TALLINN UNIVERSITY OF TECHNOLOGY, SCHOOL OF SCIENCE

DEPATMENT OF CHEMISTRY AND BIOTECHNOLOGY

Master's Thesis

Physiological Evaluation of *Rhodotorula toruloides* in Different Nutrient Environments

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Magistritöö

Rhodotorula toruloides Füsioloogiline Hindamine Erinevates Toitekeskkondades

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TOIDUTEHNOLOOGIA JA -ARDENUS

Tallinn, 2022

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…….

[allkiri ja kuupäev]

Table of Contents

Annotatsioon

Rhodotorula toruloides on õline pärm, mis toodab lipiide ja karoteene, olulisi biotehnoloogiatooteid. Käesolevas lõputöös hinnati *R. toruloides* füsioloogiat glükoosi ja ksüloosi sisaldavas keemiliselt määratletud söötmes. Lisaks kasutati lämmastiku muutusi kahe erineva C/N suhte rakendamiseks *R. toruloides* füsioloogiliseks hindamiseks. Tulemused näitavad, et *R. toruloides* kasvab glükoosil kui süsinikuallikal kiiremini kui ksüloosil nii madala (7.4) kui ka kõrge (187) C/N suhte tingimustes. Huvitav on see, et C/N suhted ei mõjuta spetsiifilist glükoosi tarbimise kiirust (mmol.gDCW^{-1}.h^{-1}), kuid ksüloosi sisaldav sööde näitab kõrgemat spetsiifilist ksüloosi tarbimise kiirust (mmol.gDCW^{-1}.h^{-1}) madala C/N suhe juures ja madal spetsiifiline ksüloosi tarbimise kiirus (gDCW^{-1}.h^{-1}) kõrgema C/N suhte korral. See tulemus näitab erinevusi *R. toruloides* glükoosi ja ksüloosi metabolismis. Lisaks näitavad glükoosiandmed, et kuigi glükoosi tarbimise erikiirus (mmol.gDCW^{-1}.h^{-1}) jääb mõlema C/N suhte puhul sarnaseks, väheneb biomassi saagis kõrge (187) C/N suhte korral 45%. Lisaks näitab ksüloosi sisaldav sööde partiikultuuride katsetes suhkrualkoholi, nimelt arabinitooli tootmist ja tarbimist. Käesolevas lõputöös saadud spetsiifilise kasvukiiruse andmed aitavad kavandada kemostaadi bioreaktori katseid uuritud C/N suhete jaoks, kus lahjendusmäär peaks jääma alla spetsiifilise kasvukiiruse, et vältida kultuuri väljapesemist katsete ajal.

Abstract

Rhodotorula toruloides is an oleaginous yeast that produces lipids and carotenes, important biotechnology products. The present thesis evaluated physiology of *R. toruloides* in glucose and xylose containing chemically defined culture medium. Moreover, the changes in nitrogen were used to deploy two different C/N ratios for physiological evaluation of *R. toruloides*. The results indicate that *R. toruloides* grows faster on glucose as carbon source than xylose in both low (7.4) and high (187) C/N ratio conditions. Interestingly, the C/N ratios show no impact on specific glucose uptake rate ($mmol.gDCW^{-1}.h^{-1}$), but xylose containing culture medium shows higher specific xylose uptake rate ($mmol.gDCW^{-1}.h^{-1}$) at low C/N ratio and low specific xylose uptake rate ($gDCW^{-1}.h^{-1}$) at higher C/N ratio. This result indicates differences in glucose and xylose metabolism in *R. toruloides*. Moreover, glucose data show that though the specific glucose uptake rate ($mmol.gDCW^{-1}.h^{-1}$) remains similar for both C/N ratios, the biomass yield decreases by 45% at high C/N ratio (187). In addition, xylose containing medium indicates production and consumption of a sugar alcohol, namely arabinitol, in the batch cultivation experiments. The specific growth rate data obtained in this thesis will help design of chemostat bioreactor experiments for the studied C/N ratios, where the dilution rate should remain below the specific growth rate to avoid culture washout during the experiments.

List of Abbreviations

G3P	Glyceraldehyde 3-phosphate
PPP	Pentose phosphate pathway
ACL	ATP-citrate lyase
TCA	Tricarboxylic acid
TAG	Triacylglyceride
C/N	Carbon to nitrogen ratio
HR	Homologous recombination
NHEJ	Non-homologous end joining
OD	Optical density
HPLC	High Performance Liquid Chromatography

Introduction

Replacing fossils-based hydrocarbon products and processes is an urgent societal imperative for sustainability of environment, food safety and life on the planet earth. Because of this imperative, a significant effort has been put into the development of cell factories aiming for more cost-efficient, sustainable, and environmentally friendly production processes than the currently existing. Microbial cell factories can be defined as microorganisms engineered to improve the natural or de novo production of biochemicals. The conventional baker's yeast *Saccharomyces cerevisiae* is considered a model cell factory that can be engineered to produce biofuel, oleochemicals and nutraceuticals [1]. The productivity of cells factories is improved by using metabolic engineering approaches such as overexpression of genes involved in the production of lipids precursors and/or knock-out of genes involved in lipid degradation processes, increasing lipid production that can be utilized for biofuels [2]. However, the increased production of biochemicals can also have an undesirable impact on the cellular physiology, reducing productivity and yields. For this reason, the recent research focus has been on identifying robust cellular factories that are native producers of different biochemicals and show robust physiology in adverse industrial culture environments. Non-conventional yeasts such as *Kluyveromyces marxianus*, *Yarrowia lipolytica*, and *Rhodotorula toruloides* are considered potential cells factories that can be the next biotechnology workhorse for biochemicals production [3-6].

In this thesis, *R. toruloides*, an oleaginous red yeast, was deployed for investigation. It naturally tends to accumulate high amount of lipids and produces carotenes that are precursor molecules for vitamin A, an essential vitamin for humans. *R. toruloides* is also remarkable in its ability to utilize a wide range of substrates including hexose and pentose sugars that are obtained from plants. Moreover, it is also highly tolerant for several environmental stressors such as pH, temperature, H_2O_2, salts and other inhibitors present in wood hydrolysates [7].

Understanding cellular physiology is relevant for developing bioprocesses. It is critical for designing novel bioprocesses that potential cell factories are evaluated in chemical defined culture medium where role of substrates, environment and products can be studied. Such investigations can be used to identify cellular regulations and potential metabolic engineering targets that can enhance productivities.

In the present thesis, the physiology of *R. toruloides* was evaluated in two different carbon sources. Moreover, changes in nitrogen source concentration were deployed for investigating impact of carbon to nitrogen ratio (C/N ratio) on growth physiology of *R. toruloides*. Growth characterization was performed in replicate batch cultures and physiology parameters were quantified by measurements of pH, biomass, and metabolites.

Briefly, the thesis provides a review of relevant literature, presented detailed experimental methodology allowing data reproducibility, discussed presented results, drives conclusions from the results and suggest future direction of research in the summary.

Aims of Study

The thesis objective was to evaluate physiology of *R. toruloides* IFO0880 *Δku70* in glucose and xylose carbon source containing chemically defined culture medium. Moreover, two different C/N ratio were deployed for evaluating potential differences between cultivation conditions that allow maximization of biomass and production formation in *R. toruloides*.

I. Review of Literature

The review of literature provides a general overview of yeast, using the baker's yeast *Saccharomyces cerevisiae* as an example of a conventional yeast, in the field of food and biotechnology. In particular, the review focuses on a non-conventional yeast, namely *R. toruloides*, for detailed description because of its use in the thesis investigation.

1. Yeast – In Food and Biotechnology

The baker's yeast is conventionally used in food and beverages for centuries. It is commonly used to make bread, and in breweries and wineries for making beer and wine, respectively [8, 9]. Yeasts are also used in prebiotics, enzyme production and for making different flavors [10, 11]. In recent decades, the advances in genetically engineering allow developing microbes into cell factories [12]. For example, engineered *S. cerevisiae* is currently used for producing biofuels and biochemicals that are traditionally obtained from fossil fuels-based hydrocarbons [13]. The processes involving micro-organisms are then called bio-based processes. In these bio-based processes, plant-based carbon can be utilized for biochemicals production instead of environmentally harmful fossil fuels-based hydrocarbons [14]. Thus, the current microbial cells factories allow bio-sustainable production of foods and chemicals, which can be beneficial for environment on the planet earth and answer to sustainability concerns arose by traditional industrial processes (Figure 1) [1, 15].

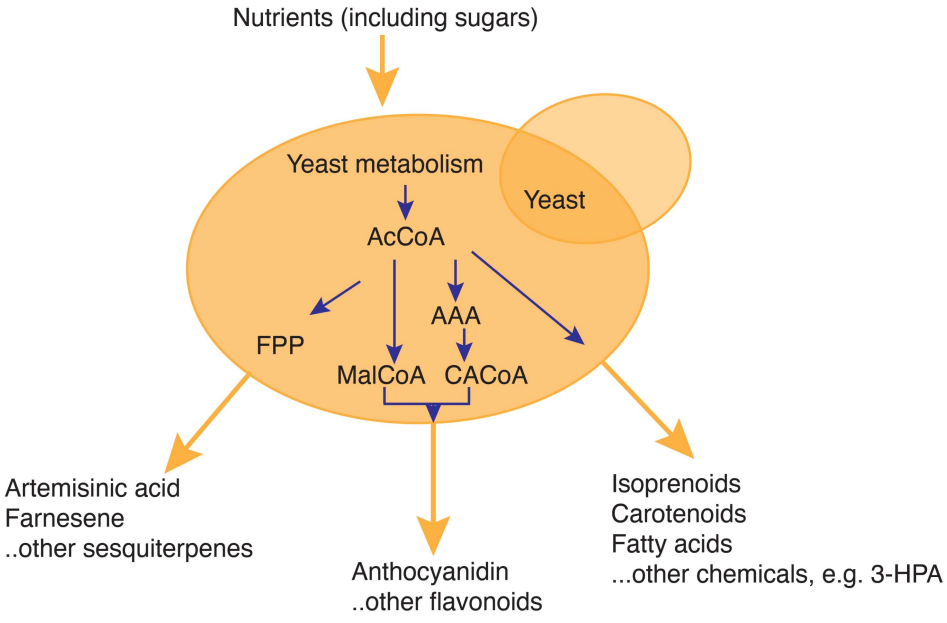

Figure 1: Cell factories concept [1]. Abbreviations: AcCoA, acetyl CoA; FPP, farnesyl-pyrophosphate; MalCoA, malonyl-CoA; CACoA, coumaryl-CoA; AAA, aromatic amino acids; 3-HPA, 3-hydroxypropionic acids.

1.1 *Rhodotorula toruloides* – A non-conventional yeast

The baker yeast is widely used in the food and beverage industry and is often considered as one of the key microbial cell factories. However, it does not naturally produce several biochemicals that are currently needed for the society such as higher concentrations of fatty acids for producing biofuels. For this reason, the non-conventional yeasts are attractive potential cell factories, being natural producers of oleochemicals, carotenoids, and other precursors molecules [16-18]. Among the non-conventional yeasts, *R. toruloides* is one of the most promising candidate cells factories for biotechnological applications [5]. The next section describes *R. toruloides* substrates and its main biotechnology products.

1.1.1. Nutrients

In comparison to conventional yeast, *R. toruloides* can consume a wide range of carbon sources including hexose and pentose sugars that can be obtained from lignocellulosic materials (Figure 2) [19-21]. Moreover, *R. toruloides* is also tolerant to environmental stressors, e.g.,

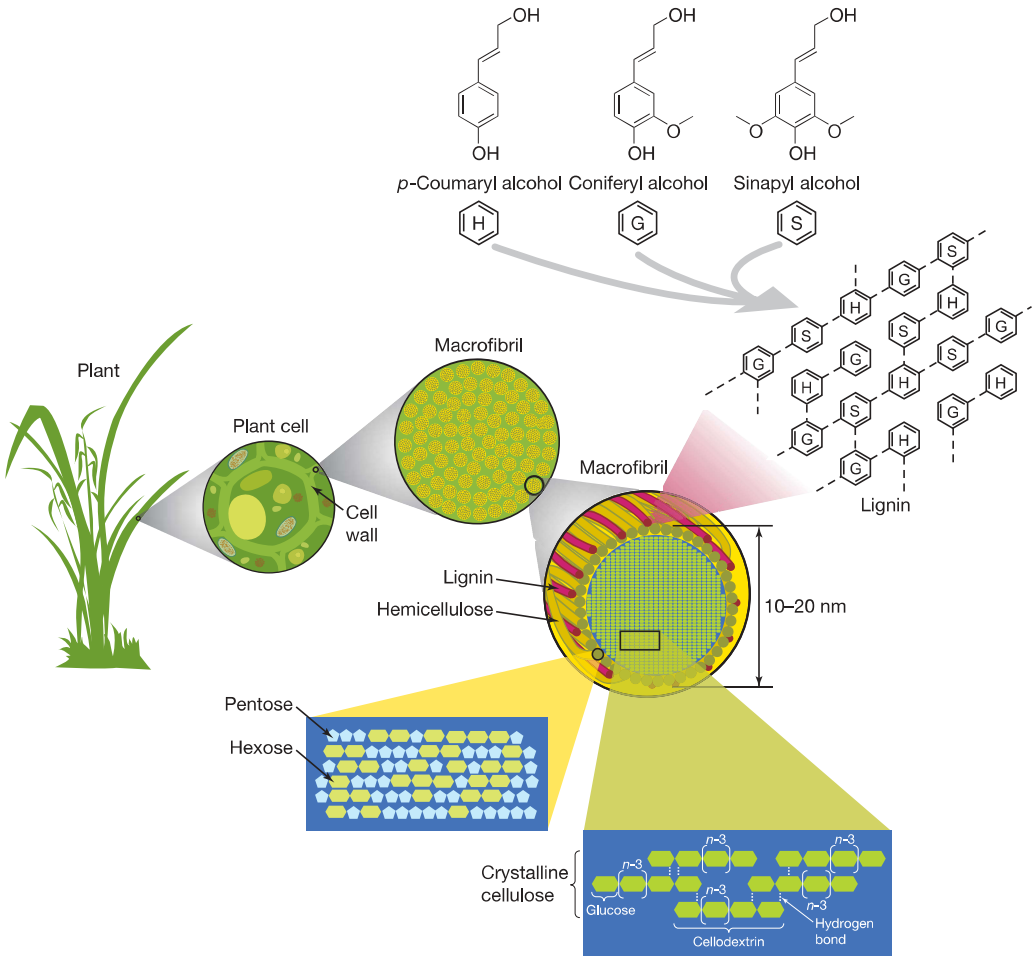

Figure 2: Structure and biochemical properties of lignocellulose [19].

pH, temperature, H_2O_2, salts, and inhibitors present in wood hydrolysates [7, 19].

1.1.2. Lipids

Cellular nutrients can be divided into macro-nutrients, e.g., carbon and nitrogen sources, and micro-nutrients, e.g., minerals and vitamins. Previous studies indicate that carbon to nitrogen ratio requirement is different when goal is maximization of biomass formation as compared to formation of biotechnology products [22]. Nitrogen limitation is implicated in increased lipid accumulation in *R. toruloides*, demonstrated by using differential interference contrast (DIC) microscopy with the fluorescent neutral lipid dye 4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene (BODIPY^{TM} 493/503) (Figure 3) [23, 24]. Further studies indicate that *R. toruloides* is able to naturally accumulate lipids (triacylglycerol) up to 65% of dry cell weight from esterification of fatty acids [25].

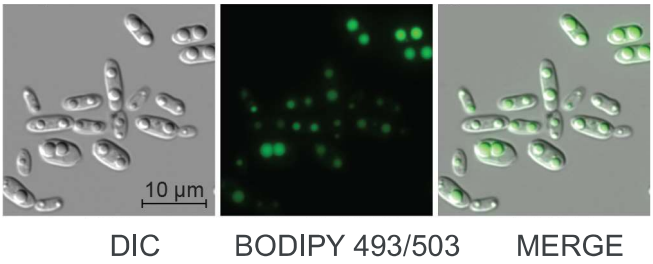

Figure 3: *R. toruloides* IFO 0880 strain showing lipid accumulation [23].

One of the reasons non-conventional yeasts accumulate more lipids than conventional yeasts is metabolic differences in producing acetyl CoA. The non-conventional yeasts contain two enzymes ATP citrate lyase (ACL) and phosphoketolase (PKL) that are lacking in conventional yeasts such as *S. cerevisiae* [26].

1.1.3. Carotenes

In addition to lipids accumulation, *R. toruloides* is native producer of carotenes, precursor molecules for vitamin A in humans. It can naturally produce β-carotene, γ-carotene, torulene, and torularhodin, which confers its distinctive pink-orange-reddish color [27]. Moreover, these chemicals also have coloring and antioxidants properties that find use in different biotechnology applications. The conditions of oxidative stress tend to increase carotenoid production due to its antioxidant properties in *R. toruloides* [28]. Additionally, the carotenoid biosynthesis genes are also stimulated by light, increasing carotenoid production in *R. toruloides* [28, 29].

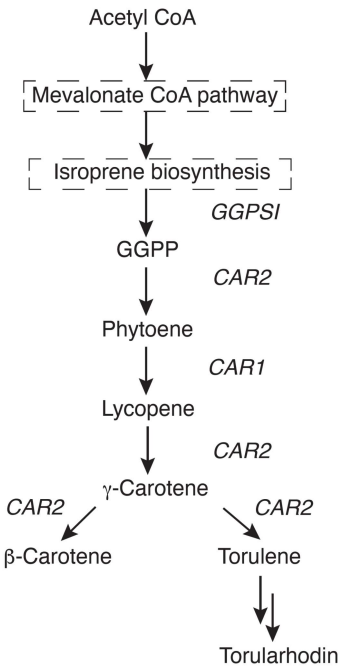

Figure 4: Carotenoid biosynthesis pathway [29]**.** Abbreviations: *GGPSI,* geranylgeranyl diphosphate synthase; *CAR2*, phytoene synthase/lycopene cyclase; *CAR1*, phytoene desaturase**.**

II. Experimental

2.1. Materials and Methods

2.1.1. Strain

The present study used *Rhodosporidium toruloides* IFO0880 *ΔKU70* strain in experiments [30]. The strain was obtained from the Joint Bioenergy Institute (California, United States) and stored according to provided instructions in 10-15% glycerol at -80 °C.

2.1.2. Medium composition

The composition of low and high C/N ratio medium (Table 1) was prepared according to previously investigated culture medium for *R. toruloides* [31]. The C/N ratio is a mass (g/g) relation between carbon coming from the carbon source (glucose, xylose) and the nitrogen source (ammonium sulfate). In case of carbon sources (glucose, xylose), carbon atoms per glucose ($C_6H_{12}O_6$) and xylose ($C_5H_{10}O_5$) represent 40% of their respective molar. In ammonium sulfate, nitrogen atoms accounts for 10.7% of the molar mass. Therefore, a concentration of 10 g/L carbon source provides approximately 4 g/L of carbon atoms concentration culture medium. Similarly, the nitrogen concentration is 0.54 g/L and 0.0214 g/L in low C/N ratio medium and high C/N ratio medium, respectively. The carbon concentration was maintained at the same level, while the nitrogen concentration, either high or low, results in C/N ratio of 7.4 and 187 in culture medium, respectively. In this study the C/N ratio was mass-based calculated, but it can also be calculated using moles. Low C/N ratio medium contained, per litre, 5 g of $(NH_4)_2SO_4$, 3 g of KH_2PO_4, 0.5 g of MgSO4·7H2O, 10 g of carbon source, in addition to 1 ml of vitamins and trace elements each as reported previously [32]. In high C/N ratio medium $(NH_4)_2SO_4$ concentration was reduced to 0.5 g/L and sulfate was compensated by adding 6.3 g of K_2SO_4. The vitamin solution contained, per liter, 0.05 g of biotin, 0.2 g of p-amino benzoic acid, 1 g of nicotinic acid, 1 g of Ca pantothenate, 1 g of pyridoxine-HCl, 1 g of thiamine-HCl and 25 g of myoinositol. Trace element solution contained, per liter, EDTA (sodium salt), 15.0 g; $ZnSO_4·7H_2O$, 4.5 g; $MnCl_2·2H_2O$, 0.84 g; $CoCl_2·6H_2O$, 0.3 g; $CuSO_4·5H_2O$, 0.3 g; $Na_2MoO_4·2H_2O$, 0.4 g; $CaCl_2·2H_2O$, 4.5 g; $FeSO_4·7H_2O$, 3.0 g; H_3BO_3, 1.0 g; and KI, 0.10 g. The pH of culture medium pH was adjusted to 5.5 ± 0.2 with addition of 2M KOH. Medium base (phosphates and sulphates) and carbon source solutions were prepared and autoclaved separately. Separately autoclaved carbon source was added afterward at 10g/L concentration.

Table 1. Culture medium compositions in per liter, providing low and high C/N ratio.

Component	Low C/N ratio	High C/N ratio
$(NH_4)_2SO_4$	5 g/L	0.2 g/L
KH_2PO_4	3 g/L	3 g/L
K_2SO_4	0 g/L	6.3 g/L
$MgSO_4 \cdot 7H_2O$	0.5 g/L	0.5 g/L
Carbon source	10 g/L	10 g/L
Vitamins	1 ml/L	1 ml/L
Trace elements	1 ml/L	1 ml/L

2.1.3 Culture conditions

Pre-inoculum was grown from glycerol stock in yeast peptone dextrose (YPD) broth at 30 °C overnight. Batch cultivations were performed in triplicates using 250 ml Erlenmeyer shaker flasks with 50 ml of medium. Medium was prepared in 250 ml bottle by mixing 190 ml of base solution, 10 ml of carbon source solution and 200 µl of vitamins and trace elements each. Cultures were incubated in a VWR Incubating Orbital Shaker (model 3500) at 30 °C and 200 RPM. The starting OD was 0.05 and 0.5 in glucose and xylose cultures, respectively.

Samples of 1 ml volume were collected into 1.5 ml Eppendorf tubes right after inoculation, at the start (10 h and 16 h) and during the exponential phase (every 2 h and 3 h) for glucose and xylose cultures, respectively, and once in the stationary phase. The optical density (O.D. 600 nm) was measured by Biochrom Ultrospec 10 Cell Density Meter and pH was measured by Mettler Toledo SevenCompact S210 pH meter for each sample. Samples were then centrifuged for 5 min at 12,000 × g to remove cells. The supernatant was collected and stored in -20 °C for extracellular metabolite quantification.

2.1.4 Metabolites quantification

Carbon sources (glucose, xylose) and extracellular metabolites (acetic acid, glycerol, arabitol, xylitol) were measured and quantified using high-performance liquid chromatography (HPLC Shimadzu LC-2050C analyzer) with RID-20A detector. Parameters used to detect organic acids and monosaccharides are reported in the Table 2.

Table 2. Metabolites quantification parameters using HPLC

	Organic Acids	**Monosaccharides**
Column	Aminex HPX-87H 300 X 7.8 mm	Rezex RPM Monosaccharide PB+2 LC column 300 x 7.8 mm
Eluent	H_2SO_4 5 mM (100%)	Water (100%)
Oven temperature	45 °C	85 °C
Running time (min)	30	60
Flow (ml/min)	0.6	0.6
UV	OFF	OFF
RID	ON - Temperature: 45 °C	ON - Temperature: 85 °C

2.1.5 Biomass Estimation

The data used to calculate the linear coefficient between dry cell weight (gDCW/L) and optical density were in-house data collected for an unpublished separate study. In this study *R. toruloides* IFO0880 *ΔKU70* strain was grown in flask using xylose as carbon source (n=3). For each sample, optical density, and dried cell mass (g) were measured from the same volume of broth. The linear coefficient between gDCW/L and OD was calculated on averaged values and equals 0.298 with R^2 higher than 0.9 (Figure 5). This coefficient was used to convert OD into biomass unit (gDCW/L) which was necessary to calculate yields and rates.

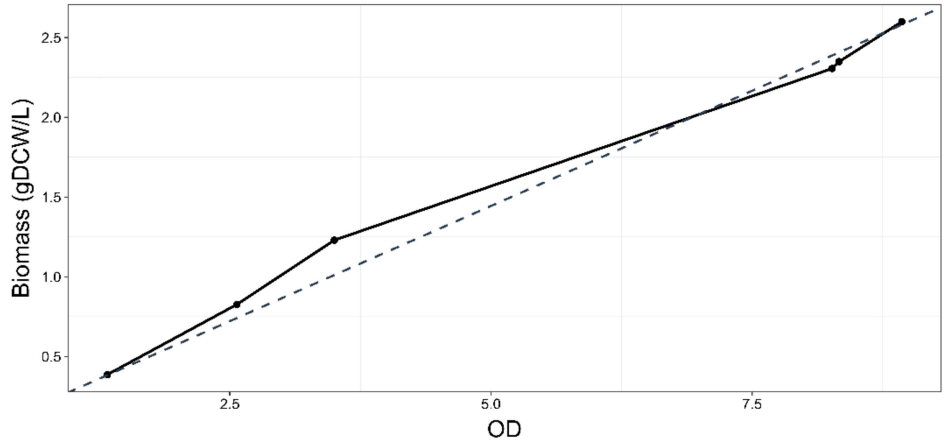

Figure 5: Calculation of the conversion factor from optical density to biomass (gDCW/L) for *R. toruloides* IFO0880 *ΔKU70*. The coefficient of the linear relationship between biomass and OD was 0.298 (R^2 >0.9) and used later to convert OD 600nm into biomass values (gDCW/L).

2.2. Data analysis

Data analysis was done in MS Excel. Specific growth rate was calculated over the exponential phase, i.e. time range where the optical density doubles every hour. Then, the optical density was converted into biomass unit (gDCW/L), log transformed and fitted using exponential linear regression. The fit was asserted using the R^2. When R^2 was at least 0.9 the exponential coefficient of the equation was reported as specific growth rate. Carbon source consumption rate was calculated by fitting carbon source concentration (mmol/L) and biomass (gDCW/L) over the exponential phase. The coefficient was then multiplied by the growth rate to get a consumption rate unit in mmol/gDW/h. Figures were prepared using R (2021.09.1) and Inkscape (1.1.1).

III. Results and Discussion

3.1. Culture media and inoculum selection

In the present thesis, two different chemically define culture media were investigated. These culture medium were based on previous studies and are referred to as media A [31] and media B [33] in the thesis. Media A and B differed by their amount of $MgSO_4 \cdot 7H_2O$ (0.5g and 2g, resp) and their nitrogen source (5g $(NH_4)_2SO_4$ and 5g NH_4Cl, resp). The motivation to choose *R. toruloides* IFO0880 Δ*KU70* strain for study was previous report on *R. toruloides* W29, indicating formation of cellular aggregates or clumps within few hours in presence of glucose as carbon source in the chemically defined medium [3]. The addition of KCl (0.2M) was suggested to prevent cellular aggregates formation in *R. toruloides* W29 [3]. Therefore, in addition to investigating two different culture media, the impact of KCl addition on *R. toruloides* IFO0880 Δ*KU70* in the presence of glucose as carbon source was also investigated. In contrast to R. toruloides W29 [3], for *R. toruloides* IFO0880 Δ*KU70* the addition of KCl decreased the specific growth rate by 20% and 14% in medium A and B, respectively (Figure 6). In absence of KCl, specific growth rate in media A and B was not significantly different but the biomass production was slighly higher at the end of the experiment in medium A (Figure 6). Moreover, *R. toruloides* IFO0880 Δ*KU70* showed no cellular aggregates formation in absence of KCl, suggesting that IFO0880 Δ*KU70* and W29 strains of *R. toruloides* may have different metabolic regulation, which may possibly be due to difference in their ploidy levels [30]. These results support the need for further characterization of *R. toruloides* IFO0880 Δ*KU70*. The media A was thus selected to run further experiments for physiological evaluation of *R. toruloides* IFO0880 Δ*KU70*.

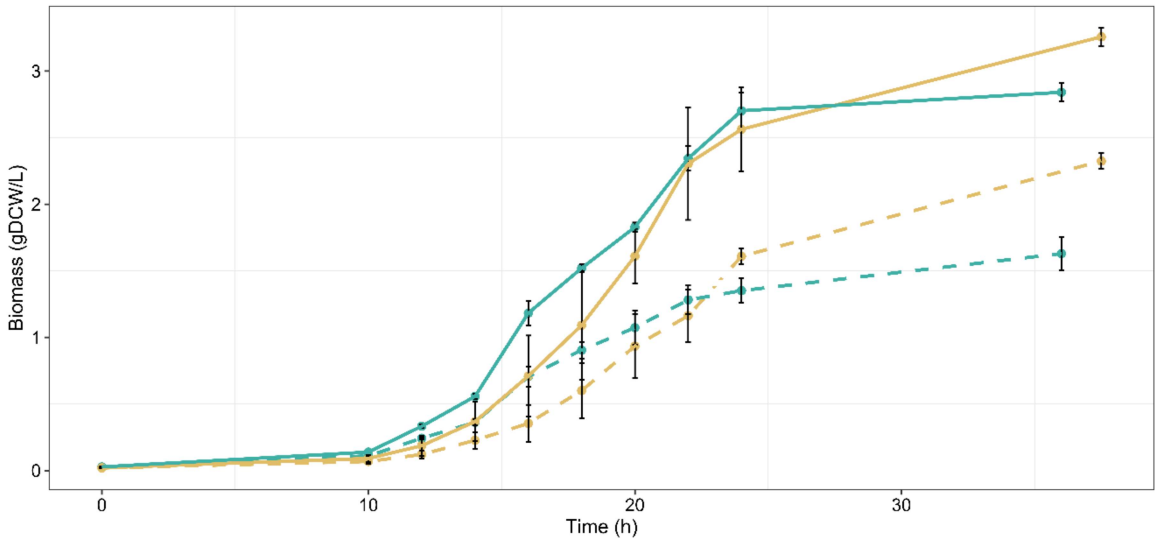

Figure 6: Selection of an optimal medium for *R. toruloides* IFO0880 *ΔKU70*. Media A and B differed by their amount of $MgSO_4 \cdot 7H_2O$ and their nitrogen source (green and yellow line, resp). In addition, both medium were supplemented with KCl to potentially prevent cells aggregation (dashed lines).

To determine the inoculum size for the batch cultivation experiments, three starting ODs (0.1, 0.05, 0.025) were investigated in low C/N ratio medium with glucose as carbon source (Figure 7). The stationary phase reached faster for culture with starting OD 0.1 and 0.05. No significant difference in the growth profile was observed between culture with starting OD 0.1 and 0.05. However, a starting OD at 0.05 was choosen to minimize the pre-inoculucum volume needed per experiment and for data reproducibility. For the experiments with xylose as carbon source, previously established starting OD at 0.5 was used for starting the batch experiments. It is worth noting that inoculum size may impact product formation by impacting the growth kinetics of cultures [34].

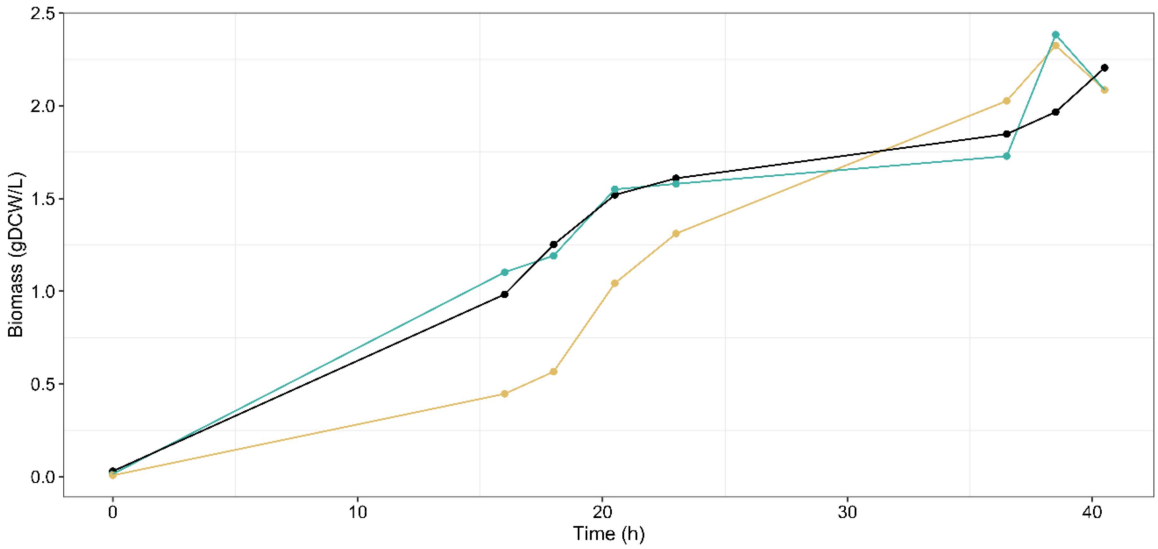

Figure 7: Pre-inoculum size determination, using glucose containing low C/N ratio media A. Three starting ODs (0.1, 0.05, 0.025) were tested to assert final biomass and growth rates remain similar regardless of the starting OD. The highest ODs (black for 0.05, blue for 0.1) reached the stationary phase earlier than the lowest starting OD (yellow line).

After determination of appropriate culture medium and inoculum size, *R. tourloides* IFO0880 *ΔKU70* was grown in batch with glucose and xylose as carbon source at high and low C/N ratios. Biomass production was monitored using optical density. Concentration of carbon sources and metabolites excreted were quantified using by HPLC. The following sections present these results and discuss the findings of the study.

3.2. Physiology in glucose

The highest specific growth rate was obtained at low C/N ratio medium with glucose as carbon source (Figure 8A, 0.26±0.01 h^{-1}). In nitrogen limited medium, specific growth rate was more than 30% lower (0.18±0.02 h^{-1}, Figure XC). In both conditions, the specific glucose consumption rate was similar 1.81±0.06 and 1.89±0.12 mmol. $gDCW^{-1}.h^{-1}$ at high and low C/N ratio medium, respectively. However, glucose was not fully depleted when cells reached the stationary phase (29h and 24h for low and high C/N ratio medium). In addition, no secreted metabolites were observed. Consequently, the decrease of pH could not be explained by the secretion of organic acid over time. The results were in line with the previously published results in which growth and glucose consumption prematurely stopped

while pH decreased from 5.5 to 3.3 [35]. In the same study, no such limitations were observed when the nitrogen source was $NaNO_3$ instead of $(NH_4)_2SO_4$. A prior study described the effect of pH on carotenoids and lipids content and composition with optimal production at pH 5 and 4, respectively [36].

Although the specific glucose consumption rate was similar between both conditions, the biomass yield on carbon source was 45% lower in nitrogen limited medium. Several changes in *R. toruloides* metabolism are known to be triggered by the depletion of nitrogen such as, increase of nitrogen machinery activity, carotenoids synthesis, de novo lipids synthesis and excretion [31]. In define medium, the biosynthesis of de novo proteins is tightly related to nitrogen source availability [31, 37].

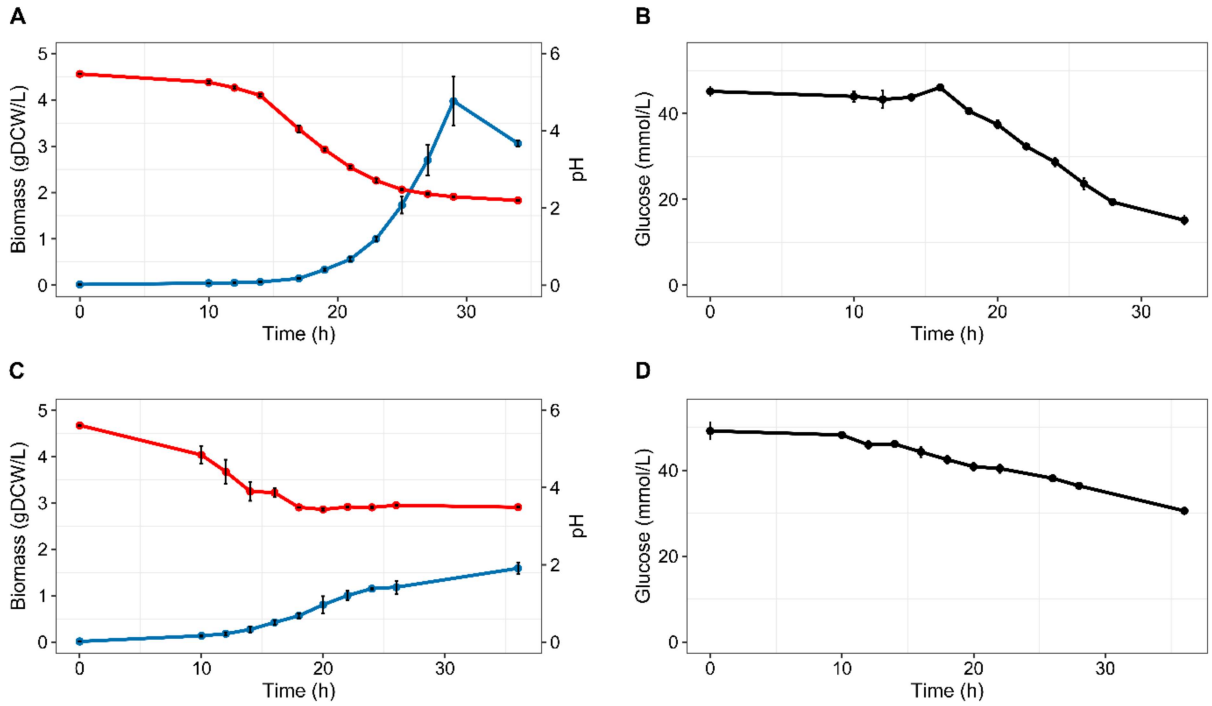

Figure 8: Glucose physiology of *R. toruloides* IFO0880 Δ*KU70* cultivated in flasks in define medium at low (panel A and B) and high (panel C and D) C/N ratio. Biomass (blue line), glucose concentration (panel B and D) and pH (red line) were monitored. Average and standard deviation were calculated from triplicates.

3.3. Physiology in xylose

Xylose is a monomeric pentose that can be chemically extracted from lignocellulosic material such as wood or agricultural waste [19]. Hydrolysates are usually a toxic environment for microorganisms but several yeasts, such as *Candida tropicalis*, *Pichia stipistis*, are able to grow, ferment the xylose contained in the hydrolysates and produce valuable byproducts [38, 39]. In this study, *R. toruloides* consumed xylose at 1.39±0.06 and 0.85±0.08 mmol. $gDCW^{-1}.h^{-1}$ at low and high C/N ratio (Figure 9 B and D, resp). The decrease of xylose consumption rate in nitrogen limited medium was paired with ~30% decrease of specific growth rate with 1.13±0.01 h^{-1} and 0.08±0.002 h^{-1} at low and high C/N ratio. Regardless the condition, xylose specific uptake rate was at least 1.3 times lower than glucose specific uptake rate. This result was coherent with previous studies where glucose was consumed 2 times faster than xylose [26]. As already observed with

glucose condition, xylose was not fully depleted when cells entered the stationary phase (28h). The acidification of the medium remains the current hypothesis although deeper analysis is required (Figure 9 A and C, resp). The biomass yields on xylose were not significantly different between low and high C/N ratio condition (0.80±0.11 gDCW/g and 0.69±0.19 gDCW/g, resp) which was not in agreement with previously published results, through C/N ratios used in the present study are different may attribute to observed differences [40].

After being consumed xylose is converted by NADPH-xylose reductase (EC 1.1.1.21) into xylitol which is either secreted or rerouted to D-arabitol or L-arabitol [41]. Although xylitol production was expected arabitol was the only metabolite detected [28]. Arabitol production occurred over the first 22h and was later co-consumed with xylose during exponential phase (Figure 9 B and D). At the moment, no clear metabolic reason could explain why *R. toruloides* secretes arabitol to reconsume it. However, the limitation of nitrogen enhanced the arabitol consumption (Figure 9D).

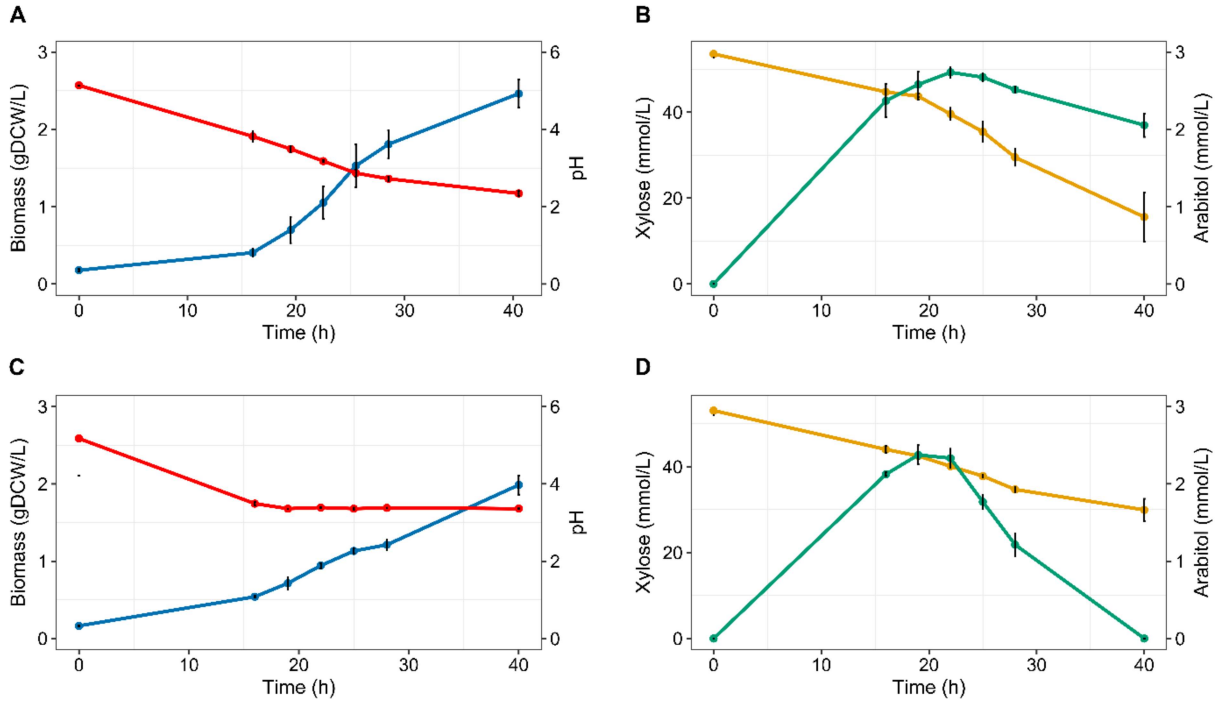

Figure 9: Xylose physiology of *R. toruloides* cultivated in flasks in define medium at low (panel A and B) and high (panel C and D) C/N ratio. Biomass (blue line), glucose concentration (panel B and D) and pH (red line) were monitored. Average and standard deviation were calculated from triplicates.

Conclusions

In this study, *R. toruloides* IFO0880 *ΔKU70* grew in batch consumed glucose and xylose at 1.81±0.06 and 1.39±0.06 mmol. $gDCW^{-1}.h^{-1}$. In nitrogen limited medium, glucose and xylose were consumed at 1.89±0.12 and 0.85±0.08 mmol. $gDCW^{-1}.h^{-1}$. Although the glucose specific uptake rate was similar in both low and high C/N ratio the biomass yield on glucose was decreased by 45% in low nitrogen medium. For both glucose and xylose, specific growth rate was lower in nitrogen limited medium (0.18±0.02 h^{-1} and 0.08±0.002 h^{-1}, respectively) than non-limited nitrogen medium (0.26±0.01 h^{-1} and 0.13±0.01 h^{-1}, respectively). In presence of xylose, *R. toruloides* IFO0880 *ΔKU70* secreted arabinitol which was re-consumed later. The results presented here are coherent with literature on other *R. toruloides* strains.

Kokkuvõte

R. toruloides IFO0880 *ΔKU70* iseloomustati loksutuskolvis (partiikultuur), kasutades süsinikuallikana glükoosi ja ksüloosi madala ja kõrge C/N suhtega. Nii süsinikuallika puhul mõjutasid pH ja lämmastiku piirang *R. toruloides* IFO0880 *ΔKU70* füsioloogiat (saagis, süsinikuallika tarbimiskiirus). Ksüloosi juuresolekul sekreteeris *R. toruloides* IFO0880 *ΔKU70* arabitooli, kuid ksülitooli ei tuvastatud. Kuna L- ja D-arabinitooli biosünteesi rada on erinev, oleks asjakohane kindlaks teha, milline isomeer toodetakse [40, 42]. Selles lõputöös erinevate süsiniku substraatide ja C/N suhete kindlaksmääratud kasvu erikiirused võivad aidata kavandada kemostaadi bioreaktori katseid, kus täielik biomassi koostise määramine (sealhulgas karotenoidide, lipiidide sisaldus) koos multiomika analüüsiga aitab mõista *R. toruloides* metabolismi regulatsioone. Lisaks täheldati katse lõpus kõigis kolbides vees lahustumatut ühendit. Sama katse viidi läbi ka *R. toruloides* W29-ga, et välistada tüvespetsiifilist füsioloogiat. Ramani spektroskoopial põhinevad esialgsed tähelepanekud viitavad karoteenide sisaldusele selles vees lahustumatus keemilises segus, kuid enne lõpliku väite esitamist *R. toruloides* võimalike karoteenide või muude sekretsiooniproduktide kohta on vajalik selle keemilise segu täielik analüüs (Lisa 1).

Summary

R. toruloides IFO0880 *ΔKU70* was characterized in shake flask (batch) using glucose and xylose as carbon source under low and high C/N ratio. For both carbon source, pH and nitrogen limitation impacted *R. toruloides* IFO0880 *ΔKU70* physiology (yield, carbon source uptake rate). In presence of xylose, *R. toruloides* IFO0880 *ΔKU70* secreted arabitol but no xylitol was detected. The biosynthesis pathway of L- and D-arabinitol being different it would be relevant to determine which isomer is produced [40, 42]. The determined specific growth rates in this thesis for different carbon substrates and C/N ratios could help design chemostat bioreactor experiments, where a complete biomass composition determination (including carotenoid, lipids content) together with multiomics analysis can help understand regulations in *R. toruloides*' metabolism. In addition, a water-insoluble compound was observed at the end of the experiment in all flasks. The same experiment was conducted with *R. toruloides* W29 to exclude a strain-specific physiology. Preliminary observations based on Raman spectroscopy suggest presence of carotenes in this water-insoluble chemical mixture, but a complete analysis of this chemical mixture would be necessary for before a definitive claim on potential carotenes or other secretion products by *R. toruloides* could be made (Annex 1).

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Appendices

Annex 1

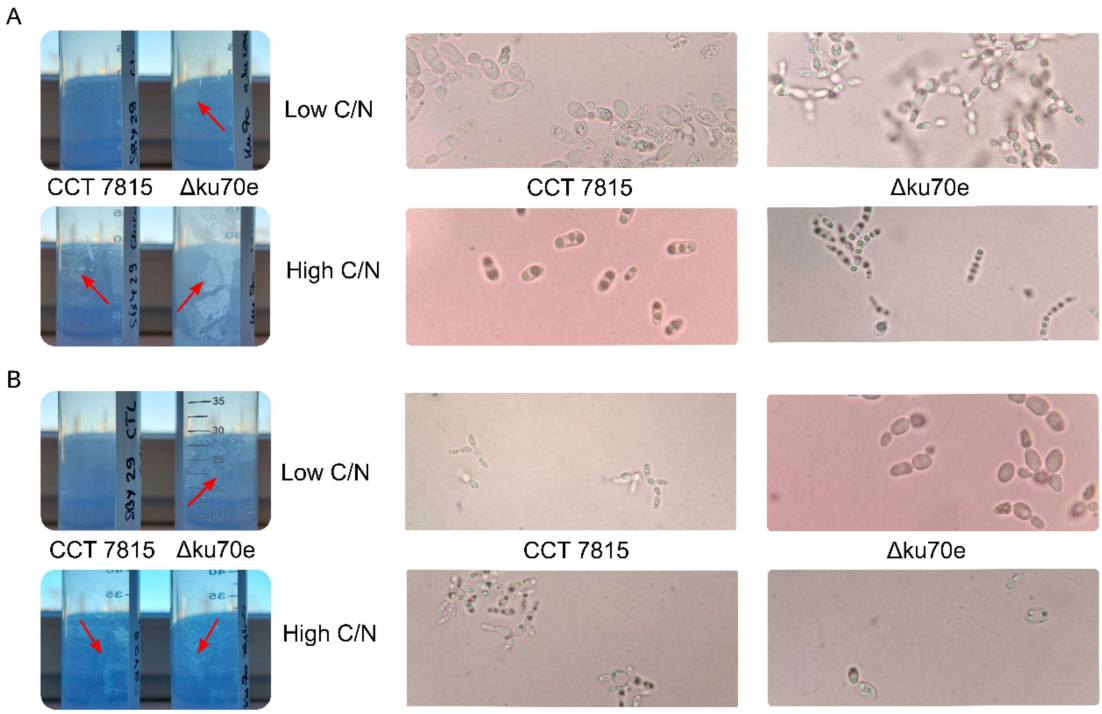

Figure 10: Macroscopic and microscopic observations of *R. toruloides* IFO0880 *ΔKU70* cultures cultivated with glucose (A) and xylose (B). For most conditions, the centrifugation of the culture revealed the presence of water-insoluble compound that adhered to the surface of the falcon (red arrow, left panel A and B). Similar artifact was also observed when using *R. toruloides* W29 strain in the same cultivation condition. Cells were observed under microscope (right panels) to attest their integrities.