BRNO UNIVERSITY OF TECHNOLOGY
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PRODUCTION OF BETA-GLUCANS AND OTHER POLYSACCHARIDES BY YEAST AND MICROALGAE

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

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1 INTRODUCTION

Yeast played an important role in human society as early as ancient Egypt and ancient Greece when they were an important component in the production of bread, or in alcoholic beverages, where they laid the foundation for the later production of wine and beer. Although fermentation processes have remained popular in today's society, these organisms are gaining in importance mainly in the pharmaceutical and molecular biology industries, which focus on research into metabolic pathways, gene expression, and genetic modification.

Occurrence is a ubiquitous organism, found mainly on sugary foods, such as fruit, and in the air or soil. Certain colonial representations are present in the human body, where diseases can lead to diseases (such as candidiasis).

"Red" yeast play an important role in this group concerning the presence of a metabolic pathway for lipophilic red-orange pigments, so-called carotenoids. These substances have strong antioxidant properties and, therefore they are used in cosmetics, pharmacy, or the food industry for food fortification. Other abundant red yeast metabolites are lipids and their possible use in the production of third-generation fuels. In this way, these organisms are also slowly but surely entering the field of environmental research.

Autophototrophic organisms are a very attractive group of microorganisms in terms of producing a wide range of metabolites only from light energy, carbon dioxide, water, and salts. In this way, the cost of the required culture medium is lower. The opposite of this positive is the low production of biomass, the need for lighting, and the need for special cultivation equipment. These requirements ultimately make the whole cultivation process more expensive.

However, important and known autotrophic organisms appear to be, their production of polysaccharides is still very little studied. In the efford of the largest possible accumulation of photosynthetic pigments or lipids, one of the most important metabolites of cellular metabolism has therefore been somehow “forgotten”.

2 THEORETICAL PART

2.1 Microbial polysaccharides

Over the last three decades, the microbial polysaccharides have been widely applied in food, chemical and pharmaceutical industries. Althrough the low cost of traditional market-dominant polysaccharides, such as cellulose and cornstarch, microbial-derived PS show the great diversity in structural and functional properties. Besides, microbial sources are favoured due to their fast and high yielding production procedures under controlled conditions. Some of these polysaccharides (e.g. starch, glycogen) are located within the cell and serve as storage compounds. Conversely, exopolysaccharides or capsular polysaccharides are synthetized mainly for protective purposes, either as a general physical barrier, or to prevent dehydratation [1, 2, 3].

2.1.1 Fungal cell wall

The cell wall is the outermost part of the cell that provides both physical protection from its environment and osmotic support. Fungal walls normally make up 10-30% of the biomass and consists of layers of about equal amounts of mannoproteins, glucan and a small part of chitin (1-2 %). The biosynthesis of the cell wall is tight regulated and depends on growth and morphogenesis of the cell [4, 5]. Before of their incorporation to the cell wall, the mannoproteins (40 %) become heavily glycosylated on *N*- and *O*-glycosylation sides when going through the secretory pathway. They can be connected directly to the "spain" of the cell wall, 1,3-*β*-glucan via alkali-sensitive binding (Pir cell wall proteins) or linked covalently through 1,6-*β*-glucan to 1,3-*β*-glucan (the glycosylphosphatidylinositol-dependent cell wall proteins) [5].

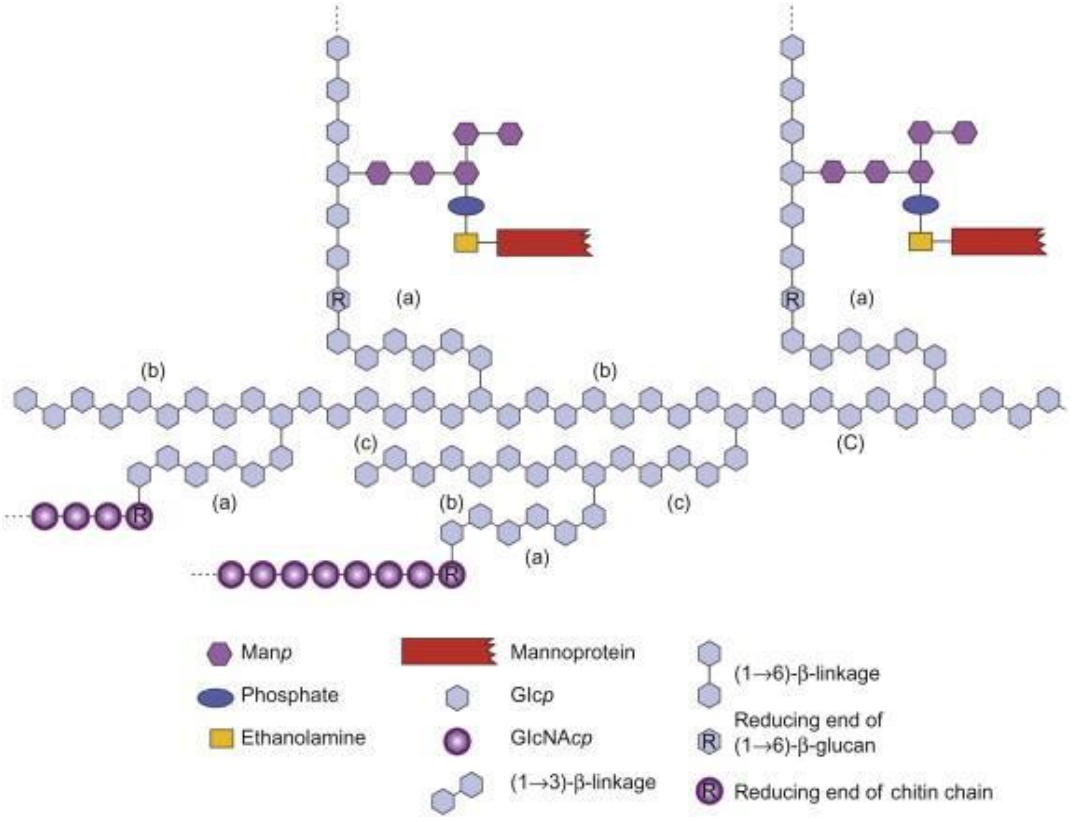

Figure 1: Composition of the cell wall of *S. cerevisiae* [4, 5]

Cell wall precursors are polymerized on the cell surface and then modified and interconnected [4, 5]. Beta-glucan synthase is the key membrane-located enzyme for beta-glucan biosynthesis since it is catalyzing the production of 1,3-β-glucan chain from UDP-glucose as a sugar precursor. This enzyme is composed of two subunits: membrane-bonded catalytic subunit and GTP-bonding regulatory subunit. It is not clear which cell protein represses the catalytic subunit, the most likely it could be Fks1p, Fks2p (catalytic proteins), or Rho1p (regulatory protein from Rho GTPase family) [6]. The enzyme geranylgeranyltransferase I (GGTase I) plays an important role in the regulation of glucan synthase since it connects the geranylgeranyl group to Rho1p in the prenylation process. Thus, prenylated protein can be anchored in the cell membrane [4].

Increased glucan production also occurs during sporulation. Diploid yeast cells enter the meiotic cycle with nutrient limitation and form haploid spores. Each spore is equiped by a special cell wall, which has a protective function against damage from the external environment. The wall contains four layers: two inner layers of β-glucan and mannan (same as for vegetative cells), the outer one composed of chitosan and a dityrosine polymer (both polymers specific for spores) [4].

In vegetative cells (1,6) -β-glucan forms 12% of cell wall polysaccharides. While (1,3) -β-glucan forms the "backbone" of the wall, (1,6) -β-polymer is a branched amorphous structure that acts as an adhesive for other building blocks of the cell wall. Despite efforts to identify the gene responsible for the production of the catalytic enzyme for the synthesis of this polysaccharide, it has not yet been identified yet. The synthesis of (1,6) -β-glucan appears to be found on the plasma membrane, but several endoplasmic reticula and Golgi apparatus proteins also perform a (for now unknown) function [4].

2.1.1.1 Beta-glucan

Beta (β)-glucans represent a widespread group of polysaccharides, consisting of D-glucose monomers linked through β-glycosidic bonds. Beta-glucans of different origin are present in different amounts and structural modifications. For example, beta-glucans of oat and barley are linear unbranched with 1,3 and 1,4-β-linkages [7, 8], beta-glucans of higher fungi and yeasts consist of 1,3-β-linked glucopyranosyl residues with small numbers of 1,6-β-linked branches 1,3- and 1,6-bonded beta-glucans [9], while beta-glucans from bacterial origin consist of cyclic 1,2-β-linkages [10]. Paramylon, callose or curdlan represent the simplest, unbranched linear chains of (1,3)-β-glucans [6]. They are also other bond-types, mostelly in the position C(O)6, and mainly in the polysaccharides like chromistan and fungal laminarin. Cyclic β-glucans occure at *Bradyrhizobium japonicum* [6]. Currently, the commercial sources of beta-glucans are higher fungi (*Rhynchelytrum repens, Lentinus edodes, Grifola frondosa, Tremella mesenterica, Tremella aurantia, Zea may, Agaricus blazei, Phellinus baummi, Pleurotus* spp.) [11], and plants barley and oat, which have been extensively used in food, feed, pharmaceutical or cosmetics industries.

According to a report by BCC Research the global beta-glucan market is expected to reach 576.28 million USD by 2025, growing at a GAGR of 7.3% from 2017 to 2025 [12]. The increasing demand for immunostimulatory polysaccharide requires investigating more yielding new sources applicable in the biotechnological industry. The baker's yeast *Saccharomyces cerevisiae* (SC) is the main microbial biotechnological source of beta-glucan due to its GRAS label. Its content in the cell wall can reach up to 50 %.

The high concentration of trehalose protects the cell from autolysis and increases its chances of surviving under adverse conditions. Trehalose protects the cell from drying out. Synergies with chaperones are involved in protein assembly, and in addition, when cells are excessively hydrated, it secretes water from the surface of the proteins and, thus, protects them from denaturation. This disaccharide and chaperone Hsp104 have been shown to be essential for refolding proteins in the cytosol as well as for repairing poorly assembled glycoproteins due to heat shock in the lumen of the endoplasmic reticulum [13].

2.1.2 Algal cell wall

Algal cells are covered by extracellular matrix (ECM, cell wall) that consists of diverse neutral and charged polysaccharides and proteins. The major fibrillar component of the cell walls of plants and many algal taxa is cellulose, the 1,4-β-glucan polymer. Cellulose is often synthetized in the plasma membrane and incorporated into the cell wall, creating microfibrils of diverse amount, layers and orientation. Many other algal taxa produce microfibrillar wall from 1,3-β-xylans, 1,4-β-mannans and heteropolymeric fibrillar polysaccharides. Another important component of the cell walls are hemicelluloses (xylans, xyloglucans, mannans) and pectins. The composition of extracellular matrix of diverse algal groups is in the Table 1 [14].

Table 1: Composition of extracellular coverings of algal group [14]

Algal group	Components of extracellular coverings
Chlorophyta	Cellulose, mannans, xyloglucans, xylans, 1,3-β-glucans, mixed linkage glucans, xylogalactorhamnans, rhamnoxylogalactogalacturonans, arabinogalactan proteins
Rhodophyta	Cellulose, xylans, mannans, sulphated mixed linkage glucans, sulphated galactans
Phaeophyta	Cellulose, alginates, fucoidans
Haptophyta	Acidic polysaccharides, proteins, calcite
Dinophyta	Cellulose
Bacillariophyta	Mannose and 1,3- β-glucans, multiple proteins, silica

The cell wall of cyanobacteria, unlike unicellular algae, does not contain cellulose and is thus better digested by humans. It consists of lipoproteins and a solid peptidoglycan (murein). Murein forms a network structure here, just like in the cell walls of bacteria. This network structure is formed by N-acetylglucosamine and acetylmuramic acid, which are linked via (1 → 4) -β glycosidic bonds. The surface of cells is also often covered with glycocalyx (mucous sheath formed by lipopolysaccharides) [14].

2.1.2.1 Beta-glucans in algae and cyanobacteria

In research into the production of beta-glucans by microalgae, not much has been published, except for *Euglena gracilis*. Single-cell *Chlamydomonas reinhardtii* is used for the biosynthesis and analysis of this polysaccharide in microalgae, although its cell wall consists almost exclusively of hydroxyproline-rich glycoproteins. Polysaccharides in photoautotrophic microorganisms are preferably studied in brown seaweed, which produces alginate, agar, or carrageenan and where their products can reach up to 74% dry matter [15, 16].

The best-known representative of beta-glucans in this case is paramylon, produced by the protozoan *Euglena gracilis*, where it forms monomorphic grains in the cytoplasm. It is a linear unbranched β- (1,3) -glucan chain of high molecular weight. Under heterotrophic conditions, this

microorganism can synthesize up to 95% of paramylon on dry matter and is thus competitive with *Saccharomyces cerevisiae*. The biosynthetic pathway here is based on the synthesis of an initiation primer on a membrane-bound synthase, and then the attachment of activated glucose (UDP-glucose) results in a chain formation. So far, the pathway is very poorly understood and remains the subject of research [6]

2.1.3 Extracellular polymeric substances

Extracellular polymeric substances are high molecular weight natural polymers that are secreted into the environment by microorganisms. These are different categories of organic molecules, such as polysaccharides, proteins, nucleic acids, (phospho) lipids, and others [17].

2.1.3.1 Exopolysaccharides

Microalgae and cyanobacteria possess a complex carbohydrate metabolic pathway that includes the ability to synthesize intracellular monosaccharides, polymeric glucan storage, and structurally complex exopolysaccharides (EPS). EPS are synthesized in phototrophic organisms according to strictly regulated and energy-intensive processes. They can be adhered to the cell or released into the medium (in the form of mucus). In the case of adhesion, they form specific envelopes and capsules, so-called polysaccharide layers covalently connected to the cell surface [18, 19].

Depending on the properties of the secreted polymers, such as molecular weight, degree of branching, and conformation, they may exhibit unique biological activity. Glucose, galactose, and fucose predominate here, but rhamnose and arabinose can also be found to a lesser extent [8].

The main parameter influencing EPS production in phototrophic organisms is the intensity of illumination. The higher it is, the greater the production of polysaccharides. In algae and cyanobacteria, carbohydrate synthesis occurs in the chloroplast and/or cytosol during the Calvin cycle, consumed by NADPH and ATP, where electrons and protons are generated during the cleavage of water in photosynthetic reactions. In the Calvin cycle, carbon dioxide is assimilated by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This enzyme carboxylates 2 molecules of 3-phosphoglycerate. In microalgae, the synthesis of polysaccharides and their sulfation takes place in the Golgi apparatus (GA) [8].

Yeast exopolysaccharides are a very little studied group of metabolites. For the time being, their industrial production is not feasible in terms of low yield. To date, enzymes and biosynthetic pathways, as well as regulatory mechanisms, have not been described [20].

In general, the optimal conditions for the yeast exopolysaccharides biosynthesis are strain depended. In most research paper, the yeast requires strong oxygenation and low temperature of the cultivation. The medium should contain suitable carbon source (sucrose, glucose), inorganic nitrogen source (ammonium sulphate) and the C/N ratio of 15:1. Chemically, the yeast exopolysaccharides are linear mannans, pullulan, glucooligosaccharides, galactooligosaccharides and other heteropolysaccharides containing α-1,2; α-1,3; α-1,6; β-1,3, and β-1,4 bonds [18, 19].

2.1.3.2 Extracellular glycolipids

Biosurfactants represent a broad spectrum of biomolecules containing both, polar and non-polar part. Glycolipids are glycosides of fatty acids containing one or more monosaccharide residues. They can be produced by some yeast, bacteria, and mycelial fungi, where the best known extracellular glycolipids are rhamnolipids, mannosylerythritol lipids, sophorolipids, cellobiose lipids, and trehalose lipids. They differ by structural diversity, biological activities with conection

to broad spectrum of application in cosmetology, medicine or agriculture. In comparison to chemical surfactants, biosurfactants possess higher biodegradability and lower toxicity [21, 22].

Among carotenogenic yeast, the genus of Rhodotorula have been reported to be potential of extracellular polyol esters of fatty acids (PEFA) producers (specifically *Rhodotorula babjevae, Rhodotorula taiwanensis*, *Rhodotorula aff. paludigena*). *Rhodotorula's* glycolipids consist of hydroxy fatty acids and sugar alcohols (D-arabitol, D-mannitol, (R)-3-hydroxymyristate, (R)-3-hydroxypalmitate, and (R)-3-hydroxystearate) [23]

2.2 Glucan analysis

Structural characterization of polysaccharides represents a challenging task due to the complexity of the molecules in terms of glycosidic bonds, branching degree, molecular weight and monosaccharide composition. Polysaccharides within the microbial cells are mostly bounded with other cellular molecules by covalent or non-covalent bonds, making the isolation of pure native samples the first crucial step for their further analysis [24].

2.2.1 Vibrational spectroscopy

FTIR spectroscopy is a frequently used method for polysaccharide analysis. It is a sensitive method for determining the anomeric configuration and position of glycosidic linkages (Figure 4) [24]. So-called "sugar region" of FTIR spectra (1200-950 cm^{-1}, CO and CC vibration of glycosidic bonds and in pyranoid rings), as well as another "anomeric region" (950-750 cm^{-1}, complex skeletal vibrations), exhibit significant differences between alpha- and beta-D-glucans with different structures [24].

The characteristic bands for (1-3)- and/or (1-6)-linked beta-D-glucans can be found near 1160, 1078, 1044 and 890 cm^{-1}, bands for (1-4), (1-6)-linked alpha-D-glucans are located near 1155, 1023, 930, 850 and 765 cm^{-1} IR bands in the 1366, 930 regions , 850, 822, 542 and 420 cm^{-1} indicate (1-3)-alpha-glucan. For relative quantification between alpha- and beta-bonds as well as to verify the purity of the beta-glucan preparation, ratios 1160/1155, 1041/1023, 1160/1023 and 889/930, 889/765 cm^{-1} should be used [24, 25, 26].

Raman scattering, another technique of vibrational spectroscopy, complementary to IR, has been applied to the structural analysis of plant, animal and microbial glucan polysaccharides - starch, glycogen, cellulose, dextran. Unfortunately, there are few articles dealing with Raman spectra [24, 26].

2.2.2 Enzymatic determination of glucans (commertial kits)

In the enzymatic assay, the first step is the hydrolysis (cell wall) and then the action of specific enzymes. Lyophilized biomass is exposed to sulfuric acid and high temperature. In alkaline medium (mainly KOH) most of the cell wall components are solubilized for further decomposition by exo-(1→3)-β- glucanase and β-glucosidase. Subsequent detection is performed spectrophotometrically, eg using a GOPOD reagent (containing glucose oxidase and peroxidase). The total glucans are thus determined. Determination of α-glucan is performed by incubation of biomass in alkaline medium (no solubilization of β-glucan) and by amyloglucosidase and invertase [25].

3 THE AIMS OF THE STUDY

The aim of presented thesis was to explore possibility of production of the β-glucan from carotenogenic yeast as unexplored source together with some algal strains, commertial higher fungi and cereals.

The experimental part solves following goals:

- Metabolic screening of yeast and microalgal strains (β-glucans, extracellular polymeric substances, intracellular lipids and pigments)
- Optimization of culture conditions for β-glucans and other high-valuable compounds
- Compare of β-glucans production within the heterotrophs and autotrophs
- Analysis of commertial higher fungi and cereals

4 EXPERIMENTAL PART

4.1 Microorganisms

Yeasts strains of the generi *Saccharomyces, Metschnikowia, Cystofilobasidium, Rhodotorula, Sporidiobolus, Dioshegia* and *Phaffia*, were purchased from the Culture Collection of Yeasts (CCY, Bratislava, Slovakia). *Aureobasidium* and *Candida* yeast were purchased from Czech Collection of Microorganisms, Masaryk University (CCM, Brno). Algal and cyanobacterial strains were obtained from Culture Collection of Autotrophic Organisms (CCALA, Třeboň, Czech Republic). The list of microorgamisms is presented in the Table 2-7. The commertial brewer yeast of *S. cerevisiae* were kindly provided by Ing. Václav Štursa (Table 4).

Table 2: List of used carotenogenic yeasts strains

Number	Microorganisms	Strain collection number
1	*Cystofilobasidium infirmominiatum*	CCY 17-18-4
2	*Cystofilobasidium macerans*	CCY 10-1-2
3	*Phaffia rhodozyma*	CCY 77-1-1
4	*Rhodotorula kratochvilovae*	CCY 20-2-26
5	*Rhodotorula mucilaginosa*	CCY 19-4-6
6	*Rhodotorula mucilaginosa*	CCY 20-9-7
7	*Rhodotorula toruloides*	CCY 62-2-4
8	*Rhodotorula glutinis*	CCY 20-2-47
9	*Rhodotorula glutinis*	CCY 20-2-22
10	*Sporidiobolus metaroseus*	CCY 19-6-20
11	*Sporidiobolus pararoseus*	CCY 19-9-6
12	*Sporidiobolus salmonicolor*	CCY 19-6-4
13	*Sporidiobolus salmonicolor (roseus)*	CCY 19-4-25
14	*Dioshegia hungarica*	CCY 18-1-3

Table 3: List of used non-carotenogenic yeasts strains

Number	Microorganisms	Strain collection number
15	*Metschnikowia pulcherrima*	CCY 29-2-149
16	*Metschnikowia pulcherrima*	CCY 29-2-147
17	*Metschnikowia pulcherrima*	CCY 29-2-129
18	*Aureobasidium pullulans*	CCM 8183
19	*Yarrowia lipolytica*	CCY 29-26-4
20	*Candida glabrata*	CCM 8270

Number	Microorganisms	Strain collection number
21	*Saccharomyces cerevisiae*	CCY 21-4-102
22	*Saccharomyces cerevisiae*	CCY 21-4-81
23	*Saccharomyces cerevisiae*	CCY 48-26
24	*Saccharomyces cerevisiae*	CCY 21-4-64
25	*Saccharomyces cerevisiae*	CCY 48-88

Table 4: List of comertial brewer yeasts

Number	Microorganisms	Fermenting temperature range [ºC]
26	Bohemian lager yeast, M84	10-15
27	Cider yeast, M02	12-28
28	Premium gold; Muntons	-
29	Safale S-04	12-25
30	Saflager W-34/70	9-22
31	Saflager S-23	9-22
32	SafAle WB-06	12-25
33	Safale US-05	12-25

Table 5: List of algal strains

Number	Microorganisms	CCALA
46	*Euglena gracilis*	349

4.2 Media and growth conditions of yeast strains

Cultivation of yeasts was performed, first, on YPD agar medium to recover frozen cultures and then in YPD broth medium to prepare inoculum. For the screening of yeasts for β-glucan, lipids and carotenoids production, inoculum was inoculated into the YPD production medium amd medium with different C/N ratios was used to test the effect of C/N ratio on β-glucan production.

For the YPD agar cultivation, yeast cells from the frozen cryopreserved stock were transferred onto Petri dishes with YPD agar (yeast extract, 10.0 g/L; peptone, 20.0 g/L; glucose 20.0 g/L; agar, 20.0 g/L) (Merck, Darmstadt, Germany) and cultivated for 72 hours at 25 °C. Inoculum was prepared by transferring 1µl of yeasts cells from YPD agar into 50 mL of sterile YPD broth medium (yeast extract, 10.0 g/L; peptone, 20.0 g/L; glucose 20.0 g/L) (Merck, Darmstadt, Germany) in Erlenmeyer flask (250 mL) and cultivated for 24 hours at 25 °C under shaking regime (100 rpm, 50 mm). To remove residual medium after cultivation, the inoculum biomass was washed with sterile water and re-suspended to the original volume. The inoculum added to the YPD broth production medium (composition see above, C/N ratio 2.13:1) and media with other C/N ratios was in the volume ratio of 1:5. The YPD production medium with different C/N ratios was composed of (g/L): N-source yeast extract, 2; KH_2PO_4, 4; $MgSO_4 \cdot 7H_2O$, 0.696 and C-source

glucose monohydrate at the C/N ratios 10:1, 40:1, 70:1 and 100:1. For the calculation of the C/N ratio, a carbon content of 40 % in glucose and a nitrogen content of 10.5 % in yeast extract were assumed. The cultivation in all production media was performed in Duetz Microtiter Plate System (Enzyscreen, Netherlands) which consists of 24-well extra deep microtiter plates (MTPs) (Fig. 2) with low-evaporation Sandwich cowers and Clamp system for mounting MTPs on the top of each other. Cultivation in the control YPD broth production medium was done for 48h and 96h at 25 °C. Cultivations in the YPD broth production media with different C/N ratios were performed for 96h at 25 °C under shaking regime (100 rpm, 50 mm). For investigation of increasing osmolarity on beta-glucan and lipids production, four yeast strains with high beta-glucan production were choose, namely *Saccharomyces cerevisiae* (CCY 21-4-102), *Cystofilobasidium infirmominiatum* (CCY 17-18-4), *Phaffia rhodozyma* (CCY 77-1-1) and *Rhodotorula kratochvilovae* (CCY 20-2-26). Media with C/N ratio 40, 70 and 100 were supplemented with sodium chloride to final concentration 0.2, 0.5, 2, 5, 8 and 11 %. MTPs were used also for the screening of the exopolysaccharide production in red yeast.

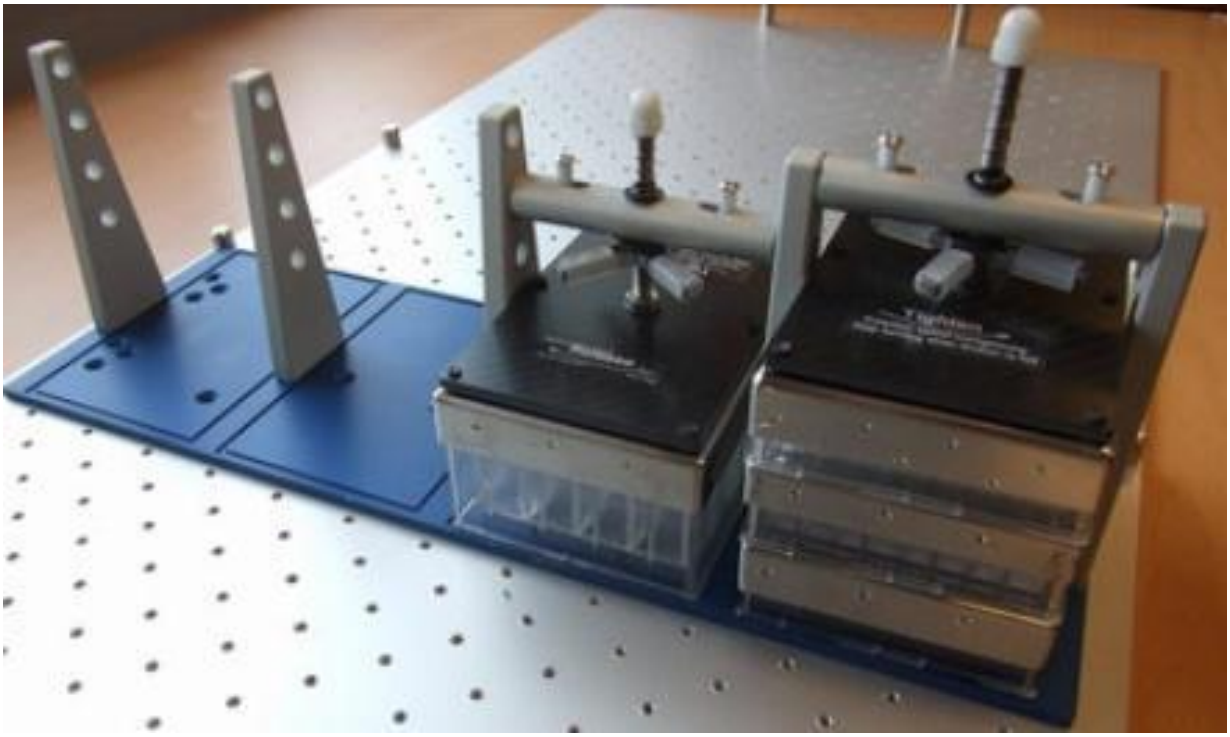

Fig 2: Duetz Microtiter Plate System (Enzyscreen, Netherlands) [50]

These four strains together with *S. pararoseus* CCY 19-9-6 were used for growth in different temperature regime (8, 15, 22 and 30 ^{o}C) at the C/N ratio of 70 and diverse nitrogen sources, where the inoculum was precultivated at the studied temperature. The used nitrogen sources were urea, ammonium sulphate, yeast extract and the combination af yeast extract and urea with the same amount of nitrogen as at C/N ratio experiment (Table 6). Cultivations were done in Erlenmeyer flasks for the time points of 48, 72, 96, 144 and 168h under shaking regime (110 rpm). All cultivation media were sterilized at 121^{o}C for 15 min.

Table 6: Composition of the media used for experiment with different temperature and C/N ratio of 70:1

MED 1		MED 2		MED 3		MED 4	
Compon.	**g/L**	**Compon.**	**g/L**	**Compon.**	**g/L**	**Compon.**	**g/L**
Glucose	40..4	Glucose	40.4	Glucose	40.4	Glucose	40.4
Yeast extract	0..5	Yeast extract	-	Yeast extract	-	Yeast extract	0.5
KH_2PO_4	4.0	KH_2PO_4	4.0	KH_2PO_4	4.0	KH_2PO_4	4.0
$MgSO_4 \cdot 7H_2O$	0.7	$MgSO_4 \cdot 7H_2O$	0.7	$MgSO_4 \cdot 7H_2O$	0.7	$MgSO_4 \cdot 7H_2O$	0.7
$(NH_4)_2SO_4$	0.7	$(NH_4)_2SO_4$	1.0	Urea	0.5	Urea	0.4

The screening of the production of exoglycolipids at *R. kratochvilovae* CCY 20-2-26 started with examination of nitrogen sources in media with glucose as a carbon source. Subsequently, different carbon sources (mannose, glycerol, xylose, galactose and lactose) were explored for production of beta-glucans, exoglycolipids and intracellular lipids. The media composition are vizualized in the Tables 7-8.

Table 7: Composition of culture media at C/N ratio 70:1 – nitrogen sources

Component	**g/L**
Glucose	40.42
KH_2PO_4	4
$MgSO_4 \cdot 7\ H_2O$	0.7
Nitrogen sources	**g/L**
Yeast extract	2
KNO_3	1.52
NH_4Cl	0.80
$(NH_2)_2SO_4$	0.9906
Urea	0.45

Table 8: Composition of culture media at C/N ratio 70:1 – different carbon sources

Component 1[g/l]	**Component 2 [g/L]**
Urea – 0.45	KNO_3 – 1.52
KH_2PO_4 - 4	KH_2PO_4 - 4
$MgSO_4 \cdot 7\ H_2O$ – 0.7	$MgSO_4 \cdot 7\ H_2O$ – 0.7

Carbon sources	g/L
Mannose	40.42
Lactose	34.94
Glycerol	37.57
Xylose	36.75

4.3 Culture conditions of *Euglena gracilis*

The 6day old cultures stocks were used for inoculation of two independent Erlenmeyer flasks by diluting the stock culture to 0.1 OD (680 nm) with fresh medium (Euglena medium or NM medium). The microalgal cultures were grown mixotrophically at 25 ºC and continuous shaking (150 rpm) and illumination or heterotrophically (10 and 20 g/L of glucose; NM medium adapted from Cramer et al. (1952) [26]) without light. After cultivation, biomass was centrifuged at 9,500 rpm for 10 min at 15°C and biomass pellet was then washed three times using distilled water. Further, biomass was freeze-dried for 48 h and, subsequently, stored at -80 °C until use.

Table 9: Composition of culture media for *Euglena gracilis*

	Euglena medium
$NaNO_3$	-
$MgSO_4 \cdot 7H_2O$	-
$CaCl_2 \cdot 2H_2O$	0.01
Yeast extract	2
Sodium acetate	1
Beef extrakt	1
Trypton	2
Chelated Iron	-
Heterotrophic culture: Euglena medium supplemented with 10 or 20 g/L of glucose	

4.3.1 Biomass determination

After cultivation, biomas sample was centrifuged at 4500 rpm for 5 min at 4°C and biomass pellet was then washed three times using 0,1% NaCl solution. Further, biomass was freeze-dried for 48 h and, subsequently, stored at -20 °C until use.

4.3.2 FAMEs analysis by GC/FID

Method FAMEs analysis from extracted biomas was used for screening of the yeast cultivated in MTPs and salt stress. Lipids from biomass in other experiments were modified to FAMEsby direct transesterification.

8.5.6 FAMEs analysis from extracted biomass

For total lipid extraction a modified Folch-method was used [27, 28]. Into 2 mL polypropylene tubes 15 ± 3 mg of freeze-dried yeast biomass was added together with 250 ± 20 mg acid-washed glass beads (710 – 1180 µm diameter, Sigma-Aldrich, USA) and 600 µL methanol. For disruption of yeast cells, the Precellys evolution homogenizer (Bertin Instruments, Germany) was used with shaking cycles of 5500 rpm (2 x 20 s) three times. The content of the PP tube was transferred into

glass reaction tube by washing it with 2400 µL solvent mixture of methanol:chloroform:hydrochloric acid (7.6:1:1 v/v). 1 mg of tridecanoid acid (C13:0) was used as internal standard and added to the reaction mixture. The glass tube was vortexed for 10 s and incubated for 1 hour at 90 °C. After following cooling to room temperature, 1 mL of distilled water and 2 mL hexane:chloroform (4:1 v/v) mixture. The separated upper hexane phase with extracted lipids was evaporated under nitrogen at 30 °C followed by addition of sodium sulphate and dissolving the FAMEs in 1.5 mL hexane containing 0.01% butylated hydroxytoluene (BHT, Sigma-Aldrich, USA). Hexane was transferred into glass vials for further GC analysis. Total lipid content (wt% of total FAMEs of the dry weight) and the fatty acid profile were performed by HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, USA) equipped with an SGE BPX70, 60.0 m x 250 µm x 0.25 µm column (SGE Analytical Science, Ringwood, Australia) and flame ionization detector (FID). Total time of the analysis was 36 min with the initial temperature of 70 °C, holding for 2 min, with increasing of 10 °C/min to 150 °C, then 6 °C/min to 230 °C. 1 µL of the sample was injected in split mode (30:1 split ratio) to inlet tempered to 280 °C. FAME standard mixture (C4 – C24; Sigma Aldrich, Germany) dissolved in hexane was used for identification of the FAMEs. Quantification was based on C13:0 internal standard and relative response factors (RRF) calculated from 5-point calibration curves of the individual FAMEs present in the standard mixture.

8.5.7 FAMEs analysis by direct transesterification

Into 2 mL glass crimp vial 10 ± 2 mg of freeze-dried yeast biomass was weight together with 1.8 mL of transesterification solvent (15% (v/v) H_2SO_4 in methanol (HPLC grade), 0.5 mg/mL C17 as a intermal standard) and the sample was incubated for 2 hours at 85 °C. After following cooling to room temperature, the full volume of vial was transferred into 4mL glass vial with addition of 0.5 mL 0.05M NaOH and 1 mL of hexane (GC grade). The mixture was vortexed for 5 min and after the phase separation, 0.1 mL upper hexane extract and 0.9 mL of pure hexane was transfered into glass vials for further GC analysis. Total lipid content (wt% of total FAMEs of the dry weight) and the fatty acid profile were performed by Thermo Scientific TRACE™ 1300 Gas Chromatograph equipped with a Zebron ZB-FAME column, 30 m x 0,25 mm x 0,20 µm and flame ionization detector (FID). Temperature gradient is presented in the Table 17. 1 µL of the sample was injected in split mode to inlet tempered to 260 °C. FAME standard mixture (C4 – C24; Sigma Aldrich, Germany) dissolved in hexane was used for identification of the FAMEs. Quantification was based on C17:0 internal standard and relative response factors (RRF) calculated from 5-point calibration curves of the individual FAMEs present in the standard mixture.

Table 10: Temperature gradient

Retention time [min]	Gradient [°C/min]	Target temperature [°C]	Hold [min]
0	-	-	-
1	0	80	1
5	15	140	0
21.7	3	190	0
25.5	25	260	1
25.5	STOP	-	-

4.3.3 Glucan content determination in yeast and mushrooms by enzyme method

The content of total glucans, β- and α-glucans in yeast freeze-dried biomass were determined according to the Yeast and Mushroom β-glucan Assay Procedure K-YBGL (Megazyme Int., Poland). To estimate the total glucans content, yeasts biomass was hydrolysed with ice-cold 12 M sulphuric acid for 2 h and then incubated for 2 h at 100 ^{o}C. Further, the neutralization of acidic hydrolysate with 200 mM sodium acetate buffer (pH 5) and 10 M KOH was done, followed by effect of enzymes exo-β-(1→3)-D-glucanase and β-glucosidase in acetate buffer (pH 4.5) for 1 hour at 40 ^{o}C. The α-glucan content was determined after enzyme hydrolysis with amylo-glucosidase and invertase. The β-glucan content was calculated from the assay kit procedure as difference between total glucans content and α-glucan content. The absorbance values for total glucans and α-glucans content were obtained spectrophotometrically at 510 nm after adding glucose oxidase/peroxidase reagent to the samples.

4.3.4 Isolation of paramylon granules from *Euglena gracilis*

Paramylon was extracted and purified according to Schwartzbach et al. (1975) [29]. 15±3 mg of freeze-dried biomass was weight into glass tube with screw and 2 mL of solution containing 1% (w/v) SDS and 5% (w/v) Na_2EDTA were added. The suspension was vortexed, incubated for 30 min at 37 ^{o}C and then centrifuged for 10 min for 1 000 x g. The SDS-Na_2EDTA treatment was repeated. The pellet was washed twice with hot glass-distilled water (70 ^{o}C). After the second wash, the suspension was centrifuged, and the granules were dried overnight at 90 ^{o}C for dry weight determination. The amount of paramylon was expressed as it's the ratio of paramylon weight per biomass (CDW).

4.3.5 Isolation of exopolysaccharides

To isolate the exopolysaccharides (EPS) released into the culture broth, supernatants obtained from 10 ml samples, after centrifugation, were treated by two volumes of cold isopropanol followed by an incubation at 4 ^{o}C. Precipitated EPS were removed by centrifugation at 6,000 g for 10 min at 4 ^{o}C, washed again with 5 mL of isopropanol and centrifuged. The samples were dried at 85 ^{o}C overnight and then freeze dried.

8.5.6 Isolation of exoglycolipids

Exoglycolipids were isolated from culture medium according to procedure from Wang et al. (2019) [22]. 20 mL of culture broth was centrifuged (6,500 rpm/5 min), the supernatant was separated and washed with ethylacetate (5 mL). The exoglycolipids contained biomass was washed with 5 mL of ethylacetate and centrifuged (6500 rpm/5min). The ethylacetate fractions were merged together, evaporated under nitrogen flow and lyophilized. The dry weight of ethylacetate extract was weighed to estimate grams of extracellular glycolipids per liter of culture.

5 RESULTS AND DISCUSSION

5.1.1 Growth, total glucan content and co-production of β-glucans and lipids

The screening of yeasts for identifying new potential producers of β-glucans lipid production was performed in a high-throughput set-up by utilizing high-throughput micro-cultivation in Duetz-MTPS. Due to the fact, that glucan production is usually performed in a low C/N ratio media [30], while lipid accumulation in yeasts is triggered by the high C/N ratio and depletion of a nitrogen source [31], media with both low and high C/N ratios were used for the screening.

Cultivation of yeasts in the media with low (10:1), moderate (40:1) and high (70:1 and 100:1) C/N ratios showed a continuous increase in a biomass yield with the increase of C/N ratio (Table 11). Thus, for most of the studied yeast strains the highest biomass yield in a range from 2.67±0.26 to 15.33±1.16 g/L was observed in the medium with the 100 C/N ratio, and strains *R. kratochvilovae* CCY 20-2-26, *R. mucilaginosa* CCY 19-4-6 and CCY 20-9-7, *R. toruloides* CCY 62-2-4 and *S. pararoseus* CCY 19-9-6 showed the highest biomass production in the medium with 70 C/N ratio (Table 11). The highest biomass yield 15.3 g/L was obtained for the strain *C. macerans* CCY 10-1-2 at 100 C/N ratio, that was nearly three times higher than for the reference β-glucans producing strain *Saccharomyces cerevisiae* CCY 21-4-102. For all *Rhodotorula* strains and strain *S. salmonicolor* CCY 19-4-25 the significant increase in the biomass production occurred from 10 to 40 C/N ratio, and from 70 to 100 C/N ratio, while little change was observed from 40 to 70 C/N ratio.

Table 11: Biomass yield for yeasts grown in the media with low and high C/N ratios

					Biomass yield (g/L)	
No	**Strain name**	**CCY**	**C/N 10:1**	**C/N 40:1**	**C/N 70:1**	**C/N 100:1**
1	*Cystofil. infirmominiatum*	17-18-4	3.80±0.30	8.37±0.31	13.06±0.43	15.19±0.91
2	*Cystofilobasidium macerans*	10-1-2	3.63±0.24	8.76±0.27	13.34±0.06	**15.33±1.16**
3	*Phaffia rhodozyma*	77-1-1	2.67±0.26	6.78±0.24	10.98±0.50	13.09±1.01
4	*Rhodotorula kratochvilovae*	20-2-26	4.15±0.68	10.23±0.17	12.05±0.29	10.11±1.37
5	*Rhodotorula mucilaginosa*	19-4-6	4.57±0.29	10.36±0.26	11.34±0.23	10.84±0.35
6	*Rhodotorula mucilaginosa*	20-9-7	4.30±0.18	9.80±0.18	9.97±0.49	8.90±1.52
7	*Rhodotorula toruloides*	62-2-4	3.65±0.10	8.70±0.20	11.80±0.61	11.70±0.80
8	*Rhodotorula glutinis*	20-2-22	3.01±0.65	7.34±0.84	11.51±1.02	12.79±0.54
9	*Rhodotorula glutinis*	20-2-47	2.78±0.37	6.93±0.51	10.13±0.16	11.31±0.43
10	*Sporidiobolus metaroseus*	19-6-20	3.67±0.25	8.88±0.05	13.42±0.12	15.11±0.93
11	*Sporidiobolus pararoseus*	19-9-6	3.83±0.44	6.81±1.30	10.88±1.15	10.80±1.88
12	*Sporidiobolus salmonicolor*	19-6-4	2.99±0.15	6.30±0.15	6.55±0.31	8.24±1.09
13	*Sporidiobolus salmonicolor*	19-4-25	3.49±0.15	5.85±0.27	5.88±0.50	5.86±0.36
14	*Dioshegia hungarica*	18-1-3	4.14±0.48	6.61±0.47	7.14±0.21	7.20±0.39

Generally, *Ascomycetes* yeast showed lower biomass production in comparison to carotenogenic yeast (Table 12), except from *Y. lipolytica* CCY 29-26-4, where the biomass content reached 14.95±1.08 g/L. The growth of other yeast strains did not exceeted the yield of 7 g/L. The lowest biomass production can be seen at strain *A. pullulans* CCM 8183, where the 0.81 g/L of biomass was achieved.

Table 12: Biomass yield for yeasts grown in the media with low and high C/N ratios

			Biomass yield (g/L)			
No	**Strain name**	**CCY**	**C/N 10:1**	**C/N 40:1**	**C/N 70:1**	**C/N 100:1**
15	*Saccharomyces cerevisiae*	21-4-102	3.59±0.09	5.25±0.36	5.32±0.74	5.94±0.56
16	*Aureobasidium pullulans*	CCM 8183	1.05±0.04	1.03±0.02	1.12±0.11	0.81±0.16
17	*Candida glabrata*	CCM 8270	2.54±0.24	5.30±0.41	6.38±0.34	6.47±0.67
18	*Yarrowia lipolityca*	29-26-4	4.49±0.54	9.53±0.62	13.57±0.33	**14.95±1.08**
19	*Metschnikowia pulcherrima*	29-2-149	3.57±0.05	5.42±0.06	5.82±0.18	6.78±0.19
20	*Metschnikowia pulcherrima*	29-2-147	3.32±0.06	4.99±0.07	5.66±0.19	5.73±0.10
21	*Metschnikowia pulcherrima*	29-2-129	3.69±0.24	5.49±0.25	6.55±0.34	6.73±0.32

It has been shown previously that variation in a C/N ratio has a significant effect on the accumulation of carbon-based intracellular metabolites (carbohydrates, lipids, pigments, etc.) [31]. We observed the increase in the glucan and lipid content at high C/N ratio for the yeast strains showing the increase in the biomass yield (Table 13, Fig. 3), with the exception of *C. macerans* CCY 10-1-2, *C. infirmominiatum* CCY 17-18-4, *S. metaroseus* CCY 19-6-20 in case of glucan and *S. salmonicolor* CCY 19-6-4 in case of lipids. For the strains *C. macerans* CCY 10-1-2, *P. rhodozyma* CCY 77-1-1 and *R. toruloides* CCY 62-2-4, the highest total glucan content was detected at low C/N ratios (Table 13). This could be due to the fact, that low C/N ratio triggering yeast proliferation and subsequently cell wall formation and carbon is transformed into protein and cell wall components, while lipogenesis is supressed. While the highest β-glucans content 38.84 ± 1.89 % (w/w) was achieved for the strain *C. glabrata* under 100 C/N ratio, and 26.96 ± 2.90% (w/w) for control strain *S. cerevisiae*. Among the carotenogenic yeast the highest total glucan content of 30.15±3.21 (w/w) with the high content of β-glucans 25.34±3.79 (w/w) was recorded for *C. infirmominiatum* CCY 17-18-4. Several other yeast strains showed very similar β-glucans content at different C/N ratios: (i) at 10 C/N ratio, strains *P. rhodozyma* CCY 77-1-1, *S. metaroseus* CCY 19-6-20, *R. kratochvilovae* CCY 20-2-26 and *C. macerans* CCY 10-1-2 had β-glucans content between 21,68% and 24,52% (w/w); (ii) at 40 C/N raio, strain *C. infirmominiatum* CCY 17-18-4 accumulated 25.34 ± 3.79% (w/w) of β-glucans; (iii) at 70 C/N ratio, strain *M. pulcherrima* CCY 29-2-149 accumulated 20.45 ± 0.86% (w/w) of β-glucans and (iv) at 100 C/N ratio, strains *M. pulcherrima* CCY 29-2-129 and *M. pulcherrima* CCY 29-2-147 showed β-glucan content of 22.35 ± 1.68% (w/w) and 21.09±1.68% (w/w), respectively. Taking into the account the high biomass yield and β-glucans content for some of the yeast strains, as for example *C. infirmominiatum* CCY 17-18-4, *R. kratochvilovae* CCY 20-2-26 and *P. rhodozyma* CCY 77-1-1, they could be considered as promising new yeast sources of the β-glucans within the carotenogenic yeast.

Most of the studied yeast strains showed the negligible presence of α-glucans, while some of them have a relatively high content of it (Table 13). The highest α-glucan content, over 5 % per dry weight, was observed for the strain *C. macerans* CCY 10-1-2, which exhibited one of the lowest β-glucans content, meaning that the ratio of α- and β-glucans in this strain was 1:2 at 100 C/N ratio, while in all other yeast strains β-glucans were in a significantly higher proportion than α-glucans (Fig. 20). The high content of α-glucans in Basidiomycetes carotenogenic yeasts could be explained by the possible presence of intracellular glucose (poly)saccharides like trehalose (α-1,1-bounded glucose) or glycogen (α-1,4- and α-1,6-glucose polymer). The lowest α-glucan content was detected for yeasts of *A. pullulans* CCM 8183, *Metschnikowia* genera and *R. mucilaginosa* strains, where it did not exceed 1.5 % of the total cell dry weight (Table 13 and 14).

Table 13: Total glucan, α- and β-glucans content (% CDW) in yeasts grown in the media with low and high C/N ratios

Strain		**C/N 10:1**	**C/N 40:1**	**C/N 70:1**	**C/N 100:1**
C. infirmominiatum CCY 17-18-4	Total glucan	26.04±0.80	30.15±3.21	24.78±1.64	23.73±2.25
	α-glucans	3.33±0.39	4.81±1.81	4.03±0.94	3.01±0.36
	β-glucans	**22.72±0.41**	**25.34±3.79**	**20.75±1.33**	**20.73±1.39**
C. macerans CCY 10-1-2	Total glucan	26.15±1.38	23.61±2.05	18.53±2.08	16.32±1.14
	α-glucans	2.68±0.37	5.19±3.30	5.08±3.30	5.02±3.25
	β-glucans	**23.43±1.60**	**18.42±2.64**	**13.45±4.42**	**11.31±3.34**
P. rhodozyma CCY 77-1-1	Total glucan	27.13±0.88	24.93±1.61	21.05±1.74	20.35±0.33
	α-glucans	2.61±0.06	2.68±0.75	2.20±0.40	2.03±0.28
	β-glucans	**24.52±0.85**	**22.28±1.05**	**20.04±0.18**	**18.55±0.15**
R. kratochvilovae CCY 20-2-26	Total glucan	23.18±1.19	23.80±1.12	21.84±0.83	18.82±0.29
	α-glucans	1.50±0.34	1.04±0.07	0.98±0.11	1.23±0.19
	β-glucans	**21.68±0.96**	**22.20±1.04**	**21.43±0.34**	**17.59±0.55**
R. mucilaginosa CCY 19-4-6	Total glucan	17.52±0.64	14.60±0.12	15.54±1.04	15.99±1.15
	α-glucans	0.87±0.27	0.49±0.09	0.66±0.10	0.73±0.04
	β-glucans	**16.65±0.67**	**14.11±0.20**	**14.88±1.08**	**15.26±1.15**
R. mucilaginosa CCY 20-9-7	Total glucan	18.18±0.06	19.17±0.67	19.91±1.21	20.31±0.95
	α-glucans	0.96±0.32	0.98±0.21	1.46±0.72	1.18±0.20
	β-glucans	**17.22±0.26**	**18.19±0.65**	**18.45±1.68**	**19.13±1.13**
R. toruloides CCY 62-2-4	Total glucan	19.26±0.84	14.74±0.25	11.75±1.22	11.81±0.58
	α-glucans	1.85±0.30	1.97±0.28	2.07±0.42	1.78±0.31
	β-glucans	**17.41±0.98**	**12.83±0.37**	**9.67±1.63**	**10.03±0.72**
R. glutinis CCY 20-2-22	Total glucan	10.35±0.54	16.02±1.27	6.63±0.28	5.56±0.36
	α-glucans	0.56±0.14	1.03±0.14	1.35±0.21	1.67±0.13
	β-glucans	**9.79±0.36**	**14.99±1.58**	**5.28±0.16**	**3.89±0.22**
R. glutinis CCY 20-2-47	Total glucan	10.23±1.02	9.37±0.34	6.08±0.28	4.13±0.32
	α-glucans	1.02±0.14	1.27±0.11	0.62±0.10	1.35±0.17
	β-glucans	**9.21±0.57**	**8.10±0.26**	**5.46±0.14**	**3.03±0.41**
S. metaroseus CCY 19-6-20	Total glucan	26.75±2.59	22.77±1.32	16.63±1.00	17.68±2.21
	α-glucans	2.60±0.30	4.50±0.36	2.62±0.15	2.92±0.23
	β-glucans	**24.15±2.89**	**18.27±1.32**	**13.99±1.04**	**14.76±2.32**
S. pararoseus CCY 19-9-6	Total glucan	14.30±1.21	16.87±1.91	15.58±0.66	14.73±0.79
	α-glucans	1.26±0.45	2.41±1.60	2.76±1.88	3.51±1.43
	β-glucans	**13.04±0.79**	**14.46±3.13**	**12.81±2.20**	**11.23±1.72**
S. salmonicolor CCY 19-6-4	Total glucan	12.90±1.25	17.12±0.92	17.96±0.50	18.95±2.00
	α-glucans	1.32±0.28	2.28±0.74	2.55±0.57	2.43±0.38
	β-glucans	**11.58±1.03**	**14.83±1.32**	**15.41±0.88**	**16.52±2.28**
S. salmonicolor CCY 19-4-25	Total glucan	15.04±0.67	14.10±0.31	14.95±0.63	17.16±1.73
	α-glucans	1.48±0.14	2.35±0.66	2.67±0.76	2.84±0.57
	β-glucans	**13.56±0.55**	**11.75±0.59**	**12.28±1.29**	**14.32±2.30**
D. hungarica CCY 18-1-3	Total glucan	13.45±1.45	15.34±0.45	11.56±0.68	9.17±0.88
	α-glucans	1.32±0.12	1.32±0.21	2.43±0.23	3.48±0.41
	β-glucans	**12.13±0.75**	**14.02±1.02**	**9.13±0.45**	**5.70±24**

Table 14: Total glucan, α- and β-glucans content (% CDW) in non-yeasts grown in the media with low and high C/N ratios

Strain		C/N 10:1	C/N 40:1	C/N 70:1	C/N 100:1
S.cerevisiae CCY 21-4-102	Total glucan	20.54±0.58	22.91±2.03	26.21±1.14	29.86±3.11
	α-glucans	2.35±0.38	2.35±0.56	3.41±0.80	2.90±0.41
	β-glucans	**18.19±0.36**	**20.57±1.54**	**22.80±0.58**	**26.96±2.90**
A. pullulans CCM 8183	Total glucan	10.45±0.55	10.90±0.55	11.72±1.23	13.54±0.74
	α-glucans	0.12±0.10	0	0.41±0.06	0.64±0.17
	β-glucans	**10.33±1.58**	**10.90±0.55**	**11.31±1.01**	**12.90±0.84**
C. glabrata CCM 8270	Total glucan	25.57±1.38	37.59±1.24	41.48±2.42	44.45±3.14
	α-glucans	3.45±0.16	3.94±0.16	5.35±0.26	5.61±0.37
	β-glucans	**22.12±1.49**	**33.65±1.32**	**36.13±1.57**	**38.84±1.89**
Y. lipolytica CCY 29-26-4	Total glucan	15.65±1.41	18.22±1.42	17.82±1.05	17.81±2.09
	α-glucans	0.84±0.12	1.07±0.13	1.89±0.27	1.89±0.07
	β-glucans	**14.81±0.84**	**17.15±1.41**	**15.93±1.24**	**15.93±1.22**
M. pulcherrima CCY 29-2-149	Total glucan	15.42±0.90	18.26±0.91	21.09±0.94	21.34±1.22
	α-glucans	0.29±0.12	0.57±0.20	0.64±0.18	1.30±0.31
	β-glucans	**15.13±1.02**	**17.69±0.79**	**20.45±0.86**	**20.04±1.12**
M. pulcherrima CCY 29-2-147	Total glucan	18.41±1.55	19.84±1.23	21.70±1.56	21.73±1.21
	α-glucans	0.35±0.04	0.42±0.04	0.77±0.26	0.64±0.11
	β-glucans	**18.06±1.54**	**19.42±1.09**	**20.93±1.80**	**21.09±0.69**
M. pulcherrima CCY 29-2-127	Total glucan	16.84±1.08	21.13±1.25	21.81±1.56	23.25±1.21
	α-glucans	0.47±0.29	0.52±0.19	1.28±0.72	0.90±0.59
	β-glucans	**16.37±1.12**	**20.61±1.09**	**20.54±1.10**	**22.35±1.68**

The decrease in glucan production for the *Cystofilobasidium* strains, as well as strains *R. toruloides* CCY 62-2-4, *R, kratochvilovae* CCY 20-2-26, and *P. rhodozyma* CCY 77-1-1 under high C/N ratios can be explained by the increased accumulation of lipids in the range from 30 up to 47 % (w/w). It is known that due to the high activity of AMP-dependent isocitrate dehydrogenase some Basidiomyctes carotenogenic yeasts produce lipids up to 70 % lipids/dry weight [31, 32].

In overall, the total lipid content, similarly as a glucan content, was increased in yeast cells when grown in the media with high C/N ratios (Fig. 3), except for the strains *R. kratochvilovae* CCY 20-2-26, *R. mucilaginosa* CCY 19-4-6 and CCY 20-9-7, *S. pararoseus* CCY 19-9-6 and *S. salmonicolor* CCY 19-4-25, where high amount of glucose (100 C/N) led to the decrease in the lipid content accompanied by the lower biomass production (Table 13). This, could be due to the osmotic pressure stress under the high glucose concentration that negatively influenced the growth of these strains.

Basidiomycetes carotenogenic red yeasts showed oleaginous properties and were able to accumulate lipids from 30 to over 47 % (w/w) while Ascomycetes non-carotenogenic yeast did not accumulate lipids more than 10% (w/w) (Fig. 4). While, it has been reported that non-carotenogenic *Metschnikowia* yeast can accumulate large amount of lipids (up to 40% w/w) [33], in the present study it did not accumulate more than 10 % (w/w) that could be due to the higher temperature and shorter cultivation time used, in comparison to the previsouly reported 15 ^{o}C and 14 days [33]. Generally, Ascomycetes yeasts were not affected by the variation in the amount of glucose in the media, and the biomass and lipid yield were unchanged.

High lipid yield in the range from 33 to 59 % (w/w) was observed for the strains *C. infirmominiatum* CCY 17-18-4, *C. macerans* CCY 10-1-2, *R. toruloides* CCY 62-2-4, *S. metaroseus* CCY 19-6-20, *R. glutinis* CCY 20-2-47 and *S. pararoseus* CCY 19-9-6 when grown under high C/N ratios. The highest lipid content 59.20 ± 1.60 % (w/w) was observed for carotenogenic strain *R. glutinis* CCY 20-2-47 (Fig. 3). Yeast strains with the highest lipid accumulation (over 45 % w/w), namely *C. macerans* CCY 10-1-2, *R. toruloides* CCY 62-2-4, *R. glutinis* CCY 20-2-47, and *S. metaroseus* CCY 19-6-20 showed low β-glucan content (10 – 14 % w/w) and can be considered as mainly lipid producers. Some yeast strains as for example *C.*

infirmominiatum CCY 17-18-4 at 100 C/N and *R. kratochvilovae* CCY 20-2-26 at 70 C/N were able to accumulate relatively high content of both, lipids (38.21 % and 37.92 % of w/w) and β-glucan (20.73 and 21.43 % of w/w) accompanying with the high biomass yield (15.19 and 12.05 g/L) and therefore could be utilised for developing co-production strategies.

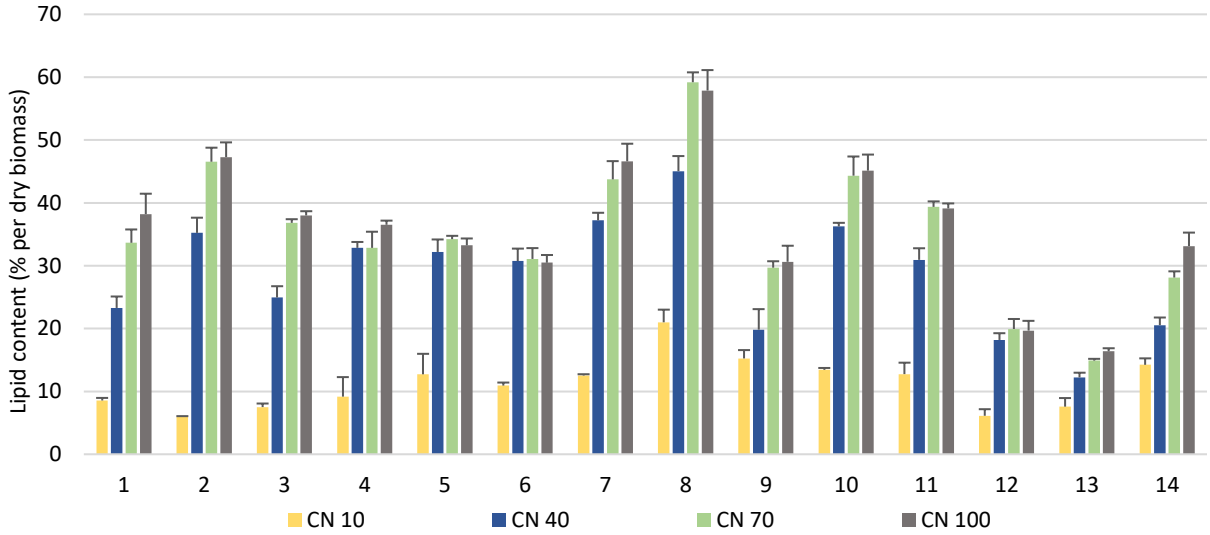

Fig. 3: Lipid content (% w/w) in carotenogenic yeasts grown in the media with low and high C/N ratios. Yeast strain numbers are described in Table 18.

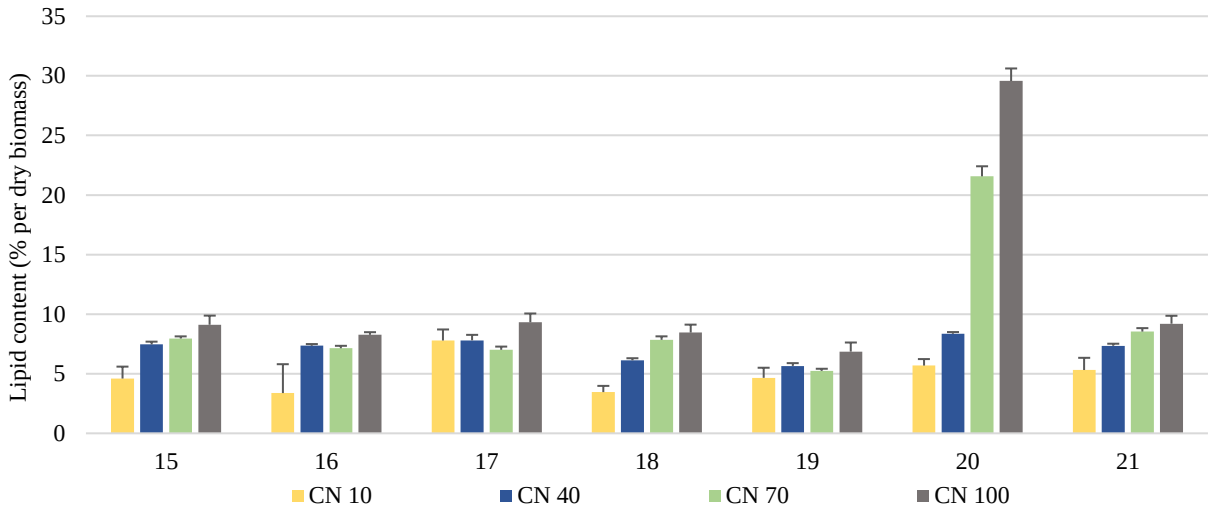

Fig. 4: Lipid content (% w/w) in Ascomycetes yeasts grown in the media with low and high C/N ratios. Yeast strain numbers are described in Table 19.

9.2. Salt stress

Cell wall remodelling is often a key response event when cells are exposed to the osmotic stress [34, 35]. Due to the fact, that β-glucans are important structural component of the cell wall, applying osmotic stress could potentially have a significant effect on their production [36, 37]. Recently, several studies reported the influence of extracellular osmolarity on β-glucans' biosynthesis in *Saccharomyces cerevisiae* where a decrease in β-glucan content at higher osmolarity was detected [38].

In this study, the sodium chloride (NaCl) was used at six different concentrations (0.2; 0.5; 2; 5; 8 and 11%) to investigate the influence of different extracellular osmolarity levels on the production of β-glucans and co-production of lipids and β-glucans [39]. The three yeast strains *C. infirmominiatum* CCY 17-18-4, *P. rhodozyma* CCY 77-1-1 and *R. kratochvilovae* CCY 20-2-26, showing the highest β-glucans and a high lipid production in this study were selected for the study. The *S. cerevisiae* CCY 19-6-4 strain was used as a control strain for β-glucans production. The supplementation of growth media with C/N ratios of 40 and 70 by NaCl at all studied concentration led to a decrease in the biomass yield for all yeast strains except for *S. cerevisiae* (Table 15). The biomass production for *S. cerevisiae* under low osmolarity conditions (0,2% and 0,5% of NaCl) in C/N ratio growth media with C/N ratios of 40, 70 and 100 was higher or very similar to the standard conditions without salt, while high osmolarity resulted in the significant decrease of biomass (Table 15). Low extracellular osmolarity (0.2% of NaCl) combined with high C/N ratio resulted in an increase or no change in the biomass yield in comparison to the standard conditions (Table 11). The biomass yield was gradually decreasing with the increased amount of NaCl in the media and high concentration of NaCl combined with high C/N ratio resulted in the lowest biomass production that was possibly due to the elevated levels of osmolarity caused by both, high NaCl and glucose content in the medium (Table 15). The strain *P. rhodozyma* CCY 77-1-1 showed the highest sensitivity to the applied osmotic stress, and it growth was highly inhibited in the presence of NaCl 2% and higher (Table 15).

Table 15: The effect of different osmolarity levels on biomass yield in strains *C. infirmominiatum* CCY 17-18-4 (1), *P. rhodozyma* CCY 77-1-1 (6), *R. kratochvilivae* CCY 20-2-26 (7), *S. cerevisiae* CCY 21-4-102 (11)

Strain	C/N	0.2% NaCl	0.5% NaCl	2% NaCl	5% NaCl	8% NaCl	11% NaCl
	40	7.44±0.04	7.38±0.19	7.08±0.11	6.38±0.07	5.93±0.07	4.81±0.61
1	**70**	11.40±0.10	11.47±0.35	10.77±0.07	10.28±0.07	9.13±0.49	4.54±0.84
	100	15.25±0.39	14.88±0.11	11.40±2.84	11.33±0.05	8.90±0.59	4.73±0.24
	40	5.62±0.41	5.56±0.08	2.24±0.16	1.05±0.14	0.70±0.16	0.68±0.07
6	**70**	8.86±0.61	8.69±0.27	2.59±0.21	1.06±0.09	0.87±0.16	0.64±0.10
	100	12.35±1.21	11.11±1.48	2.73±0.19	1.00±0.03	0.78±0.23	0.66±0.13
	40	9.04±0.31	8.57±0.30	8.44±0.07	7.52±0.10	5.71±0.25	3.71±0.09
7	**70**	11.76±0.81	11.26±0.73	10.70±0.04	8.47±0.08	5.80±0.46	3.90±0.39
	100	11.52±0.74	10.83±0.75	10.48±0.04	8.24±0.06	5.69±0.42	3.69±0.47
	40	5.45±0.11	5.26±0.20	5.03±0.07	3.25±0.04	2.71±0.16	1.78±0.13
11	**70**	5.94±0.14	5.70±0.31	4.97±0.06	3.82±0.05	3.03±0.29	1.69±0.04
	100	6.32±0.40	5.71±0.47	4.95±0.09	4.00±0.03	3.04±0.28	1.57±0.06

The effect of extracellular osmolarity on the total glucan, β- and α-glucan content in yeasts cells was strain-specific and differed depending on the level of osmolarity and C/N ratio used (Table 16). Thus, low (0.2 and 0.5% of NaCl) and in some cases moderate (2 and 5%) levels of osmolarity combined with C/N ratios of 40, 70 and 100 led to an increase in the total glucan and β-glucan content in comparison to the standard conditions (Table 16, Table 11). For example, the addition of 0.2% NaCl caused increase in β-glucan production up to 32.15 ± 0.81 (w/w) in *C. infirmominiatum* CCY 17-18-4 (Table 16), that is about 21 % more than in the standard conditions and significantly higher than for the control strain *S.cerevisiae* CCY 21-4-102 (Table 14). In the case of strain *P. rhodozyma* CCY 77-1-1, β-glucans content was increased at all osmolarity levels

when combined with C/N ratios of 70 and 100, while it showed an inhibiting effect on production of β-glucans in other yeast strains.

Table 16: Glucans production on media supplemented with sodium chloride (% od DCW)

Strain	C/N	% w/w	0.2% NaCl	0.5% NaCl	2% NaCl	5% NaCl	8% NaCl	11% NaCl
1	40	Total Glucan	35,65±0,86	34,10±1,91	32,87±1,75	22,71±1,67	21,65±0,97	20,80±1,56
		Alpha-Glucan	3,50±0,40	3,55±0,39	3,07±0,32	2,96±0,22	3,03±0,48	2,63±0,43
		Beta-Glucan	32,15±0,81	30,55±1,84	29,80±1,43	19,75±1,87	18,62±0,67	18,17±1,55
	70	Total Glucan	27,15±1,26	25,42±0,38	21,11±0,44	23,33±1,07	19,72±0,56	21,07±0,96
		Alpha-Glucan	3,16±0,21	3,11±0,30	2,79±0,10	3,87±1,16	2,70±0,12	2,79±0,77
		Beta-Glucan	23,99±1,06	22,32±0,63	18,32±0,52	19,46±1,54	17,03±0,66	18,28±1,23
	100	Total Glucan	21,83±0,27	21,64±1,47	23,16±0,45	19,95±1,58	17,96±0,82	21,52±2,16
		Alpha-Glucan	3,04±0,29	3,41±0,36	4,42±1,29	3,04±0,18	3,20±0,03	3,11±0,77
		Beta-Glucan	18,78±0,49	18,24±1,11	18,73±1,71	16,91±1,40	14,76±0,82	18,40±2,02
6	40	Total Glucan	31,69±1,97	32,51±0,51	24,47±1,76	27,75±0,99	29,05±0,46	26,25±0,87
		Alpha-Glucan	4,37±2,58	3,25±0,52	3,44±0,17	3,05±0,45	3,25±0,98	2,72±0,56
		Beta-Glucan	27,32±4,55	29,26±1,02	21,03±1,93	24,70±0,60	25,80±1,26	23,53±1,44
	70	Total Glucan	24,27±2,24	24,30±0,97	26,60±2,10	27,18±0,81	25,65±0,74	24,27±1,37
		Alpha-Glucan	2,42±0,35	2,71±0,32	3,05±0,24	2,69±0,30	3,24±0,66	2,46±0,89
		Beta-Glucan	21,85±2,07	21,59±0,65	23,55±2,31	24,49±0,96	22,41±0,20	21,81±0,69
	100	Total Glucan	24,97±1,16	27,56±1,29	24,29±1,21	28,11±0,87	26,74±0,83	26,02±1,06
		Alpha-Glucan	2,32±0,35	2,73±0,57	3,09±0,13	2,58±0,50	2,87±0,36	2,81±0,88
		Beta-Glucan	22,65±1,19	24,83±1,86	21,20±1,20	25,53±1,28	23,87±0,46	23,21±1,17
7	40	Total Glucan	24,13±2,17	23,53±2,53	17,08±1,09	13,29±0,96	14,67±0,67	12,23±1,90
		Alpha-Glucan	1,25±0,28	1,19±0,11	1,13±0,26	0,91±0,14	1,19±0,14	0,84±0,23
		Beta-Glucan	22,88±2,37	22,34±2,53	15,95±0,85	12,38±1,04	13,47±0,61	11,39±1,67
	70	Total Glucan	18,97±1,45	20,32±0,10	16,41±1,00	11,81±1,09	14,48±1,85	14,84±2,38
		Alpha-Glucan	1,12±0,33	0,83±0,38	0,93±0,31	0,68±0,13	0,90±0,24	1,14±0,19
		Beta-Glucan	17,84±1,20	19,49±0,44	15,48±1,30	11,14±1,05	13,58±1,62	13,69±2,20
	100	Total Glucan	20,36±0,46	18,18±1,00	13,03±0,53	12,08±0,67	15,68±0,29	9,42±0,38
		Alpha-Glucan	1,20±0,24	1,07±0,24	0,68±0,11	0,59±0,07	1,05±0,31	0,81±0,20
		Beta-Glucan	19,16±0,64	17,11±0,78	12,35±0,63	11,49±0,63	14,63±0,31	8,62±0,34
11	40	Total Glucan	30,40 ± 0,72	29,87±1,69	23,95±2,51	23,76±2,93	19,24±1,12	16,31±1,29
		Alpha-Glucan	1,49±0,18	1,31±0,35	2,05±0,32	1,21±0,17	0,67±0,21	0,35±0,13
		Beta-Glucan	28,91±0,62	28,56±1,37	21,90±2,81	22,55±2,77	18,57±0,96	15,93±1,45
	70	Total Glucan	29,16±1,40	27,29±0,36	31,08±2,42	25,83±2,12	18,07±0,45	16,94±2,13
		Alpha-Glucan	1,69±0,39	1,54±0,42	2,19±0,32	1,50±0,10	0,52±0,20	0,58±0,16
		Beta-Glucan	27,47±1,03	25,75±0,57	28,89±2,40	24,33±2,15	17,56±0,58	16,36±2,24
	100	Total Glucan	33,38±0,93	31,55±2,43	30,06±2,11	24,32±0,92	21,67±0,29	15,59±2,03
		Alpha-Glucan	2,06±0,19	1,13±0,47	1,75±0,31	1,03±0,09	1,05±0,32	0,50±0,14
		Beta-Glucan	31,32±0,93	30,42±2,02	28,31±2,27	23,29±0,84	20,62±0,10	15,10±1,92

9.3. Influence of the temperature and media composition to β-glucan production

Each of the selected yeast species was cultured in four types of media, differing in composition, resp. nitrogen source (Table 6). Cultivation was carried out in a tempered shaker, ensuring a constant temperature (8, 15, 22, and 30 °C) throughout cultivation. The glucan measurement takes place at higher biomass content, namely at 168 h (8 °C), 144 h (15 °C), 96 h (22 °C), and 144 h (30 °C).

For all studied media, the combination of ammonium sulphate and urea (MED 4 – Table 6) shows the increase in β-glucan at *S. cerevisiae* CCY 21-4-102, *C. infirmominiatum* CCY 17-18-4 and *P. rhodozyma* CCY 77-1-1 (29.12, 12.32 and 23.48 % of DCW respectively). Concerning *R. kratochvilovae* CCY 20-2-26, the urea as the only nitrogen source exhibit the highest yield (MED 3) of β-glucan. Conversely, the biomass decreases due to low temperature.

Table 17: The effect of the temperature (8 °C) and media composition to β-glucan production (% of DCW)

8 °C **168 h**	*S. cerevisiae* CCY 21-4-102	*C. infirmominiatum* CCY 17-18-4	*P. rhodozyma* CCY 77-1-1	*R. kratochvilovae* CCY 20-2-26
MED 1	26.05±1.58	6.45±0.89	21.74±0.76	20.97±1.08
MED 2	12.59±1.06	5.84±0.67	20.83±0.99	21.01±0.20
MED 3	14.01±1.10	11.72±0.55	15.94±0.45	**22.95±2.14**
MED 4	**29.12±2.37**	**12.32±1.04**	**23.48±2.14**	16.45±1.12

The temperature of 15 °C has a positive effect on *C. infirmominiatum* CCY 17-18-4 (21.94 %, MED 3), *P. rhodozyma* CCY 77-1-1 (29.10 %, MED 3) and *R. kratochvilovae* CCY 20-2-26 (27.19 %, MED 1). The β-glucan content decreased at *S. cerevisiae* CCY 21-4-102. At most of the studied strains (except of *S. cerevisiae* CCY 21-4-102) the increase of the β-glucan production can be seen, but also the decrease in biomass content.

Table 18: The effect of the temperature (15 °C) and media composition to β-glucan production

15 °C **144 h**	*S. cerevisiae* CCY 21-4-102	*C. infirmominiatum* CCY 17-18-4	*P. rhodozyma* CCY 77-1-1	*R. kratochvilovae* CCY 20-2-26
MED 1	16.71±1.25	12.49±0.26	23.68±1.90	**27.19±2.11**
MED 2	13.44±0.73	**21.94±1.49**	28.43±1.05	25.07±3.74
MED 3	14.98±1.06	21.09±2.01	**29.10±2.64**	17.54±1.69
MED 4	**17.03±1.84**	15.86±0.61	28.39±0.55	21.61±1.72

The temperature of 22 °C seemst to be optimal for *C. infirmominiatum* CCY 17-18-4 (26.08±2.03 % of β-glucan on DCW) (Table 19). Oppositelly, the production was inhibited at *S. cerevisiae* CCY 21-4-102, *P. rhodozyma* CCY 77-1-1, *R. kratochvilovae* CCY 20-2-26 when compared with the temperature of 15 and 8 °C (Table 17 and 18).

Table 19: The effect of the temperature (22 °C) and media composition to β-glucan production

22 °C **96 h**	*S. cerevisiae* CCY 21-4-102	*C. infirmominiatum* CCY 17-18-4	*P. rhodozyma* CCY 77-1-1	*R. kratochvilovae* CCY 20-2-26
MED 1	9.54±1.62	10.39±0.20	**24.76±1.05**	13.17±1.02
MED 2	5.09±0.18	**26.08±2.03**	21.15±1.39	9.92±0.45
MED 3	**11.99±0.39**	19.25±1.13	22.72±0.67	**18.06±0.65**
MED 4	10.01±0.72	8.83±0.36	23.46±0.41	16.71±1.14

Table 20 presents the β-glucan content at 30 °C, which was highly inhibited in all culture media and at all studied strains. Thus, the temperature of 30 °C is not benefitial for biotechnological β-glucan production.

Table 20: The effect of the temperature (30 °C) and media composition to β-glucan production

30 °C **144 h**	*S. cerevisiae* CCY 21-4-102	*C. infirmominiatum* CCY 17-18-4	*P. rhodozyma* CCY 77-1-1	*R. kratochvilovae* CCY 20-2-26
MED 1	9.35±0.68	7.59±0.24	**6.08±0.74**	**5.22±0.16**
MED 2	**10.41±1.05**	4.80±0.06	3.98±0.09	3.07±0.04
MED 3	7.12±0.17	**12.45±1.37**	5.15±0.33	2.66±0.13
MED 4	4.89±0.50	3.77±0.43	4.07±0.58	2.38±0.72

In general, increasing the culture temperature decreased the β- glucan content at all studied strains (Tables 17-20). At *C. infirmominiatum* CCY 17-18-4 the highest biomass content occurred at 8 °C, 11.14 g/L, which corresponds with the information from Culture Collection of Yeast, where the optimal conditions for growth is 15 °C. Opossitely, the β-glucan yield was optimal at 22 °C, where it reached 26.08 % w/w (ammonium sulphate as a single nitrogen source), which was even higher than on medium with yeast extract. The highest β-glucan production was measured at *S. cerevisiae* CCY 21-4-102 – 29.12±2.37 % w/w (8 °C, combination of urea and yeast extract), but the biomass was declined (only about 3 g/L). The optimal biomass production was at 22 °C (11 g/L) on medium with urea, but the β-glucan content decrease to 11.99±0.39 % w/w. The highest biomass contents were measured at *R. kratochvilovae* CCY 20-2-26 (12 – 15 g/L) when cultured on medium with urea as the single nitrogen source. For this strain the optimal temperature combined with β-glucan and biomass yield is 22 °C (18.06±0.65 % of DCW**)**.

There are no direct informations about correlation between temperature, medium composition and β-glucan content at carotenogenic yeast. Most of the research is focused on bacteria, where low temperature can increase the production of extracellular polysaccharides (the same for yeast) or surviving of the yeast during fermentation process [40, 41].

9.4. Exopolysaccharides production

Some microbial strains produce a broath spectrum of extracellular metabolites, such as proteins, DNA, RNA, lipids etc. Particularly, many yeasts synthetize and secrete exopolysaccharides (EPS), biopolymers comprised of monosugar units, and it is related to their secondary metabolism. The biosynthesis and structure depend on many factors, which include the composition of the culture medium and fermentation conditions such as pH, temperature and oxygen concentration [42]. Here, the screening of 11 carotenogenic strains for possibility of EPS biosynthesis were explored. The strains were cultured at MTPs at triplicates on media supplemented with glucose and sucrose, as the most optimal carbon sources according to literature [42].

From all studied yeast only one strain exhibited the biosynthesis of extracellular polysaccharides, namely *Sporidiobolus pararoseus* CCY 19-9-6, with amount of 2.17 g/L (medium with glucose) and 2.28 g/L (medium with sucrose).

After the choice of production strain (*S. pararoseus* CCY 19-9-6), the culture conditions for enhanced production of the exopolysaccharides were explored. From the Table 21, the increase of glucose caused higher yield of EPS, 1.21±0.17 g/L. Ammonium sulphate (AS) or combination AS with yeast extract decreased the bioproduction.

The low temperature (8 °C) with combination of higher C/N ratio exhibited greater accumulation of polysaccharides, on the contrary, the temperature of 30 °C inhibits the secretion of the polymer into culture media.

Another important factor, the pH, has a big influence for EPS biosynthesis, where slightly acidic medium (pH 4) increased in the production to 2.35 g/L. The cause can be the hydrolysis of polysaccharide from cell surface. In highy acidic conditions (pH 3 and 2) the biosynthesis of EPS was inhibited but also the biomass.

Surprisingly, the 2% NaCl has the positive effect on EPS biosynthesis, where the highest yield was achieved, 3.17±0.07 g/L.

Table 21: Exopolysaccharides production in *S. pararoseus* CCY 19-9-6

	EPS [g/L]
Yeast extract (YE), glucose, C/N 40	0.46±0.05
Yeast extract, glucose, C/N 70	0.68±0.08
Yeast extract, glucose, C/N 100	**1.21±0.17**
Ammonium sulphate (AS), glucose, C/N 40	0.20±0.03
Ammonium sulphate, glucose, C/N 70	0.41±0.02
Ammonium sulphate, glucose, C/N 100	0.59±0.07
YE/AS, glucose, C/N 100	0.76±0.12
YE, saccharose, C/N 100	0.86±0.06
YE, glycerol, C/N 100	0.48±0.03
Temperature – 8 °C	
YE, glucose, C/N 40	1.26±0.07
YE, glucose, C/N 70	0.99±0.05
YE, glucose, C/N 100	**1.92±0.10**
Temperature – 30 °C	
YE, glucose, C/N 40	0
YE, glucose, C/N 70	0
YE, glucose, C/N 100	0
pH	
pH 2	0.59±0.02
pH 3	1.83±0.13
pH 4	**2.35±0.08**
pH 7	1.62±0.16
pH 8	1.52±0.09
pH 9	1.26±0.11
NaCl	
2% NaCl	**3.17±0.07**
5% NaCl	1.5±0.12
11% NaCl	0.51±0.01

9.5. Exoglycolipids, intracellular lipids and β-glucan biosynthesis at *R. kratochvilovae*

Secretion of extracellular glycolipids has been already reported at *Rhodotorula* strains [22, 23]. Here, for optimization of culture conditions for enhancement of the exoglycolipid biosynthesis the yeast *Rhodotorula kratochvilovae* CCY 20-2-26 was choosen, which was the only strain that exhibited the presence of "oil droplets" on the surface of the culture medium.

9.9.1 Influence of different nitrogen sources on growth and metabolism of *Rhodotorula kratochvilovae* CCY 20-2-26

In the first step, *R. kratochvilovae* CCY 20-2-26 grew in the presence of various nitrogen sources such as potassium nitrate, yeast extract, ammonium sulfate, ammonium chloride, and urea. Here, glucose was used as a carbon source. The conditions and composition of the culture media are presented in chapter 4.2 (Tables 7 and 8).

Figure 5 shows biomass production with different nitrogen sources. Potassium nitrate, yeast extract, and urea appear to be the most effective sources of nitrogen. Yields for these sources reached a value of about 9.0 g/L of biomass. The highest biomass yield was obtained using potassium nitrate as a nitrogen source in the 144h hour of cultivation, namely 9.5 g/L of biomass. On the contrary, ammonium sulphate and ammonium chloride were evaluated as the worst sources of nitrogen, therefore these sources were no longer used in the following experiments. In general, the highest biomass production was recorded after 96 and 144 hours.

Jiru et al. (2017) state ammonium sulfate in combination with yeast extract and peptone as the best nitrogen source. When cultivated in bioreactors, the amount of biomass reached values of about 17.0 g/L, which is a value higher than ours, but the possible cause is due to the different way of cultivation. As other sources of nitrogen, Jiru et al. (2017) used ammonium chloride again in combination with yeast extract and peptone, but biomass yields were slightly lower there, about 15.0 g/L. As in our experiment, ammonium chloride was a less suitable source of nitrogen [43].

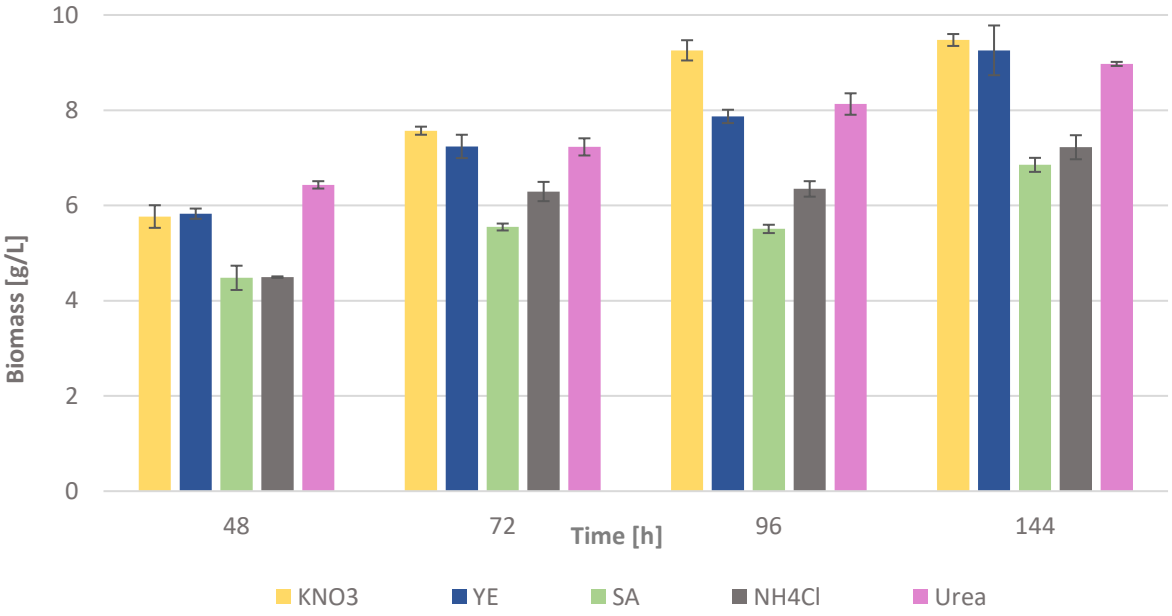

Fig. 5: Biomass production at different nitrogen sources at C/N ratio 70:1

Diverse nitrogen sources affected differently the glucan composition in yeast biomass (Table 22). Pottasium nitrate, yeast extract and urea decreased the beta-glucan yield through the time. Opositelly, the ammonium sulphate reached maximum in the end of cultivation, 17.60±1.84 % w/w.

Table 22: Glucan content in yeast biomass cultivated on different nitrogen sources (% CDW)

N source	Time	48 h	72 h	96 h	144 h
Pottasium nitrate	Total glucan	24.22±2.14	22.14±1.54	17.26±1.03	16.55±0.38
	Alpha-glucan	2.16±0.21	3.83±0.23	2.02±0.06	2.16±0.04
	Beta-glucan	**22.05±2.21**	**18.31±1.83**	**15.24±1.24**	**14.39±0.77**
Yeast extract	Total glucan	27.11±1.54	23.79±1.36	18.46±1.15	14.30±1.03
	Alpha-glucan	2.12±0.13	2.36±0.20	1.76±0.02	1.69±0.01
	Beta-glucan	**24.99±1.69**	**21.43±1.57**	**16.70±1.33**	**12.62±1.11**
Ammonium sulphate	Total glucan	15.12±0.37	16.59±0.89	17.56±0.67	18.46±1.59
	Alpha-glucan	2.71±0.48	2.62±0.11	1.02±0.04	0.86±0.15
	Beta-glucan	**12.41±1.02**	**13.98±1.04**	**16.54±1.00**	**17.60±1.84**
Urea	Total glucan	23.45±1.00	22.86±1.05	16.65±0.44	17.94±1.07
	Alpha-glucan	2.51±0.33	2.71±0.21	1.98±0.03	1.01±0.09
	Beta-glucan	**20.94±1.25**	**20.15±1.30**	**14.67±0.59**	**16.93±1.13**
Ammonium chloride	Total glucan	17.18±0.19	18.69±0.49	15.49±0.16	12.61±0.41
	Alpha-glucan	2.38±0.04	2.98±0.06	2.15±0.14	1.32±0.12
	Beta-glucan	**14.80±0.81**	**15.71±0.66**	**13.33±0.85**	**11.30±0.75**

Figure 6 shows the biosynthesis of exoglycolipids, where the best sources of nitrogen were also ammonium nitrate, yeast extract, and urea, while the worst sources were again ammonium sulfate and ammonium chloride. Amplitudes were reached at 144 hours using potassium nitrate, where the concentration of exoglycolipids reached up to 1.8 g/L. The lowest yield was observed at 72 and 144 hours of cultivation using ammonium sulfate, only 0.1 g/L.

In potassium nitrate, a slight decrease in the amount of exoglycolipids was observed between 72 and 144 hours, and thus at 96 hours of cultivation, the yield value decreased. In the case of ammonium chloride, the culture also reached the highest value at 96 hours of cultivation, namely 0.3 g/L, and at 144 hours the value was lower. Yields with urea then increased over time from 0.6 g/L at 48 hours of cultivation to 1.6 g/L at 144 hours.

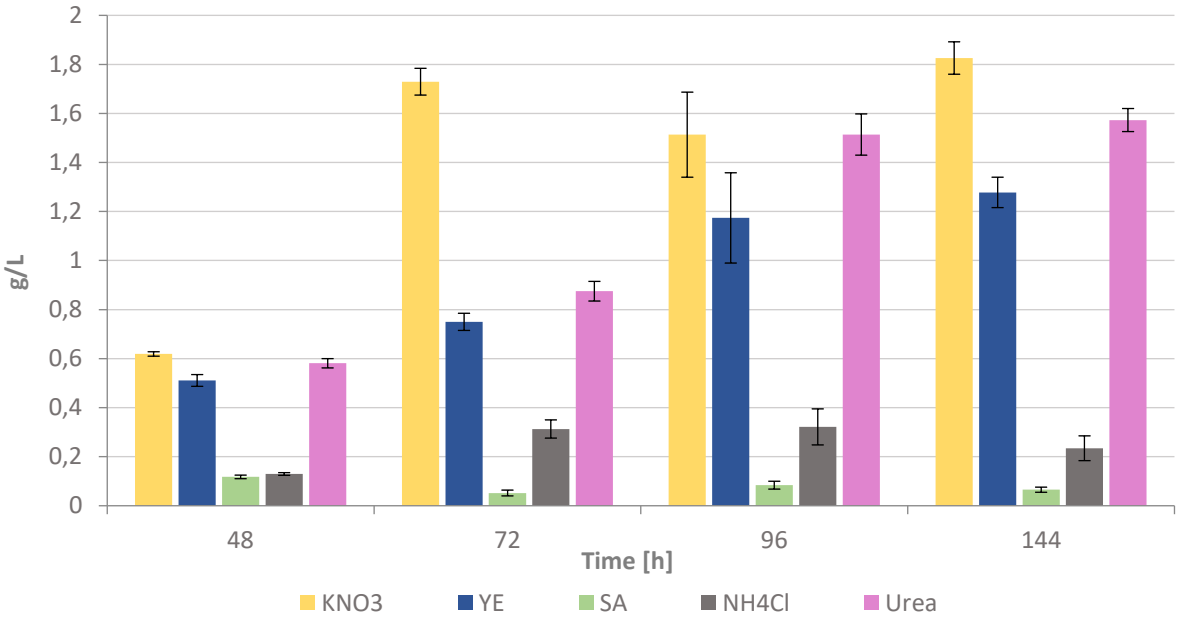

Fig. 6: Extracellular glycolipids yield at media with different nitrogen sources

9.9.2 Influence of different carbon sources on growth and metabolism of *Rhodotorula kratochvilovae* CCY 20-2-26

The next step of the experiment was the selection of suitable carbon sources, which were selected according to drop tests from the experiments of the diploma thesis from previous study [44], when the optimal carbon source were mannose, lactose, glycerol, and xylose. For other monosaccharides, the growth was inhibited and therefore they were not included in this thesis [44].

Potassium nitrate and urea were chosen as the nitrogen source, as they showed the highest yields of biomass, intracellular lipids, and mainly exoglycolipids in the previous experiment. High yields were also observed for the yeast extract, which is, however, a demanding source due to its price compared to the two selected above.

The worst source in the experiment was lactose, which is the least suitable source of carbon for yeast, probably due to the absence of lactase to break down disaccharide into usable monosaccharides (Fig. 7). The biomass yield was maximally only about 1.0 – 1.5 g/L. Lactose was followed, also as a less suitable carbon source, by glycerol, which showed values slightly above 2.0 g/L of biomass, and these values were very similar throughout the whole time horizon of cultivatiom. Xylose appears very similar to glycerol, which showed slightly better results with 3.0 g/L of biomass.

The best carbon source was unrivaled mannose, which at the beginning of the experiment showed values of about 6.0 g/L, up to 10.0 g/L of biomass in the 144th hour of cultivation.

Thus, it can be assessed that urea and mannose appear to be the best sources of carbon and nitrogen for biomass production over time.

In the previous experiment, using urea as a nitrogen source, a maximum yield of about 9.0 g/L was obtained, when the carbon source was glucose. Here it can be observed that even better results (10.0 g/L) were obtained with the same nitrogen and mannose source as the carbon source. Thus, it is clear that mannose is an even better source of carbon than glucose for this experiment, although the difference is not significant.

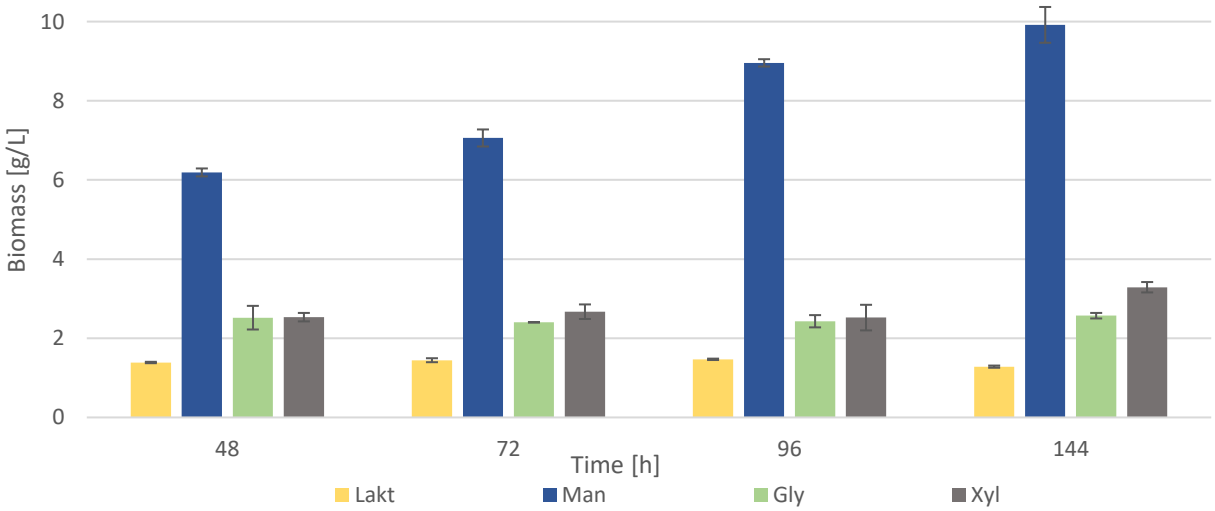

Fig. 7: Biomass production with diverse carbon sources and with urea as nitrogen source

Table 23 shows the production of total, α- and β-glucan in yeast biomass cultured at different saccharides and glycerol with combination of urea. The biomass content for lactose was too low for glucan measurements.

The urea with combination of mannose, glycerol and xylose decreases the β-glucan content when compared to previous experiment with glucose. At mannose as a carbon source, glucan yield dropped from 17.96 to 10.54 (% CDW), probably due to accumulation of other metabolites. Opositelly, glycerol and xylose showed slighty increase at β-glucan production in the end of cultivation.

Table 23: Total, α- and β-glucan content at diverse carbon sources with combination of urea

C source	Time	**48 h**	**72 h**	**96 h**	**144 h**
Lactose	Total glucan Alpha-glucan **Beta-glucan**	-	-	-	-
Mannose	Total glucan	17.96±0.68	11.83±0.43	11.12±1.57	10.54±0.49
	Alpha-glucan	4.21±0.12	1.23±0.02	0.84±0.11	0.67±0.14
	Beta-glucan	**13.75±0.99**	**10.59±0.76**	**10.28±1.83**	**9.87±0.67**
Glycerol	Total glucan	5.64±0.22	5.19±0.33	5.28±0.24	6.31±0.60
	Alpha-glucan	1.82±0.07	1.00±0.04	0.28±0.02	0.95±0.21
	Beta-glucan	**3.83±0.54**	**4.19±0.67**	**5.00±0.37**	**5.37±0.80**
Xylose	Total glucan	4.56±0.16	9.88±1.02	8.87±0.76	8.75±0.55
	Alpha-glucan	1.60±0.01	1.60±0.09	1.28±0.04	1.71±0.13
	Beta-glucan	**2.96±0.39**	**8.28±1.13**	**7.59±0.91**	**7.04±0.76**

The graph below (Fig. 8) shows the production of exoglycolipids using different carbon sources and a single nitrogen source - urea. The situation is repeated by analogy, as with the production of biomass with different carbon sources. Thus, the most suitable carbon source for the production of exoglycolipids is mannose, leaving the remaining sources far behind. Using mannose, values of up to 1.4 g/L of exoglycolipids were achieved.

The next suitable source appears to be glycerol, where the maximum yield was reached after 96 hours of cultivation, slightly above 0.2 g/L of exoglycolipids. Other sources showed very low values compared to mannose, around 0.1 g/L.

The concentration of exoglycolipids was slightly lower in this experiment (formerly 1.8 g/L, now 1.4 g/L) when compared to the previous experiment. Thus, glucose in combination with urea appears to be a slightly more suitable source for the production of exoglycolipids.

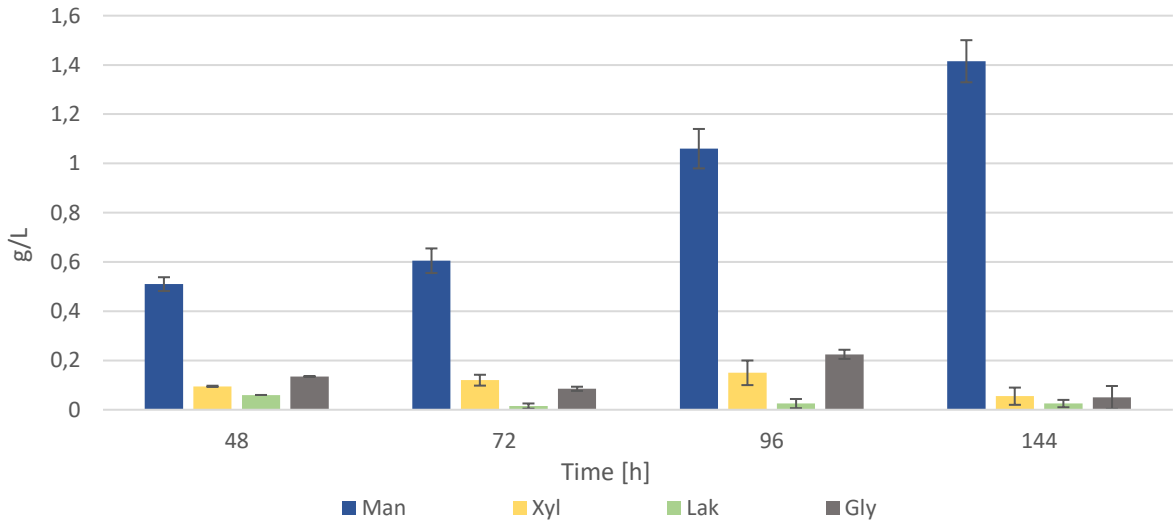

Fig. 8: Extracellular glycolipids yield at media with urea as a single nitrogen source

At the experiment with the potassium nitrate (Fig. 9), the best source of carbon is again mannose, specifically at 144 hours, when the yield reached up to about 9.5 g/L of biomass. From the beginning of the cultivation, the yield increased rapidly. This fact indicates that mannose is a suitable source of carbon for yeast in combination with KNO_3 when even with a short exposure of the studied strain to the thus assembled medium resultend in 7.2 g/L biomass after 48h.

In general, potassium nitrate is a less suitable source of nitrogen than urea, because, in the previous experiment, mannose values reached up to about 10.0 g/L, which is the maximum for biomass across of all experiments performed. However, comparable biomass values, i.e. around 9.5 g/L, were obtained in an experiment in which the carbon source was glucose and the nitrogen source, already mentioned, potassium nitrate.

The second most suitable carbon source here was xylose, which reached values of about 6.2 g/L at the end of the cultivation.

Here, too, it is possible to compare the amount of biomass with an experiment using urea and xylose - the values were lower for this combination, only about 3.5 g/L than for the combination of urea and mannose. Thus, it can be concluded that the combination of urea with mannose shows better results than mannose with potassium nitrate, but on the contrary, xylose, as a carbon source, works better in combination with potassium nitrate than with urea.

The worst carbon sources here are glycerol and especially lactose. While glycerol reached at least 3.38 g/L during cultivation, lactose showed much worse results, a maximum of 0.61 g/L.

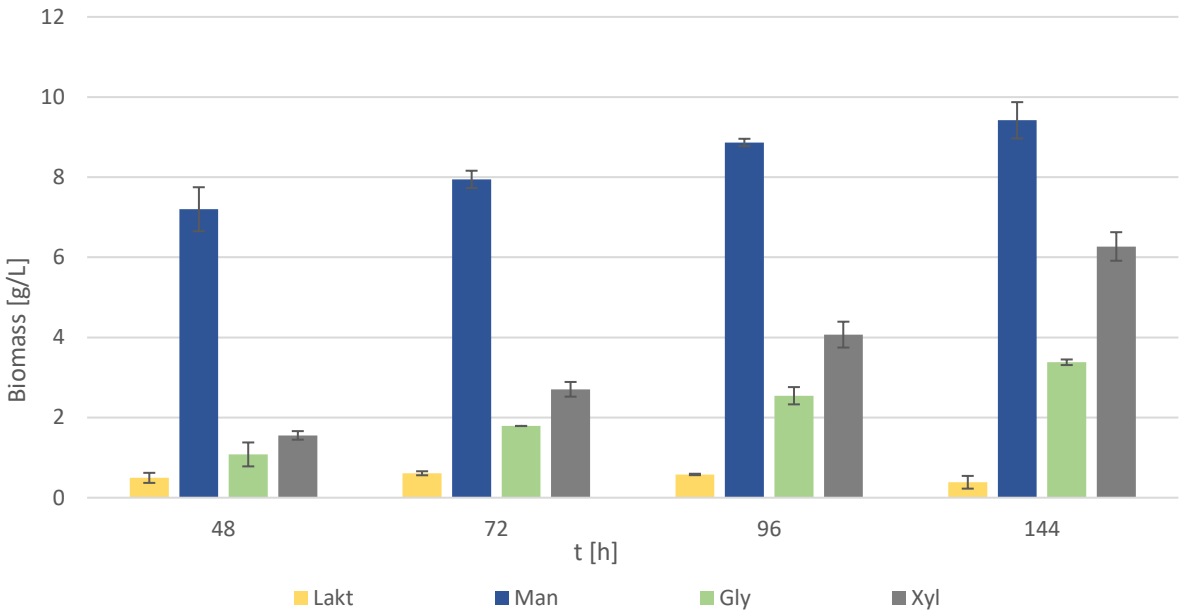

Fig. 9: Biomass production with diverse carbon sources and with potassium nitrate as nitrogen source

The Table 24 shows the production of total, α- and β-glucan in yeast biomass cultured at different carbon sources combined with potassium nitrate as a nitrogen source. Again, the biomass content for lactose was too low for glucan measurements.

When compared with experiment with urea, a rapid increase in β-glucan yield occurred when cultured on mannose. At 144h of cultivation, the high β-glucan content was accompanied with

biomass production of 9.42 g/L. On the contrary, the glycerol shows the production only 2.69±0.34 % of DCW.

Table 24: Total, α- and β-glucan content at diverse carbon sources with combination of potassium nitrate

C source	Time	48 h	72 h	96 h	144 h
Lactose	Total glucan Alpha-glucan **Beta-glucan**	-	-	-	-
Mannose	Total glucan	18.86±1.08	17.43±1.30	20.70±0.29	21.53±1.46
	Alpha-glucan	1.73±0.13	1.81±0.06	2.57±0.42	2.07±0.13
	Beta-glucan	**17.13±1.24**	**15.62±1.67**	**18.14±0.58**	**19.46±1.85**
Glycerol	Total glucan	8.64±0.36	7.17±0.15	5.39±0.41	7.24±0.20
	Alpha-glucan	2.03±0.08	2.56±0.10	2.31±0.06	4.55±0.08
	Beta-glucan	**6.61±0.49**	**5.36±0.41**	**3.08±0.67**	**2.69±0.34**
Xylose	Total glucan	16.57±0.99	16.43±0.44	15.23±1.00	13.80±1.05
	Alpha-glucan	2.49±0.21	2.85±0.13	3.38±0.32	3.80±0.04
	Beta-glucan	**14.08±1.05**	**13.58±0.81**	**11.85±1.45**	**10.00±1.27**

Figure 10 shows the amount of biosynthesized exoglycolipids when cultured on a medium with different carbon sources and a single nitrogen source – potassium nitrate. Again, the best carbon source is mannose, which achieved the highest yields, up to about 2.0 g/L in 144 hours of cultivation.

Compared with the previous experiment (with urea), potassium nitrate appears to be a more suitable source for exoglycolipid biosynthesis, given that in the previous experiment the maximum values were only about 1.4 g/L exoglycolipids.

For lactose and glycerol, the exoglycolipids production did not exceed the value of 0.2 g/L.

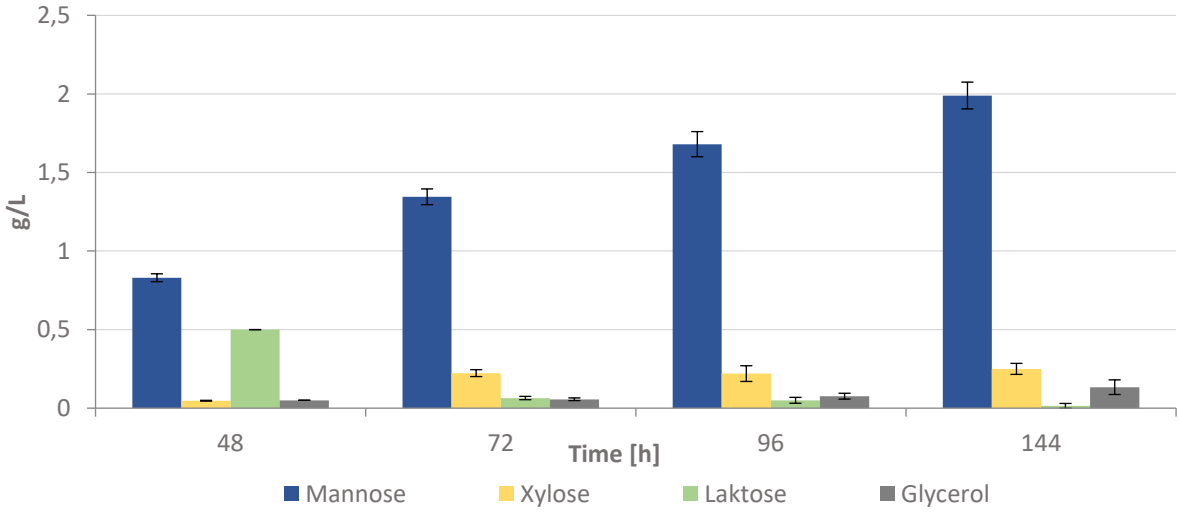

Fig. 10: Extracellular glycolipids yield at media with potassium nitrate as a single nitrogen source

9.9.3 Influence of different carbon:nitrogen ratio on growth and metabolism of *Rhodotorula kratochvilovae* CCY 20-2-26

The C/N ratio is a very important criterion for achieving maximum production of selected metabolites. Regarding its influence on biomass production, it can be said that the higher the C/N

ratio, the higher the biomass yields we are able to achieve, mainly due to the accumulation of specific biomolecules [32].

The graph in Figure 11 describes the dependence of biomass production and different concentrations of mannose as a carbon source. Potassium nitrate, previously experimentally tested, was chosen as the nitrogen source with the C/N ratios of 20:1, 40:1, 100:1 and 120:1 (C/N 70:1 was used in previous experiments).

The highest biomass yields were achieved at the highest concentration of mannose, in the 192nd hour of cultivation (around 15.23 g/L). The situation is relatively favorable even with a C/N ratio of 100:1, where the highest yield was achieved in the 168th hour of cultivation, with a value exceeding 14.0 g/L of biomass.

For C/N ratios of 20: 1 and 40: 1, the yields were significantly lower. As for the ratio of 40:1, the highest yield was obtained here at the 144th hour of cultivation (7.16 g/L), then the value rather decreased.

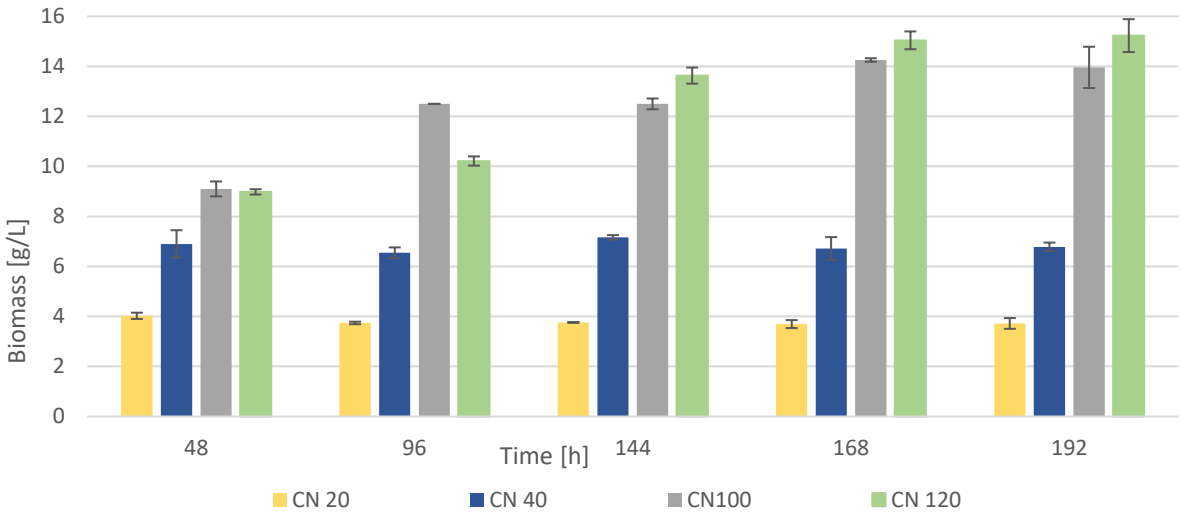

Fig. 11: Biomass production with diverse C/N ratio

The Table 25 shows the production of total, α- and β-glucan in yeast biomass cultured at different C/N ratios, where the C/N ratio 40:1 shows the highest β-glucan yield, 12.83±1.23 % of DCW. Taking together, the C/N ratio of 70:1 (Table 25) shows the best results for β-glucan production, but the C/N ratio 120 exhibited better biomass, intracellular lipids and exoglycolipids yield, thus, is better for biotechnological application.

Table 25: Total, α- and β-glucan content at diverse C/N ratios

C/N ratio	Time	**48 h**	**96 h**	**144 h**	**168 h**
	Total glucan	12.18±0.55	10.28±0.16	8.04±0.11	8.64±0.39
20	Alpha-glucan	1.33±0.02	0.98±0.08	1.49±0.09	1.26±0.12
	Beta-glucan	**10.85±0.68**	**9.30±0.23**	**6.54±0.25**	**7.38±0.63**
	Total glucan	20.82±1.20	16.55±1.36	15.28±1.08	14.51±1.05
40	Alpha-glucan	2.36±0.33	1.07±0.05	1.95±0.03	1.68±0.47
	Beta-glucan	**18.45±1.26**	**15.48±1.41**	**13.33±1.19**	**12.83±1.23**
	Total glucan	22.02±0.84	15.11±1.64	13.18±0.28	11.84±0.91
100	Alpha-glucan	2.26±0.14	1.01±0.91	1.14±0.01	1.59±0.06
	Beta-glucan	**19.75±0.91**	**14.10±1.33**	**12.04±0.42**	**10.25±0.98**
	Total glucan	22.93±0.79	13.29±1.32	16.21±0.83	13.06±1.22
120	Alpha-glucan	2.17±0.17	1.21±0.37	1.45±0.05	1.73±0.51
	Beta-glucan	**20.75±0.85**	**12.08±1.49**	**14.76±0.95**	**11.33±1.34**

As mentioned previously, cultures that are deficient in nitrogen produce intensively metabolites, such as the exoglycolipids. Therefore, the difference between low and high C/N ratios is obvious, up to 12 times the production is higher at a C/N ratio of 120:1 than at a ratio of 20:1 (Fig. 12).

The highest value for the C/N ratio of 120: 1 was reached at the 192nd hour of cultivation. A very similar situation can be seen with the C/N ratio of 100:1.

The situation was many times worse for the low C/N ratios (20:1 and 40:1), where the very lowest value of the whole experiment was reached at 96 hours, for the lowest selected ratio, namely 0.05 g / l.

Compared to our previous experiment, the exoglycolipids production was higher than with the C/N ratio 70:1. Thus, the C/N ratio played an important role in the production of exoglycolipids, especially in its increase.

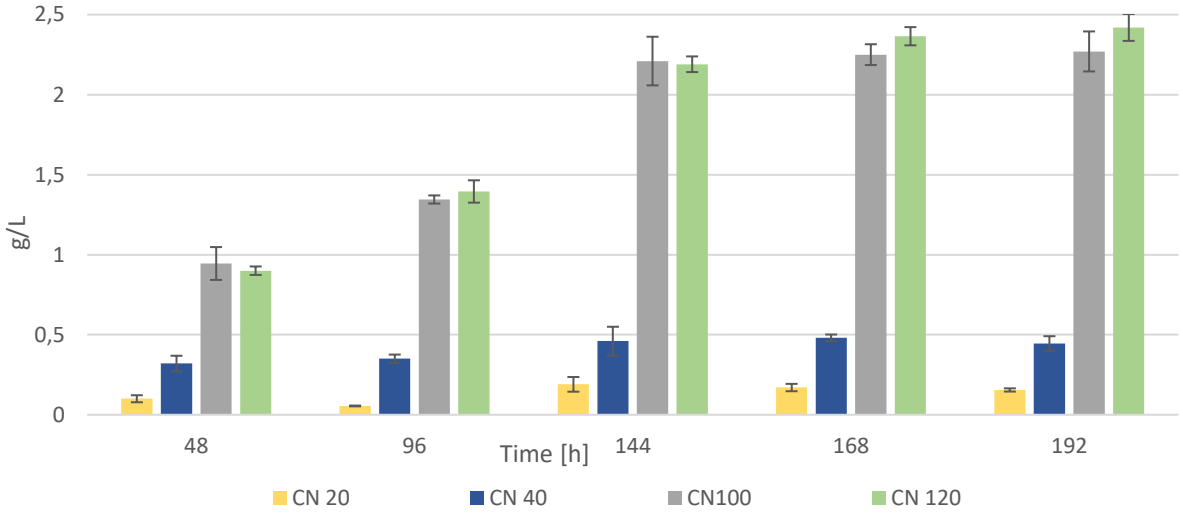

Fig. 12: The exoglycolipid production with different C/N ratios

9.6. Beta-glucan diversity at *Saccharomyces* collection strains and commertial brewer yeast

Four *Saccharomyces cerevisiae* strains from Culture Collection of Yeasts and eight commertial brewer yeast were used in this study to compare productivity of beta-glucan as a diversity among *Saccharomyces* genera. Collection yeast were choosed to cover different types of the technological use, like wine production (CCY 21-4-81), beer industry (CCY 48-26), baker industry (CCY 21-4-64), and sugar rafinery (CCY 48-88). The commertial brewery yeasts represented the top-fermenting (Safale S-04, Safale S-05, Safbrew WB-06), bottom-fermenting (Bohemian Lager M84, Saflager S-23, Saflager W-34/70), Premium gold yeast, and cider yeast (M02) samples, and were kindly provided, the same as spent biomass after brewing (Safale S-04 and Saflager W-34/70) by Ing. Václav Štursa from Brno University of Technology, Faculty of Chemistry. The culture conditions were the same as for the screening of the carotenogenic yeast in the Chapter 4.2, and biomass was harvested after 96 hours of cultivation.

For biotechnological application the biomass plays the crutial role right after the valuable metabolite production. In this part of the thesis, the biomass yield was in a range from 2.39±0.24 up to 11.78±0.67 g/L. Here, in the nutrient-rich YPD medium, the significant increase occured at the commertial samples of Safale US-05, Cider yeast M02, Bohemian lager yeast M84, SafAle

WB-06, collection *S. cerevisiae* CCY 48-88, and, the highest (11.78±0.67 g/L), at the strain *S. cerevisiae* CCY 21-4-64, which is baker strain from Libaň, Czech Republic. After exposure this yeast into medium with different C/N ratios, the biomass dropped up to 50 % suggesting the composition of the medium is not optimal for the growth. The lowest growth was presented at *S. cerevisiae* CCY 48-26 collection brewer strain where the biomass reached maximum of 3.00±0.15 g/L at the C/N ratio of 100, and, the same as the Saflager W-34/70 and Saflager S-23, show no significant difference between used medium. It is important to note that experiment was performed in Erlenmeyer flasks, thus it is not comparable with high biomass yields from reported batch or fed-batch fermentor cultivations where it can be achieved more than 100 g/L [45].

In general, these is no significant difference between growth of top-fermenting and bottom-fermenting yeast as a two different type of brewing yeast. The biomass production is strain-depended and shows no consistency among diverse biotechnological applications.

Table 26: Biomass yield for biotechnologically important yeast from *Saccharomyces* genera

		Biomass yield (g/L)			
Strain name	**CCY**	**YPD**	**C/N 40:1**	**C/N 70:1**	**C/N 100:1**
Saccharomyces cerevisiae	21-4-81	9.83±0.49	5.56±0.73	6.03±0.62	6.44±1.01
Saccharomyces cerevisiae	48-26	2.50±0.16	2.39±0.24	2.49±0.17	3.00±0.15
Saccharomyces cerevisiae	21-4-64	11.78±0.67	5.56±0.15	5.74±0.30	6.02±0.22
Saccharomyces cerevisiae	48-88	11.09±0.43	6.35±0.37	6.92±0.45	6.63±0.43
Bohemian lager yeast, M84	-	11.10±1.04	7.27±0.26	6.99±0.03	9.21±1.06
Cider yeast, M02	-	10.58±0.53	6.59±0.19	6.06±0.59	8.54±0.66
Premium gold; Muntons	-	6.75±0.06	4.06±0.44	4.12±0.11	3.84±0.09
Safale S-04	-	6.50±0.28	3.71±0.03	3.70±0.33	3.60±0.10
Saflager W-34/70	-	6.26±0.09	5.99±0.22	5.81±0.06	6.42±0.62
Saflager S-23	-	7.24±0.73	6.30±0.81	6.16±1.01	7.55±0.23
SafAle WB-06	-	10.09±0.99	7.60±0.08	7.64±0.55	7.96±0.48
Safale US-05	-	10.34±1.04	6.93±0.33	6.40±0.06	7.69±0.25

From all studied yeast from *Saccharomyces* genera the highest beta-glucan content was detected at *S. cerevisiae* CCY 21-4-102 (26.96±2.90 % w/w) (Table 27). *S. cerevisiae* CCY 48-88 isolated from sugar refinery shows similar results (24.62±1.56 % w/w) at C/N ratio 40. The yield of beta-glucan from other studied *Saccharomyces* strains ranged from 2 – 17 % w/w. The increase in glucose content in the culture media has no significant effect on β-glucan yield, except the Cider yeast M02, where the C/N 100 doubled the glucan content when compared to other ratios. Interesingly, the brewery yeast showed high amount of α-glucans when compared to collection strains. The α-glucan content at Cider yeast represented 83 % of total glucan. The Saflager W-34/70, Saflager S-23, Bohemian lager M84, Safale S-04 and Safale US-05 showed the highest α-glucan yield, over 13 % w/w when cultured at different C/N ratios.

In this thesis, the spent biomass from Safale-S04 and Saflager W-34/70 (Table 27) after brewery process was analysed with the 4.42 and 8.07 % w/w of β-glucan.

Table 27: Total glucan, α- and β-glucans content (% CDW) in yeasts grown in the YPD media and media with low and high C/N ratios

Strain		YPD	C/N 40:1	C/N 70:1	C/N 100:1
S. cerevisiae CCY 21-4-81	Total glucan	7.43±0.22	14.51±1.23	15.35±0.81	17.27±0.46
	α-glucans	1.57±0.06	3.60±0.14	3.02±0.09	2.86±0.21
	β-glucans	**5.86±0.64**	**10.91±1.36**	**12.33±0.93**	**14.41±0.55**
S. cerevisiae CCY 48-26	Total glucan	9.21±0.12	18.21±0.55	14.87±0.34	13.80±1.02
	α-glucans	0.58±0.09	1.39±0.06	0.67±0.11	0.66±0.17
	β-glucans	**8.63±0.20**	**16.82±0.84**	**14.02±0.38**	**13.14±1.24**
S. cerevisiae CCY 21-4-64	Total glucan	8.14±0.61	18.54±1.01	17.73±1.02	17.10±0.55
	α-glucans	0	6.64±0.22	3.61±0.13	3.46±0.24
	β-glucans	**8.14±0.61**	**11.90±1.35**	**14.12±1.14**	**13.64±0.69**
S. cerevisiae CCY 48-88	Total glucan	9.76±1.64	27.98±1.29	22.75±1.87	25.09±1.47
	α-glucans	1.06±0.94	3.37±0.44	1.08±0.18	1.08±0.03
	β-glucans	**8.70±1.33**	**24.62±1.56**	**21.67±2.03**	**24.00±1.53**
Bohemian lager yeast, M84	Total glucan	11.92±0.88	21.01±1.21	22.15±0.20	22.65±0.61
	α-glucans	1.59±0.39	14.62±0.72	16.03±0.31	17.49±0.37
	β-glucans	**10.34±0.85**	**6.40±1.63**	**6.12±0.48**	**5.17±1.08**
Cider yeast, M02	Total glucan	11.21±0.58	12.44±0.59	14.64±0.03	19.04±0.88
	α-glucans	0.87±0.31	10.35±0.18	9.32±0.02	7.65±0.24
	β-glucans	**10.34±0.72**	**2.09±0.88**	**5.32±0.09**	**11.39±1.03**
Premium gold; Muntons	Total glucan	7.40±0.25	21.70±0.67	19.89±0.50	17.72±0.51
	α-glucans	1.56±0.10	9.94±0.14	9.01±0.57	9.72±0.13
	β-glucans	**5.84±0.46**	**11.76±0.55**	**10.87±0.88**	**8.00±0.82**
Safale S-04	Total glucan	9.05±0.44	20.59±0.45	19.75±0.63	18.96±0.44
	α-glucans	2.33±0.51	13.87±0.21	12.45±0.76	11.22±0.19
	β-glucans	**6.71±0.68**	**6.72±0.75**	**7.03±1.09**	**7.74±0.68**
Saflager W-34/70	Total glucan	5.64±0.26	26.38±0.73	21.67±0.31	19.29±0.25
	α-glucans	0.37±0.01	13.74±0.48	12.06±0.66	11.15±0.43
	β-glucans	**5.27±0.49**	**12.64±1.06**	**9.61±0.59**	**8.14±0.59**
Saflager S-23	Total glucan	7.18±0.70	22.95±0.25	23.58±0.81	24.94±1.33
	α-glucans	0.96±0.05	11.41±0.37	12.95±0.13	**16.35±0.21**
	β-glucans	**6.22±0.76**	**11.54±0.88**	**10.63±0.92**	**8.60±1.24**
SafAle WB-06	Total glucan	12.44±1.03	22.97±0.11	19.48±0.66	19.34±1.06
	α-glucans	0.91±0.02	8.85±0.02	7.39±0.17	8.48±0.22
	β-glucans	**11.54±1.08**	**14.12±0.14**	**12.09±0.69**	**10.86±1.30**
Safale US-05	Total glucan	7.66±0.05	30.26±1.00	20.50±0.39	24.87±0.36
	α-glucans	1.41±0.12	15.14±0.05	15.45±0.22	15.80±0.14
	β-glucans	**6.24±0.16**	**5.12±1.13**	**5.04±0.55**	**9.07±0.42**
Spent biomass from Safale S-04	Total glucan	14.84±0.67			
	α-glucans	10.42±0.41	-	-	-
	β-glucans	**4.42±0.79**			
Spent biomass from Saflager W-34/70	Total glucan	13.69±1.00			
	α-glucans	5.62±0.23	-	-	-
	β-glucans	**8.07±1.04**			

9.7. Metabolic activity of *Euglena gracilis*

Euglana gracilis is protozoic algal strain known for its beta-glucan (called as paramylon) accumulation as the reserve polysaccharide. Here, the mixotrophic and heterotrophic cultivations were performed to evaluate growth and paramylon biosynthesis in Erlenmeyer flasks (culture conditions – chapter 4.3).

The biomass content reached maxima at 192h hour of cultivation for all culture conditions with the maximum yield of 2.79 g/L on media supplied with 10 g/L of glucose (Fig. 13). During the first 24 h the biomass shows similar production within the hetherotrophic growth, but for the mixotrophic stayed very low from the beginning till the end of cultivation.

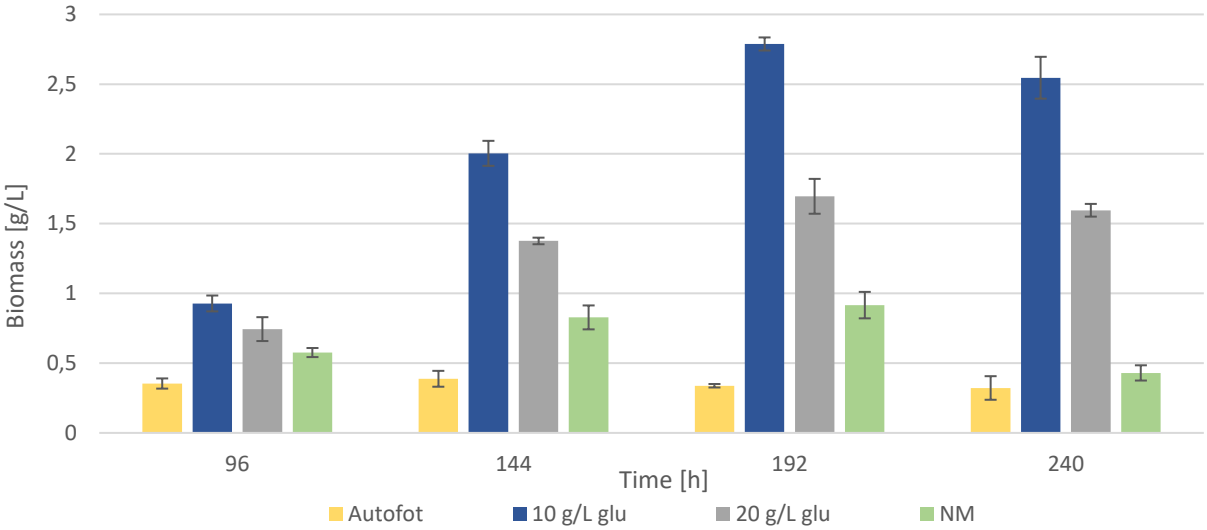

Fig 13: Biomass content of *Euglena gracilis* CCALA 349

The paramylon content isolated from *Euglena gracilis* biomass ranged from 11.9 – 69.29 % of CDW (Fig. 14). The paramylon yield increased rapidly in the medium supplemented with 10 g/L of glucose up to 70 % od DCW, whereas the lowest concentration can be found at growth on light illumination with acetate as a carbon source. The glucose content of 20 g/L lead to decrease the β-glucan content to 61 % of DCW which together with lower biomass yield represent unfavourable conditions for biotechnological purposes. The composition of other type of culture medium was adapted from Cramer et al. (1952) [26], where the authors stated the highest yields. However, the CCALA 349 strain shows biomass yield below 1 g/L. In general, these types of media are cost-uneffective when compared with yeast, therefore there is need to use waste substrates to alleviate the culture price.

Among the literature, *Euglena gracilis* can accumulate up to 85 % paramylon of DCW. But such a high biosynthesis is caused mostelly by non-photosynthetic mutant strains [46].

Isolation of paramylon granules is simple and quick process due to the absence of the cell wall and presence of pellicle made up of a protein layer instead, when compared to fungal biomass. Mixing the biomass with sodium dodecylsulphate solution cause the rupture of the cells and release of granules which are insoluble in water [47]. On the contrary, yeast β-glucan is a part of the thick cell wall with is covalently bounded to mannan and chitin. Therefore, isolation and subsequent purification of fungal β-glucan represent challenging task for the future.

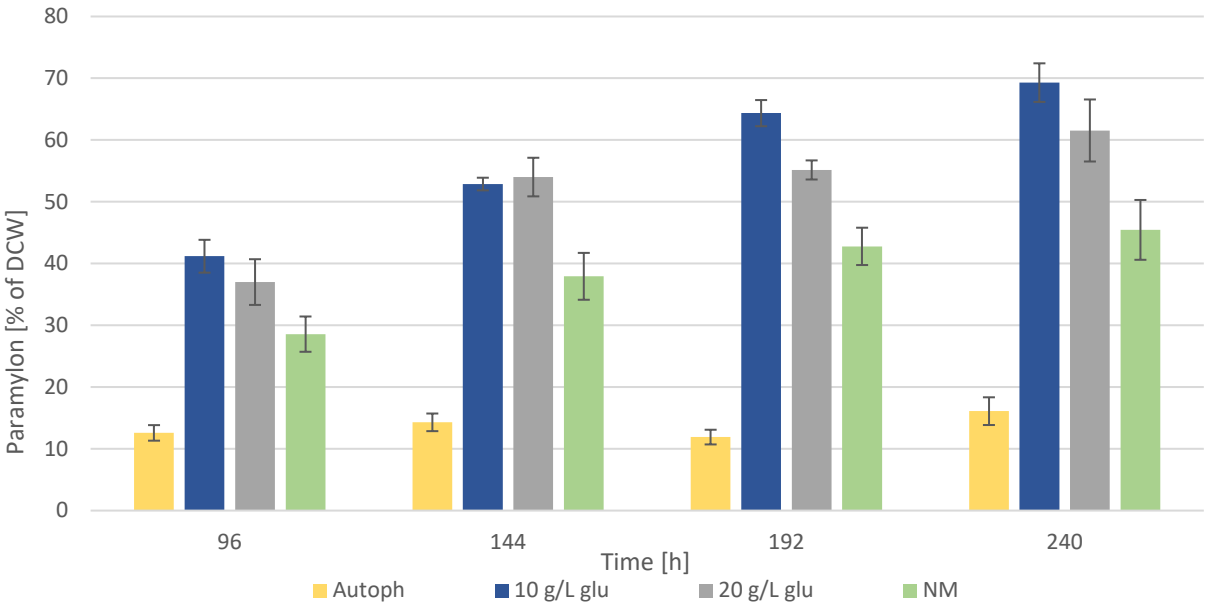

Fig 14: Paramylon accumulation in *Euglena gracilis* CCALA 349

The commercial cultivation of *E. gracilis* began in 2007, after the success of its outdoor mass cultivation and improvement of the harvesting and drying methods. The commercialization of Euglena production is based on the strategy of "5Fs of Biomass," which refers to the development and production of commercial products including food, fiber, feed, fertilizer, and fuel from biomass [48]. Nowadays, Japan-based company, Euglena, is using these microalgae to develop foods and cosmetics as well as conducting research for the production of biofuels. Euglena's sales in 2018 reached $133 million, according to The Investor and the big challenging plan is to produce biofuel for airplanes flying into Tokio for the Summer Olympic Games [49].

6 CONCLUSIONS

The main objective of this work was to optimize the culture conditions for β-glucan production together with intracellular lipids, extracellular polymeric substances and pigments at diverse yeast, and microalgal strains. To enhance the yield of desired metabolites, the broath range of culture conditions were tested, from osmotic stress, temperature to nitrogen and carbon sources.

By applying a high-throughput screening approach, several carotenogenic Basidiomycetes yeast strains were identified as new sources of β-glucans, where the most promising results were obtained for strains *C. infirmominiatum* CCY 17-18-4, *R. kratochvilovae* CCY 20-2-26 and *P. rhodozyma* CCY 77-1-1. Further, several yeast strains showed a high co-production potential, for example strain *C. infirmominiatum* CCY 17-18-4 was able to accumulate 20.73 % (w/w) of β-glucans and 38.21 % (w/w) of lipids, the strain *R. kratochvilovae* CCY 20-2-26 was able to accumulate 38.21 % (w/w) of lipids and 20.73 % of β-glucans, accompanied with the high biomass yield (15.19 g/L). It was that increase in C/N ratio led to an increase in biomass, lipid and β-glucans production for several yeast strains.

Osmotic stress caused by sodium chloride had a negative effect on the biomass and lipid production but positively affected β-glucans production. Osmolarity combined with C/N ratios of 40, 70 and 100 led to an increase in the total glucan and β-glucan content in comparison to the standard conditions. The addition of 0.2% NaCl caused increase in β-glucan production up to 32.15 ± 0.81 (w/w) in *C. infirmominiatum* CCY 17-18-4.

The temperature is the key component affecting the biomass yield in yeasts. The highest β-glucan production was measured at *S. cerevisiae* CCY 21-4-102 – 29.12±2.37 % w/w (8 ^{o}C, combination of urea and yeast extract), but the biomass was declined (only about 3 g/L). The optimal biomass production was at 22 ^{o}C (11 g/L) on medium with urea, but the β-glucan content decrease to 11.99±0.39 % w/w. The highest biomass contents were measured at *R. kratochvilovae* CCY 20-2-26 (12 – 15 g/L) when cultured on medium with urea as the single nitrogen source. The optimal temperature combined with β-glucan and biomass yield is 22 ^{o}C (18.06±0.65 % of DCW**)**.

In other experiment, the production of extracellular polysaccharides was tested. From all studied yeast only one strain exhibited the biosynthesis of EPS, namely *Sporidiobolus pararoseus* CCY 19-9-6, with amount of 2.17 g/L (medium with glucose) and 2.28 g/L (medium with sucrose). Suprisingly, the 2% NaCl has the positive effect on EPS biosynthesis where the highest yield was achieved, 3.17±0.07 g/L.

Secretion of extracellular glycolipids has been already reported at *Rhodotorula* strains. Here, for optimization of culture conditions for enhancement of the exoglycolipid biosynthesis the yeast *Rhodotorula kratochvilovae* CCY 20-2-26 was chosen. The best culture conditions to produce biomass (15.23 g/L), intracellular lipids (68 % of DCW), exoglycolipids (2.42 g/L) and β-glucan (11.33 % od DCW) are mannose as a carbon source and potassium nitrate as a nitrogen source (C/N 120:1).

The paramylon content isolated from *Euglena gracilis* biomass ranged from 11.9 – 69.29 % of CDW. The paramylon yield increased rapidly in the medium supplemented with 10 g/L of glucose up to 70 % od DCW, and 2.79 g/L of biomass.

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Řešená problematika:	Experimentální část diplomové práce, optimalizace kultivace kmenů řasy *Haematococcus pluvialis* ve fotobioreaktorech
Místo:	University of Life Sciences, Ås, Norsko
Doba pobytu:	1.10.2017 – 16.12.2017
Řešená problematika:	Možnosti produkce beta-glukanů kvasinkami a jejich analýza pomocí FTIR-HTS spektroskopie
Místo:	University of Huelva, Huelva, Španělsko
Doba pobytu:	30.05.2018 – 30.07.2018
Řešená problematika:	Možnosti analýzy chlorofylu, karotenoidů a fykobiliproteinů extremofilní sinici *Croocchosidiopsis sp.*
Místo:	University of Life Sciences, Ås, Norsko
Doba pobytu:	12.08.2018 – 4.11.2018
Řešená problematika:	Vliv stresových podmínek na produkci beta-glukanů
Místo:	University of Life Sciences, Ås, Norsko
Doba pobytu:	1.6.2019 – 1.8.2019
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