.7	4.9
RT	24	16.9	14.3	54.4	11.0	2.8
R. toruloides	48	17.7	11.5	64.3	4.5	1.0
CCY 62-02-04	72	14.2	9.7	69.2	5.2	0.8
	96	11.8	7.2	71.7	8.8	nd.
RMa	24	17.1	5.3	48.8	25.3	3.6
R.mucilaginosa	48	16.0	3.9	62.9	14.8	1.4
CCY 20-7-28	72	14.9	3.2	64.2	14.8	1.5
	96	14.3	2.6	63.6	16.4	1.3
RMb	24	13.8	5.0	48.3	25.8	7.2
R. mucilaginosa	48	10.8	9.5	67.0	10.4	2.2
CCY 20-9-7	72	10.0	8.4	69.6	9.3	1.9
	96	9.2	4.3	70.6	12.7	1.9
RMc	24	16.3	10.8	42.4	24.5	5.5
R. mucilaginosa	48	17.1	11.6	57.3	11.7	1.8
CCY 20-7-31	72	16.1	12.5	58.8	10.7	nd.
	96	16.0	10.2	60.0	12.1	nd.
RMd	24	19.0	8.7	38.8	24.1	nd.
R. mucilaginosa	48	16.8	8.0	58.7	12.9	nd.
CCY 19-4-6	72	15.3	8.0	59.8	11.3	2.3
	96	14.4	6.2	63.9	12.0	0.4
SP	24	15.2	5.0	28.7	46.3	4.7
S. pararoseus	48	16.5	4.5	41.3	35.2	0.4
CCY 19-9-6	72	18.3	4.7	44.5	30.7	1.1
	96	18.3	4.5	42.6	30.6	1.1
SS	24	9.1	2.0	48.8	34.7	5.4
S. samonicolor	48	8.8	1.6	55.4	31.0	3.2
CCY 19-4-25	72	9.2	1.6	57.0	29.5	2.7
	96	8.6	1.8	56.4	30.2	2.6
SM	24	15.1	7.3	59.4	14.3	3.6
S. metaroseus	48	18.7	7.9	48.6	22.4	1.2
CCY 19-6-20	72	19.2	5.6	50.3	22.8	0.9
	96	18.5	4.8	51.9	22.5	0.7
CI	24	11.2	3.6	47.1	35.1	2.9
C.infirmominiatum	48	13.3	2.9	46.8	35.5	1.5
CCY 17-18-4	72	15.3	2.1	42.4	24.9	14.2
	96	15.5	1.7	42.8	25.6	13.1
CM	24	15.4	6.7	57.6	16.7	3.6
C. macerans	48	18.3	5.5	46.7	28.1	1.3
CCY 10-1-2	72	19.0	4.3	50.2	24.7	1.0
	96	18.6	3.8	51.3	23.6	0.8
PR	24	16.7	8.7	52.9	20.0	1.6
P. rhodozyma	48	18.8	7.2	50.1	21.6	1.0
CCY 77-1-1	72	20.4	6.0	52.1	19.5	0.6
	96	19.6	6.2	52.3	19.5	0.5

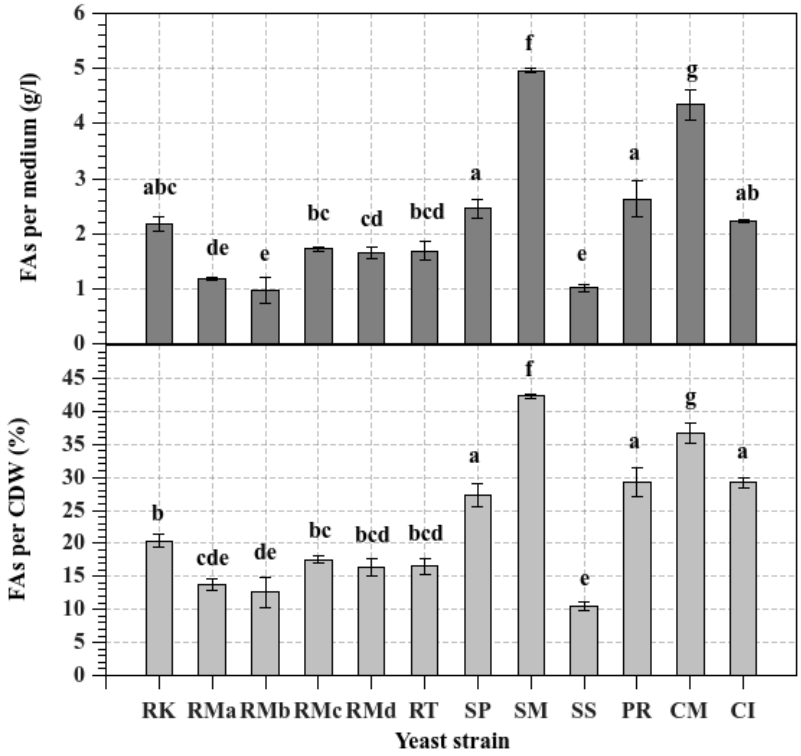

Fig. 6.45: Fatty acid production after 96 hours of cultivation, upper graph represents FAs production per medium, graph below represents production of FAs in CDW, letter indexes above the columns a, b, etc. mean homogenous groups (ANOVA, Tukey test, α=0.05)

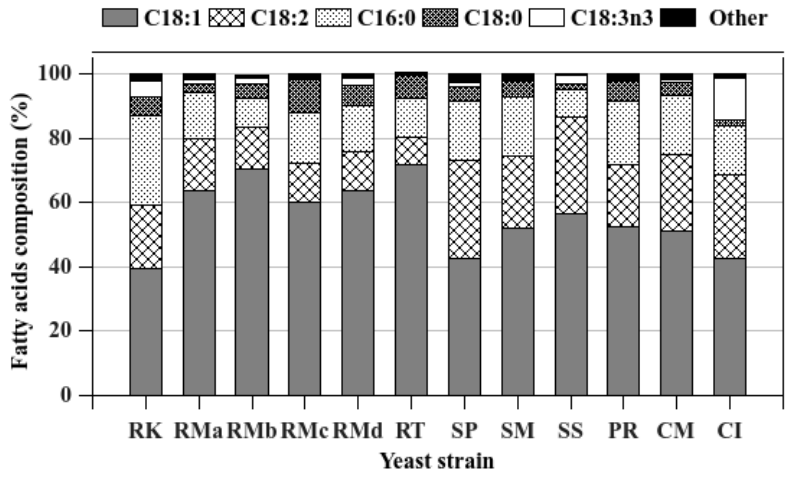

Fig. 6.46: Fatty acid composition expressed as % of total fatty acids per biomass after 96 hours of cultivation (mg/g)

6.5.3 Synthesis of carotenoids and their profile

To evaluate carotenoid production properties, the sorting proposed in the study of El-Banna et al. [122] was used. The ability to produce carotenoids was categorized as follows: low amount < 0.100 mg/g of CDW, the average for 0.101-0.500 mg/g of CDW and high amount > 0.500 mg/g of CDW. Accordingly, the best-producing yeast strains in this study belong to the category with high carotenoid production. The results summarised in Fig. 6.47 correspond to the cultivation time with the highest accumulated amounts of carotenoids. Data on carotenoid productions during cultivation time are included in the Tab 6.15. The highest carotenoid-producing yeast strains were *R. mucilaginosa* CCY 19-04-06 (RMd), *R. toruloides* CCY 62-02-04 (RT), *S. pararoseus* CCY 19-09-06 (SP), *S. metaroseus* CCY 19-6-20 (SM), *C. macerans* CCY 10-01-02 (CM), and *C. infirmominiatum* CCY 17-18-04 (CI). The aforementioned strains produced carotenoids in the range 0.8 - 2.1 mg/g of CDW. The best of these was *R. mucilaginosa* CCY 19-04-06 (RMd) followed by *S. pararoseus* CCY 19-09-06 (SP) and *R. toruloides* CCY 62-02-04 (RT). The rest of the species would fall into the average range, according to the sorting used. In addition to the production of carotenoids per CDW, another important parameter reflecting biomass growth in the medium is the production of carotenoids expressed in mg/l. The best carotenoid producers were *R. mucilaginosa* CCY 19-04-06 (RMd), *R. toruloides* CCY 62-02-04 (RT), *S. pararoseus* CCY 19-09-06 (SP) which produced 21 mg/l, 17 mg/l and 15 mg/l of carotenoids respectively. These productions were even higher than the ones reported in the studies of Tkáčová, Zhao or Sharma [128, 111, 159].

Lycopene is a precursor for the biosynthesis of carotenoids as well as β-carotene, γ-carotene, torulene, and torularhodin, which are the major carotenoids of *Rhodotorula*, *Sporobolomyces*, and *Cystofilobasidium* species [15]. The main carotenoid produced by *P. rhodozyma* is astaxanthin, which is synthesised from β-carotene that is not entirelydepleted and remains accumulated as a second major carotenoid [160].

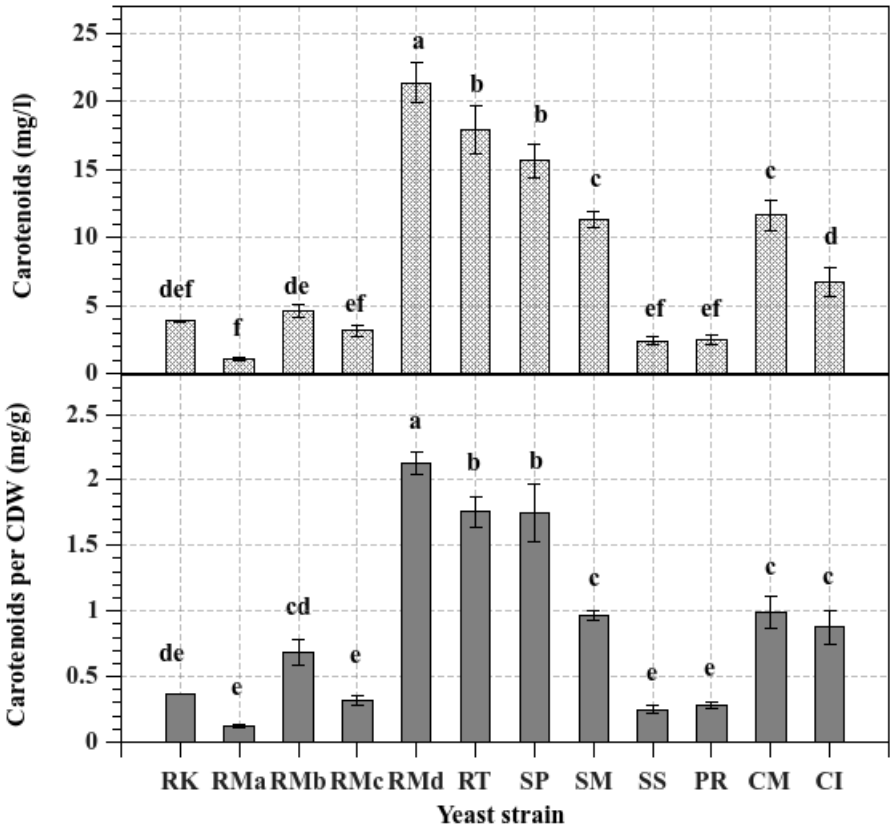

Fig. 6.47: Carotenoid production and composition after 96 hours of cultivation (strain Rmb - 48 hours of cultivation), the upper graph represents carotenoid production per medium, graph below represents production of carotenoids per biomass mg g-1 of CDW, letter indexes above the columns a, b, etc. mean homogenous groups (ANOVA, Tukey test, α=0.05)

Tab. 6.15: Carotenoid productions during cultivation time

Yeast strain	Time (h)	Total carotenoids per CDW (mg/g)	Total carotenoids per Medium (mg/l)
RK	24	0.110 ± 0.103	0.575 ± 0.527
R. kratochvilae	48	0.481 ± 0.005	3.958 ± 0.231
CCY 20-2-26	72	0.364 ± 0.015	3.756 ± 0.120
	96	0.364 ± 0.003	3.862 ± 0.077
RT	24	0.425 ± 0.021	2.552 ± 0.039
R. toruloides	48	1.379 ± 0.101	11.560 ± 1.139
CCY 62-02-04	72	1.400 ± 0.048	13.900 ± 0.281
	96	1.755 ± 0.121	17.924 ± 1.813
RMa	24	0.211 ± 0.058	0.556 ± 0.183
R.mucilaginosa	48	0.236 ± 0.036	1.535 ± 0.202
CCY 20-7-28	72	0.175 ± 0.027	1.485 ± 0.044
	96	0.123 ± 0.015	1.062 ± 0.083
RMb	24	0.160 ± 0.002	0.541 ± 0.038
R. mucilaginosa	48	0.685 ± 0.095	4.553 ± 0.474
CCY 20-9-7	72	0.428 ± 0.053	3.281 ± 0.403
	96	0.356 ± 0.004	2.786 ± 0.127
RMc	24	0.179 ± 0.039	0.497 ± 0.102
R. mucilaginosa	48	0.334 ± 0.020	2.363 ± 0.168
CCY 20-7-31	72	0.320 ± 0.008	2.864 ± 0.020
	96	0.317 ± 0.036	3.123 ± 0.406
RMd	24	0.686 ± 0.074	1.743 ± 0.166
R. mucilaginosa	48	2.404 ± 0.560	15.588 ± 4.047
CCY 19-4-6	72	2.082 ± 0.189	19.187 ± 2.911
	96	2.174 ± 0.085	21.683 ± 1.465
SP	24	0.494 ± 0.068	1.961 ± 0.143
S. pararoseus	48	1.073 ± 0.166	6.587 ± 0.618
CCY 19-9-6	72	1.280 ± 0.052	11.350 ± 0.344
	96	1.781 ± 0.218	15.902 ± 1.254
SS	24	0.149 ± 0.022	0.485 ± 0.121
S. samonicolor	48	0.262 ± 0.007	1.856 ± 0.028
CCY 19-4-25	72	0.230 ± 0.025	1.960 ± 0.245
	96	0.245 ± 0.030	2.367 ± 0.289
SM	24	0.310 ± 0.029	1.316 ± 0.294
S. metaroseus	48	0.610 ± 0.060	4.914 ± 0.234
CCY 19-6-20	72	0.881 ± 0.100	9.028 ± 1.269
	96	0.961 ± 0.038	11.357 ± 0.594
CI	24	0.210 ± 0.114	0.577 ± 0.370
C.infirmominiatum	48	0.688 ± 0.062	2.739 ± 0.035
CCY 17-18-4	72	0.768 ± 0.249	5.235 ± 2.158
	96	0.875 ± 0.129	6.685 ± 1.040
CM	24	0.633 ± 0.110	1.913 ± 0.290
C. macerans	48	0.791 ± 0.212	6.042 ± 1.194
CCY 10-1-2	72	0.879 ± 0.020	9.411 ± 0.374
	96	0.988 ± 0.119	11.658 ± 1.131
PR	24	0.044 ± 0.014	0.180 ± 0.058
P. rhodozyma	48	0.158 ± 0.038	0.802 ± 0.232
CCY 77-1-1	72	0.221 ± 0.017	1.633 ± 0.057
	96	0.278 ± 0.023	2.474 ± 0.391

In this study four *R. mucilaginosa* strains were used but only three of them produced β-carotene, γ-carotene, torulene, and torularhodin. The strain *R. mucilaginosa* CCY 19-04-06 (RMd) produced mainly lycopene. In *Rhodotorula* sp. is lycopene cyclised by bifunctional enzyme phytoene synthase/lycopene cyclase to form β-carotene, which is the substrate for subsequent enzymatic reactions leading to β-carotene, torulene and torularhodin [15, 161]. The N-terminal domain of this protein encodes mainly the lycopene cyclase while the C-terminal domain of this protein encodes phytoene synthase activity [160]. When the lycopene cyclase is inhibited the lycopene cannot be cyclised to form γ-carotene, this lead to accumulation of lycopene. The inhibition of lycopene cyclase in *Rhodotorula* sp. can be induced by addition of nicotine into production media. The inhibition causes increased production of lycopene and decreased production of β-carotene, torulene and torularhodin [69]. Since no inhibitors of lycopene cyclase were used, it is possible that *R. mucilaginosa* CCY 19-04-06 (Rmd) possesses some mutation in the part of phytoene synthase/lycopene cyclase encoding the N-terminal domain of this enzyme.

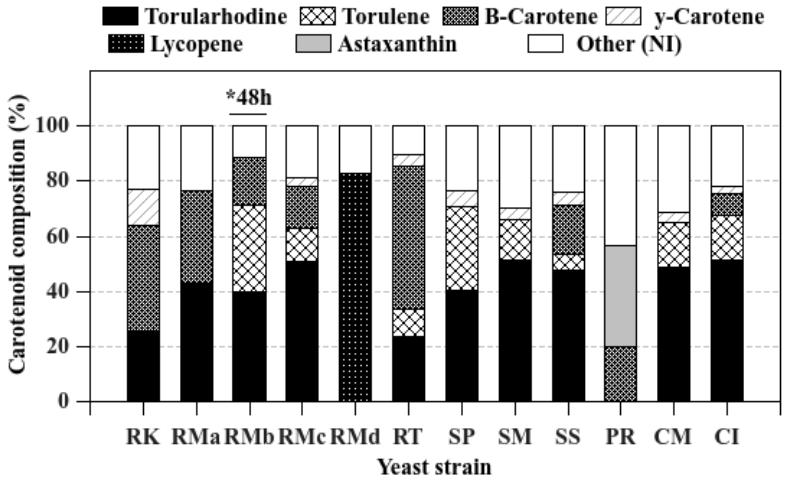

Fig. 6.48: Carotenoid composition expressed as % of total carotenoids per biomass after 96 hours of cultivation (mg/g), *48 h – the best production after 48 hours of cultivation

R. mucilaginosa CCY 19-04-06 (RMd) produced 21 mg/l of total carotenoids, of which lycopene constituted more than 80 %. *R. mucilaginosa* strains CCY 20-09-07 (RMa), CCY 20-07-31 (RMb), and CCY 20-07-28 (RMc), *Sporobolomyces*, and *Cystofilobasidium* species produced mainly torularhodin ranging from 40 to 50 % of the total carotenoid amount, but β-carotene and γ-carotene were not always accumulated. Strains *S. pararoseus* CCY 19-09-06 (SP), *S. metaroseus* CCY 19-6-20 (SM), and *C. macerans* CCY 10-01-02 (CM) did not accumulate β-carotene at all. In contrast, *R. kratochvilae* CCY 20-02-26 (RK) and *R. toruloides* CCY 62-02-04 (RT) produced β-carotene as the main carotenoid, forming 38 % and 51 % of total carotenoids, respectively. The carotenoid composition can be found in Fig. 6.48 . The carotenoid composition during cultivation time is listed in Tab. 6.16.

Tab. 6.16: Carotenoid composition during cultivation time

Yeast strain	Time (h)	Carotenoids composition (%)*						
		β-car.	γ-carotene	Torulene	Torular.	Lycopene	Astax.	NI
RK	24	60.0	20.9	-	-	-	-	19.1
R. kratochvilae	48	38.9	18.1	5.7	22.7	-	-	14.6
CCY 20-2-26	72	35.0	16.4	-	25.9	-	-	22.7
	96	38.2	13.1	-	25.7	-	-	23.0
RT	24	49.3	8.3	-	-	-	-	42.4
R. toruloides	48	38.5	11.7	11.5	21.5	-	-	16.8
CCY 62-02-04	72	41.5	8.6	14.8	24.2	-	-	10.9
	96	51.6	4.2	10.0	23.6	-	-	10.5
RMa	24	33.4	-	-	42.6	-	-	24.0
R.mucilaginosa	48	33.9	3.2	9.2	37.3	-	-	16.3
CCY 20-7-28	72	20.9	2.8	2.5	27.4	-	-	46.4
	96	33.5	-	-	43.0	-	-	23.5
RMb	24	24.0	-	-	57.7	-	-	18.3
R. mucilaginosa	48	-	17.5	31.4	39.6	-	-	11.4
CCY 20-9-7	72	17.0	4.0	20.0	43.4	-	-	15.6
	96	14.8	3.2	12.3	50.7	-	-	18.9
RMc	24	22.9	-	-	53.1	-	-	24.1
R. mucilaginosa	48	17.1	4.2	16.7	46.1	-	-	15.9
CCY 20-7-31	72	17.0	4.0	20.0	43.4	-	-	15.6
	96	14.8	3.2	12.3	50.7	-	-	18.9
RMd	24	-	-	-	-	73.9	-	26.1
R. mucilaginosa	48	-	-	-	-	86.2	-	13.8
CCY 19-4-6	72	-	-	-	-	84.2	-	15.8
	96	-	-	-	-	83.0	-	17.0
SP	24	-	11.0	8.6	48.2	-	-	32.2
S. pararoseus	48	-	8.1	20.8	42.1	-	-	29.0
CCY 19-9-6	72	-	5.8	14.2	50.0	-	-	30.0
	96	-	5.8	30.4	40.1	-	-	23.6
SS	24	-	5.6	13.0	58.2	-	-	23.2
S. samonicolor	48	20.3	8.0	13.2	38.6	-	-	19.8
CCY 19-4-25	72	18.6	5.7	8.1	44.3	-	-	23.3
	96	17.5	4.8	6.0	47.6	-	-	24.1
SM	24	-	7.6	-	55.2	-	-	37.2
S. metaroseus	48	-	7.0	15.5	48.0	-	-	29.4
CCY 19-6-20	72	-	5.6	15.9	49.0	-	-	29.5
	96	-	3.9	14.8	51.5	-	-	29.8
CI	24	9.9	4.5	-	61.5	-	-	24.1
C.infirmominiatum	48	13.2	5.9	14.0	46.0	-	-	20.9
CCY 17-18-4	72	10.0	6.3	14.5	47.6	-	-	21.5
	96	8.0	2.5	16.1	51.3	-	-	22.1
CM	24	-	7.5	10.9	52.9	-	-	28.7
C. macerans	48	-	7.8	24.0	44.0	-	-	24.3
CCY 10-1-2	72	-	5.9	20.8	45.2	-	-	28.2
	96	-	4.0	16.3	48.5	-	-	31.2
PR	24	-	-	-	-	-	48.6	51.4
P. rhodozyma	48	18.8	-	-	-	-	37.1	44.1
CCY 77-1-1	72	19.8	-	-	-	-	36.3	43.9
	96	19.8	-	-	-	-	36.9	43.3

β-carotene = β-car., γ-carotene = γ-car., torularhodin = Torular., Astaxanthin = Astax

NI = Non-identified carotenoids, * composition of total carotenoids per CDW

6.5.4 The ergosterol production

Metabolic pathways serving for the synthesis of fatty acids, ergosterol, as well as carotenoids use acetyl-CoA as a precursor [39, 51]. Without the sterol synthesis yeast cells are not viable. On the contrary, excess of sterols is toxic for the yeast cells since it cannot be metabolised. Therefore, its homeostasis is maintained through free sterol esterification with fatty acids by acyltransferases, by down-regulation of sterol biosynthesis or by sterol acetylation with its subsequent secretion in a form of sterol acetates into production media [50]. Sterol acetylation is catalysed by alcohol acyltransferases. The acetylated sterols are then transported to the plasma membrane where they are bound by pathogen-related proteins. Pathogen-related proteins retain sterol acetates soluble and facilitate their secretion [47].

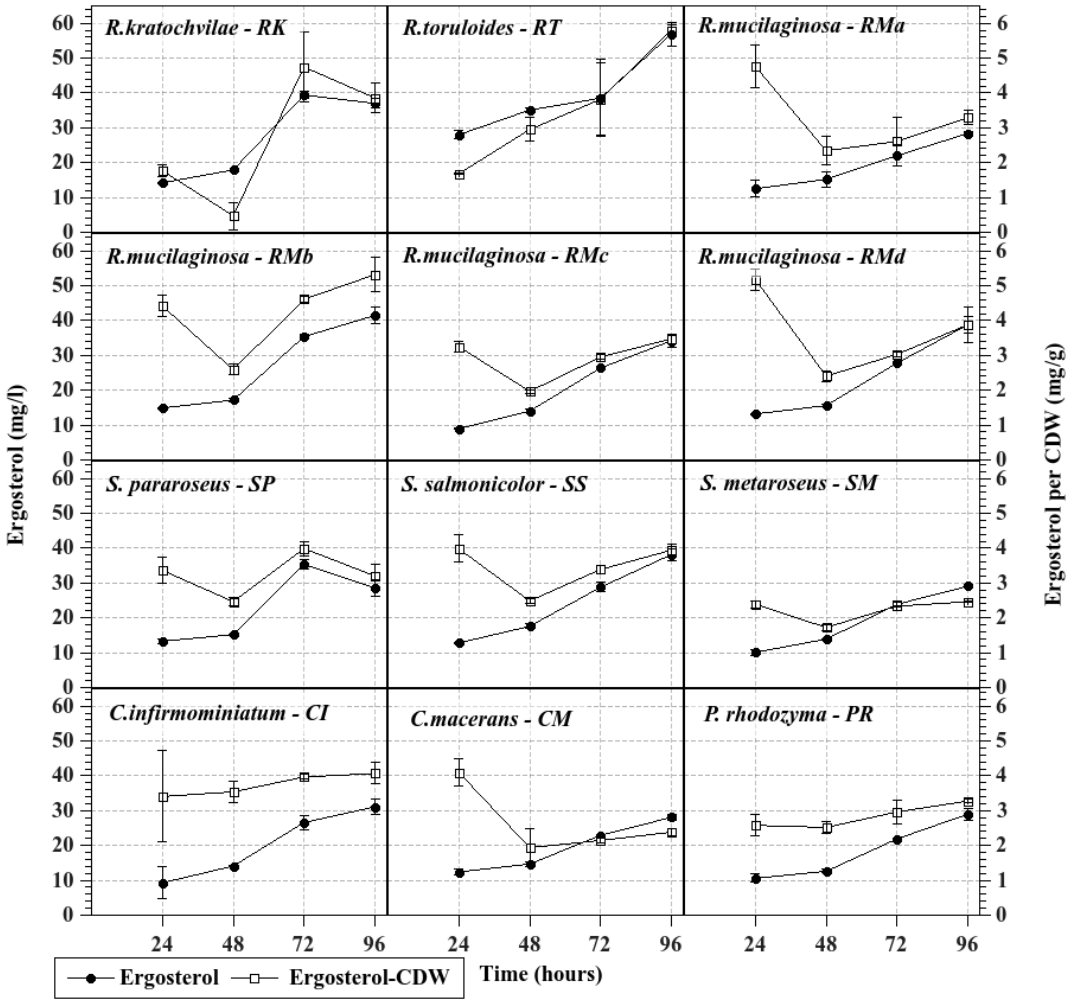

Fig. 6.49: Production of ergosterol during cultivation time.

The ergosterol production was evaluated at evaluated at 24, 48, 72 and 96 hours of cultivation. Considering the amount of ergosterol per culture medium was the production of ergosterol the greatest at the end of the cultivation, while the production per CDW differed during cultivation

time in some yeast strains. In species *R. mucilaginosa* sp., *S. salmonicolor*, and *C. macerans*, the amount of accumulated ergosterol significantly decreased after 48 hours (ANOVA, Tukey, $P < 0.05$) of cultivation (see Fig. 6.49). The decreased amount of ergosterol could be connected to ergosterol homeostasis kept in balance by the above mentioned sterol regulation mechanisms. With increasing cultivation time ergosterol again accumulated in increased amounts. Other yeast strains did not show such a dramatic decrease of accumulated ergosterol.

In *Rhodotorula* sp. the ergosterol production at 96 hours of cultivation was in the range 28 – 58 mg/l (3.3 – 5.7 mg/g). The best producers of ergosterol were strains *R. mucilaginosa* CCY 20-07-31 (RMb) and *R. toruloides* CCY 62-02-04 (RT) which produced 5.7 mg/g (58.0 mg/l) and 5.3 mg/g (41.5 mg/l) of CDW respectively. These strains had the best production of all. The *Sporobolomyces* and *Cystofilobasidium* sp. produced ergosterol after 96 hours of cultivation in the range 28.1 – 38.2 mg/l (2.4 – 4.1 mg/g) with the best producer *S. salmonicolor* 19-4-25 (SS). Volumetric production of intracellular metabolites, such as ergosterol is connected to biomass production. All examined yeast strains had very good biomass production and even the lowest ergosterol production was very good in comparison to the study of Villarreal or to the study of Szotkowski [46, 162].

The results of this study show that carotenogenic yeasts have a great potential for industrial production of nutritionally essential fatty acids and carotenoids. The quality and quantity of fatty acids and carotenoids were specific to particular yeast strain. The greatest potential for essential fatty acid production have lesser-known carotenogenic yeasts strains *S. metaroseus* CCY 19-6-20 (SM) (4.9 g/l of FAs) and *C. macerans* CCY 10-01-02 (CM) (4 g/l of FAs) and particularly *C. infirmominiatum* CCY 17-18-04 (CI) (2.2 g/l of FAs), which besides oleic and linoleic fatty acids produced also α-linolenic acid (13 % of FAs). The aforementioned yeast strains produced also high amounts of carotenoids formed mainly by torularhodin and by torulene. Lycopene is one of the most valuable carotenoid, especially because of its complicated organic synthesis. In this study, there was remarkable production of lycopene observed. In particular, strain *R. mucilaginosa* CCY 19-04-06 (RMd), produced large amounts of carotenoids (21 mg/l), mainly represented by lycopene. The very high amounts of carotenoids were also produced by strains *R. toruloides* CCY 62-02-04 (RT) (17.9 mg/l) and *S. pararoseus* CCY 19-09-06 (SP) (15.9 mg/l).

7 CONCLUSIONS

Present Doctoral Thesis was focused mainly on metabolic characterization of carotenogenic yeasts as suitable candidates for industrial applications. Carotenogenic yeasts can produce fatty acids as well as carotenoids and ergosterol, but only some strains are capable to produce high yields of these chemicals. To fulfil this task the molecular methods as well as omic-methods were needed for detailed characterization of chosen yeast strains. Therefore, present Doctoral Thesis was focused in detail on production properties of carotenogenic yeast strains within Sporidiobolales and Cystofilobasidiales. The yeast strains were exposed to nutritional limitation, carotenoid biosynthesis inhibition, chemical and physical mutagenesis and were genetically modified using biolistic method. The experimental part was divided into five sections with following conclusions:

Chapter aimed on **the diversity of *R. mucilaginosa* species** was focused on intraspecies variability regarding different production properties of *R. mucilaginosa* species. *R. mucilaginosa* is one of the most widespread carotenogenic yeast species which can survive even in extreme conditions and therefore it could be a suitable candidate for industrial exploitation. Phenotype variability is an important parameter regarding yeast strain adaptation to unfavorable conditions. In this experiment, seven yeast strains of *R. mucilaginosa* with different production properties and with four different micro and minisatelite profiles, were studied. *R. mucilaginosa* yeast strains proved to have different production properties regarding lipid and carotenoids production. These strains can provide high biomass content and high yields of carotenoids.

In the chapter focused on **fatty acid production under nitrogen limitation**, were studied properties of one strain of *R. toruloides* and four different strains of *R. mucilaginosa*. In this experimental part of the study the impact of three different carbon sources (glucose, xylose and glycerol) was also studied. The nitrogen limitation is a stress condition frequently reported to increased lipid accumulation in oleaginous yeasts. Combination of nitrogen limitation and cheap substrate should be less economically demanding. The result of these experiments proved that nitrogen limitation has positive impact only to some carotenogenic yeast species. While yeast *R. topruloides* CBS 6016 proved to have exceptional production properties at high C/N ratio (180), strains of *R. mucilaginosa* CBS 2402, 2403 and 2405 proved to have better fatty acids productions at low C/N ratios (30, 60) due to low biomass yields. The usage of carbon is different in these species. The *R. toruloides* uses carbon in nitrogen depletion mainly for fatty acids accumulation leading to decreased biomass production (FF-CDW), while strains of *R. mucilaginosa* sp. CBS 2402, 2403 and 2405 accumulated only slightly increased amounts of fatty acids within cells and responded by severe decrease in cell proliferation, which led to decreased biomass production. Also only two of studied strains *R. toruloides* CBS 6016 and *R. mucilaginosa* CBS 2402 were capable to efficiently utilize xylose, and only strain *R. toruloides* CBS 6016 was capable to efficiently utilize glycerol. The best producent with the best response to nitrogen limitation and all used carbon sources was *R. toruloides*.

Chapter **genetic modification of *R. toruloides* using biolistic method** was focused on preparation of genetically modified fatty acids overproducing strain. Since tools for genetic manipulation in carotenogenic yeasts are not yet very developed, these experiments mainly aimed on the possibility to obtain genetically modified strains of *R. toruloides*. The outcome of this experiment

was successful preparation of the two genetically modified clones of *R. toruloides*, which could be used in subsequent experiments aiming at overexpression of other genes responsible for fatty acid overproduction in the same clone. To obtain fatty acid overproducing clones the following genes should be also cloned: Citrate lyase (ACL) as a supplier of acetyl-CoA, gene for Acetyl-CoA carboxylase (ACC), which is responsible for carboxylation of Acetyl-CoA to malonyl-CoA. The GPD1 and DGA1 clones in combination with above mentioned genes overexpressed together could create carbon flux invested into lipid accumulation where GPD1 would supply sufficient source of glycerol, which would be acylated in faster rate by overexpressed DGA1 and the corresponding supply of fatty acids to assembly TG. It would be also necessary to examine higher number of genetically modified clones and optimize conditions leading to increased fatty acids accumulation.

Impact of inhibitors, physical and chemical mutagenes on metabolite production was employed on yeast strains *R. toruloides* CCY 62-02-04. This strain was ideal candidate for random mutagenesis, because it is declared by CCY collection to be haploid. To prepare mutant strains of *R. toruloides* was necessary to firstly verify selection method using DPA. Therefore, firstly was this strain exposed to different concentrations of DPA in combination with low (C/N 16) and high (C/N 80) C/N ratios. The results of these experiments may support the idea of different regulation of carotenoid synthesis under combination of DPA and C/N ratio. Also, in the next part was *R. toruloides* exposed to chemical and physical mutagens, resulting in successfully prepared carotenoid overproducing mutants with increased resistance to DPA.

The last part of thesis was focused on **variability of metabolites produced by some yeasts within Sporidiobolales and Cystofilobasidiales**.This is the only chapter which is not focused mainly on *R. toruloides* and *R. mucilaginosa* species. To explore production properties of other carotenogenic yeast species, a simple cultivation experiment aiming for metabolite analysis was employed. The carotenogenic yeast are diverse group of unrelated organisms and therefore their production properties significantly differ. This study also proved that production properties can significantly differ even among yeasts of the same species. Furthermore, this study proved that even in non-nitrogen limiting conditions can be achieved extraordinary productions of fatty acids as well as carotenoids. The best production properties including carotenoid as well as fatty acid productions proved to have strains *S. metaroseus* 19-6-20, *C. macerans* CCY 10-1-2, *C. infirmominiatum* CCY 17-18-4, *R. mucilaginosa* CCY 19-4-6, *R. toruloides* CCY 62-02-04, and *S. pararoseus* CCY 19-9-6. Furthermore, the lycopen production by the strain *R. mucilaginosa* was detected, and also was detected increased production of α-linoleic acid by the strain *C. infirmominiatum*.

The result of this Doctoral Thesis represent a detailed description of metabolite production among studied carotenogenic yeast strains, which could be used in scaled up experiments leading to their industrial application as well as a valuable information for the genetic engineering of carotenogenic yeasts.

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8 LIST OF ABBREVIATIONS

AA/Bis	acrylamide N,N'-methylene-bisacrylamide
ACAT	acyl-CoA-cholesterol acyltransferase
ACCI	acetyl-CoA carboxylase
AFTOL	Assembling the Fungal Tree of Life
AMP	adenosine monophosphate
ATM	*Agrobacterium tumefaciens* mediated transformation
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
C/N	carbon nitrogen ratio
CrtB	bifunctional enzyme phytoene synthase/lycopene cyclase
CrtI	enzyme phytoene desaturase
CrtS	astaxanthin synthase
crtX	carotenoid oxygenase
CYTB	cytochrome B
DAG	diacylglycerol
DGGE	Denaturation Gradient Gel Electrophoresis
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DO	dissolved oxygene level
DPA	diphenylamine
DTT	dithiothreitol
EMS	ethyl methanesulphonate
ER	Endoplasmic reticulum
FA	fatty acids
FAME	fatty acid methylesters
FAS1/2	fatty acid synthase genes ½
GC	gas chromatography
GC-FID	gas chromatography flame ionization detector
GL	glycerollipids
GLF	glycerolphospholipids
HMG-CoA	(3S)-3-hydroxy-3-methylglutaryl-CoA
HPLC	high performance liquid chromatography
HPLC-PDA	high performance liquid chromatography – photodiod array detector
IPP	isopentenyl pyrophosphate

ITS1/2	internal transcribed spacers ½
LD	lipid droplet
LSU	large subunit
MALDI-TOF	Matrix-Assisted Laser Ionisation Desorption - Time of Flight Detector
MCM7	minichromosome maintenance protein
MMS	methyl methanesulphonate
Mt	mitochondria
NHEJ	non-homolougous end-joining
NTG	N-methyl-N′-nitro-N-nitrosoguanidine
Nu	nucleus
ORF	open reading frame
PA	phosphatidic acid
PCR	polymerase chain reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PL	phospholipids
PUFA	polyunsaturated fatty acids
Px	peroxisomes
RNA	ribonucleic acid
RPB1/2	subunits of RNA polymerase ½
SE	sterylesters
SSRs	simple sequence repeats
T-DNA	transferred DNA
TEF1	translation elongation factor 1-alpha
TG	triacylglycerols
Ti plasmid	tumor-inducing
UPGMA	unweighted pair group method algorithm
UV	ultraviolet radiation
YCC	yeast cloning cassette
YNB	yeast nitrogen base

9 SUPPLEMENTARY MATERIALS

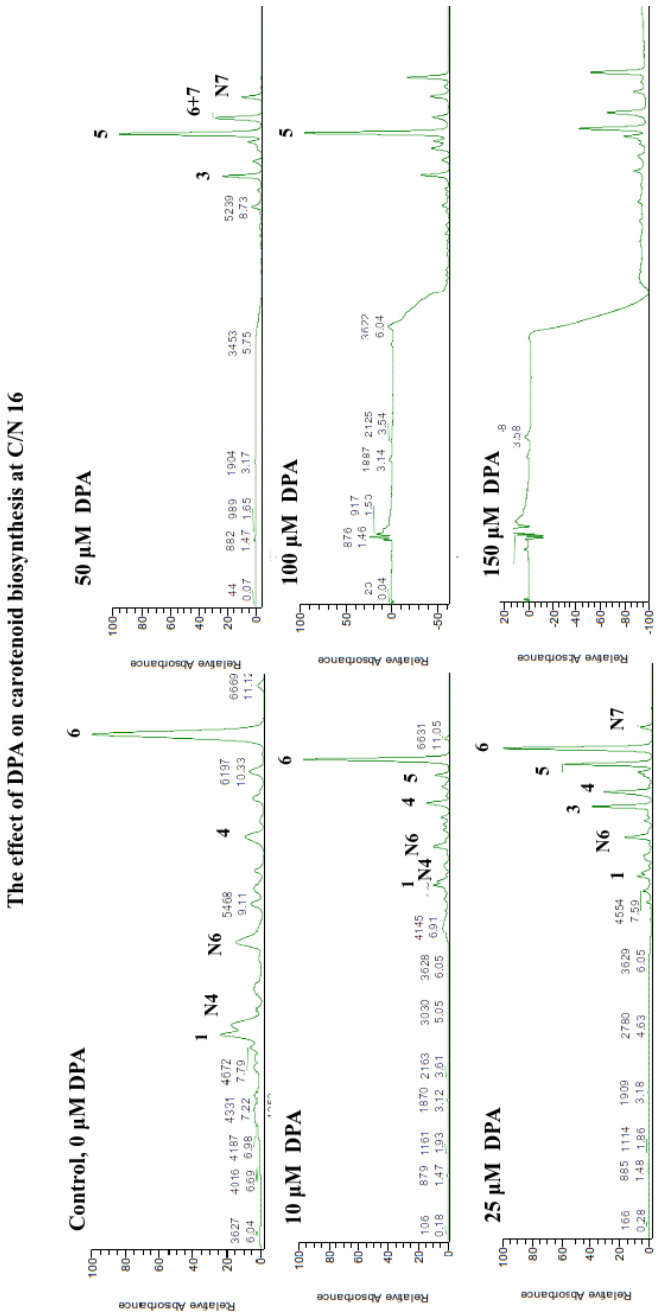

Fig. 9.1: Chromatograms with carotenoids profiles of the original strain *R. toruloides* CCY 62-02-04 under DPA ihibition at C/N 16

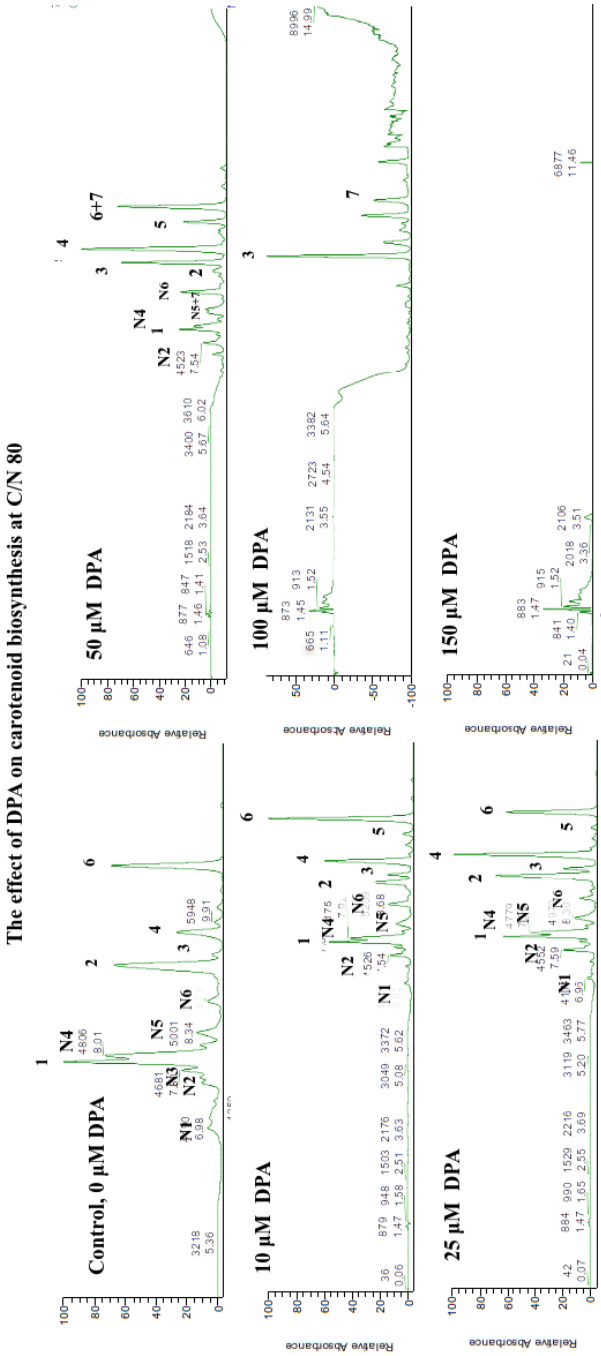

Fig. 9.2: Chromatograms with carotenoids profiles of the original strain *R. toruloides* CCY 62-02-04 under DPA ihibition at C/N 80

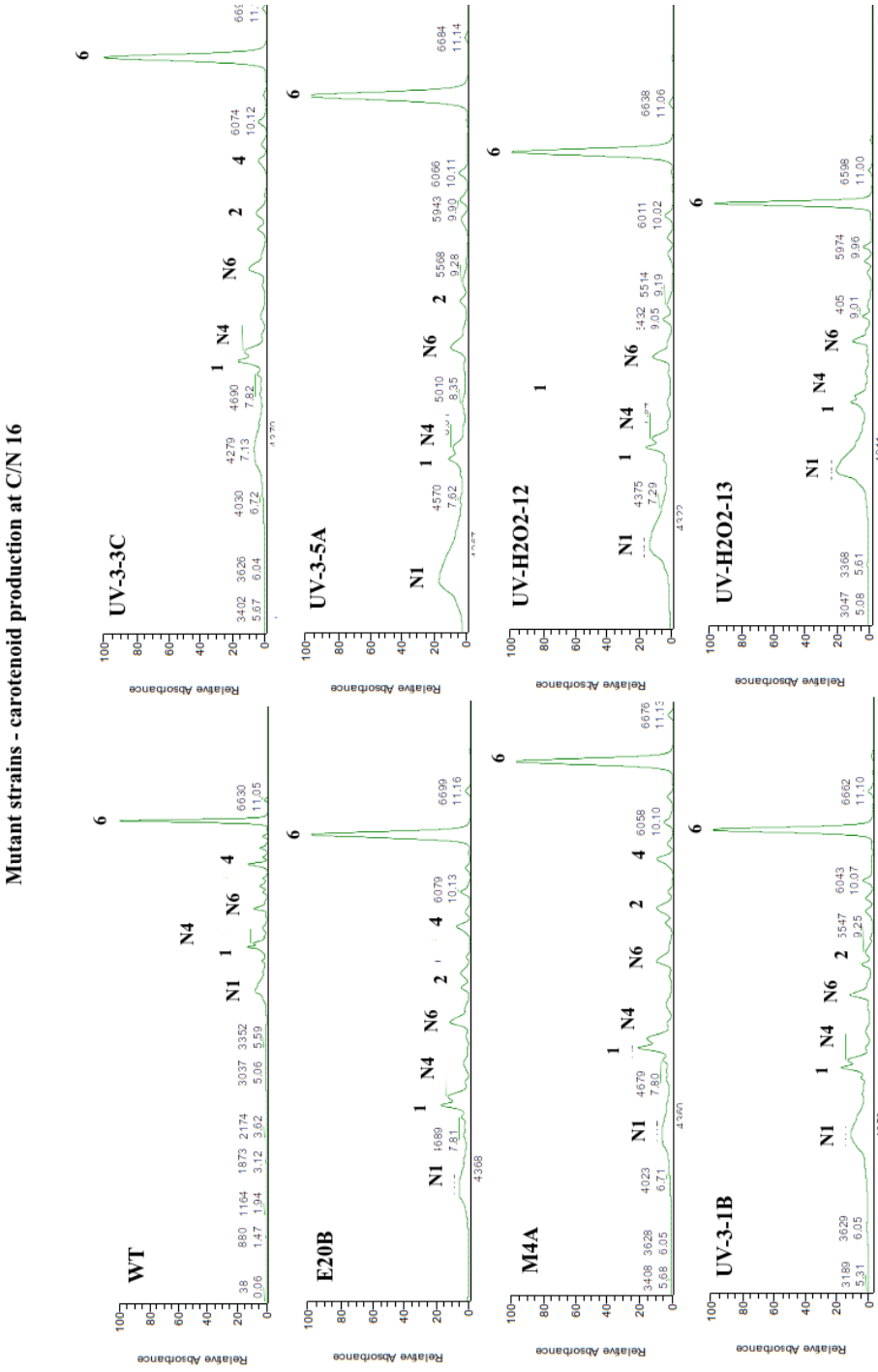

Fig. 9.3: Chromatograms with carotenoids profiles of prepared mutant strains at C/N 16

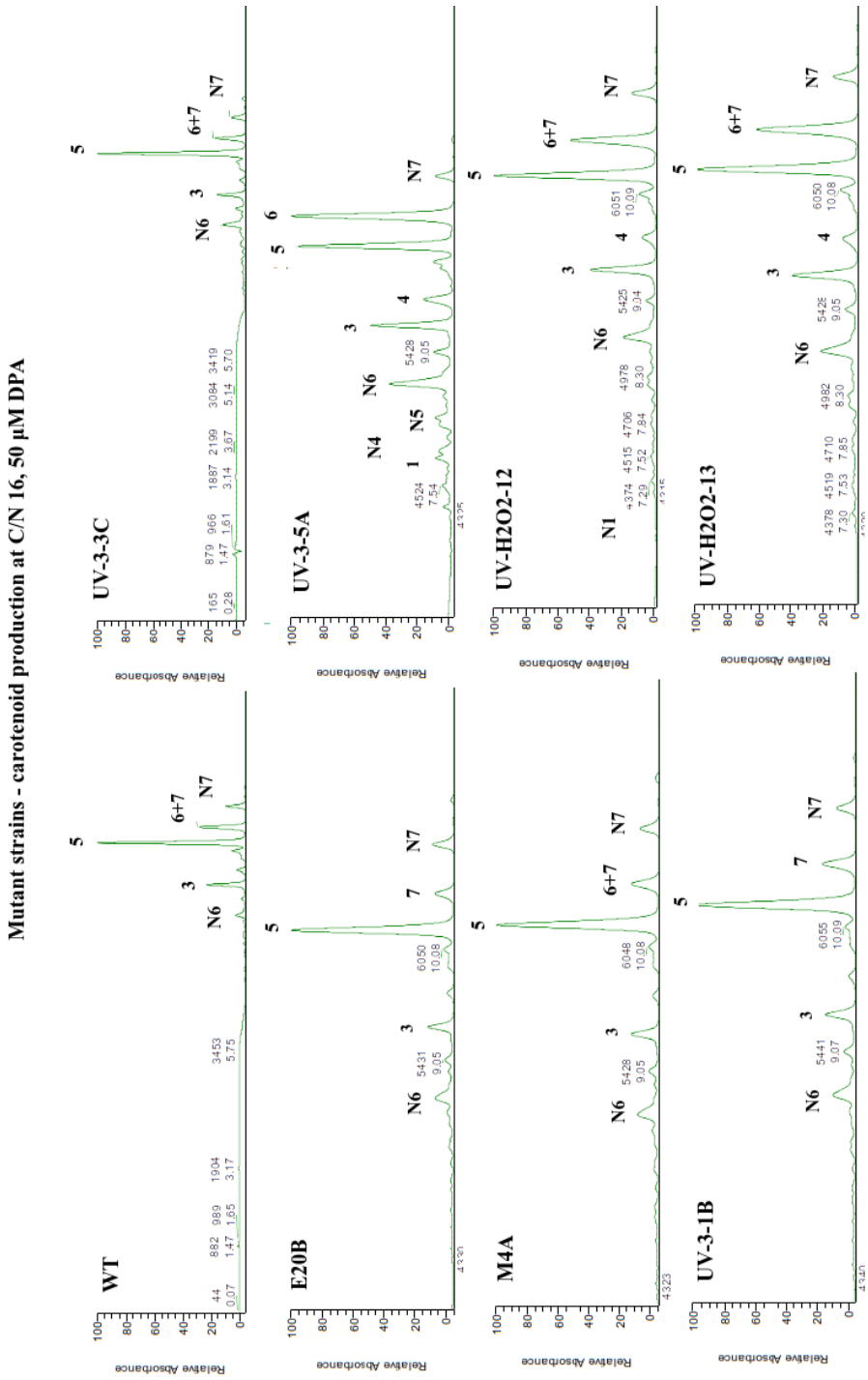

Fig. 9.4: Chromatograms with carotenoids profiles of prepared mutant strains at C/N 16, 50µM DPA concentration

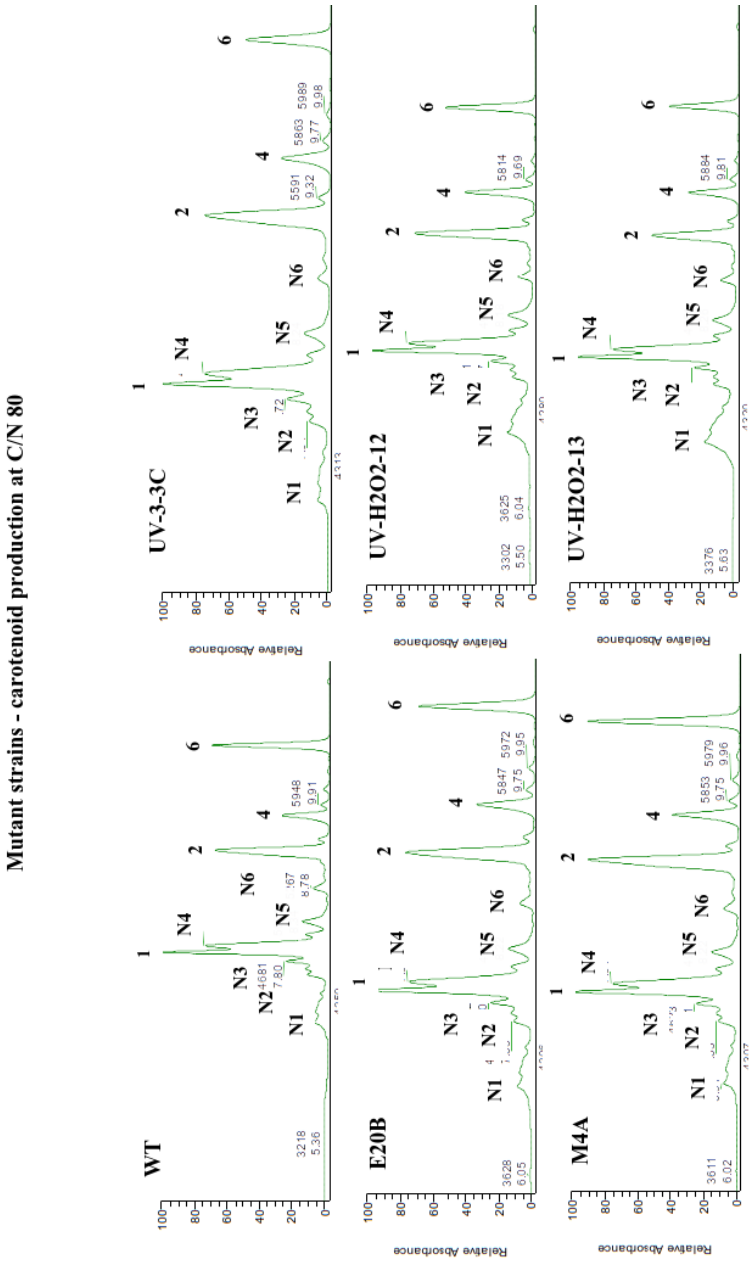

Fig. 9.5: Chromatograms with carotenoids profiles of prepared mutant strains at C/N 80

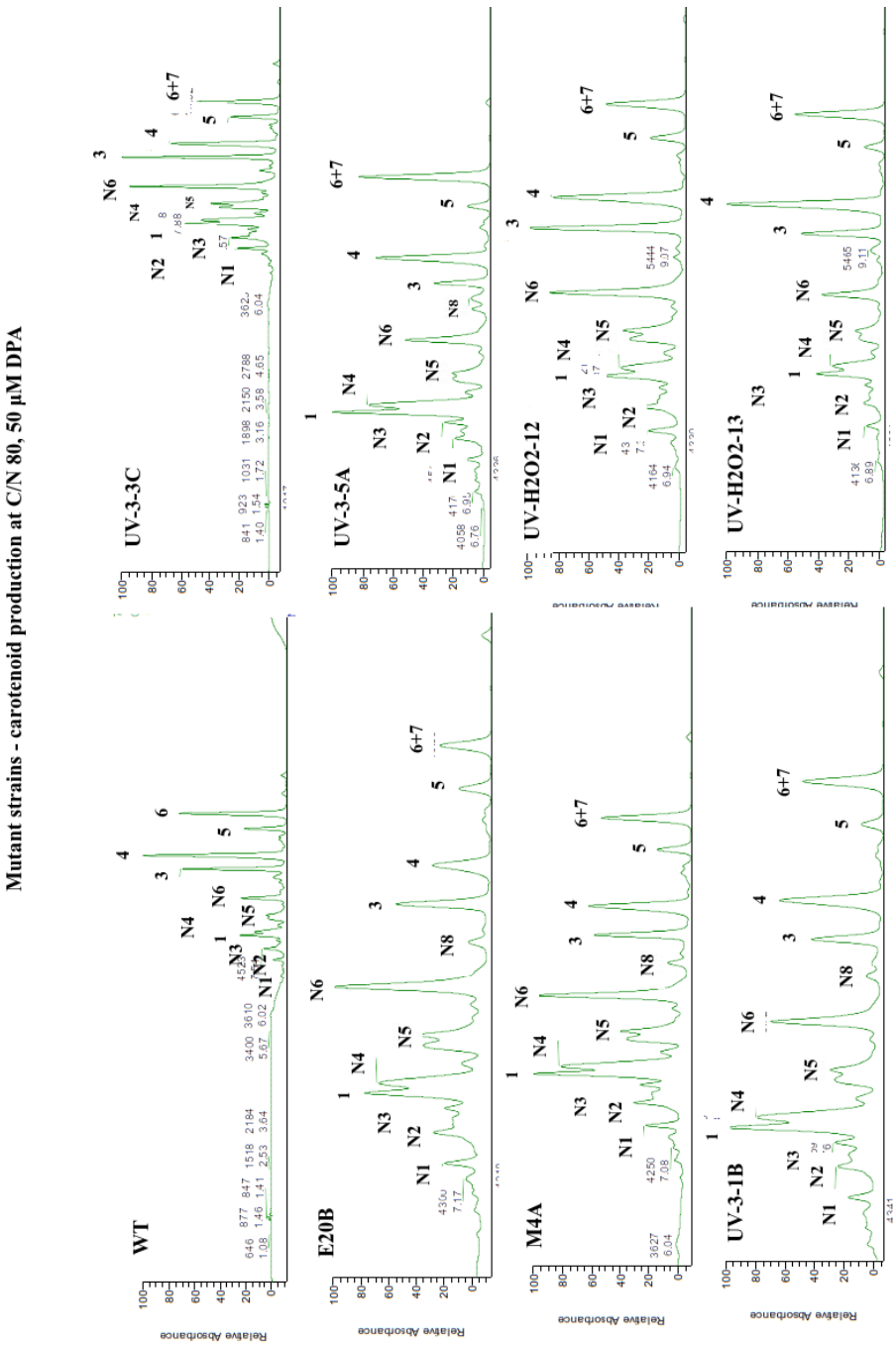

Fig. 9.6: Chromatograms with carotenoids profiles of prepared mutant strains at C/N 80, 50µM DPA concentration

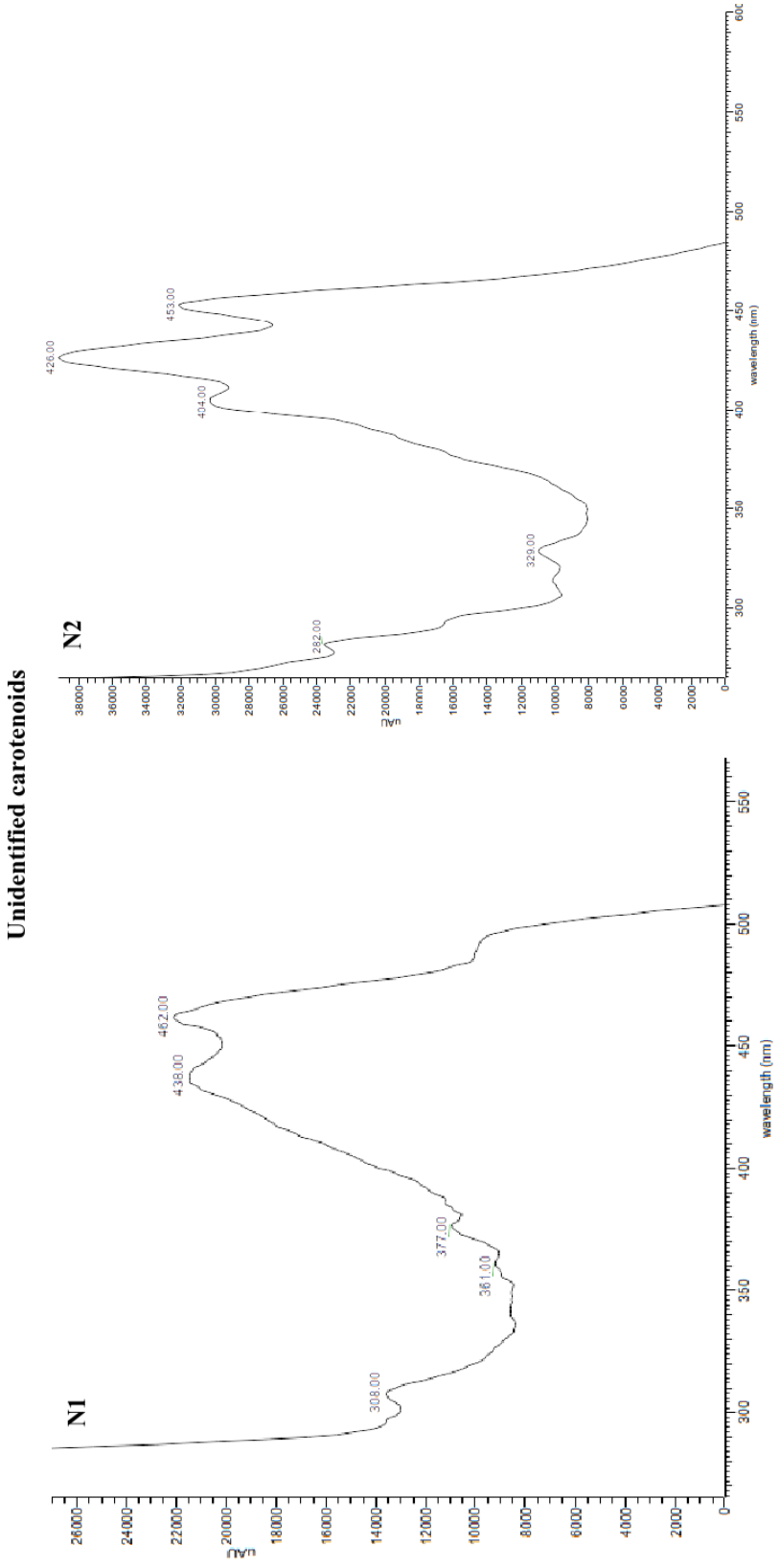

Fig. 9.7: UV-VIS spectrum of unknown carotenoids N1 and N2

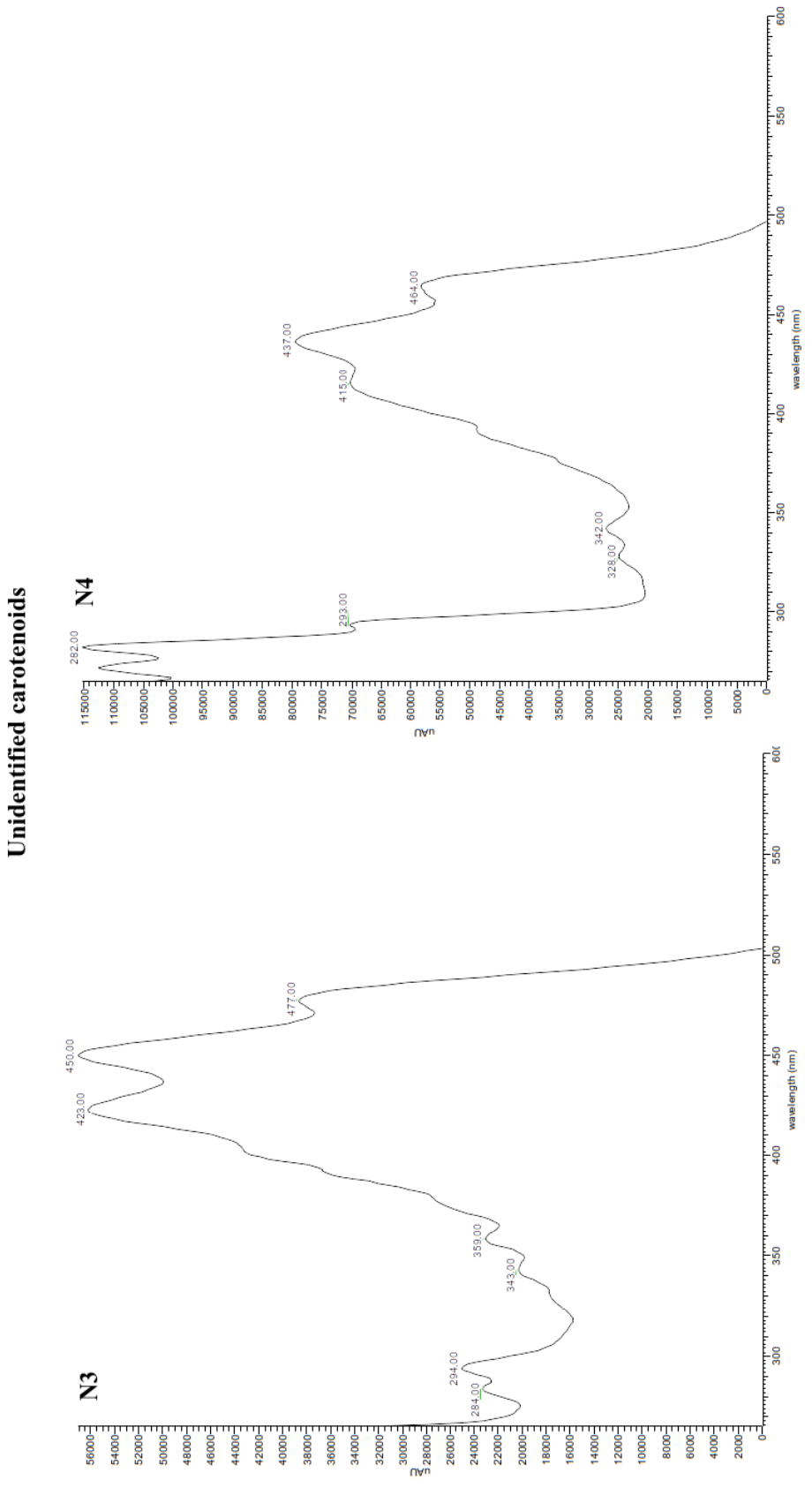

Fig. 9.8: UV-VIS spectrum of unknown carotenoids N3 and N4

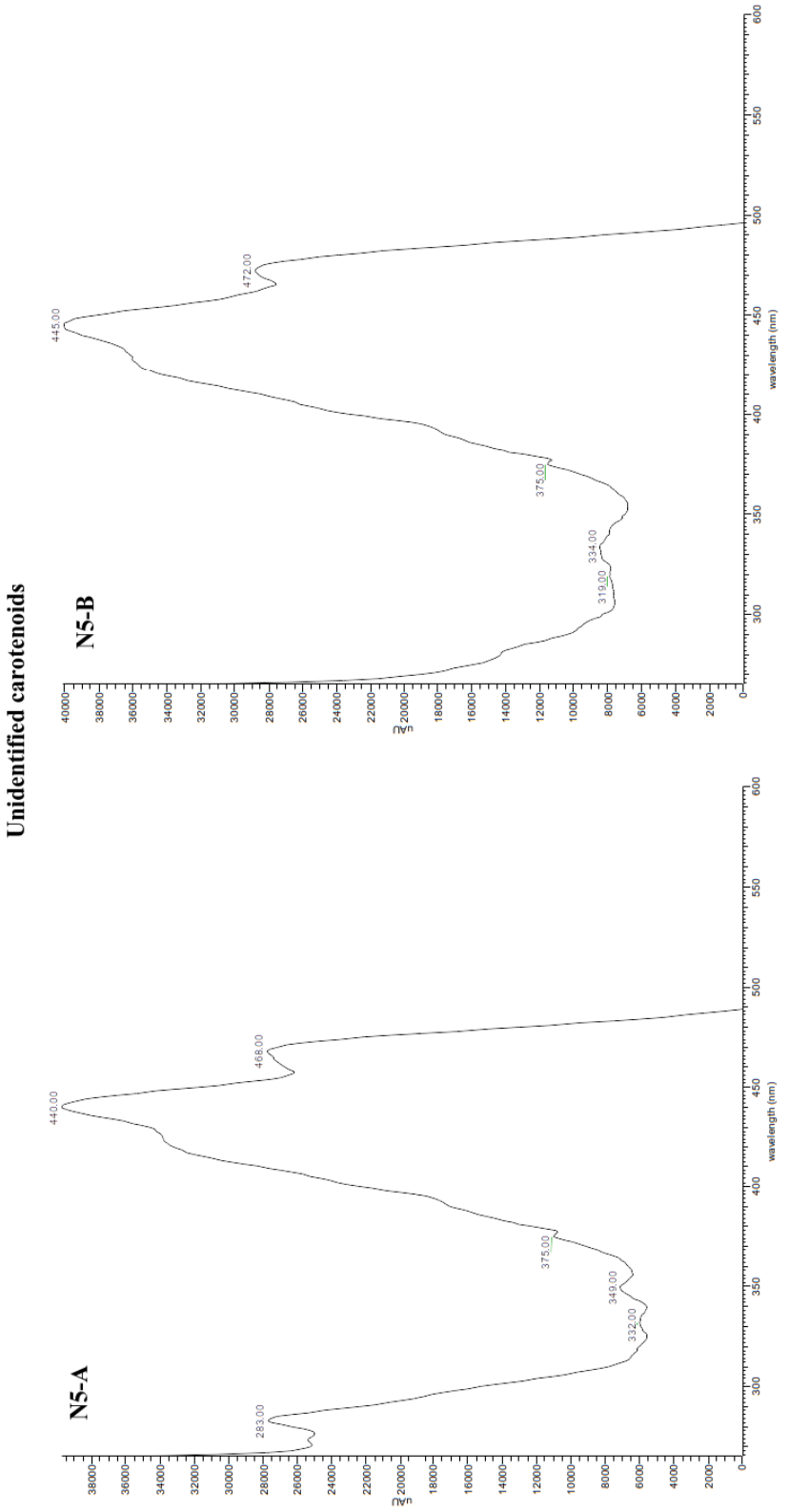

Fig. 9.9: UV-VIS spectrum of unknown carotenoids N5-A and N5-B

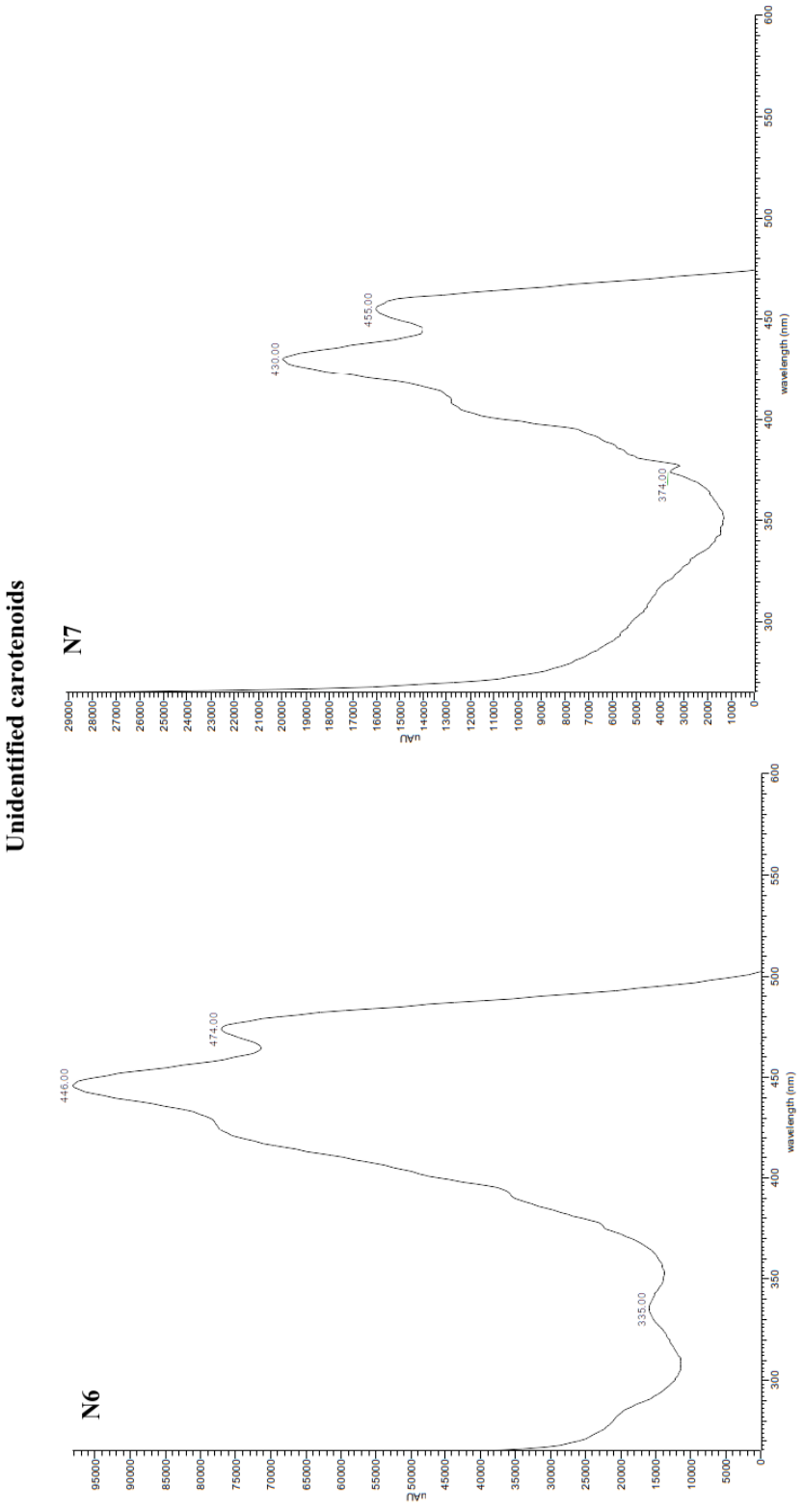

Fig. 9.10: UV-VIS spectrum of unknown carotenoids N6 and N7

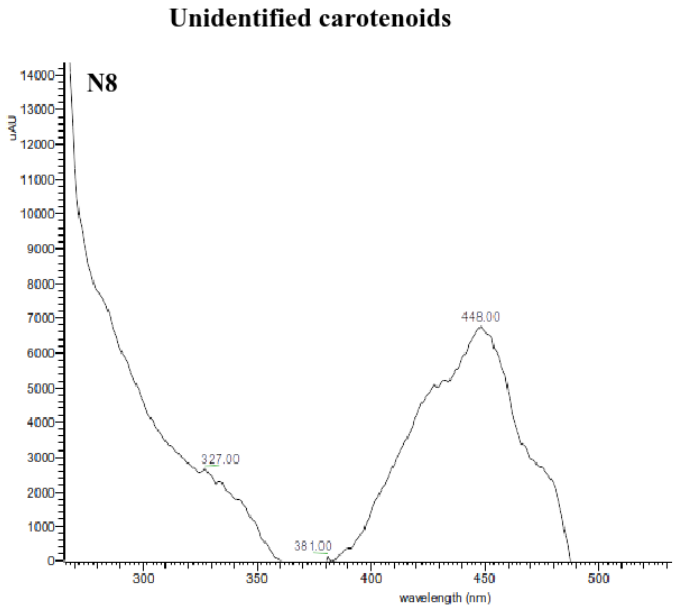

Fig. 9.11: UV-VIS spectrum of unknown carotenoid N8

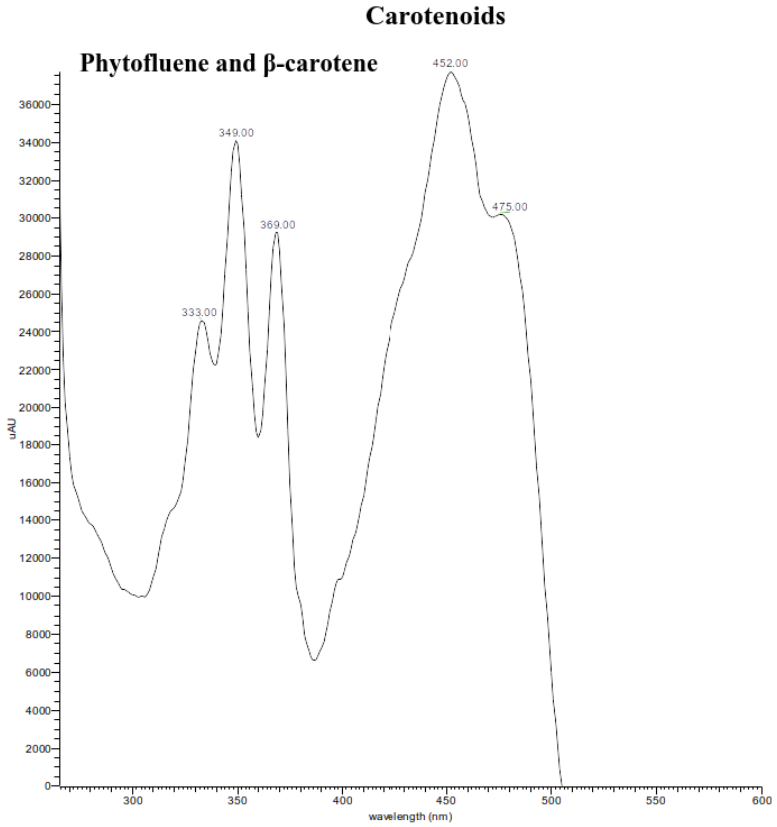

Fig. 9.12: UV-VIS spectrum of β-carotene and phytofluene

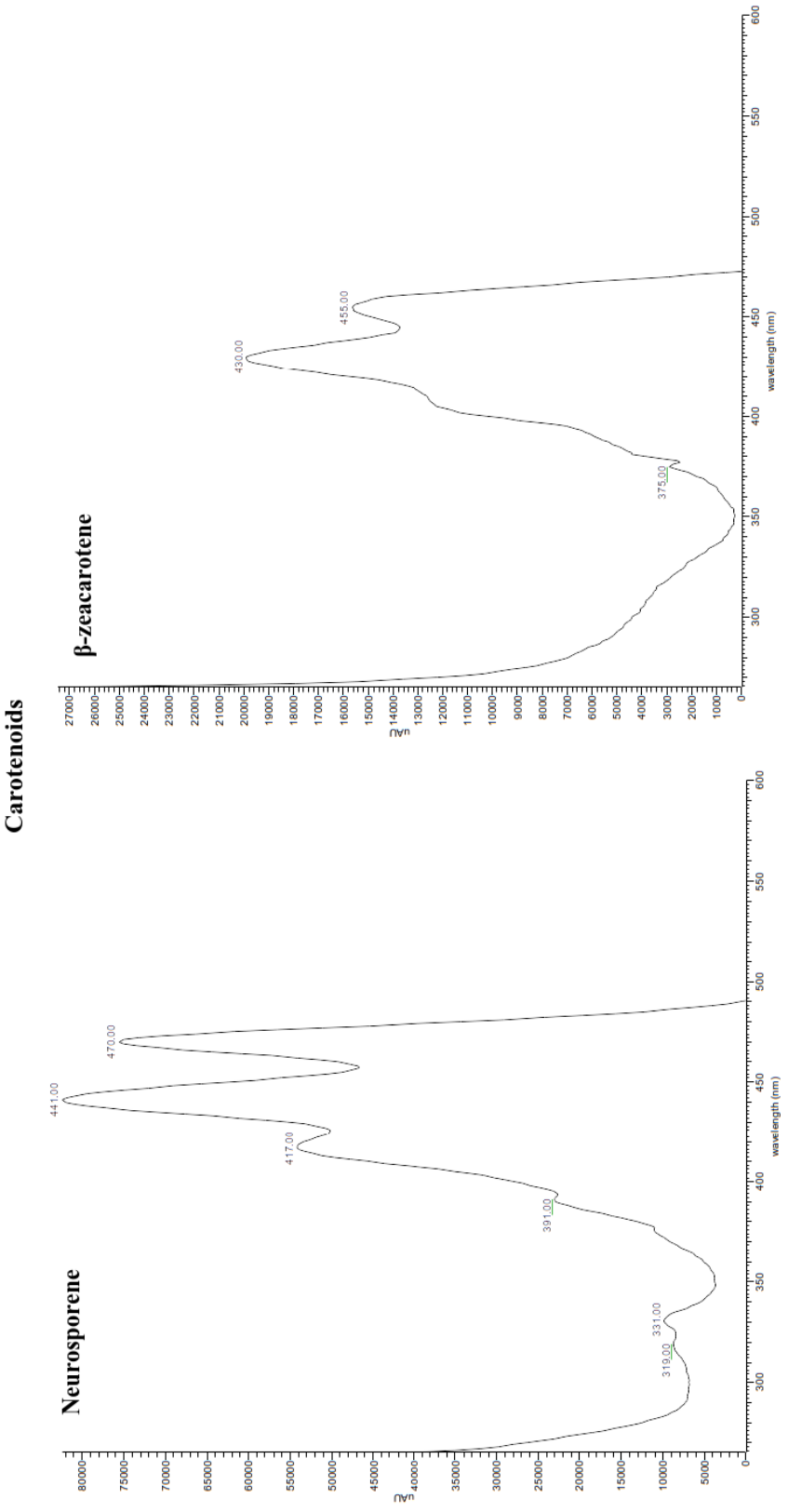

Fig. 9.13: UV-VIS spectrum of β-zeacarotene and neurosporene

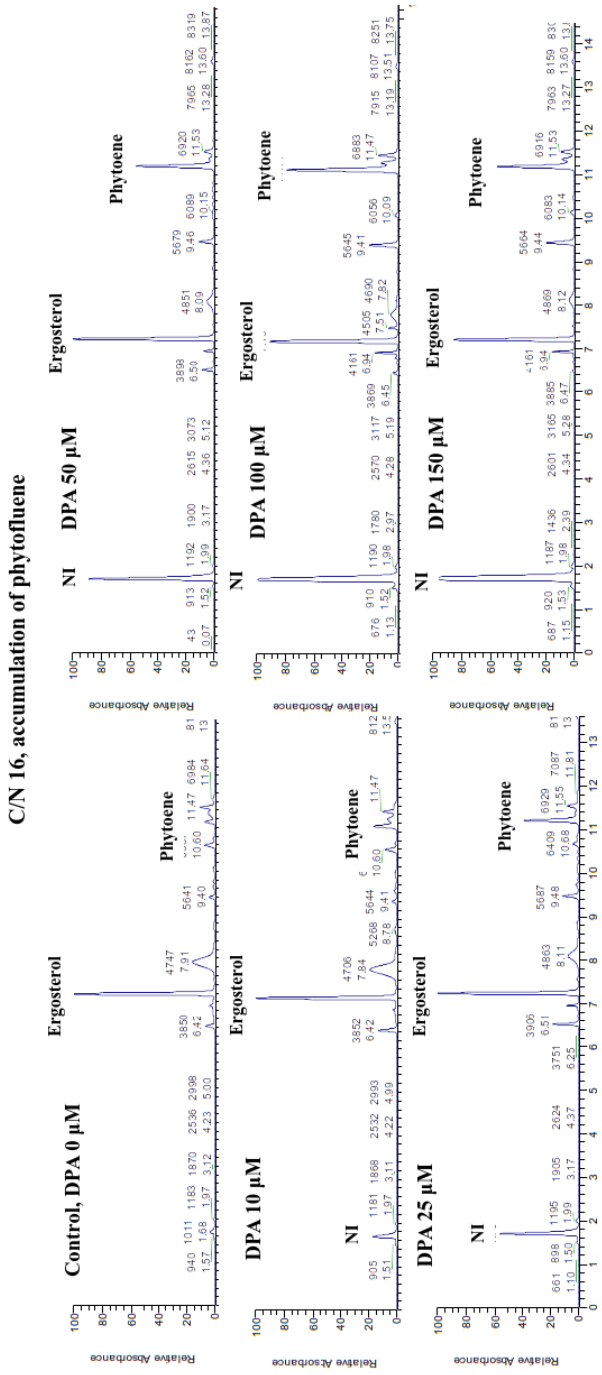

Fig. 9.14: Chromatograms with sterols profiles of the original strain *R. toruloides* CCY 62-02-04 under DPA ihibition at C/N 16

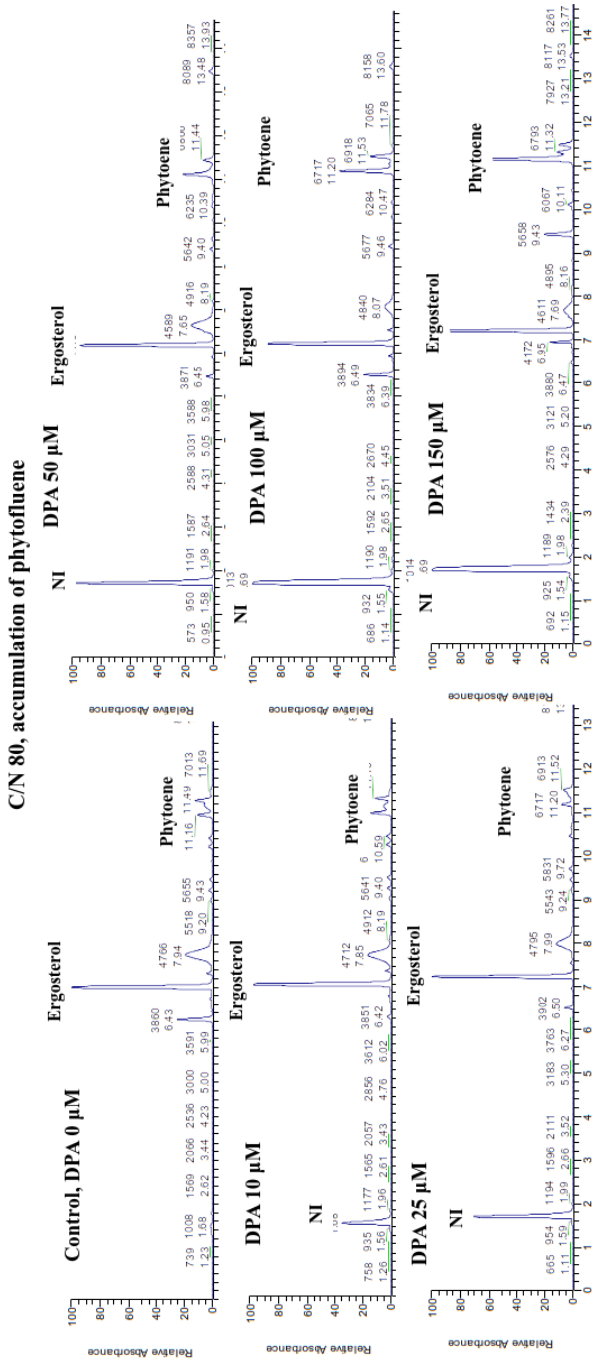

Fig. 9.15: Chromatograms with sterols profiles of the original strain *R. toruloides* CCY 62-02-04 under DPA ihibition at C/N 80

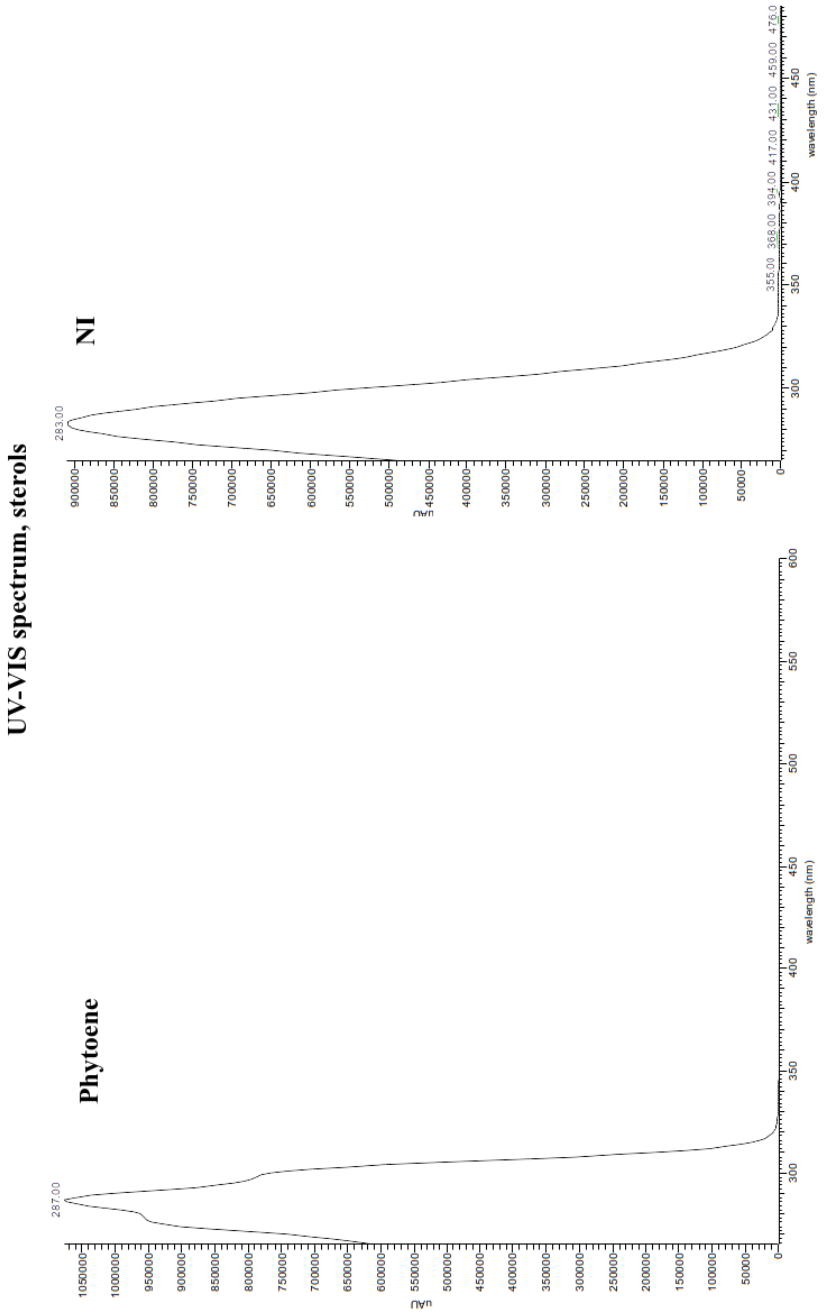

Fig. 9.16: UV-VIS spectrum of phytoene and unknown sterol - NI

ŽIVOTOPIS

Jméno a příjmení: Iveta Kostovová

Vzdělání

01/09/2000–31/05/2008 **Úplné střední odborné vzdělání s maturitou**

Taneční konzervatoř, Brno

01/09/2005–31/05/2008 **Vyšší odborné vzdělání**

Taneční konzervatoř, Brno, zakončené absolutoriem, titul DiS

02/09/2008–31/05/2011 **Bakalářské studium**
Vysoké učení technické v Brně, Fakulta chemická, Ústav chemie potravin a biotechnologií, Brno, zakončené státní zkouškou, titul Bc.

Téma práce: Možnosti využití odpadních lipidů a glycerolu k produkci karotenoidů kvasinkami

01/09/2011–31/05/2013 **Magisterské studium**
Vysoké učení technické v Brně, Fakulta chemická, Ústav Chemie potravin a biotechnologií, Brno, zakončené státní zkouškou, titul Ing

Téma práce: Charakterizace karotenogenních kvasinek pomocí molekulárních technik

01/09/2013– nyní **Doktorské studium – kombinovaná forma studia**

Vysoké učení technické v Brně, Fakulta chemická, Ústav chemie potravin a biotechnologií, Brno

Stáže

20/07/2012–31/08/2012 **Odborná stáž**

Contipro group a.s., Department of Metabolic Engineering, Dolní Dobrouč

Genetická modifikace bakterie *Streptococcus zooepidemicus*

16/03/2015–30/09/2015 **ERASMUS stáž**

INRA, Institut Micalis, Grignon (Francie)

Produkce lipidů pomocí kvasinek druhů *Rhodotorula mucilaginosa* a *Rhodotorula toruloides* na různých zdrojích

uhlíku, genetická modifikace kvasinky *R. toruloides* pomocí biolistické metody.

02/06/2016–30/09/2016 **ERASMUS stáž**

University of Graz, Yeast Genetic and Molecular Biology Group, Graz (Rakousko)

Vývoj a optimalizace metody HPLC pro karotenoidní pigmenty, práce s fermentory, produkce a izolace mikrobiálních lipidů a karotenoidů, kultivace karotenogenních kvasinek

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Petrik, S., Marova, I., Haronikova, A. **Kostovova, I**., Breierova, E., Production of biomass, carotenoid and other lipid metabolites by several red yeast strains cultivated on waste glycerol from biofuel production – a comparative screening study. *Annals of Microbiology,* 63, (2013). https://doi.org/10.1007/s13213-013-0617-x

Užitný vzor:

Hároniková, A.; Márová, I.; **Kostovová, I.;** Vysoké učení technické v Brně: *Suchá biomasa karotenogenních kvasinek druhu C.capitatum CCY10-1-2 pro krmivářské účely*. Užitný vzor CZ 28679, zapsán 2015.

Konferenční příspěvky – sborníky s plným uvedením textu:

Márová, I.; Petrik, S., Hároniková, A., **Kostovová, I**., OBRUČA, S. *Production of carotenoids and other lipidic compounds by red yeasts cultivated on some lignocellulose waste substrates.* Book of Abstracts; BioTech 2014 and 6th Czech- Swiss Symposium with Exhibition. 1. Praha: 2014. s. 147-148. ISBN: 978-80-7080-887- 0.

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Konferenční příspěvky - abstrakty:

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