BRNO UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMISTRY

Ing. Iveta Kostovová

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

Doctoral thesis statement for obtaining the academic title of "Doctor", abbreviated to "Ph.D."

BRNO 2021

Candidate: Ing, Iveta Kostovová
Institute of Food Chemistry and Biotechnology,
FCH BUT,Purkyňova 118, 61200 Brno

Supervisor: prof. RNDr. Ivana Márová, Csc
Institute of Food Chemistry and Biotechnology, FCH BUT,
Purkyňova 118, 61200 Brno

Oponents:

The doctoral thesis statement was distributed on:.....The defense of the doctoral thesis will be held on ..... at ..... before the Board for the Defence of the Doctoral Thesis in the meeting room No. ..... of the Faculty of Chemistry of the BUT in Brno.

Those interested may get acquainted with the doctoral thesis concerned at the Dean Office of the Faculty of Chemistry of the BUT in Brno, at the Department for Science and Research, Purkyňova118, 61200 Brno.

…...….....
Chairman of the Board for the Defence of
the Doctoral Thesis
Faculty of Chemistry of the BUT in Brno
,Purkyňova 118, 61200 Brno

Contents

1 Introduction **5**

2 Theory **6**

2.1 Carotenogenic yeasts and their taxonomy 6

2.1.1 Taxonomy of carotenogenic yeast 6

2.2 Yeast lipid metabolites 7

2.2.1 Carotenoids 7

2.2.2 Yeast lipids 8

2.3 Factors influencing lipid metabolite production 9

2.3.1 Nutritional limitations 9

2.3.2 Impact of physical and chemical mutagenes 9

2.4 Molecular methods 9

2.4.1 Yeast identification methods 10

2.4.2 Yeast genetic modification 10

3 Objectives **12**

4 Methods **13**

4.1 Microorganisms 13

4.2 Media and cultivation conditions 13

4.2.1 Composition of used media 13

4.2.2 Conditions of cultivation for diversity of *R. mucilaginosa* species 14

4.2.3 Cultivation conditions for nitrogen limitation and genetic modification experiments 14

4.2.4 Cultivation conditions for metabolite inhibition and mutagenesis experiments 15

4.2.5 Cultivation conditions for variability within Sporidiobolales and Cystofilobasidiales 15

4.3 Extraction of lipid compounds 15

4.3.1 Methanol-chloroform extraction 15

4.4 Analysis of yeast metabolites 16

4.4.1 Carotenoid and ergosterol analysis 16

4.4.2 Fatty acid analysis 16

4.5 Molecular methods 17

4.5.1 Yeast identification using PCR-DGGE analysis 17

4.5.2 Vector preparation and genetic modification 18

4.6 Mutagenesis experiments 19

4.6.1 Chemical and physical mutagenesis 19

5 Results and discussion **20**
5.1 Diversity of *Rhodotorula mucilaginosa* species 20
5.1.1 Molecular characterization of yeast strains 20
5.1.2 Biomass, TG, carotenoids and ergosterol productions . . 22
5.2 Fatty acids production under nitrogen limitation 23
5.2.1 Impact of nitrogen limitation and carbon source 24
5.3 Genetic modification of *R. toruloides* CBS 6016 using biolistic method 28
5.3.1 Vector construction and genetic modification 28
5.3.2 Fatty acids production by genetically modified strains . . 29
5.4 Impact of inhibitors, physical and chemical mutagenes on metabolite production 32
5.4.1 Metabolite production under DPA inhibition and nitrogen limitation 32
5.4.2 Mutagenesis 36
5.4.3 Carotenoid production of mutant strains 36
5.5 Variability of metabolites produced by yeasts within Sporidiobolales and Cystofilobasidiales 38
5.5.1 Fatty acids accumulation 38
5.5.2 Fatty acids composition 38
5.5.3 Synthesis of carotenoids and their profile 39

6 Conclusions **42**

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.

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.

Theory

2.1 Carotenogenic yeasts and their taxonomy

Carotenogenic yeasts grow as pigmented colonies and are for this reason known as "red yeasts"[1]. 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 [2]. 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 and animal feed industries [2].

2.1.1 Taxonomy of carotenogenic yeast

Carotenogenic yeasts mainly belong to subdivision Basidiomycotina, to the subphyla of Puccniomycotina, Ustilaginomycotina and Agaricomycotina [3]. The most known carotenogenic yeasts are spread within subhylums Pucciniomycotina and Agaricomycotina [4, 5]. The most known carotenoid forming yeasts within *Pucciniomycotina* are the yeast of genus *Rhodotorula* and *Sporobolomyces* [6]. The carotenogenic yeasts most commonly used in biotechnology belong to the genus *Rhodotorula, Rhodosporidiobolus* and *Sporobolomyces*, that are placed in the order of Sporidiobolales of the class Microbotriomycetes [4].

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 [5]. 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 [5].

Sporobolomyces

The genus *Sporobolomyces* now includes *Sporobolomyces* species and their sexual counterparts *Sporidiobolus*. In some conditions, they form pseudohyphae or true hyphae. Interestingly, they can form balistoconidia [3]. The shape of the cells can be ellipsoid, spindle, or cylindrical. The asexual reproduction is mediated by budding. The well-known members of this genus are: *Sporobolomyces roseus*, *Sporobolomyces pararoseus*, *S. salmonicolor*, *S. metaroseus*, and *S. jonsonii* [4, 3].

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* [4]. In some species can be observed sexual reproduction. 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. Some *Rhodotorula* species are exceptionally good at lipid accumulation, lipids can form almost 70 % of their biomass dry weight at certain conditions [7]. The most well-known species of the genus are *Rhodotorula toruloides, R. glutinis R. kratochvilovae, R. babjevae, R. graminis*, and *R. diobovata* [4].

Cystofilobasidium

The most well-known species within the genus *Cystofilobasidium* are *Cystofilobasidium capitatum, C. infirmominiatum, C. macerans, C. bisporidii* and *C. ferigula*. The reproduction can be asexual or sexual (in some species) [3]. The asexual reproduction can occur in a form of polar or bipolar budding. Mostly are *Cystofilobasidium* species mesophilic, exceptionally they can be psychrophilic. They are able to produce β-carotene, γ-carotene, torulene and torularhodin. Typical is also the production of CoQ-9 (Coenzyme Q-9) and CoQ-10 [3, 8, 5].

Phaffia

The genus *Phaffia* currently contains only one species *Phaffia rhodozyma* [5]. 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 [9]. The vegetative reproduction is done by budding [3]. 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 [9]. They have the capacity to perform sugar fermentation. Typical is the production of CoQ-10 [5, 3].

2.2 Yeast lipid metabolites

2.2.1 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 [10, 11].

Yeast carotenoids

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 [6]. Interestingly, *Phaffia rhodozyma* produces as the main carotenoid astaxanthin, β-carotene and echinenone, production of torulene and torularhodin was also reported [12].

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 [13]. 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 [14].

Astaxanthin is carotenoid which is responsible for the red color of lobsters, shrimps, crabs, and especially salmons. The molecular formula of astaxanthin is $C_{40}H_{52}O_4$ [15]. 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*.

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 majority of these studies used rats as a model system [6].

2.2.2 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 [16]. 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

[17]. 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. [16].

2.3 Factors influencing lipid metabolite production

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.

2.3.1 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. 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 ratios. With increasing C/N ratio is reported increased production of torulene and torularhodin and decreased production of PUFA in carotenogenic yeasts [18].

2.3.2 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 [19, 20, 21, 22].

2.4 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.

2.4.1 Yeast identification methods

Traditionally were used morphological methods for yeast and fungal identification. However, in some lineages of fungi, morphological character can be contentious or problematic even for trained mycologist [23]. Nowadays are therefore in mycology used short DNA sequence markers frequently based on ribosomal gene sequences for fungal identification [5, 4, 24, 23].

Ribosomal barcoding

The sequences of yeast ribosomal genes (rDNA) exhibit a sufficient level of variation to serve as short DNA sequence markers. 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, 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. [25, 23]. 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. 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 [3, 26, 24, 23].

2.4.2 Yeast genetic modification

In yeast cloning is frequently used a yeast cloning cassette (YCC) that can be prepared as a part of the chosen plasmid. Plasmid can be propagated in *E. coli*. 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. 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 [27, 28, 29]

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. Microparticles are then accelerated to a high speed with a special apparatus called a particle gun [30, 31]. In case of yeast transformation are 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 [31].

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

Methods

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.

Table 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 Media and cultivation conditions

4.2.1 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.

Table 4.2: 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				

4.2.2 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.

4.2.3 Cultivation conditions for nitrogen limitation and genetic modification experiments

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.

4.2.4 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. For every cultivation experiment was the strain inoculated on agar plates containing solid YPD medium. 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.

4.2.5 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.

4.3 Extraction of lipid compounds

4.3.1 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.

4.4 Analysis of yeast metabolites

4.4.1 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.

4.4.2 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 (Thermo-Quest 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.

4.5 Molecular methods

4.5.1 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. [23]. 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. The used concentrations of denaturants (urea and formamide) were 50, 60, 70, and 80%. 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).

4.5.2 Vector preparation and genetic modification

Vector preparation

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. Amplified genes DAG1 and GPD1 were after digestion 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 (see chapter 5.3, Fig. 5.8). 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. 4.3.

Table 4.3: 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 *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. Then, the suspension of golden beads (60 mg/ml) 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 15 μl resulting suspension of prepared golden beads was transferred to biolistic membranes. 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)

4.6 Mutagenesis experiments

4.6.1 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). 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.

Results and discussion

5.1 Diversity of *Rhodotorula mucilaginosa* species

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.

5.1.1 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* [32]. 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. 5.1 and Tab. 5.2.

Table 5.1: 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*

Table 5.2: 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 [23]. The PCR products were used for sequence analysis by optimized DGGE method. Except *R.mucilaginosa* species were also analyzed other carotenogenic yeast strains (Tab. 5.2, 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%.

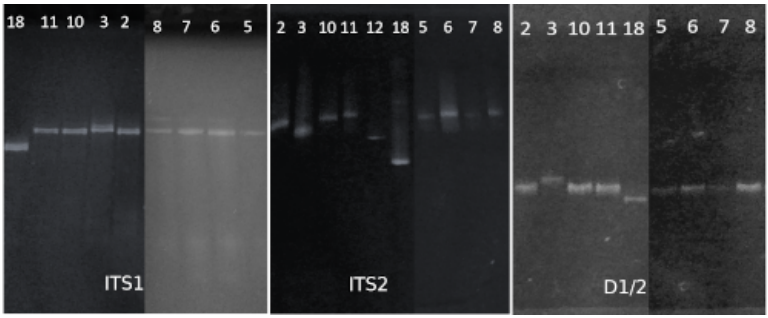

Figure 5.1: DGGE gel profiles of ITS1 (range denaturants: 60-80%), ITS2 (range of denaturants: 50-70%) and D1/2 regions of rDNA (range of denaturants: 50-80%), 3 -*R. glutinis*, 18- *R. toruloides*, numbers 2,5,6,7,8,10, and 11 represents different *R.mucilaginosa* strains (see Tab. 5.1 and Tab. 5.2)

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. 5.1). The results of DGGE analysis supported previous results of yeast identification by sequencing [32], where only strains *R. toruloides* and *R.glutinis* proved different DGGE profiles. 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 the genome and dominantly inherited [33]. 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 [34]. 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

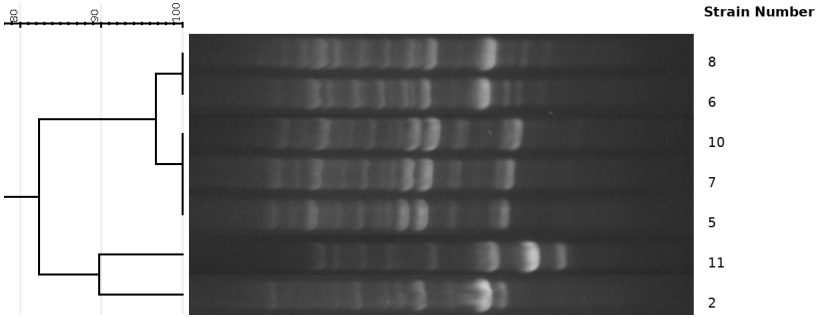

Figure 5.2: The GTG_5 microsatellite length polymorphism based dendrogram of *R.mucilaginosa* strains, numbers correspond to Tab. 5.1

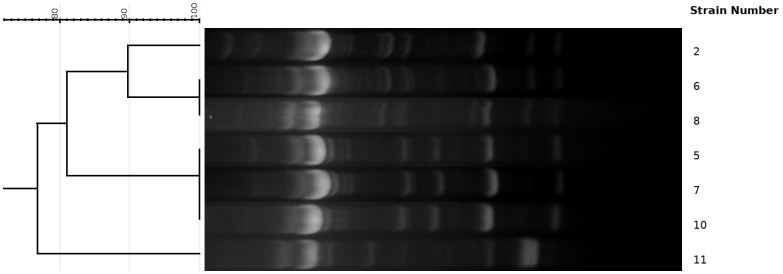

Figure 5.3: The M13 minisatelite length polymorphism based dendrogram of *R.mucilaginosa* strains, numbers correspond to Tab. 5.1

with specific band profiles (see Fig. 5.2 and 5.3). 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.

5.1.2 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 [6]. 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

[35, 36]. The best carotenoid producing strain in this study was able to produce almost 2.19 mg/g of carotenoids per biomass.

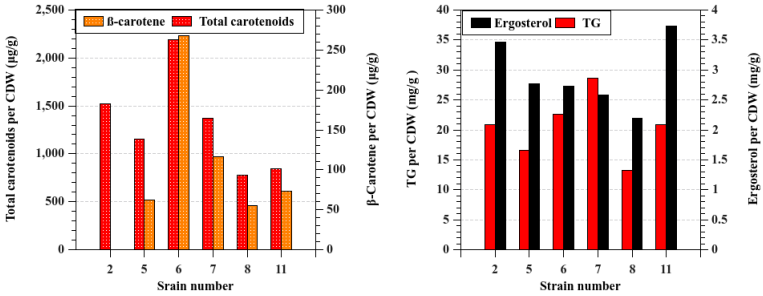

Figure 5.4: Metabolite production at 72 hours of cultivation. Numbers 2, 5, 6, 7, 8, 10, and 11 belong to *R.mucilaginosa* strains (designation can be see in Tab. 5.1), the results correspond to the average values calculated from the two independent experiments

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 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. The highest accumulated amount of ergosterol reached strains number 2 and 11 (more than 3 mg/g, see Fig. 5.4), 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. Intraspecies variability among used strains based on micro and minisatelite sequence analysis may correspond to their different genotypes.

5.2 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. 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 [36]. 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 [37]. 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 [38]. The essential parameters for fatty acid production are the selection of suitable yeast strains and optimized cultivation conditions [39]. 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 [39]. Nitrogen limitation is a stress condition leading to increased lipid accumulation in oleaginous yeasts [40, 16, 41, 42]. 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.

5.2.1 Impact of nitrogen limitation and carbon source

For cultivation on glucose were selected following 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 [43, 44].

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. 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. 5.5). 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 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.

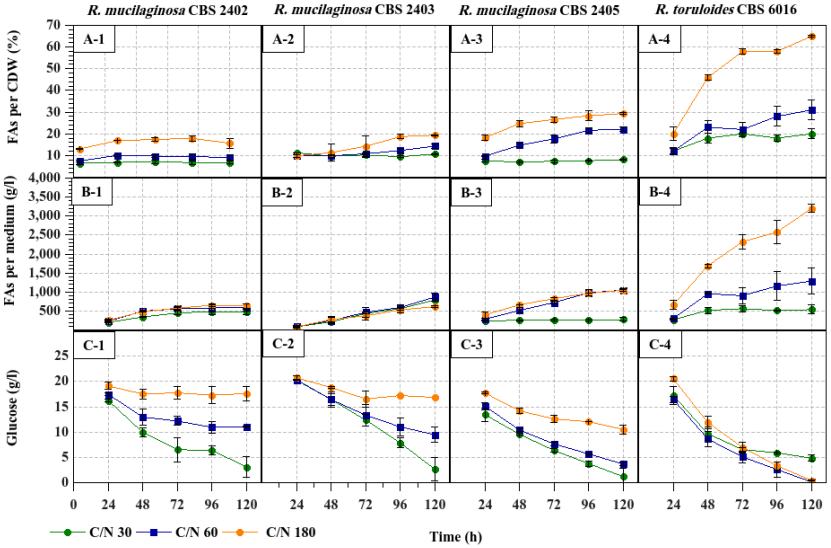

Figure 5.5: Productions of FAs and glucose consumption in time. A(1-4) - FAs production per CDW, B(1-4) - FAs production per medium, C(1-4) - glucose consumption. (A-C) - 1-3 corresponds to strain *R. mucilaginosa* CBS 2402, 2403 and 2405, (A-C) - 4 corresponds to strain *R. toruloides* CBS 6016

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 [45].

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 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 in 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.

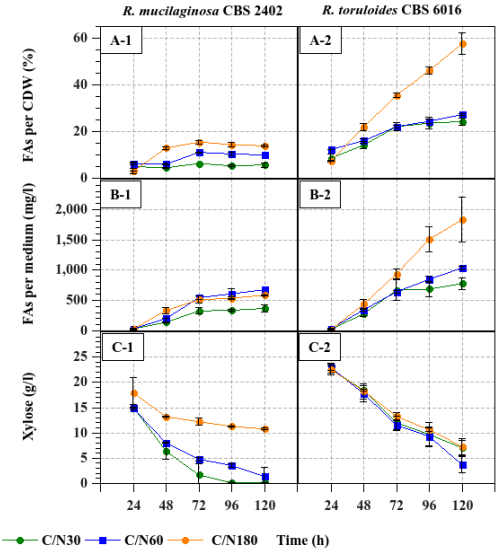

Figure 5.6: Productions of FAs and xylose consumption in time. A(1-2) - FAs production per CDW, B A(1-2)-FAs production per medium, C A(1-2) - xylose consumption. (A)-1-2 corresponds to strain *R. mucilaginosa* CBS 2402, (A-C)-2 corresponds to strain*R. toruloides* CBS 6016

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 of Tiukova et al. [46].

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. 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. 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. 5.6). *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 [45].

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.

Glycerol was as well as xylose more difficult substrate than glucose for *R. toruloides* CBS 6016. The increased C/N ratio and glycerol caused delayed FAs accumulation within CDW (see Fig. 5.7). Possibly, was nitrogen depleted later in medium with glycerol or xylose, than on medium containing glucose [46].

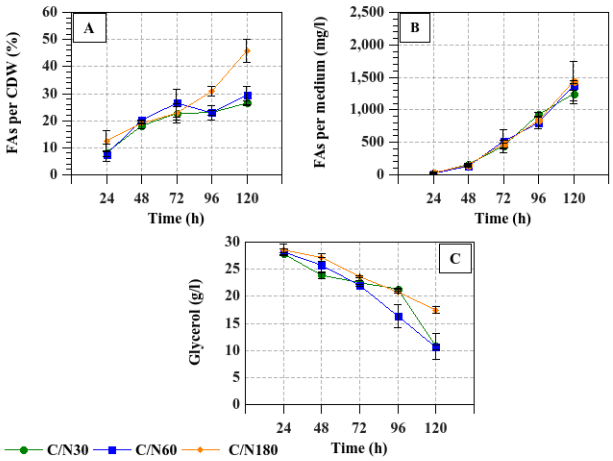

Figure 5.7: Productions of FAs and glycerol consumption in time by strain *R. toruloides* CBS 6016

The capability of *R. toruloides* CBS 6016 to accumulate high yields of FAs in nitrogen limiting conditions corresponds to the behavior of oleaginous microorganism [47]. 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 % (see Fig. 5.7).

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. [48], Li [49], and Singh et al. [50].

5.3 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 [51].

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 overexpression. 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 [52, 53].

5.3.1 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.

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. 5.8. After transformation by biolistic method were transformed yeasts grown on YNB plates with nourseotricine. Transformed yeasts were verified by two PCR reactions, where first of them included primers for pTEF promoter and a part of the 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. 5.9.

Verification PCR was done for four selected clones for each overexpressed gene. As a positive control was used plasmid bearing the corresponding expres-

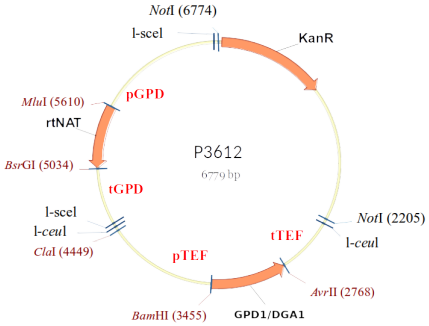

Figure 5.8: Map of modified plasmid used for vector preparation

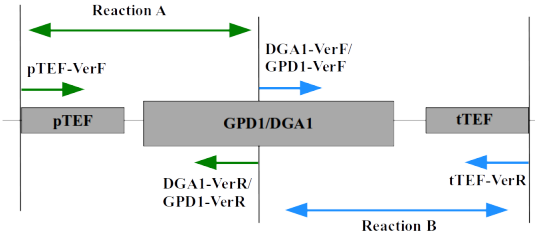

Figure 5.9: Scheme of verification PCR reaction, pTEF - TEF promotor sequence, tTEF - TEF terminator sequence, GPD1/DGA1 - inserted genes, small arrows represents locations of verification primers and big arrows visualize amplified regions (reaction A/B)

sion 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. 5.10). To examine impact of genetic manipulation on lipid production DGA1 clone 1 and GPD1 clone 4 were used for subsequent cultivation experiments.

5.3.2 Fatty acids production by genetically modified strains

Chosen clones were cultivated on YNB medium with glucose at C/N ratio 180. The chosen clones proved significantly better biomass production then the control WT strain, the results can be seen in Fig. 5.11. 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 had proved to have slower growth during 48 hours of cultivation, but after 72 hours the biomass production

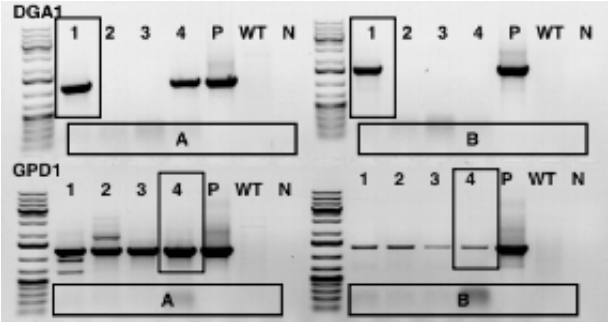

Figure 5.10: Results of verification PCR, numbers 1- 4 represents chosen clones, P – plasmid bearing expression cassettes (DGA1 or GPD1), WT – wild type strain of *R. toruloides*, N – negative control

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.

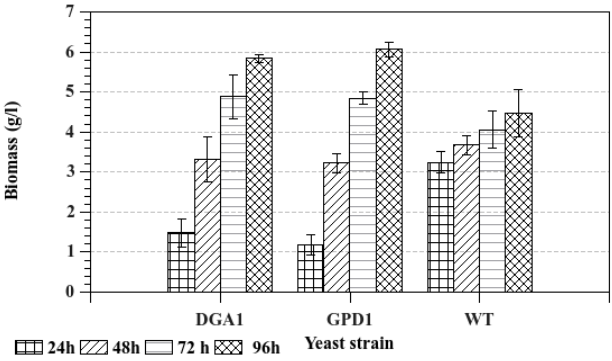

Figure 5.11: Biomass growth during cultivation time by wild type and transformants

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. 5.12). 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 [52].

The diacylglycerol acyltransferase (DAG1) is taking part in the terminal step

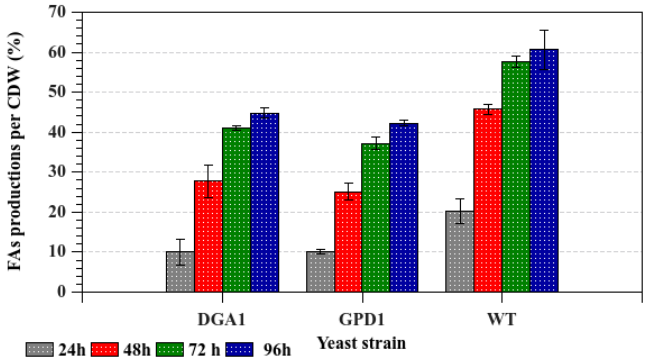

Figure 5.12: Productions of FAs during cultivation time by wild type and transformants

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 [54]. 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 [53]. 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).

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.

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

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. Generally, exploitation of random mutagenesis targeting increased carotenoid productions have difficulties in selection of overproducing mutants. In yeasts or algae are frequently used inhibitors of carotenoid synthesis as cinnamyl alcohol, thymol, dimethyl phthalate, veratrol, or diphenylamine (DPA) [55]. Inhibitors of carotenoid biosynthesis frequently affect activity of phytoene desaturase (PDS). 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 resistant mutants form yellow, pink or red colonies [55, 56].

DPA is an inhibitor with sufficient carotenoid biosynthesis inhibition and minimal impact on biomass production [55, 56]. 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.

5.4.1 Metabolite production under DPA inhibition and nitrogen limitation

The DPA inhibition lead in *Rhodotorula* sp. to decreased activity of phytoene desaturase [56]. But the general resistance to DPA is species specific and therefore prior usage, the experimentally defined DPA concentration is needed [55, 56]. Also, the impact of the C/N ratio in combination 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.

Impact of DPA in combination with different C/N ratios

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*) [3] or *R. glutinis* species [56]. 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*. *R. toruloides* CCY 62-02-04 was able to grow without severe inhibition of growth even in DPA concentrations 100 and 150 μM. W ith 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 [57, 56].

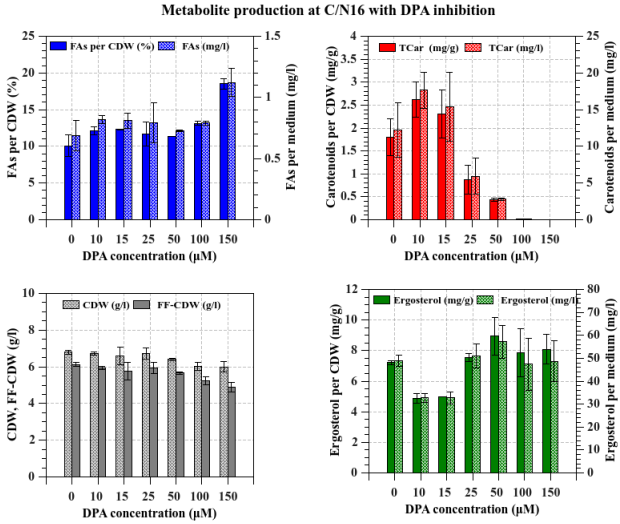

Figure 5.13: Metabolite production by *R. toruloides* at C/N 16 under DPA inhibition

Increased C/N ratio (80) 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 conditions were detected in-

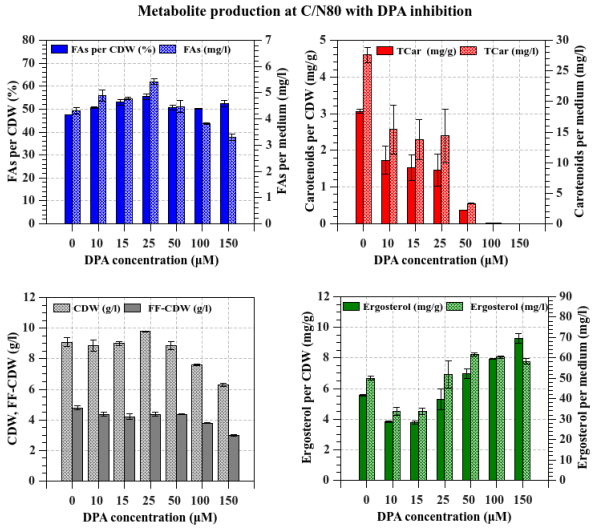

Figure 5.14: Metabolite production by *R. toruloides* at C/N 80 under DPA inhibition

creased production of carotenoids in comparison to control. Furthermore, ergosterol content in CDW dramatically decreased. The same effect was observed also at C/N 80.

Fatty acids and carotenoid composition

The composition of fatty acids and carotenoids is visualized in Fig. 5.15. 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 [56].

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

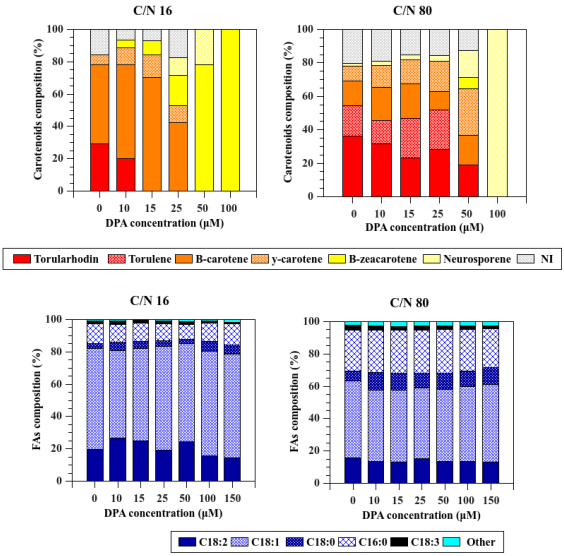

Figure 5.15: Impact of nitrogen limitation and DPA inhibition to fatty acids and carotenoids composition

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. Therefore, the enzyme phytoene desaturase *crtI* is probably the most inhibited enzyme under DPA inhibition. 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 [56]. 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 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*) [40]. 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 [6]. 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.[56].

5.4.2 Mutagenesis

The greatest decrease in survivals was after 3 min of UV 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 [58]. EMS mutagen was used in two amounts 50 and 75 µl and after incubation of cell cultures with mutagens. The survival rate 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 %.

5.4.3 Carotenoid production of 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).

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 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. 5.16).

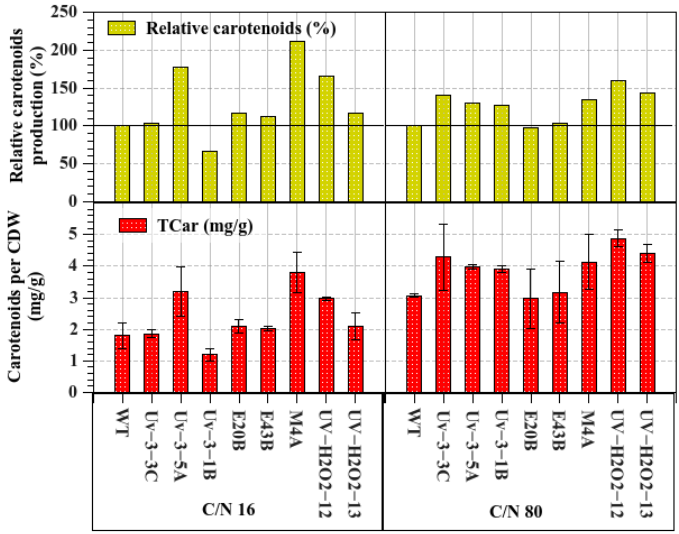

Figure 5.16: Relative carotenoids production and carotenoid productions at C/N 16 and C/N 80.

Table 5.3: 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 [35]. 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.

5.5 Variability of metabolites produced by 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*.

5.5.1 Fatty acids accumulation

The most dramatic accumulation of FAs, was observed in strains *C. macerans* CCY 10-01-02 (CM) and *S. metaroseus* CCY 19-6-20 (SM). Fig. 5.17 shows productions of FAs at 96 hours of cultivation. *R. mucilaginosa* strains had the lowest FA production. 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. 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 [59, 60]. 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).

5.5.2 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. 5.18 correspond to the highest accumulated amounts of FAs, obtained after 96 hours of cultivation. 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 [61, 44].

Sporobolomyces species (previously known as *Sporidiobolus*) [62] accumulated mainly oleic acid (42-56 %), with linoleic acid being the second most accumulated fatty acid (22-30 %).

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.

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.

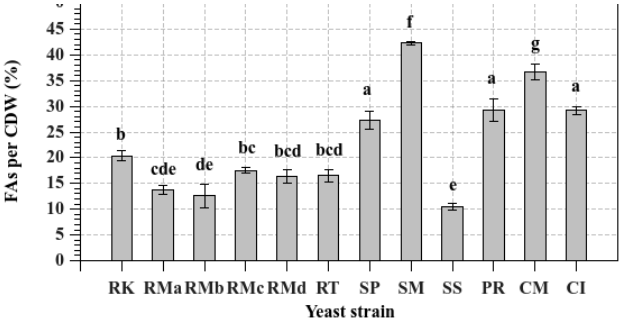

Figure 5.17: Fatty acid production after 96 hours of cultivation, graph represents production of FAs in CDW, letter indexes above the columns a, b, etc. mean homogenous groups (ANOVA, Tukey test, α=0.05)

5.5.3 Synthesis of carotenoids and their profile

To evaluate carotenoid production properties, the sorting proposed in the study of El-Banna et al. [35] 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

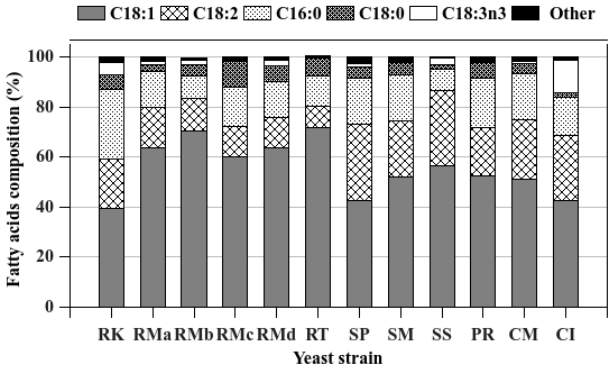

Figure 5.18: Fatty acid composition expressed as % of total fatty acids per biomass after 96 hours of cultivation (mg/g)

carotenoid production. The results summarised in Fig. 5.19 correspond to the cultivation time with the highest accumulated amounts of carotenoids. 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 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 (more than 80 % of total carotenoids). *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. 5.20.

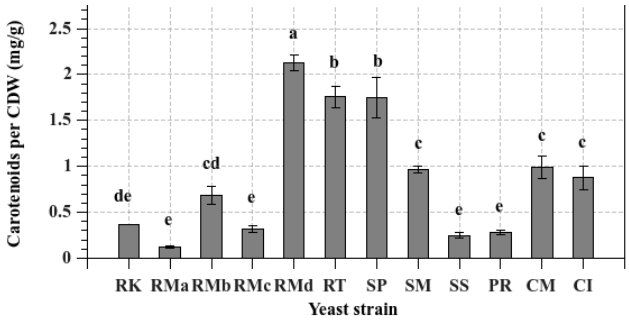

Figure 5.19: Carotenoid production and composition after 96 hours of cultivation (strain Rmb - 48 hours of cultivation), graph represents production of carotenoids per biomass mg/g of CDW, letter indexes above the columns a, b, etc. mean homogenous groups (ANOVA, Tukey test, α=0.05)

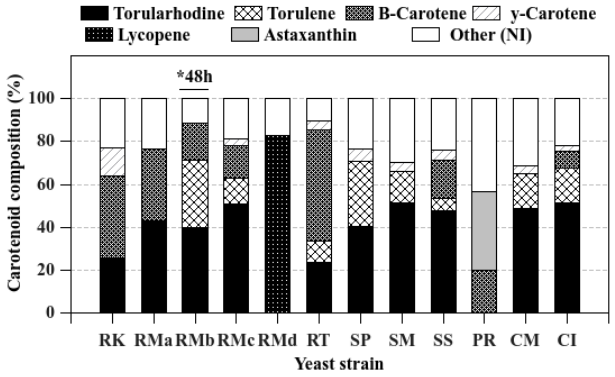

Figure 5.20: Carotenoid composition expressed as % of total carotenoids per biomass after 96 hours of cultivation (mg/g), *48 h – the best production after 48 hours of cultivation

Conclusions

Present Doctoral Thesis was focused mainly on metabolic characterization of carotenogenic yeasts as suitable candidates for industrial applications. 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* yeast strains proved to have different production properties regarding lipid and carotenoids production. Intra-species variability among suspected strains brings interesting prove of their different genotypes.

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*. The nitrogen limitation has positive impact only to *R. topruloides* CBS 6016 regarding FAs accumulation. 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.

Chapter **genetic modification of *R. toruloides* using biolistic method** 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.

Impact of inhibitors, physical and chemical mutagenes on metabolite production was employed on yeast strains *R. toruloides* CCY 62-02-04. The results of these experiments 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.

The last part of thesis was focused on **variability of metabolites produced by some yeasts within Sporidiobolales and Cystofilobasidiales**. 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. Also, the lycopen production by the strain *R. mucilaginosa* was detected.

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.

Bibliography

[1] Ilaria Mannazzu, Sara Landolfo, Teresa Lopes da Silva, and Pietro Buzzini. Red yeasts and carotenoid production: outlining a future for non-conventional yeasts of biotechnological interest, nov 2015. ISSN 15730972.

[2] Chong Mei John Koh, Yanbin Liu, Moehninsi, Minge Du, and Lianghui Ji. Molecular characterization of KU70 and KU80 homologues and exploitation of a KU70-deficient mutant for improving gene deletion frequency in Rhodosporidium toruloides. *BMC Microbiology*, 14(1): 50, feb 2014. ISSN 14712180. doi: 10.1186/1471-2180-14-50.

[3] C. P. Kurtzman, Jack W. Fell, and T. Boekhout. *The yeasts : a taxonomic study*. Elsevier Science, 2011. ISBN 9780080931272.

[4] Q. M. Wang, A. M. Yurkov, M. Göker, H. T. Lumbsch, S. D. Leavitt, M. Groenewald, B. Theelen, X. Z. Liu, T. Boekhout, and F. Y. Bai. Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. *Studies in Mycology*, 81(January 2016):149–189, jun 2015. ISSN 01660616. doi: 10.1016/j.simyco.2015.12.002.

[5] X. Z. Liu, Q. M. Wang, M. Göker, M. Groenewald, A. V. Kachalkin, H. T. Lumbsch, A. M. Millanes, M. Wedin, A. M. Yurkov, T. Boekhout, and F. Y. Bai. Towards an integrated phylogenetic classification of the Tremellomycetes. *Studies in Mycology*, 81:85–147, jun 2015. ISSN 01660616. doi: 10.1016/j.simyco.2015.12.001.

[6] Anna M. Kot, Stanisław Błazejak, Iwona Gientka, Marek Kieliszek, and Joanna Bryś. Torulene and torularhodin: "New" fungal carotenoids for industry?, dec 2018. ISSN 14752859.

[7] Jose Manuel Ageitos, Juan Andres Vallejo, Patricia Veiga-Crespo, and Tomas G. Villa. Oily yeasts as oleaginous cell factories. *Applied Microbiology and Biotechnology*, 90(4):1219–1227, may 2011. ISSN 0175-7598. doi: 10.1007/s00253-011-3200-z.

[8] Ivana Cavello, Agustín Albanesi, Dante Fratebianchi, and et al. Garmedia. Pectinolytic yeasts from cold environments: novel findings of Guehomyces pullulans, Cystofilobasidium infirmominiatum and Cryptococcus adeliensis producing pectinases. *Extremophiles*, 21(2):319–329, mar 2017. ISSN 14334909. doi: 10.1007/s00792-016-0904-0.

[9] Nicolás Bellora, Martín Moliné, Márcia David-Palma, Marco A. Coelho, Chris Todd Hittinger, José P. Sampaio, Paula Gonçalves, and Diego Libkind. Comparative genomics provides new insights into the diversity, physiology, and sexuality of the only industrially exploited tremellomycete: Phaffia rhodozyma. *BMC Genomics*, 17(1):901, dec 2016. ISSN 14712164. doi: 10.1186/s12864-016-3244-7.

[10] G Britton. Structure and properties of carotenoids in relation to function. *The FASEB Journal*, 9(15):1551–1558, dec 1995. ISSN 0892-6638. doi: 10.1096/fasebj.9.15.8529834.

[11] M. Kaczor, A. , Baranska and K. Czamara. Carotenoids: Nutrition, Analysis and Technology. In Malgorzata Baranska Agnieszka Kaczor, editor, *Carotenoids*, page 320. John Wiley & Sons, Ltd, 1 edition, jan 2016. ISBN 978-1-118-62224-7.

[12] Sören Gassel, Jürgen Breitenbach, and Gerhard Sandmann. Genetic engineering of the complete carotenoid pathway towards enhanced astaxanthin formation in Xanthophyllomyces dendrorhous starting from a high-yield mutant. *Applied Microbiology and Biotechnology*, 98(1): 345–350, jan 2014. ISSN 01757598. doi: 10.1007/s00253-013-5358-z.

[13] Delia B Rodriguez-Amaya. *A Guide to Carotenoid Analysis in Foods*. ILSI Press, 2001. ISBN 9781578810727.

[14] Alan Mortensen. Carotenoids and other pigments as natural colorants. *Pure and Applied Chemistry*, 78(8):1477–1491, jan 2006. ISSN 1365-3075. doi: 10.1351/pac200678081477.

[15] Javier Avalos and M. Carmen Limón. Biological roles of fungal carotenoids. *Current Genetics*, 61(3):309–324, aug 2015. ISSN 14320983. doi: 10.1007/s00294-014-0454-x.

[16] Birgit Ploier, Günther Daum, and Uroš Petrovič. Molecular mechanisms in yeast carbon metabolism: Lipid metabolism and lipidomics. In Jure Piškur and Concetta Compagno, editors, *Molecular Mechanisms in Yeast Carbon Metabolism*, pages 169–215. Springer Berlin Heidelberg, Berlin, Heidelberg, apr 2014. ISBN 9783642550133. doi: 10.1007/978-3-642-55013-3_8.

[17] Aruna Kumar, Aarti Sharma, Kailash C Upadhyaya, and Kailash C. Upadhyaya. Vegetable Oil: Nutritional and Industrial Perspective. *Current Genomics*, 17(3):230–240, mar 2016. ISSN 13892029. doi: 10.2174/1389202917666160202220107.

[18] Teresa Braunwald, Lisa Schwemmlein, Simone Graeff-Hönninger, William Todd French, Rafael Hernandez, William E. Holmes, and Wilhelm Claupein. Effect of different C/N ratios on carotenoid and lipid production by Rhodotorula glutinis. *Applied Microbiology and Biotechnology*, 97(14):6581–6588, jul 2013. ISSN 01757598. doi: 10.1007/s00253-013-5005-8.

[19] John A. Bon, Timothy D. Leathers, and Radheshyam K. Jayaswal. Isolation of astaxanthin-overproducing mutants of Phaffia rhodozyma. *Biotechnology Letters*, 19(2):109–112, nov 1997. ISSN 01415492. doi: 10.1023/A:1018391726206.

[20] Mohammad Reza Nasri Nasrabadi and Seyed Hadi Razavi. Optimization of β-carotene production by a mutant of the lactosepositive yeast Rhodotorula acheniorum from whey ultrafiltrate. *Food Science and Biotechnology*, 20(2):445–454, apr 2011. ISSN 12267708. doi: 10.1007/s10068-011-0062-1.

[21] Raffaela Cutzu, Annalisa Coi, Fulvia Rosso, Laura Bardi, Maurizio Ciani, Marilena Budroni, Giacomo Zara, Severino Zara, and Ilaria Mannazzu. From crude glycerol to carotenoids by using a Rhodotorula glutinis mutant. *World Journal of Microbiology and Biotechnology*, 29(6):1009–1017, jun 2013. ISSN 09593993. doi: 10.1007/s11274-013-1264-x.

[22] Chaolei Zhang, Hongwei Shen, Xibin Zhang, Xue Yu, Han Wang, Shan Xiao, Jihui Wang, and Zongbao K. Zhao. Combined mutagenesis of Rhodosporidium toruloides for improved production of carotenoids and lipids. *Biotechnology Letters*, 38(10):1733–1738, oct 2016. ISSN 15736776. doi: 10.1007/s10529-016-2148-6.

[23] Huzefa A Raja, Andrew N Miller, Cedric J Pearce, and Nicholas H Oberlies. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community, 2017. ISSN 15206025.

[24] D. Vu, M. Groenewald, S. Szöke, G. Cardinali, U. Eberhardt, B. Stielow, M. de Vries, G.J.M. Verkleij, P.W. Crous, T. Boekhout, and V. Robert. DNA barcoding analysis of more than 9 000 yeast isolates contributes to quantitative thresholds for yeast species and genera delimitation. *Studies in Mycology*, 85:91–105, sep 2016. ISSN 01660616. doi: 10.1016/j.simyco.2016.11.007.

[25] David S. Hibbett, Manfred Binder, and Joseph F. et al. Bischoff. A higher-level phylogenetic classification of the Fungi. *Mycological Research*, 111(5):509–547, may 2007. ISSN 09537562. doi: 10.1016/j.mycres.2007.03.004.

[26] Sameh Magdeldin. *Gel Electrophoresis – Advanced Techniques*. InTech, apr 2012. ISBN 9789535104575. doi: 10.5772/2688.

[27] Gerold Barth and Claude Gaillardin. *Yarrowia lipolytica: Biotechnological Applications*. 1996. ISBN 978-3-642-38319-9. doi: 10.1007/978-3-642-79856-6_10.

[28] Alexander Idnurm, Andy M Bailey, Timothy C Cairns, Candace E Elliott, Gary D Foster, Giuseppe Ianiri, and Junhyun Jeon. A silver bullet in a golden age of functional genomics: the impact of Agrobacterium-mediated transformation of fungi. *Fungal Biology and Biotechnology*, 4(1):6, 2017. ISSN 2054-3085. doi: 10.1186/s40694-017-0035-0.

[29] Hong Wei Pi, Marimuthu Anandharaj, Yi Ying Kao, Yu Ju Lin, Jui Jen Chang, and Wen Hsiung Li. Engineering the oleaginous red yeast Rhodotorula glutinis for simultaneous β-carotene and cellulase production. *Scientific Reports*, 8(1):10850, dec 2018. ISSN 20452322. doi: 10.1038/s41598-018-29194-z.

[30] BR Glick and JJ Pasternak. *Molecular Biotechnology: Principles and Applications of Recombinant DNA Third Edition*. 2010. ISBN 9781555814984. doi: 10.1128/9781555819378.

[31] Giuseppe Ianiri, Sandra A.I. Wright, Raffaello Castoria, and Alexander Idnurm. Development of resources for the analysis of gene function in Pucciniomycotina red yeasts. *Fungal Genetics and Biology*, 48(7):685–695, 2011. ISSN 10871845. doi: 10.1016/j.fgb.2011.03.003.

[32] I. Kostovova. Characterisation of carotenogenic yeasts using molecular techniques. *Brno University of Technology, Department of Food Science and Biotechnology*, page 86, 2013. URL `https://www.vutbr.cz/www_base/zav_prace_soubor_verejne.php?file_id=63276`.

[33] Robert J. Kokoska, Lela Stefanovic, Hiep T. Tran, Michael A. Resnick, Dmitry A. Gordenin, and Thomas D. Petes. Destabilization of Yeast Micro- and Minisatellite DNA Sequences by Mutations Affecting a Nuclease Involved in Okazaki Fragment Processing (rad27) and DNA Polymerase δ (pol3-t). *Molecular and Cellular Biology*, 18(5):2779–2788, may 1998. ISSN 0270-7306. doi: 10.1128/mcb.18.5.2779.

[34] M. A. Pérez, F. J. Gallego, I. Martínez, and P. Hidalgo. Detection, distribution and selection of microsatellites (SSRs) in the genome of the yeast Saccharomyces cerevisiae as molecular markers. *Letters in Applied Microbiology*, 33(6):461–466, dec 2001. ISSN 02668254. doi: 10.1046/j.1472-765X.2001.01032.x.

[35] Amr Abd El-Rhman El-Banna, Amal Mohamed Abd El-Razek, and Ahmed Rafik El-Mahdy. Isolation, Identification and Screening of Carotenoid-Producing Strains of Rhodotorula glutinis. *Food and Nutrition Sciences*, 03(05):627–633, may 2012. ISSN 2157-944X. doi: 10.4236/fns.2012.350862.35086.

[36] Anna M. Kot, Stanisław Błażejak, Agnieszka Kurcz, Iwona Gientka, and Marek Kieliszek. Rhodotorula glutinis—potential source of lipids, carotenoids, and enzymes for use in industries. *Applied Microbiology and Biotechnology*, 100(14):6103–6117, jul 2016. ISSN 14320614. doi: 10.1007/s00253-016-7611-8.

[37] Anuj K. Chandel, Silvio Silvério da Silva, and Om V. Singh. Detoxification of Lignocellulose Hydrolysates: Biochemical and Metabolic Engineering Toward White Biotechnology, aug 2013. ISSN 19391234.

[38] Seraphim Papanikolaou, Stylianos Fakas, Michel Fick, Isabelle Chevalot, Maria Galiotou-Panayotou, Michael Komaitis, Ivan Marc, and George Aggelis. Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. *Biomass and Bioenergy*, 32(1): 60–71, jan 2008. ISSN 09619534. doi: 10.1016/j.biombioe.2007.06.007.

[39] Seraphim Papanikolaou and George Aggelis. Lipids of oleaginous yeasts. Part II: Technology and potential applications, aug 2011. ISSN 14387697.

[40] Zhiwei Zhu, Sufang Zhang, Hongwei Liu, Hongwei Shen, Xinping Lin, Fan Yang, Yongjin J. Zhou, Guojie Jin, Mingliang Ye, Hanfan Zou, and Zongbao K. Zhao. A multi-omic map of the lipid-producing yeast Rhodosporidium toruloides. *Nature Communications*, 3(1):1112, jan 2012. ISSN 20411723. doi: 10.1038/ncomms2112.

[41] Stylianos Fakas. Lipid biosynthesis in yeasts: A comparison of the lipid biosynthetic pathway between the model nonoleaginous yeast Saccharomyces cerevisiae and the model oleaginous yeast Yarrowia lipolytica. *Engineering in Life Sciences*, 17(3):292–302, mar 2017. ISSN 16180240. doi: 10.1002/elsc.201600040.

[42] Lulu Chang, Xin Tang, Hengqian Lu, Hao Zhang, Yong Q. Chen, Haiqin Chen, and Wei Chen. Role of AMP deaminase during fatty acids accumulation in oleaginous fungus Mortierella alpina. *Journal of Agricultural and Food Chemistry*, page acs.jafc.9b03603, aug 2019. ISSN 0021-8561. doi: 10.1021/acs.jafc.9b03603. URL `http://pubs.acs.org/doi/10.1021/acs.jafc.9b03603`.

[43] Jana Tkáčová, Tatiana Klempová, and Milan Čertík. Kinetic study of growth, lipid and carotenoid formation in β-carotene producing Rhodotorula glutinis. *Chemical Papers*, 72(5): 1193–1203, may 2018. ISSN 2585-7290. doi: 10.1007/s11696-017-0368-4.

[44] Anna M. Kot, Stanisław Błażejak, Marek Kieliszek, Iwona Gientka, Joanna Bryś, Lidia Reczek, and Katarzyna Pobiega. Effect of exogenous stress factors on the biosynthesis of carotenoids and lipids by Rhodotorula yeast strains in media containing agro-industrial waste. *World Journal of Microbiology and Biotechnology*, 35(10):157, oct 2019. ISSN 0959-3993. doi: 10.1007/s11274-019-2732-8.

[45] Ines Ayadi, Hafedh Belghith, Ali Gargouri, and Mohamed Guerfali. Utilization of Wheat Bran Acid Hydrolysate by Rhodotorula mucilaginosa Y-MG1 for Microbial Lipid Production as Feedstock for Biodiesel Synthesis. *BioMed Research International*, 2019, 2019. ISSN 23146141. doi: 10.1155/2019/3213521.

[46] Ievgeniia A. Tiukova, Jule Brandenburg, Johanna Blomqvist, Sabine Sampels, Nils Mikkelsen, Morten Skaugen, Magnus O. Arntzen, Jens Nielsen, Mats Sandgren, and Eduard J. Kerkhoven. Proteome analysis of xylose metabolism in Rhodotorula toruloides during lipid production. *Biotechnology for Biofuels*, 12(1):1–17, 2019. ISSN 17546834. doi: 10.1186/s13068-019-1478-8.

[47] Katrin Ochsenreither, Claudia Glück, Timo Stressler, Lutz Fischer, and Christoph Syldatk. Production strategies and applications of microbial single cell oils, oct 2016. ISSN 1664302X.

[48] Marilyn G. Wiebe, Kari Koivuranta, Merja Penttilä, and Laura Ruohonen. Lipid production in batch and fed-batch cultures of Rhodosporidium toruloides from 5 and 6 carbon carbohydrates. *BMC Biotechnology*, 12(1):26, dec 2012. ISSN 14726750. doi: 10.1186/1472-6750-12-26.

[49] Yonghong Li, Zongbao (Kent) Zhao, and Fengwu Bai. High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. *Enzyme and Microbial Technology*, 41(3):312–317, aug 2007. ISSN 01410229. doi: 10.1016/j.enzmictec.2007.02.008.

[50] Parul Singh, Hea Jong Chung, In Ah Lee, Roshan D'Souza, Hyeon Jin Kim, and Seong Tshool Hong. Elucidation of the anti-hyperammonemic mechanism of Lactobacillus amylovorus JBD401 by comparative genomic analysis. *BMC genomics*, 19(1):292, 2018. ISSN 14712164. doi: 10.1186/s12864-018-4672-3.

[51] Young Kyoung Park, Jean Marc Nicaud, and Rodrigo Ledesma-Amaro. The Engineering Potential of Rhodosporidium toruloides as a Workhorse for Biotechnological Applications. *Trends in Biotechnology*, 36(3):304–317, mar 2018. ISSN 18793096. doi: 10.1016/j.tibtech.2017.10.013.

[52] Thierry Dulermo and Jean-Marc Nicaud. Involvement of the G3P shuttle and β-oxidation pathway in the control of TAG synthesis and lipid accumulation in Yarrowia lipolytica. *Metabolic engineering*, 13(5):482–91, 2011. ISSN 1096-7184. doi: 10.1016/j.ymben.2011.05.002.

[53] Shuyan Zhang, Masakazu Ito, Jeffrey M. Skerker, Adam P. Arkin, and Christopher V. Rao. Metabolic engineering of the oleaginous yeast Rhodosporidium toruloides IFO0880 for lipid overproduction during high-density fermentation. *Applied Microbiology and Biotechnology*, 100(21):9393–9405, 2016. ISSN 14320614. doi: 10.1007/s00253-016-7815-y.

[54] Pirapan Polburee, Takao Ohashi, Yung Yu Tsai, Thitinun Sumyai, Noppon Lertwattanasakul, Savitree Limtong, and Kazuhito Fujiyama. Molecular cloning and overexpression of DGA1, an acyl-CoA-dependent diacylglycerol acyltransferase, in the oleaginous yeast rhodosporidiobolus fluvialis DMKU-RK253. *Microbiology (United Kingdom)*, 164(1):1–10, 2018. ISSN 14652080. doi: 10.1099/mic.0.000584.

[55] Namthip Chumpolkulwong, Toshihide Kakizono, Shiro Nagai, and Naomichi Nishio. Increased astaxanthin production by Phaffia rhodozyma mutants isolated as resistant to diphenylamine. *Journal of Fermentation and Bioengineering*, 83(5):429–434, jan 1997. ISSN 0922338X. doi: 10.1016/S0922-338X(97)82996-0.

[56] Fabio M. Squina and Adriana Z. Mercadante. Influence of nicotine and diphenylamine on the carotenoid composition of Rhodotorula strains. *Journal of Food Biochemistry*, 29(6):638–652, dec 2005. ISSN 01458884. doi: 10.1111/j.1745-4514.2005.00030.x.

[57] Luis M. Ducrey Sanpietro and M. R. Kula. Studies of astaxanthin biosynthesis in Xanthophyllomyces dendrorhous (Phaffia rhodozyma). Effect of inhibitors and low temperature. *Yeast*, 14(11):1007–1016, aug 1998. ISSN 0749503X. doi: 10.1002/(SICI)1097-0061(199808)14:11<1007::AID-YEA307>3.0.CO;2-U.

[58] John F. T. Spencer and Dorothy M. Spencer. Mutagenesis in Yeast. In Wei Xiao, editor, *Yeast Protocols*, volume 53, pages 17–38. Humana Press, New Jersey, 1996. ISBN 0091-679X (Print)\r0091-679X (Linking). doi: 10.1385/0-89603-319-8:17.

[59] Jieni Lian, Manuel Garcia-Perez, and Shulin Chen. Fermentation of levoglucosan with oleaginous yeasts for lipid production. *Bioresource Technology*, 133:183–189, apr 2013. ISSN 09608524. doi: 10.1016/j.biortech.2013.01.031.

[60] Mohamed Guerfali, Ines Ayadi, Nadia Mohamed, and et al. Triacylglycerols accumulation and glycolipids secretion by the oleaginous yeast Rhodotorula babjevae Y-SL7: Structural identification and biotechnological applications. *Bioresource Technology*, 273:326–334, feb 2019. ISSN 09608524. doi: 10.1016/j.biortech.2018.11.036.

[61] Tamene Milkessa Jiru, Laurinda Steyn, Carolina Pohl, and Dawit Abate. Production of single cell oil from cane molasses by Rhodotorula kratochvilovae (syn, Rhodosporidium kratochvilovae) SY89 as a biodiesel feedstock. *Chemistry Central Journal*, 12(1):91, dec 2018. ISSN 1752-153X. doi: 10.1186/s13065-018-0457-7.

[62] Hector Urbina and M. Catherine Aime. A closer look at sporidiobolales: Ubiquitous microbial community members of plant and food biospheres. *Mycologia*, 110(1):79–92, jun 2018. ISSN 15572536. doi: 10.1080/00275514.2018.1438020.

ŽIVOTOPIS

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* a 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, produkce a izolace mikrobiálních lipidů a karotenoidů, kultivace karotenogenních kvasinek

PŘEHLED PUBLIKAČNÍ ČINNOSTI

Publikace v časopisech:

Kostovová, I., Byrtusová, D., Rapta, M. et al. The variability of carotenoid pigments and fatty acids produced by some yeasts within Sporidiobolales and Cystofilobasidiales. *Chem. Pap*. (2021). https://doi.org/10.1007/s11696-021-01567-1

Marova, I., Haronikova, A., Petrik, S., Obruca, S., **Kostovova, I.**, Production of carotenoids, ergosterol and other lipidic compounds by red yeasts cultivated on lignocellulose waste substrates, *New Biotechnology,* 2014, roč. 31, č. 5, s. 210-212. ISSN: 1871-6784. https://doi.org/10.1016/j.nbt.2014.05.991.

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.

Kostovová, I.; Márová, I.; Hároniková, A.; Petrik, S. The Production Stability of Mutant Red Yeasts Adapted to Glycerol as a Substrate. In *Studentská konference. Brno: FCH*, 2014. s. 294-304. ISBN: 978-80-214-5078- 3.

Hároniková, A.; Benešová, P.; **Kostovová, I**.; Petrik, S.; Márová, I. The possibilities of using carotenoid- rich yeast biomass cultivated on whey substrates. In *Hygiena a technologie potravin, XLIII. Lenfeldovy a Hoklovy dny - Sborník přednášek a posterů. Brno: 2013*. s. 136-139. ISBN: 978-80-7305-664- 3.

Márová, I.; Hároniková, A.; Petrik, S.; **Kostovová, I**.; Rapta, M. Cystofilobasidium capitatum - red yeast for potential biotechnological application?. In *1st International*

Conference on Chemical Technology ICCT 2013 Sborník příspěvků. Mikulov: 2013. s. 1-4. ISBN: 978-80-86238-37- 1.

Konferenční příspěvky - abstrakty:

Kostovová, I.; Roubalová, M.; Pokrývková, Z.; Byrtusová, D.; Szotkowski, M.; Hlaváček, V.; Márová, I. Improvement of lipid compound prooduction by red yeasts though random mutagenesis. *44th Annual Conference on Yeast. Bratislava: Slovak Academy of Sciences, Bratislava*, 2017. s. 58-58.

Kostovová, I.; Roubalová, M.; Pokrývková, Z.; Rapta, M.; Márová, I. Effect of Random Mutagenesis on Lipid Compounds Production by Carotenogenic yeast. *Abstract book, 13th Yeast lipid conference*. Paris: 2017. s. 92-92.

Kostovová I., Rapta M., Szotkowski M., Zavan Marques A.B., Dulermo R., Nicaud J.M., Márová I.: Experimental study of lipidic compound production by red yeasts. 43rd Annual Conference on Yeasts, May 10 – 13, 2016, Smolenice, Slovakia. Boo of abstracts, p. 31. ISSN 1336-4839.

Kostovová, I.; Hároniková, A.; Petrik, S.; Márová, I. Optimisation of DGGE nested PCR for red yeasts characterisation. 4*1st Annual Conference on Yeast. Bratislava: Slovak Academy of Sciences, Bratislava* 2014. s. 55-55.

Kostovová, I.; Márová, I.; Hároniková, A.; Petrik, S. The Production Stability of Mutant Red Yeasts Adapted to Glycerol as a Substrate. *Studentská odborná konference Chemie je život , Sborník abstraktů. Brno: Vysoké učení technické v Brně, Fakulta chemická, Purkyňova 464/118*, 612 00 Brno, 2014. s. 73-73. ISBN: 978-80-214-5077- 6.

Kostovová, I.; Hároniková, A.; Hlaváček, V.; Petrik, S.; Márová, I. Rapeseed cake residue as a waste substrate for valuable metabolites production. *The 15th European Meeting on Environmental Chemistry, EMEC15, BOOK OF ABSTRACT. Brno: Brno University of Technology,* 2014. s. 103-103. ISBN: 978-80-214-5073- 8.

Matoušková, P.; Hároniková, A.; Petrik, S.; **Kostovová, I.;** Márová, I. Growth and production properties of red yeast cultivated on lignocellulose waste substrates. Book of Abstracts - V *International Conference on Environmental, Industrial and Applied Microbiology - BioMicroWorld2013. 2013. Madrid: 2013*. s. 339-339.