Rhodotorula toruloides' Exopolysaccharides: Production, Optimization and Characterization

Bachelor thesis

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Applied Chemistry and Gene Technology

Tallinn 2024

Rhodotorula toruloides'i Eksopolüsahhariidid: Tootmine, Optimeerimine ja Iseloomustus

Bakalaureusetöö

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Tallinn 2024

Autorideklaratsioon

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Table of contents

ABSTRACT

One of the key objectives of the United Nations' sustainable development goals is to, identify the unsustainable products or processes, and develop sustainable solutions in their place. Hydrocarbon-driven chemical industry's transformation by biotechnology is one of the key transformative areas of societal level change and developmental opportunities, creating future jobs in a bio-based economy. The fundamental to this envisioned biotechnology-based transformation is the development and deployment of microbial cell factories which are specialized systems in which microorganisms replace chemical catalysis with biocatalysis, producing specific biochemicals. These systems exploit the inherent metabolic flexibility of microorganisms to synthesize target compounds. Among these microorganisms, yeasts are outstanding for their versatility, robustness, and ease of genetic manipulation.

In this thesis, we used an oleaginous yeast, *Rhodotorula toruloides* that can accumulate large amounts of lipids. However, its potential to produce other industry-relevant chemicals, such as exopolysaccharides (EPS), has not been extensively investigated. Therefore, this thesis focused on investigating potential of *R. toruloides* for EPS production.

In this thesis, we not only identified experimental conditions which are permissive for EPS production but also a condition that is prohibitive for EPS production. The results showed that the dry cells weight increased with the increase in glucose concentration in the culture medium, but EPS production appears decoupled from increased glucose supply, indicating a complex regulation for EPS production in *R. toruloides*. We obtained a maximum of 2.6 g/L of final EPS, using 100 g/L of glucose and 5 g/L ammonium sulfate. However, the increased glucose supply resulted in lower EPS yields, suggesting a potential role of carbon catabolite repression in *R. toruloides*. Finally, the EPS characterization using FTIR and GC-MS showed that the EPS was a carbohydrate with glucose, mannose, and galactose repeating units in its structure.

In conclusion, this thesis lays the foundation for further research on EPS production optimization, engineering EPS composition and deploying it for application in different fields, such as in the production of bioplastics, food, and drugs.

ANNOTATSIOON

ÜRO säästva arengu üks peamisi eesmärke on teha kindlaks mittesäästvad tooted või protsessid ja luua nende asemele säästvad lahendused. Süsivesinikupõhise keemiatööstuse ümberkujundamine biotehnoloogia abil on üks peamisi ühiskondliku tasandi muutuste ja arenguvõimaluste transformatiivseid valdkondi, mis loob tulevasi töökohti biopõhises majanduses. Selle kavandatud biotehnoloogial põhineva ümberkujundamise aluseks on mikroobirakutehaste väljatöötamine ja kasutuselevõtt, mis on spetsialiseerunud süsteemid, milles mikroorganismid asendavad keemilise katalüüsi biokatalüüsiga, tootes spetsiifilisi biokeemilisi aineid. Need süsteemid kasutavad mikroorganismidele omast metaboolset paindlikkust sihtühendite sünteesimiseks. Nende mikroorganismide hulgas on pärmid silmapaistvad oma mitmekülgsuse, vastupidavuse ja geneetilise manipuleeritavuse poolest.

Käesolevas väitekirjas kasutasime õlipärmi Rhodotorula toruloides, mis suudab akumuleerida suures koguses lipiide. Siiski ei ole põhjalikult uuritud selle potentsiaali toota muid tööstuses olulisi kemikaale, näiteks eksopolüsahhariide (EPS). Seetõttu keskenduti käesolevas töös R. toruloides'i potentsiaali uurimisele EPS tootmiseks.

Käesolevas väitekirjas me ei tuvastanud ainult eksperimentaalseid tingimusi, mis lubavad EPS tootmist, vaid ka tingimusi, mis on EPSi tootmise jaoks ebasoodsad. Tulemused näitasid, et rakkude kuivmass suurenes koos glükoosisisalduse suurenemisega kasvukeskkonnas, kuid EPSi tootmine näib olevat lahutatud suurenenud glükoosivarustusest, mis viitab EPSi tootmise keerulisele regulatsioonile R. toruloides'is. Kasutades 100 g/l glükoosi ja 5 g/l ammooniumsulfaati, saime maksimaalselt 2,6 g/l lõplikku EPSi. Suurenenud glükoosivarustuse tulemuseks oli siiski madalam EPSi kogus, mis viitab süsinikukataboliidi repressiooni võimalikule rollile R. toruloides'is. Lõpuks näitas EPSi iseloomustus FTIR ja GC-MS abil, et EPS oli süsivesik, mille struktuuris olid glükoosi, mannoosi ja galaktoosi korduvad üksused.

Kokkuvõtteks paneb see väitekiri aluse edasistele uuringutele EPSi tootmise optimeerimise, EPSi koostise konstrueerimise ja selle rakendamise kohta erinevates valdkondades, näiteks bioplastide, toiduainete ja ravimite tootmisel.

ABBREVIATIONS

MCF	Microbial Cell Factories
EPS	Exopolysaccharide
HoPS	Homopolysaccharides
HePS	Heteropolysaccharides
YPD	Yeast Extract Peptone Dextrose
EDTA	Ethylenediaminetetraacetic acid
HPLC	High-Performance Liquid Chromatography
GC-MS	Gas chromatography - Mass Spectrometry
FTIR	Fourier Transform Infrared

1. **INTRODUCTION**

Historically dependent on hydrocarbon-based resources, the chemical industry is a major contributor to environmental pollution, largely due to the production and use of non-recyclable toxic chemicals and synthetic polymers in various manufacturing processes and products[1]. This dependence is unsustainable and highlights the need to find sustainable processes and products using biotechnology. For example, biopolymers, derived from renewable biological sources such as plants, algae, and microbes, offer a viable alternative to non-recyclable polymers. These bio-derived materials offer the potential for complete biodegradability while reducing the environmental impact of the chemical industry[2]. Among these, microbial sources are particularly promising due to their potential to serve as efficient, scalable microbial cell factories. These cell factories are either natural producers or can be engineered to produce a wide range of biochemicals using the fermentation of natural materials and waste products in bioreactors.

Microbial cell factories (MCF) are specialized systems in which microorganisms produce specific biochemicals either naturally or through metabolic engineering methods. These systems exploit the inherent metabolic flexibility of microorganisms to synthesize valuable compounds, from pharmaceuticals to biopolymers, under controlled bioreactor conditions[3]. Yeasts stand out for their versatility, robustness, ease of genetic manipulation, and, in some cases, well-understood metabolic pathways[4]. For instance, Rhodotorula toruloides (R. toruloides) is an oleaginous yeast known for its ability to accumulate large amounts of lipids[5,6]. However, beyond lipid production, its potential to produce other industry-relevant chemicals, such as exopolysaccharides (EPS), has not been extensively investigated. Therefore, this dissertation research focuses on the identification of EPS production conditions, study effect of glucose and nitrogen utilization on EPS production, and characterize R. toruloides' EPS.

Initially, the thesis focuses on identifying EPS production conditions in R. toruloides. Thereafter, it focuses on understanding the role of nutrient concentrations, particularly carbon and nitrogen, in EPS production. Finally, the thesis aims to quantify produced EPS and characterize its composition, which can help the understanding of EPS structure and functional properties, essential for any future applications.

2. LITERATURE REVIEW

2.1. Sustainability Opportunities

To meet the United Nations sustainability goals[7], there is an urgent need to transform the chemical industry's unsustainable processes, such as the use of environment-polluting fossil fuels to produce chemicals and products, into sustainable bioprocess. Biotechnology harnesses bioprocesses and organisms to develop technologies and products that enable sustainability on our planet[8,9]. Using MCF, it is possible to develop technologies and products that can reduce the negative impact of the chemical industry by providing sustainable alternatives to fossil-fuels based processes and products. Biotechnology can help reduce toxic chemicals and products, for example, by allowing a transition from fossil fuels to biofuels in a green and sustainable economy (Figure 1)[10–14].

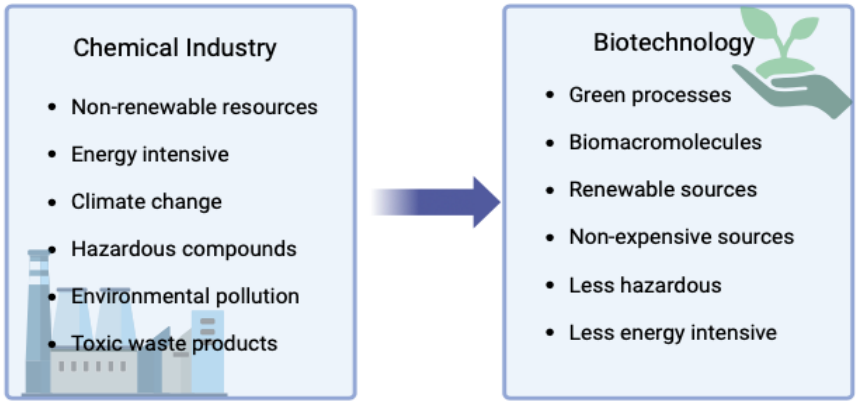

Figure 1. Chemical industry to biotechnology

2.2. Microbial cell factories

Microbial cell factories are engineered microorganisms, such as bacteria or yeast, modified to produce specific chemicals. These microorganisms are engineered using various molecular biology techniques to convert renewable resources, such as renewable resources—lignocellulosic biomass or carbon dioxide, into valuable products (Figure 2)[3].

Microbial fermentation methods use strains to catalyze the synthesis of various compounds, including vitamins, carboxylic acids, biofuels, and enzymes. Therefore, the development of MCF-based bioprocesses to obtain diverse products is a field with high growth potential, helping achieve sustainability using biotechnology[15].

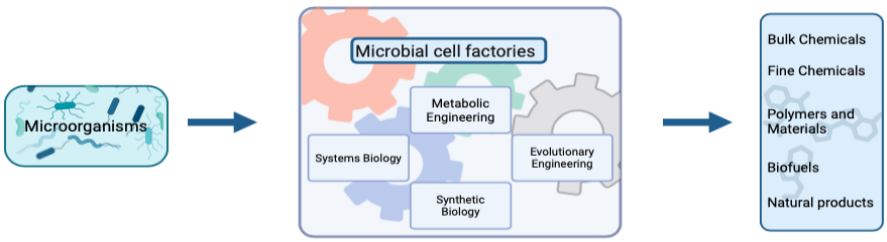

Figure 2. Microbial cell factories scheme[15]

2.3. Biopolymers and their uses

Biopolymers, or bio-based polymer composites, are polymers derived from natural sources, either directly from living organisms such as plants, animals, and microorganisms or from their metabolic processes (Figure 3)[16,17]. They can be classified into three primary categories depending on their origin: polysaccharides, nucleic acids, and proteins. All three of them play key roles in living systems. Recognized for their sustainable and biodegradable properties, biopolymers are increasingly favored in some industrial sectors due to growing environmental concerns and regulatory pressures. This shift is particularly pronounced in the medical and food industries, where biopolymers not only enhance the efficacy of drug delivery systems, improving the performance of bioactive molecules for more effective disease treatment[18], but also offer a green alternative to synthetic and chemical-based polymers, reducing carbon dioxide emissions, municipal solid waste, and dependence on non-renewable petroleum-based resource[19]. Bioplastics, a subset of biopolymers, are synthesized and degraded by various organisms, they also have distinct advantages over petroleum-based plastics, including their sustainability and lack of toxic effects on hosts (Figure 3)[20,21].

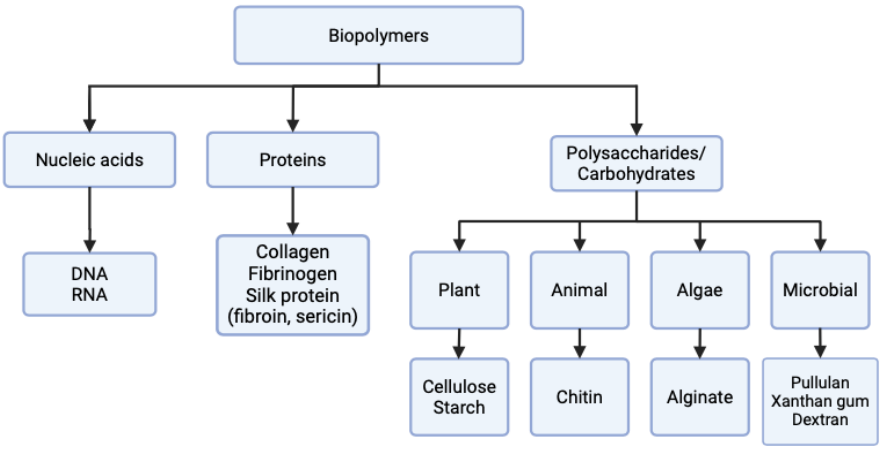

Figure 3. A schematic overview of the most common natural biopolymers

Some biopolymers have industry-relevant applications due to their desirable properties such as biocompatibility and gelling ability[22]. In the food industry, they serve as viscosity enhancers, gelling agents, and stabilizers[23]. In environmental applications, they aid in bioremediation processes and wastewater treatment[24]. In cosmetics and pharmaceuticals, biopolymers contribute to product texture, stability, and moisturizing properties[25]. Their potential as excipients and in tissue engineering to create targeted drug delivery systems and scaffolds to support tissue growth and regeneration is being explored. This diverse biopolymer usage underscore the motivation of the present thesis because of their indispensability in advancing sustainable industrial practices and addressing pressing global challenges[26].

2.4. Polysaccharides: properties and production

2.4.1. Polysaccharides

Polysaccharides perform a variety of essential functions in living organisms, including energy storage, structural support, lubrication, and cell signaling[27]. Polysaccharides are structural molecules formed by the condensation of monosaccharide units through O-glycosidic bonds. This bond is formed by a dehydration reaction between the hemiacetal hydroxyl group of one sugar (glycosyl donor) and the hydroxyl group of another sugar (glycosyl acceptor), Figure 4 shows different examples of polysaccharide structures and the glycosidic bonds between the monosaccharides. Unlike nucleic acids and proteins, which have specific types of bonds (phosphodiester and peptide bonds), polysaccharides have a variety of stereo- and regio-types of glycosidic bonds.[28]

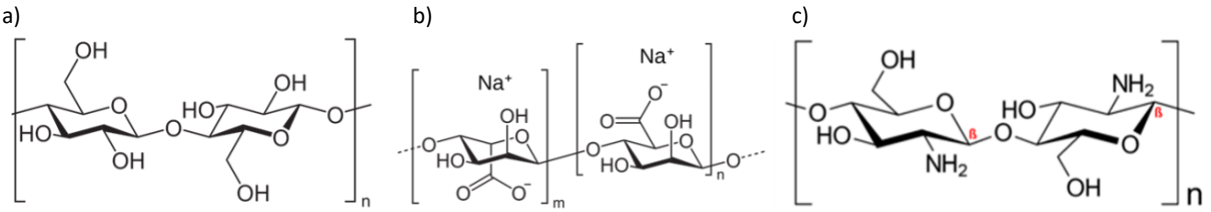

Figure 4. Examples of polysaccharides and their structures: a) cellulose; b) alginate; c) chitin[29]

2.4.2. Microbial exopolysaccharides

Unlike plants-based polysaccharides, microbial polysaccharides have advantaged of on-demand production using bioreactors. Therefore, there is an urgent need to investigate microbial exopolysaccharides, which are complex biopolymers produced by microorganisms, such as yeast and bacteria, either secreted into their environment or synthesized extracellularly. These substances act as protective barriers or adhesives and are critical in biofilm formation[30,31]. These extracellular polysaccharides vary in their relationship to the cell, leading to different classifications such as slime, capsular, or micro capsular polysaccharides, depending on their location and function relative to the bacterial or yeast cell[32].

For instance, *Aureobasidium pullulans* is a yeast-like fungus known for producing pullulan, a water-soluble EPS[33]. This fungus has both yeast and mycelial forms and can produce melanin, which makes separation of *pullulan* difficult[34]. *Pullulan* is known for its solubility, structural flexibility, and as a homopolysaccharide composed of maltotriose units linked by α-(1,6)-glycosidic linkages[35]. It's used in various industries due to its non-toxic, biocompatible, and safe properties. In the medical field, it is used in everything from plasma expanders to produce gene therapy components due to its non-immunogenic nature. In food and cosmetics, it acts as a film former and adhesive[36]. The fungal genus *Candida* has a versatile ability to produce EPS with different sugar compositions. Among *Candida* species, *Candida albicans* produces a water-soluble fraction, a mannoprotein ß-glucan complex, which contributes to biofilm formation and poses a challenge in the management of hospital-acquired infections[37]. Another EPS-producing *Candida* species is *C. utilis*, which synthesizes glucomannan with various glycosidic linkages and shows promising health benefits, including antioxidant and anti-

mutagenic properties. In addition, strains of *C. famata* and *C. guilliermondii* are also capable of EPS synthesis, with the EPS content varying depending on the carbon source in the culture media[38].

The biosynthesis of EPS, whether homopolysaccharides (HoPS) or heteropolysaccharides (HePS), involves similar biochemical pathways in different species of microorganisms[39]. There are two main groups of enzymes involved in the production of EPS, which are enzymes that synthesize basic sugar nucleotides such as glucose-1-phosphate and fructose-6-phosphate, which are also used by other metabolic pathways in the cell. Another group of EPS-specific enzymes, such as glycosyltransferases and acetyltransferases, regulates polymerization and export[40]. The production of EPS by microbes and yeast is influenced by several environmental and cultural conditions, such as pH, temperature, the ratio of carbon to nitrogen in the medium, and oxygen levels. These factors can affect the amount of EPS produced and its chemical composition. For example, different carbon sources can lead to changes in the composition of EPS, as seen in certain strains of *Lactobacillus*[26,41,42].

2.4.3. *Rhodotorula toruloides*

Rhodosporidium toruloides, also known as *Rhodotorula toruloides* in some classifications, is a red yeast that exhibits both yeast and mycelial forms. First isolated in 1922 in Dalian, China, this organism can grow in environments including soil, seawater, acid wastewater, pine wood pulp, and plant leaves. *R. toruloides* is classified within the phylum *Basidiomycota* and is characterized by its ability to metabolize different carbon and nitrogen sources, from simple sugars to complex organic acids and amino acids[43]. *Rhodotorula toruloides* is particularly noted for its ability to synthesize oleochemicals, including fatty acid esters and sesquiterpenes, which can be used as advanced biofuels[5]. In addition to fuels, this yeast can also produce high-value carotenoids such as torulene and torularhodin, which have antioxidant, anticancer, and antimicrobial properties[44], highlighting its potential for a sustainable industrial biotechnology by converting biomass into various high-value products[6]. Some *Rhodotorula* species can produce polysaccharides with significant biological activities. In particular, *Rhodotorula mucilaginos*a, a marine yeast, has been investigated for its ability to produce polysaccharides with immunomodulatory properties[45]. Another example is *Rhodotorula glutinis* strain K-24, which is known to produce a highly viscous polysaccharide under acidic conditions[46].

The potential for EPS production by *R. toruloides* is an intriguing area of biotechnological research, largely due to the organism's robust metabolic capabilities and adaptability to diverse environmental conditions. Although traditionally known for lipid and carotenoid synthesis, the literature suggests that it has the potential to synthesize complex sugars as EPS.

3. THESIS AIMS

1. Identification of exopolysaccharides producing and non-producing conditions in *R. toruloides*.
2. Understanding the role of glucose and nitrogen utilization in *R. toruloides*' EPS production.
3. *R. toruloides'* EPS quantification and its compositional characterization.

4. MATERIALS AND METHODS

4.1. Culture medium: composition and preparation

The pre-inoculum consisted of YPD medium. The YPD medium composition, prepared in milli-Q water, included: yeast extract 10 g/L, >95%; peptone from meat (bacteriological grade), and 20g/L; D(+)-Glucose monohydrate 20 g/L.

The chemically defined culture media, used in experiments, was prepared in Milli-Q water, where per liter composition was: 3 g/L KH_2PO_4 (Fisher Scientific, US); 2-5 g/L $(NH_4)_2SO_4$ (Fisher Scientific, US) (Table 1); 0.5 g/L $MgSO_4 \cdot 7H_2O$, >=99% (Fisher Scientific, US); 20-120 g/L D(+)-Glucose monohydrate, >99.5% (Carl Roth GmbH, Germany) (Table 1) and C_2H_6O 96% v/v. Vitamins and trace elements added 1 ml/L, prepared and filter sterilized separately in the following way: vitamin solution per liter of Milli-Q water (pH = 6.5): biotin, 0.05 g; p-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g; and myoinositol (AppliChem GmbH, Germany), 25 g. Trace elements solution per liter of Milli-Q water: EDTA, 15 g; $ZnSO_4.7H_2O$, 4.5 g; $MnCl_2.2H_2O$, 0.84 g (or $MnCl_2.4H_2O$, 1.03 g); $CoCl_2.6H_2O$, 0.3 g; $CuSO_4.5H_2O$, 0.3 g; $Na_2MoO_4.2H_2O$, 0.4; H_3BO_3, 1 g; KI, 0.10 g.[47]

The culture media was sterilized in two separate Pyrex™ bottles, where the first contained salts solution and the second contained D(+)-Glucose monohydrate solution, together making a final volume of 250 mL. The heat sterilization was done at 121 °C, 1.2 bar, for 15 min in autoclave (Systec V-95, Systec, USA). and the solutions cooled to room temperature, the solutions were mixed and the vitamins and trace elements solutions were added, all steps were done under sterile conditions.

The experimental conditions were varied using the C/N ratio (in mols) which was calculated using the following formula:

$$C/N\ ratio\ = \left(\frac{N_C\ \times\ [C-source]}{MM_C}\right) \div \left(\frac{N_N\ \times\ [N-source]}{MM_N}\right)$$

Where,

- N_c and N_N are the number of mol of atoms of carbon and nitrogen per mol of the carbon and nitrogen sources, respectively;
- [C-source] and [N-source] are the concentrations in mol/L of the carbon and nitrogen sources, respectively;
- MM_c and MM_N are the molar mass for the carbon and nitrogen sources, respectively.

4.2. Culture and cultivation conditions

For the inoculum, the yeast *Rhodotorula toruloides* (genotype - CCT 0783) was used for its potential to produce EPS. The culture glycerol stocks were stored at -80 °C, thawed on ice prior to inoculation. The pre-culture experiments were done in a 50-mL Falcon tube containing a 5 mL YPD medium. The culture was cultivated overnight at 30 °C and 200 rpm in a rotary incubator shaker (Excella E25, New Brunswick

Scientific, US) and used as inoculum for the experiments with chemically defined medium. The optical density (OD600) was measured to ensure that the cells were sufficient to provide a starting optical density of 0.1 at OD600 nm. The experiments in 25 mL chemically defined medium were performed in a rotary incubator at 200 rpm and 30 °C using 250 mL sterile shaker flasks (Erlenmeyer flasks with filter screw caps). The cultivation experiments were performed for 120 hrs. All preparations and further sampling were performed under a HEPA-filtered positive pressure flow hood.

4.3. High-Performance Liquid Chromatography

1 mL of medium was collected in 2 mL Eppendorf tubes and subsequently transferred to HPLC vials. The sample was filtered and diluted if needed. Glucose and ethanol in the chemical solution were quantified by HPLC (model LC-2030C Plus, Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-20A, Shimadzu, Kyoto, Japan). Samples were placed on the sample rack at 4 °C and aliquots of 20 µL were automatically injected onto an Aminex HPX-87H ion exclusion column (Bio-Rad, US). Elution was carried out in isocratic mode with H_2SO_4 (5 mmol/L) as the mobile phase with a flow rate of 0.6 mL/min at 45 °C. The temperature in the detector was also set to 45 °C.

4.4. pH measurement

For initial pH value measurement, 1 mL of culture medium was collected in 2 mL Eppendorf tubes and the pH was measured in a pH meter (HACH HQ30d, METTLER TOLEDO, USA). For final pH measurements, the cell culture broth was centrifuged for 5 min and 15200 rpm to remove the cells. The supernatant was collected for measuring the pH value.

4.5. Determination of dry-cell weight

To obtain the biomass' dry weight, samples were vortexed, pipetted onto previously weighed filter paper (0.22 µm) and filtered using a vacuum filtration system. Cells were dried in a microwave oven (at 900 W) for 2-3 min (30 sec intervals). Filter papers with cells were placed in small Petri dishes and kept in a desiccator until weighed the next day. The dry weight of the samples was determined gravimetrically and expressed as gDCW/L.

4.6. Exopolysaccharides

4.6.1. Extraction and quantification

The culture solution was transferred to 50-mL Falcon tubes and centrifuged at 6000 rpm for 30 minutes at 4 °C. The supernatant containing the suspended polysaccharides was then carefully separated from the cell pellet. To precipitate the EPS, ethanol (96% v/v) was added to the supernatant in proportion to 2 parts of ethanol for 1 part of the supernatant. The Falcon tubes, now containing the mixture of supernatant and ethanol, were stored in a cold room at 4 °C for 24 hours to facilitate the precipitation of the EPS. After 24 hours, the EPS had formed a distinct layer in the middle of the tubes. Using tweezers, this middle layer was carefully collected for further processing and analysis. The EPS obtained was placed in an incubator at 37 °C until no change in mass was observed, indicating the liquid had completely evaporated.

4.6.2. Exopolysaccharides Structure Characterizations

4.6.2.1. Fourier Transform Infrared (FTIR) Spectroscopy

For the FTIR analysis, dried EPS samples were carefully positioned in an FTIR spectrometer IRTracer-100 (Shimadzu, Kyoto, Japan). The spectra were recorded between 4000 and 400 cm^{-1} with spectral resolution of 6 cm^{-1} and aperture of 5 mm. The peaks detected by the software (LabSolutions, Shimadzu, Kyoto, Japan) were compared to the literature to identify functional groups.

4.6.2.2. Monosaccharide composition

The determination of the EPS monosaccharide composition was performed following the methods from Hamacek, Lahtvee, and Kumar (2023)[48] and adapted from Hamidi et al. (2020)[49]. Briefly, the EPS samples were dissolved and hydrolyzed in trifluoroacetic acid (2 mol/L) for 90 minutes in a dry bath at 120 °C. The solvent was evaporated with a stream of nitrogen. Finally, the derivatization of the sample and GC-MS analysis was performed according to Pierre et al. (2014)[50] with equal volumes of pyridine and BSTFA: TMCS (99:1). The resulting solution was mixed at room temperature and injected into GC-MS. The injection temperature was 250 °C. The oven temperature was 150 °C initially, then it was increased by 10 °C/min until 200 °C, which was kept for 35 minutes. Ionization was performed with a trap temperature of 150 °C and the target ion was set to 40-650m/z.

5. RESULTS AND DISCUSSION

5.1. Experiment design

The identification of control conditions is critical for the evaluation of investigated parameters and was motivation for identifying exopolysaccharide (EPS) production and non-production conditions. This was hypothesized for identification by deploying different carbon sources, namely glucose and ethanol, that utilize different metabolic pathways and therefore can result in EPS producing and non-producing conditions (Table 1). In addition, we aimed at increasing the carbon-to-nitrogen ratios in the culture medium (Table 2) to evaluate whether the increase in carbon availability enhances the EPS production. Finally, the determination of EPS composition was considered to understand its chemical profile through analytical methods.

Table 1. Effect of carbon source and initial ammonium sulfate concentration in EPS production

Experiment	Glucose (g/L)	Ethanol (g/L)	$(NH_4)_2SO_4$ (g/L)	C/N ratio
G20-N5	18.17	0	5	8.00
G20-N2	18.80	0	2	20.70
E20-N5	0	19.668	5	11.28
E20-N2	0	18.651	2	26.75

Table 2. Effect of glucose concentration on EPS production

Experiment	Glucose (g/L)	$(NH_4)_2SO_4$ (g/L)	C/N ratio
G20-N5	18.17	5	8.00
G40-N5	35.47	5	15.62
G60-N5	53.68	5	23.64
G80-N5	72.56	5	31.96
G100-N5	89.40	5	39.38
G120-N5	109.00	5	48.01

5.3. Exopolysaccharide producing and non-producing conditions

As planned, the initial experiments were performed to determine the production and non-production conditions:

- In the first experiment, the medium contained 5 g/L ammonium sulfate and the glucose concentration was measured as 18 g/L. The results in Figure 5 show the concentration of EPS and dry cell weight.
- In the second experiment, the culture containing ethanol as the carbon source was analyzed. The medium contained 20 g/L ethanol and 5 g/L ammonium sulfate. Despite the growth of cells and the ability of *R. toruloides* to use ethanol for its metabolism, no EPS was detected in the studied condition (ND).

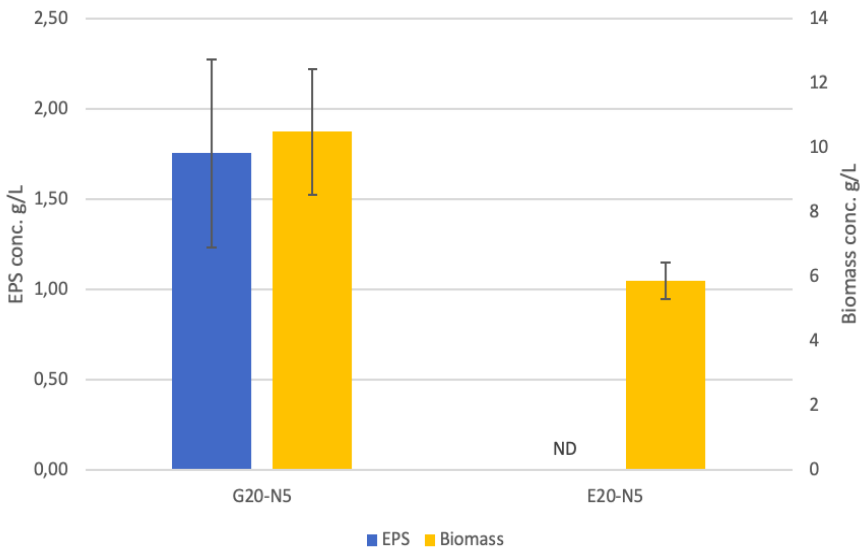

Figure 5. Comparison between EPS production (g/L) and dry cell weight for different carbon sources (glucose and ethanol)

As hypothesized at the experiment design phase, the difference in EPS production for different carbon sources can be attributed to the metabolic pathways activated by the presence of glucose as a carbon source in the chemical medium. Glucose is metabolized via the glycolysis pathway, generating cellular energy ATP but moreover provide precursor molecules for macromolecular synthesis, such as EPS. The previous studies indicated that under energy-rich conditions, certain yeasts and bacteria can produce EPS[51]. However, ethanol is metabolized via gluconeogenesis pathway in metabolism, which seems to primarily contribute to biomass formation instead of such byproducts as EPS formation (Figure 6).

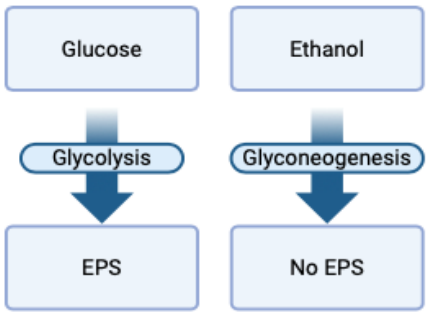

Figure 6. Use of cellular metabolism for the identification of EPS producing and non-producing conditions

5.4. Effect of C/N ratio concentration on exopolysaccharide production

To evaluate whether the increase in carbon availability changes the EPS production, we deployed various C/N ratio conditions, where ammonium sulfate was either at 2 g/L or 5 g/L. The glucose concentration was increased from 20 to 120g/L in the culture medium.

We quantified the changes in the biomass dry cell weight and EPS concentration after 120 hours of cultivation for different C/N ratios (Figure 7). The data analysis indicates that an increase in the glucose concentration, corresponding with increased C/N ratio, in the culture medium (and the C/N ratio increases), increases biomass until the glucose concentration reaches 100 g/L. We expected that an increase in the glucose concentration in the medium will cause a corresponding increase in the EPS concentration until the glucose concentration reaches 100 g/L; however, the EPS concentration decreased. Our results suggest that an increase in the glucose concentration leads to an increase in biomass but do not increase the EPS production similarly, indicating a more complex regulation for the EPS production than a simple growth associated production formation.

Moreover, the studied Figure 7 changes in nitrogen concentration (G20-N2) failed to show a significant (p-value > 0.05) effect on cell growth and EPS production when compared to the G20-N5 condition, indicating that nitrogen is not yet a limiting nutrient under these conditions studied.

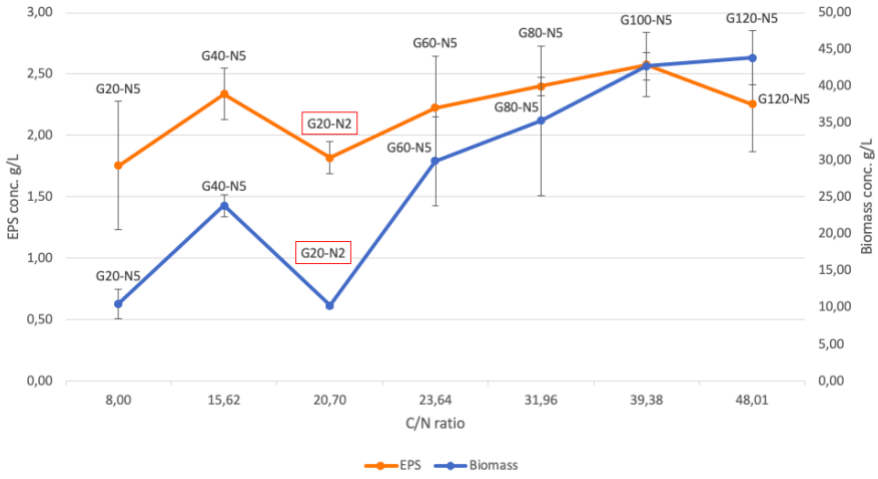

Figure 7. Concentration of dry cell weight and EPS as a function of the C/N ratio

To further examine relationship between the biomass formation and EPS production, we invested the EPS yield on biomass (Figure 8). We observed that the increase in C/N ratio, results in lower EPS yields, indicating a potential role of carbon catabolite repression for EPS production in *R. toruloides* [52].

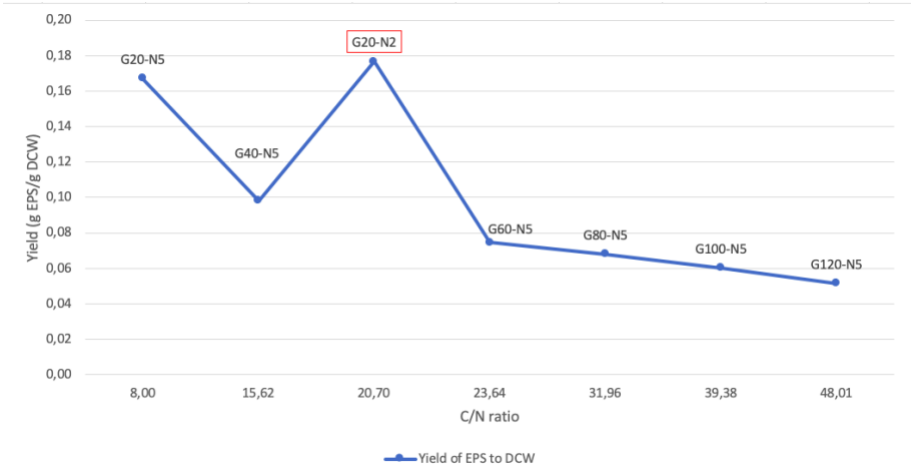

Figure 8. Yield of EPS to dry cell weight

5.5. pH changes in *R. toruloides*

An interesting observation in the study relates to a significant drop in the final measured pH (Figure 9). Notably, the final pH value from the cell culture broth had a significant increase in acidity, despite no organic acids being detected in samples by HPLC analysis. Despite variations in experimental conditions, no significant variation in initial and final pH values was observed between experiments, indicating it may be related to intrinsic ion maintenance mechanisms in *R. toruloides*.[47,53]

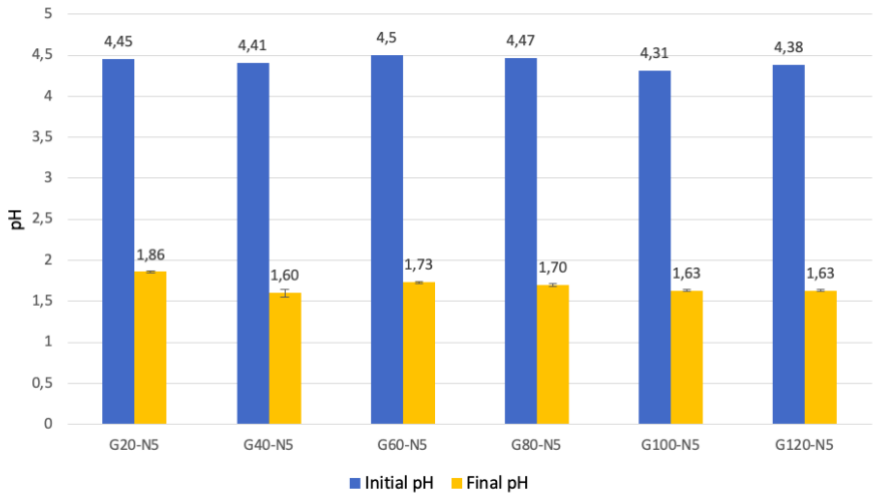

Figure 9. Initial and final pH values for different experimental conditions

It is one of the possibilities that the decrease in pH value might be due to the production of weak organic acids that are not detectable by the RID in HPLC. Another possibility relates to ammonium ions and protons exchange playing a role, necessitated by cellular mechanism responsible for the cytosolic pH maintenance in *R. toruloides*.[53,54] A preliminary support for the latter hypothesis is observed when we used a much lower nitrogen concentration of 0.5 g/L, where pH remained above 2.5 (Annex 3).

5.6. Characterization of exopolysaccharides

For understanding chemical bonds and the monomeric unit composition of *R. toruloides'* EPS, we extracted the EPS and characterized it using FTIR spectroscopy. It allowed qualitative identification of

chemical bonds in the polysaccharide., Furthermore, we performed GC-MS analysis, obtaining the monosaccharide composition of EPS.

A representative FTIR spectrum of the EPS analysis shows no changes in the spectra under different studied conditions (Annex 1). The spectrum shows a peak at 3323 cm^{-1} for -OH stretching. The double-peak at 2930 and 2887 cm^{-1} were identified as aliphatic -C-H stretching. The peak at 1635 cm^{-1} is regarded to be either -O-H bending from water bound to the polysaccharide chains and/or C=O stretching. Finally, two strong bands are observed at 1061 and 1025 cm^{-1}, which represents -CH2-O-CH2- stretching. Therefore, the typical carbohydrate peaks described previously confirm that the product obtained during the extraction is a polysaccharide[49,55,56]. For the monosaccharide composition of the EPS, the polysaccharide chains were hydrolyzed into monosaccharides, the hydroxyl groups were then derivatized into acetate to increase the volatility for analysis. In these results shows that the EPS can be mainly classified as a 'glucomannan' since it contains glucose, mannose, and galactose in the proportion of 79:17:4, respectively (Figure 10). This implies that *R. toruloides* can generate precursor molecules using glucose that polymerize to form the EPS structure, demonstrating used carbon in experiments is likely in excess for complete use in biomass formation and therefore leads to EPS as a byproduct formation.

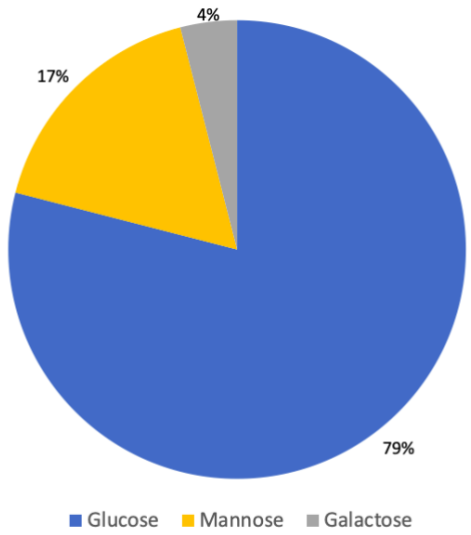

Figure 10. Proportion of detected monosaccharides by GC-MS after hydrolysis and derivatization of EPS

6. SUMMARY

This thesis aimed to identify exopolysaccharide-producing and non-producing conditions in *R. toruloides*, focusing on the carbon and nitrogen. First, we identified experimental conditions in which EPS is produced (with glucose as a carbon source) and conditions in which EPS is not synthesized (when ethanol is the carbon source). The results suggest that EPS production might be related to the glycolysis pathway, as opposed to the gluconeogenesis pathway which did not favor EPS synthesis. The use of different metabolic pathways indicates that the glycolysis produces precursor monosaccharides that can be polymerized into EPS in *R. toruloides*. Secondly, it was observed that the amount of yeast biomass increased with the increase in glucose concentration in the culture media. EPS production did not follow the same trend, indicating that the flux of carbon toward EPS production is not completely controlled by the availability of glucose. Therefore, further optimization of parameters is necessary to increase the EPS titer. We preliminarily tested whether an increased C/N ratio by reducing nitrogen would affect EPS production and results indicate that it indeed increases EPS yield. In the future, different carbon sources instead of glucose, and cultivation conditions such as temperature and stress responses could be considered for EPS production in *R. toruloides*, since these were reported to increase EPS production in other microbes[49]. Finally, the EPS characterization showed that the product obtained from the extraction process was a carbohydrate that contained glucose, mannose, and galactose. In conclusion, this thesis work lays the foundation for further research on EPS production optimization, engineering EPS composition and deploying it for application in different fields, such as in the production of bioplastics, food, and drugs.

7. KOKKUVÕTE

Käesolevas väitekirjas kasutasime õlipärmi Rhodotorula toruloides, mis suudab akumuleerida suures koguses lipiide. Siiski ei ole põhjalikult uuritud selle potentsiaali toota muid tööstuses olulisi kemikaale, näiteks eksopolüsahhariide (EPS). Seetõttu keskenduti käesolevas töös R. toruloides'i potentsiaali uurimisele EPS tootmiseks.

Käesolevas väitekirjas me ei tuvastanud ainult eksperimentaalseid tingimusi, mis lubavad EPS tootmist, vaid ka tingimusi, mis on EPSi tootmise jaoks ebasoodsad. Tulemused näitasid, et rakkude kuivmass suurenes koos glükoosisisalduse suurenemisega kasvukeskkonnas, kuid EPSi tootmine näib olevat lahutatud suurenenud glükoosivarustusest, mis viitab EPSi tootmise keerulisele regulatsioonile R. toruloides'is. Kasutades 100 g/l glükoosi ja 5 g/l ammooniumsulfaati, saime maksimaalselt 2,6 g/l lõplikku EPSi. Suurenenud glükoosivarustuse tulemuseks oli siiski madalam EPSi kogus, mis viitab süsinikukataboliidi repressiooni võimalikule rollile R. toruloides'is. Lõpuks näitas EPSi iseloomustus FTIR ja GC-MS abil, et EPS oli süsivesik, mille struktuuris olid glükoosi, mannoosi ja galaktoosi korduvad üksused.

Kokkuvõtteks paneb see väitekiri aluse edasistele uuringutele EPSi tootmise optimeerimise, EPSi koostise konstrueerimise ja selle rakendamise kohta erinevates valdkondades, näiteks bioplastide, toiduainete ja ravimite tootmisel.

8. ACKNOWLEDGEMENTS

This work is financially supported by the Estonian Research Council team grant for developing a novel 3D-printable cell factory platform for growth-decoupled oleochemical production (PRG1101). I would to thank Prof. Petri-Jaan Lahtvee for providing me the opportunity to be a part of the BioEng Lab.

I thank my supervisors, Rahul Kumar and Henrique Sepulveda Del Rio Hamacek for the thesis planning, results' discussion, and feedback on writing. Finally, I thank the BioEng lab for the practical support during my thesis work.

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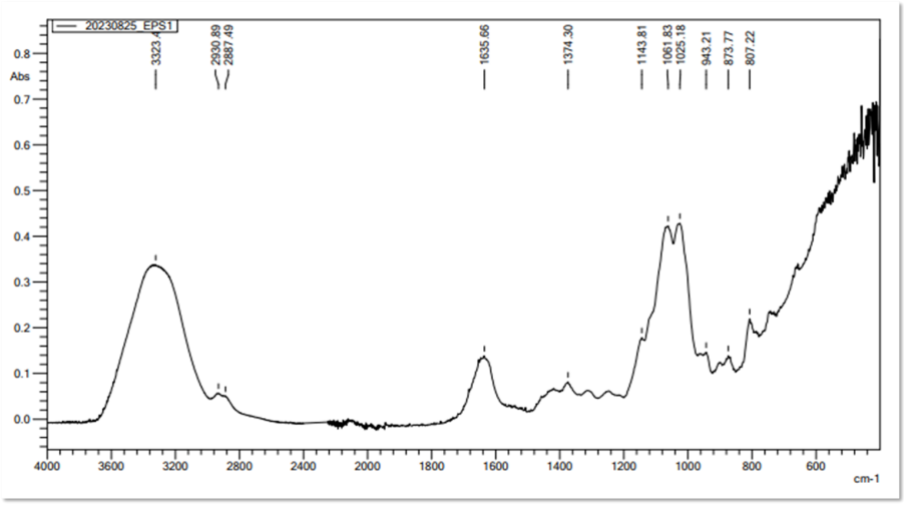
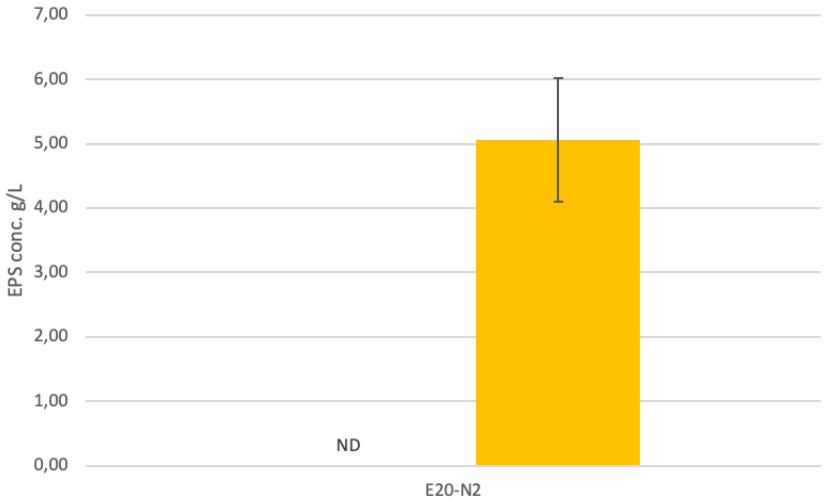
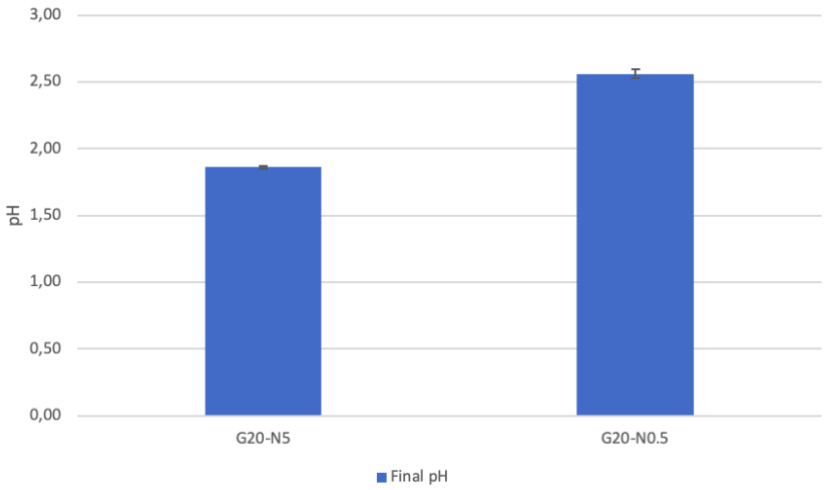
10. SUPPLEMENTARY INFORMATION



Annex 1. FTIR spectrum and its peaks of an EPS sample



Annex 2. Comparison between EPS production (g/L) and dry cell weight for 20 g/L of ethanol and 2 g/L of ammonium sulfate



Annex 3. Effect of nitrogen concentration on the final pH in *R. toruloides* cultivation after 120 h

Annex
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