RHODOSPORIDIUM TORULOIDES AS ROBUST YEASTS FOR ADVANCED BIOFUEL PRODUCTION USING WOOD HYDROLYSATE

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ABSTRACT

In response to increasing global energy demand as well as greenhouse emissions from petroleum fuels, sustainable and renewable sources have been intensely researched to produce biofuel. For instance, microbial lipids have been recognized as a potential feedstock for biofuel production due to their similarities with vegetable oils in terms of fatty acids. Typically, microorganisms capable of accumulating more than 20% lipids are known as oleaginous microorganisms. These microorganisms can thrive on various renewable substrates and biochemically convert excess carbon into lipids. One such example of a renewable substrate is lignocellulosic biomass, which produces hydrolysate containing hexoses and pentoses sugars upon pre-treatment and saccharification, and thus could be employed as a potential substrate for microbial lipids. However, wood hydrolysate presents several challenges such as low consumption of pentose sugars, and the presence of microbial growth inhibitors such as furans, organic acids and phenols. In this sense, *Rhodosporidium toruloides*, an oleaginous yeast, could be employed to produce lipids due to its ability to accumulate 50-70% of lipids, consume C5 sugars, and tolerate inhibitors. Thus, the present thesis explores the ability of *R. toruloides*-1588 to thrive on undetoxified hydrolysate derived from forestry residues (hardwood and softwood sawdust) and accumulate lipids. Additionally, several strategies were employed to increase the lipid titer such as carbon and nitrogen ratios, fed-batch fermentation, and carbohydrate supplementation such as crude glycerol, which resulted in maximum lipid accumulation of 56.3% (*w/w*) along with more than 90% consumption of carbohydrates. A life cycle assessment has been also performed to identify the hotspots in terms of energy consumption, greenhouse gas emission, and waste produced during the lipid production process. Lastly, the strain was accessed for its ability to thrive on microbial growth inhibitor such as furfural and use it as an energy source. Based on the above findings, the current dissertation concludes that *R. toruloides*-1588 can thrive on undetoxified wood hydrolysate, accumulate lipids that can serve as a feedstock for biofuel production and provide aid in the further development of biorefinery industries.

Keywords: Wood hydrolysate, *Rhodosporidium toruloides*, essential fatty acids, biodiesel, sustainability, inhibitor tolerance, scale-up, life cycle assessment.

GRAPHICAL ABSTRACT

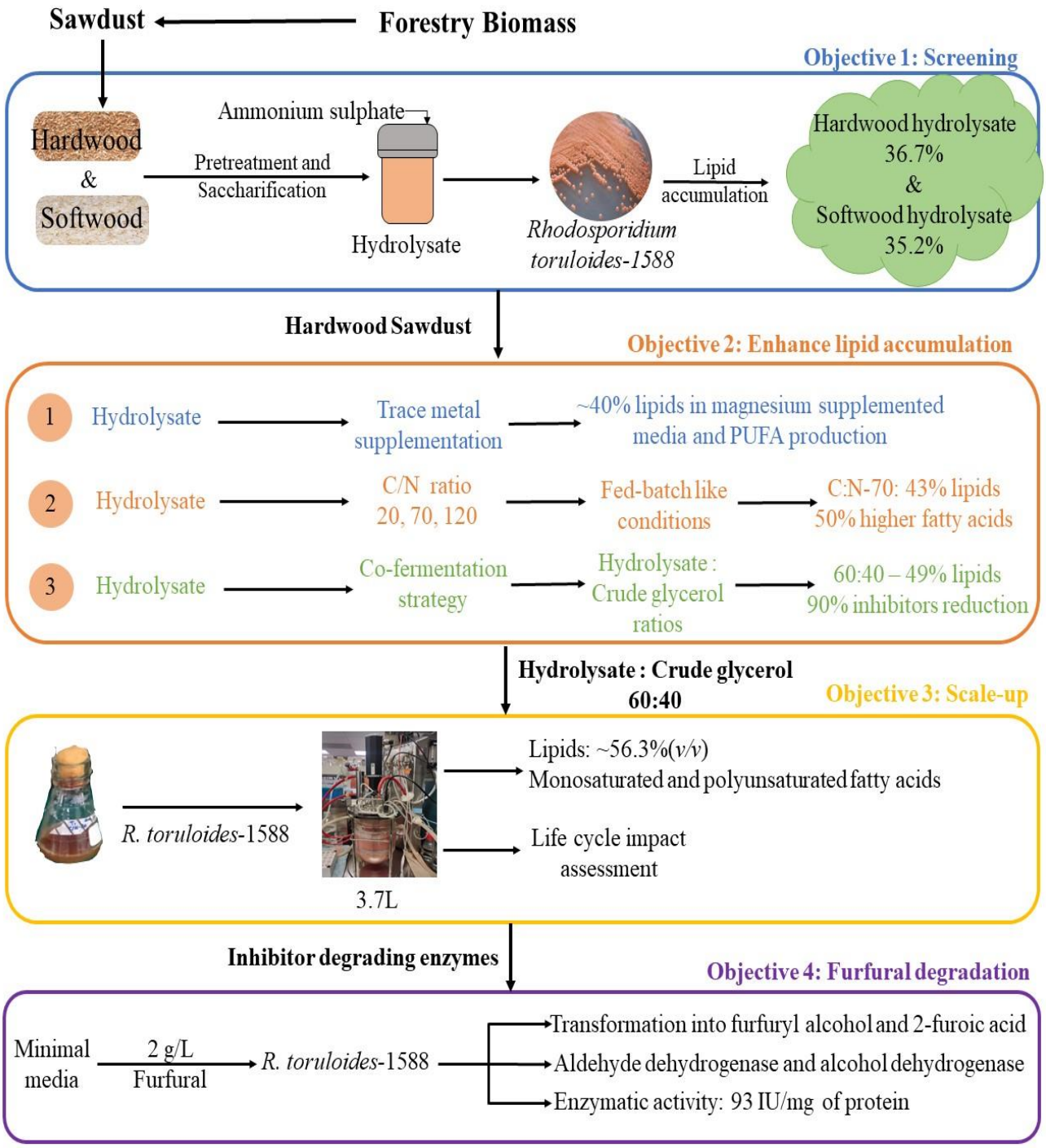

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACL	ATP-citrate lyase
ACR	Fatty acyl-CoA reductase
AD	Aldehyde decarbonylases
ADO	Aldehyde reformulating oxygenase
AFEX	Ammonia fiber explosion
ALD4	Aldehyde dehydrogenase 4
AP	Apple pomace
ATCC	American type culture collection
ATP	Adenosine triphosphate
BIS	Bisabolene synthase
C/N	Carbon: nitrogen ratio
CN	Cetane number
CO	Carbon monoxide
CO_2	Carbon dioxide
COVID-19	Coronavirus disease of 2019
CRISPER -Cas9	Clustered regularly interspaced short palindromic repeats and CRISPER-associated protein 9
DAG	Diacylglycerol
DCW	Dry cell weight
DGA	Diacylglycerol acyltransferase
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicyclic acid
ELO	Elongase-1
ER	Endoplasmic reticulum
FAD2	Fatty acid desaturase
FAME	Fatty acid methyl ester
FAS	Fatty acid synthesis
GC	Gas chromatography
GND1	Phosphogluconate dehydrogenase
H_2	Hydrogen gas
HDO	Hydrodeoxygenation
HMF	5-hydroxymethylfurfural
HPLC	High-performance liquid chromatography
HW	Hardwood
ICDH	Isocitrate dehydrogenase
IEA	International energy agency
KCS	3-ketoacyl-CoA synthases
LCA	Life cycle assessment
LC-MS	Liquid chromatography-mass spectroscopy

LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MCFA	Medium-chain fatty acids
ME	Malic enzyme
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaOH	Sodium hydroxide
NRRL	Northern regional research laboratory
OD	Optical density
ORP	Oxidation reduction Potential
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PDA	Potato dextrose agar
RNA	Ribonucleic acid
RPE1	Ribulose 5-phosphate epimerase
SW	Softwood
TAG	Triacylglycerol
TEAB	Triethylammonium bicarbonate
VLCFA	Very long-chain fatty acids
YM	Yeast-Malt medium
YNB	Yeast nitrogen-based media

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CHAPTER 1: BACKGROUND

1. INTRODUCTION

With the increasing population, the demand and consumption of fuel have increased exponentially. Hence, it is imperative to find an alternative that can replace fossil fuels while maintaining a sustainable and green environment [1]. Alternately, biofuels such as bioethanol, biodiesel or biobutanol have been widely accepted as a potential replacement for conventional fuels due to their ability to be produced from a variety of renewable sources [2]. Currently, for commercial-scale production, biofuels are being produced from vegetable oils due to their high availability and easy conversion steps. However, its conflict with food raised the food vs fuel debate [3]. In this sense, microbial lipids could be a potential alternative as it resemblances with vegetable oils in terms of fatty acid composition, and thus can be considered as a non-food oil alternative feedstock for biofuel production [4]. Microorganisms capable of accumulating more than 20% lipids of their total dry cell weight are known as oleaginous microorganisms [4]. In general, microbial-based lipids can be produced using different renewable sources such as domestic, agricultural or industrial waste [5]. In particular, lignocellulosic biomass has been extensively exploited over the past decade due to its ability to produce carbohydrate-rich media and its availability as forestry biomass, crop residues and industrial residues [6], [7]. In terms of Canada, it has more than 35% of land covered with forest with an annual residue production such as chips or sawdust of approximately 2.3 million tonnes/year [8]. Hence, it could be a potential substrate for microbial-based lipid production.

In general, lignocellulosic biomass is the complex between lignin (26-31%), cellulose (41-46%), and hemicellulose (25-32%), hence, it requires pretreatment and enzymatic saccharification to release the fermentable sugars such as glucose, xylose, arabinose and lactose [9, 10]. This sugar-rich hydrolysate is then employed as a substrate for microorganisms. Additionally, pretreatment of lignocellulosic biomass also leads to the production of several toxic compounds such as furans, organic acids, and phenols which hinders the ability of the microorganisms to grow and accumulate lipids. Hence strain capable of tolerating inhibitors as well as accumulating lipid is highly desirable. Recently, *Rhodosporidium toruloides* have been identified as a workhorse for the biorefinery industry due to their ability to accumulate 50-70% of lipids, tolerate inhibitors and consume hexoses and pentoses sugars [11], [12], [5]. However, studies of *R. toruloides* are still limited to using undetoxified wood hydrolysate as a substrate and require further investigation to increase the lipid titer and identify the potential impact of the lipid production process on the

environment. In this sense, the dissertation will investigate the lipid accumulation ability of *R. toruloides* using hydrolysate derived from forestry residues.

The Dissertation is divided into different chapters starting from the introduction (Chapter1); review of the literature (Chapter 2); research gap, hypothesis, objectives and originality (Chapter 3); exploring the ability of *Rhodosporidium toruloides*-1588 to accumulate lipids (Chapter 4); strategies to enhance the lipid accumulation in *R. toruloides*-1588 (Chapter 5); identification of inhibitor degrading enzymes (Chapter 6); and life cycle assessment of lipid production (Chapter 7). Briefly, Chapter 2 provides a wide perspective of *R. toruloides*, lipid production technologies, different types of culture media and their advantages and disadvantages. Furthermore, the scope of the forestry industry in Canada and its residues as a potential feedstock for microbial lipid production are presented. Following, the research gap, hypothesis, objectives and originality of the study are explained in Chapter 3. Chapter 4 explores the ability of *R. toruloides*-1588 to thrive on undetoxified hydrolysate derived from forestry residues. Chapter 5 provides an extensive analysis of different strategies to increase the lipid accumulation in *R. toruloides*-1588 such as trace metal supplementation, carbon to nitrogen ratio studies, development of Fed-batch-like conditions and co-fermentation of wood hydrolysate and crude glycerol. Based on inhibitor degradation results from previous Chapter studies, Chapter 6 investigates the ability of *R. toruloides*-1588 to use furfural as a carbon source as well as identification of enzymes responsible for its degradation. Lastly, in Chapter 7, the life cycle assessment of the lipid accumulation process was performed to identify the potential impact on the environment in terms of global warming, ecotoxicity and human toxicity. In nutshell, this dissertation illustrates the following aspect of the study: i) utilization of forestry-derived hydrolysate as a media for microbial lipid accumulation; ii) *R. toruloides*-1588 can transform the furfural into non-toxic compounds as well as accumulate lipids and carotenoids, and iii) life cycle assessment is necessary to find the hot-spots in the production process that could impact the environment if scaled-up further without any modifications. In this sense, this research demonstrates the scope of creating new methods to increase lipid titer as well as utilization of undetoxified hydrolysate as a medium to produce biofuel feedstock. It will also help in the valorization of forestry residue into value-added bioproducts while endorsing renewable biofuel industries.

CHAPTER 2: LITERATURE REVIEW

2. REVIEW OF LITERATURE

2.1. Biofuels: an alternative approach

Petroleum fuel is one of the vital energy sources used all over the world. More than 70% of the oil is consumed directly by the transportation sector. Due to the massive increase in the utilization of petroleum the world might run out of fossil fuel-based oil by the year 2070 [4]. Besides, its widespread usage has also brought global warming and health concerns due to the release of greenhouse and toxic gases such as carbon monoxide, carbon dioxide, methane, and chlorofluorocarbons. In this sense, renewable biofuel has received considerable attention due to its ability to replace fossil fuels as well as meet growing energy demand. Several countries, such as Canada, the USA, the United Kingdom and France, have developed numerous policies to decrease the usage of fossil fuel-based energy, reduce greenhouse gas emissions and promote biofuel production from renewable sources such as lignocellulosic biomass, energy crops, as well as domestic and industrial residues [5]**.** As per the sustainable developmental goal report, it has been estimated that more than 700 policies have been implemented. Over 83 countries around the world have set up a 10-year framework for sustainable production and consumption strategies. For instance, under renewable energy directives, European Union aims to reduce greenhouse gas emissions by 55% and increase by 32% in renewable targets [13]. Likewise, International Energy Agency (IEA) has launched the "Methane Tracker 2020" initiative to track the total methane emissions from oil and gas operations as they are the second-largest sector contributing to global warming. A decrease of 5.35% in methane emissions was seen in 2020 due to the coronavirus (COVID-19) pandemic, however, in 2021, again 5% increase in methane emission has already been observed as economies recovered from the shock [14]. Hence, robust policies and initiatives should be taken into consideration to tackle this worldwide problem as well as an increase in biofuel production.

Table 1 illustrates the comparative differences based on production time, cost, energy density, and blending requirement, between biofuels and conventional fuel. In general, biofuel can be produced using different ways such as (i) transesterification of fatty acids from vegetable oils and free fatty acids; (ii) hydrotreatment of animal fats and vegetable oils; (iii) microbial-based biomass conversion or carbon-containing sources such as lignocellulosic biomass, domestic or industrial waste; and (iv) thermochemical conversion of lignocellulosic biomass into syngas [15]. Figure 1 shows the different procedures of renewable biofuel production. Over the past decade, biofuel

production has been subject to constant transformation, for instance, reduction in biofuel production cost, utilization of renewable feedstocks, and process improvement to obtain maximum biofuel yield are being prioritized. Different types of feedstocks can be used for biofuel production, which has been further divided into different biofuel generations. For instance, first-generation biofuels are made using starch or sugar-based feedstocks as well as from vegetable oils [16]. The major flaw of the first-generation biofuel was the use of edible, plant-based biomass resulted in the rise of the food vs fuel debate [17, 18]. As a solution to this problem, the second generation of biofuel production includes the use of non-food feedstock, such as lignocellulosic biomass [10]. Lignocellulosic biomass can be converted either chemically or biochemically into biofuel using microorganisms as an intermediate [5]. Biofuel production from these types of lignocellulosic biomass would reduce the environmental, food, and energy crisis. On the other hand, third-generation biofuels include algae-based biofuel production [19], while fourth-generation biofuel includes genetic and metabolic engineering of microorganisms to increase their growth and lipid accumulation [20]. Moreover, the large-scale production of third and fourth-generation biofuel is surrounded by several complications such as lower biomass production, substrate availability, high upstream and downstream cost as well as potential health and environmental concerns [20].

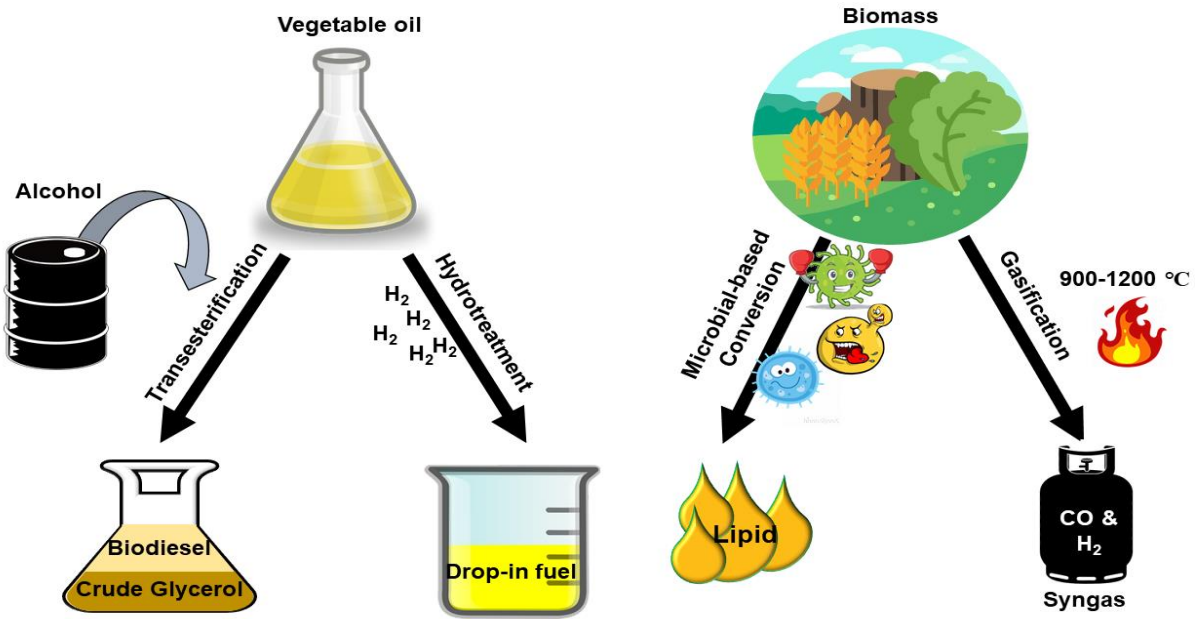

Figure 1. Different types of biofuels producing techniques.

Table 1. Comparison between biofuels and conventional fuel

Specification	Biofuels				Conventional Fuel
	Bioethanol	**Biobutanol**	**Biodiesel**	**Drop-in fuels**	
Substrate	They can be produced from renewable resources such as molasses, rice bran, sorghum, or a lignocellulosic waste of forestry by the action of microorganisms				Naturally occurring fuels are produced from the geological process
Period	Requires 5-6 days	6-7 days of production time	Production time 6-7 days	Requires 6-7 days	Requires hundreds of millions of year
Energy Density (*MJ/L)	19.6	29.2	30.53	32 - 36	34.6
By-products	12% less greenhouse gas emission than diesel	Reduced emission of hydrocarbon and carbon dioxide	41% less greenhouse gas emission than conventional gasoline	Very little or no emission of greenhouse gas.	High greenhouse gas and sulfur dioxide emission
Production Cost	Cheap, in general, requires $ 1.74 per gallon	Expensive, microorganism such as *Clostridium* sp. requires an anaerobic condition	Expensive, because of transesterification cost as well as purification cost	The high conversion cost of vegetable oil and the need to develop engineered microbial strain	The cost of gasoline production is ~95 cents
Blending	10% of bioethanol and 90% of petrol	10 – 16% can be used in gasoline	Can be blended with petroleum diesel (i.e., 80-98%)	Neither blending nor engine modification is required.	Can be used directly in vehicles

*Megajoule/litre

In this sense, lignocellulosic biomass could be a renewable and alternative option for biofuel production. The following points can be taken into consideration for advanced biofuels production i.e., i) utilization of renewable feedstock (i.e., lignocellulosic biomass) which does not interfere with food security; (ii) production using new conversion technologies; (iii) media optimization, supplementation, zero waste production and life cycle assessment; (iv) does not require blending and should be compatible with existing motor engines. Moreover, biofuel production using lignocellulosic biomass has been strongly encouraged by several countries such as Canada, the USA, England, France, Brazil, and the Netherlands. Likewise, the forestry industry in Canada represents 6% of the total energy supply [8]. This resulted in a increased interest of academic researchers, and government and private industries to develop the research for advanced biofuel production using forestry-based lignocellulosic biomass. In this sense, forestry in Canada holds enormous potential for biofuel production.

2.2. Lignocellulosic biomass: Forestry in Canada

In general, lignocellulosic biomass is a complex between lignin (26-31%), cellulose (41-46%), and hemicellulose (25-32%). It generally requires a pre-treatment to break the lignocellulosic complex followed by enzymatic saccharification to convert the polymeric sugars into their monomeric forms such as glucose, xylose, arabinose, lactose, galactose and fructose [21]. The sugar-rich hydrolysate produced can be employed as culture media for microorganisms to grow and accumulate lipids. However, the major criterion of the biorefinery industry is the availability of lignocellulosic biomass throughout the year. There are multiple sources of lignocellulosic biomass that can be utilized to produce biofuels such as sugarcane bagasse, corn stover, sawdust, wood pellets, rice bran etcetera. Likewise, the forest industry is the primary source of lignocellulosic biomass in Canada. Canada is the second-largest country in the world with 35% of land covered with forests and possessing 10% of the total forests [8]. Canada has different types of trees, such as spruce (47.3%), poplar (13.1%), pine (11.9%), fir (7.4%), hemlock (5.8%), birch (3.3%), maple (3%) and others. The feedstocks are available in the form of harvest residues, sawdust, and chips from sawmills and industrial residues. Most of the mills generally use wood residues to co-generate electricity and heat required for different processes. It was estimated that 70% of the generated residue is either converted to value-added products or energy and the rest of the 30% residue with the conversion efficiency of >90% could lead to the production of 6000 million L of biofuel, which will be three times higher than the current biofuel production in Canada [8].

In addition to the development of clean and green technology, researchers have also focused on the production of value-added products such as lignin derivatives and phenolic compounds from lignocellulosic biomass to boost the country's economy. Likewise, due to the enormous potential of Canadian forestry, biorefinery concepts are being reconsidered. Hence, lignocellulosic biomass can be employed to develop a green and sustainable process for biofuel production.

2.3. Pretreatment Strategies

In general lignocellulosic biomass requires a pre-treatment to break down the lignocellulosic links and release the cellulose and hemicellulose followed by enzymatic hydrolysis of these polymeric sugars into monomeric sugars [22]. Over the past years, several pre-treatment techniques have been explored to break down the lignin matrix, which are broadly categorized into three different methods i.e., physical, chemical and biological methods [9]. Additionally, combinations of these pre-treatment methods are also available. The physical method uses mechanical energy to break the lignocellulosic biomass into smaller pieces and increases its porosity [23]. For instance, ball-milling, grinding, mechanical comminution, hydrothermal pre-treatment, and steam explosion are some examples of physical pre-treatment processes [24]. Unlike physical pre-treatment, chemical processes require either acid or base for the pre-treatment of lignocellulosic biomass and generally performed at high temperatures (>120℃) for 20 – 30 minutes. Chemicals such as hydrochloric acid, oxalic acid, sulfuric acid, phosphoric acid, acetic acid, citric acid, and tartaric acid have been widely used for the acid-based pre-treatment of the lignocellulosic biomass [25]. Similarly, alkaline chemicals such as sodium hydroxide, potassium hydroxide, lime, urea, ammonia, sodium carbonate, calcium hydroxide, and methylamine are also commonly exploited in the pre-treatment [9]. Apart from these pre-treatment methods, interest in using biological pre-treatment has also increased over the past few years. This is so because the process does not require chemicals, is easy to perform and requires less energy. The biological method requires ligninolytic enzyme-producing bacteria or fungi to degrade the lignocellulosic complex resulting in the release of sugars in the solution [26]. Regardless of the process, the foremost goal of pre-treatment is to break down the lignocellulosic complex and to enhance the polymeric sugar accessibility for enzymes by increasing the surface area or porosity of the biomass [27].

Among several pre-treatment methods, commonly employed pre-treatment processes for lignocellulosic biomass are sulfuric acid-based treatment, sodium hydroxide-based pre-treatment,

steam explosion, ammonia fiber explosion (AFEX) and biological pre-treatment method using white-rot fungi. Depending on the type of pre-treatment, several toxic compounds are also generated in the hydrolysates which include furans, organic acids and phenols [28]. These compounds are known to inhibit microbial growth by interfering with the metabolic pathways of the microbial system and ultimately hinder sugar consumption and product formation ability of microorganisms [29, 30]. Hence, microbial strain capable of consuming both C5 and C6 sugars as well as tolerating inhibitors is highly desirable.

2.4. *Rhodosporidium toruloides*: an overview

Rhodosporidium toruloides, basidiomycetes yeast, also known as oleaginous yeast, have been isolated from soil, dry leaves, conifers and wood pulp [31]. Its ability to accumulate 50-70% lipid of its total dry weight as well as the tolerance to inhibitors has attracted a lot of attention [5, 28]. *R. toruloides* tends to accumulate lipid either during the excess of carbon or during nitrogen limitation [5, 32]. Additionally, *R. toruloides* is also known as red yeast because of its ability to produce carotenoids such as astaxanthin, β-carotene, and torularhodin. These compounds are known as high-value antioxidant compounds in the pharmaceutical and food industries [33]. *R. toruloides* have been reported to thrive on different types of carbon sources such as glucose, crude glycerol, xylose, fructose, vegetable waste and industrial residues [12].

In general, during the presence of excess nutrients in the media, the yeast diverts the flux of carbon from energy production to lipid (TAGs) synthesis [34, 35]. Nitrogen starvation leads to the inhibition of isocitrate dehydrogenase (ICDH) causing citrate accumulation in mitochondria which then takes part in fatty acid synthesis (FAS) as shown in Figure 2. In general, excess citrate is cleaved into oxaloacetate and acetyl-CoA by the ATP-citrate lyase (ACL) [36]. Acetyl-CoA along with palmitoyl-CoA and stearoyl-CoA are then directed towards the FAS complex which further relocates to the endoplasmic reticulum, where it undergoes NADPH-dependent desaturation and is then utilized for TAGs production. TAGs synthesis then yields diacylglycerol (DAG), phosphatidic acid (PA), and lysophosphatidic acid (LPA) through the Kennedy pathway and finally stored as lipid droplets. NADPH for lipid synthesis is generally provided through the oxidative pentose phosphate pathway or during the conversion of malate into pyruvate by the malic enzyme (ME) [34]. Recently, it was shown that the oxidative pentose phosphate pathway is the

primary NADPH source for the biosynthesis of fatty acid in *Y. lipolytica* [37] and presumably in *R. toruloides.* [38].

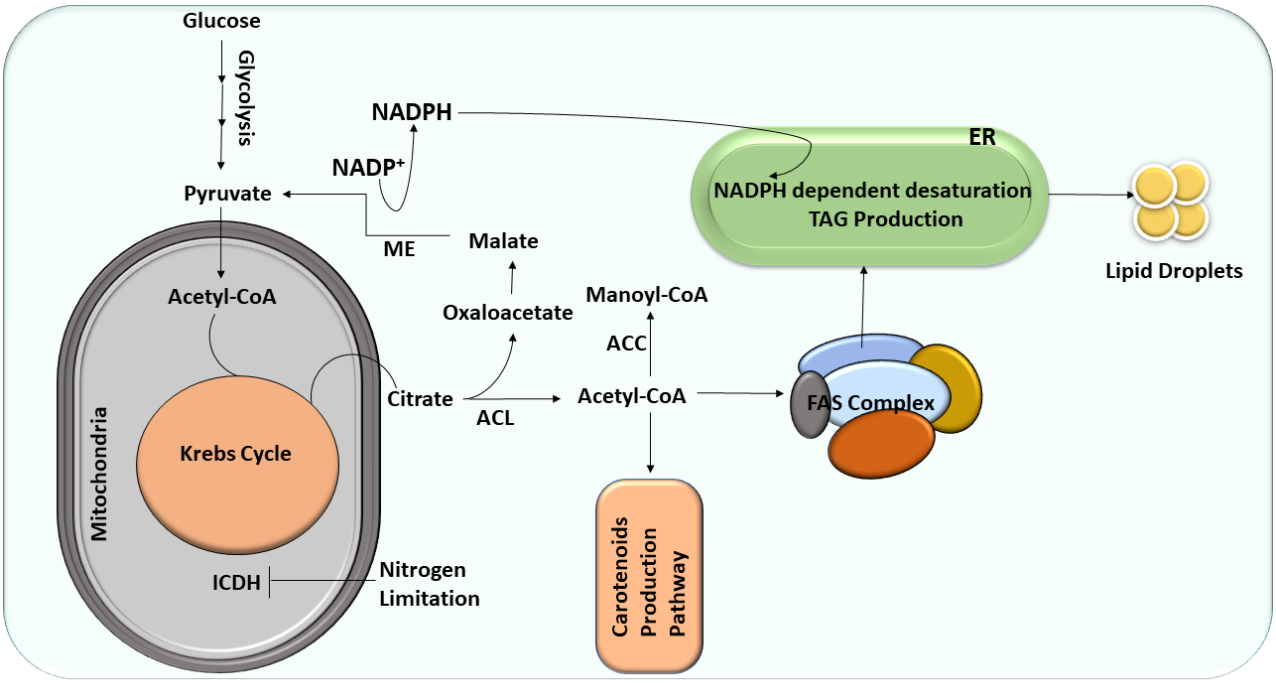

Figure 2. An overview of the lipid accumulation pathway, modified from Zhu, et al. [39]. ER: Endoplasmic Reticulum; ICDH: Isocitrate dehydrogenase; ME: Malic enzyme; ACL: ATP-citrate lyase; ACC: Acetyl-CoA carboxylase; FAS: Fatty acid synthesis; TAG: Triacylglycerol.

2.4.1. Microbial lipids and their application

Microbial lipids include triacylglycerols (TAGs), glycolipids, steryl esters, and phospholipids. Microbial lipids are known to have similarities with plant oils. For instance, fatty acid of microbial lipid is generally composed of C16 (palmitic acid), C18 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (linolenic acid) [40], while plant oil such as soybean oil, olive oil, palm oil, corn oil, also consists of C16, C18, C18:1, C18:2 fatty acids [41]. The fatty acid composition generally depends on the type of microorganisms, growth conditions, lipid inducers and carbohydrates [41]. Oleaginous microorganisms can also produce polyunsaturated fatty acids such as eicosapentaenoic acid, docosahexaenoic acid and eicosatetraenoic acid. However, TAG constitutes the main synthesized component (~70-80%) inside the microbial cells [42].

Microbial lipids have many impressive applications not only in the biofuel industry but also in the chemical, medical, and food industries. For instance, gamma-linolenic acid has anti-cancer properties, while eicosapentaenoic acid and docosahexaenoic acid can be employed for hormonal balance, eye functioning, and the cardiovascular system [43, 44]. Lipids could chemically or biochemically be converted into a broad range of biofuels such as fatty alkanes, fatty acid methyl esters, fatty alcohol, and fatty aldehydes [42]. Work has been performed for the bioconversion of various carbon sources into lipids such as *Jerusalem artichoke* [45], rice hulls [46], switchgrass [47], sweet sorghum [48], cassava starch [49], kraft pulp [50], *Cassia fistula* L. fruit pulp [51], CO_2 [52], crude glycerol [53, 54], rice bran [55], and wood waste [56]. The major challenges in lipid production include the cost of substrates and their annual availability, type of microorganism, the extent of their lipid accumulation, carbohydrate consumption, presence of toxic compounds and the requirement of genetic or metabolic engineering for bioconversion of lipid into drop-in fuels (Figure 3). The various factors affecting lipid accumulation in microorganisms are discussed in detail in the following sections.

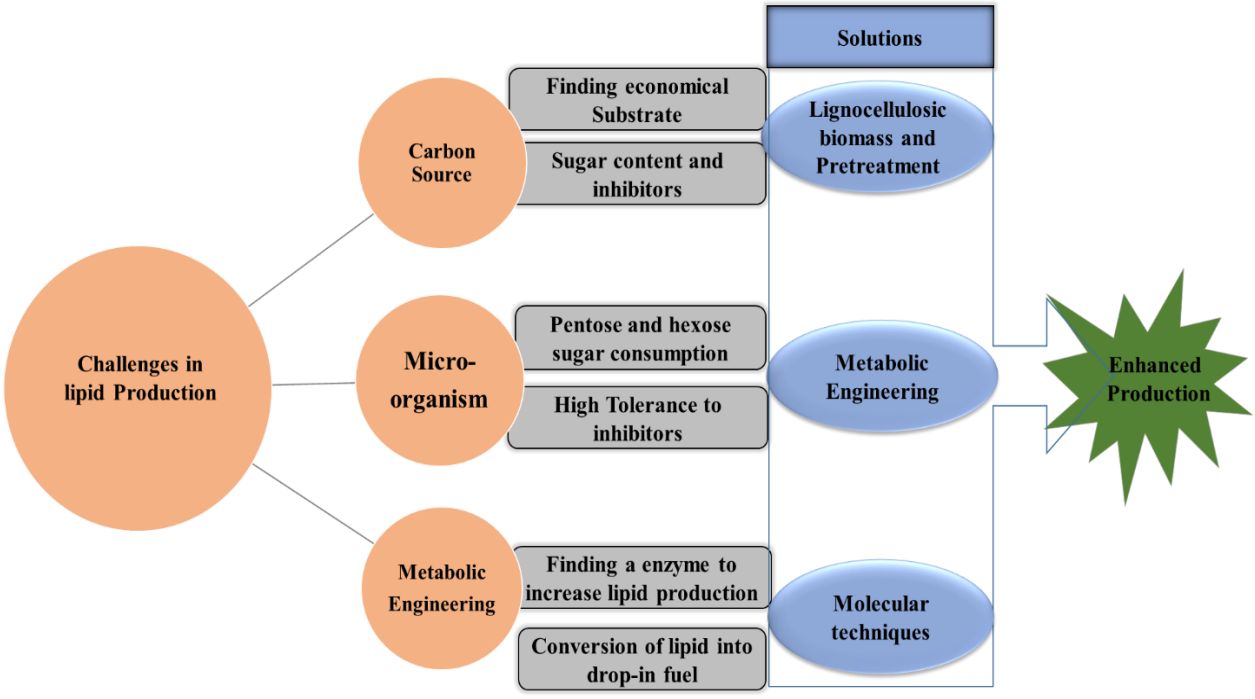

Figure 3. Demonstration of potential challenges and solutions in lipid production.

2.5. Factors affecting the lipid application

2.5.1. Type of substrates

The type and cost of the substrate have a significant impact on the economics of lipid production. Various types of carbon sources, such as glucose, xylose, galactose, lactose, glycerol, and organic acids can be utilized for lipid production. Generally, glucose is an ideal carbon source for most microorganisms. For instance, *R. toruloides* was reported to accumulate 71.9 g/L of lipids, achieved biomass of 106.5 g/L and glucose to lipid conversion yield of 0.23 g/g, after 134 hours of fermentation [57]. Several *Rhodosporidium* species were reported to grow on glucose, such as *Rhodosporidium babjevae, Rhodosporidium fluviale, Rhodosporidium paludigenum, Rhodosporidium kratochvilovae,* and *Rhodosporidium sphaerocarpum*, with the lipid content of more than 50% of their total dry cell weight [58].

However, the use of pure glucose for large-scale lipid production is not feasible; hence, there is a need to find another economical carbon source. Other than glucose, glycerol has also been intensively investigated for lipid production. Glycerol is the major by-product of the biodiesel industry and its consumption for microbial lipid accumulation is considered advantageous for the industry [40]. The utilization of crude glycerol may help in decreasing the biodiesel production cost and accomplish the concept of a zero-waste production process. Several studies have been conducted on using crude glycerol as media for lipid accumulation [59-61]. For instance, *Rhodosporidium* sp. was able to grow on crude glycerol under fed-batch fermentation conditions and reached the maximum biomass of 41 g/L and lipid of 24.6 g/L [62] then grown on glucose media (biomass – 37.2 g/L and lipid – 24 g/L) [63].

Likewise, the utilization of lignocellulosic biomass as a carbon substrate for biofuel production has also drawn much attention. Naturally, hydrolysate derived from lignocellulosic biomass consists of a mixture of carbons such as hexose and pentose sugars, that can be employed for microbial-based lipid accumulation [5]. The complete consumption of both hexose and pentose sugars is one of the important factors to achieve maximum lipid titer and reduce the biofuel production cost. Moreover, the behaviour of different sugar consumption was observed to be strain-dependent. For instance, *R. toruloides* CCT 0783 was found to utilize both glucose and fructose at the same rate [64], while Patel, et al. [51] reported the simultaneous consumption of fructose (17.98 g/L), glucose (29.45 g/L), xylose (3.87 g/L). However, the consumption of

arabinose was not seen when *R. toruloides* As. 1389 was grown in a mixture of arabinose, xylose, and glucose [65]. Table 2 represents the lipid accumulation by different types of microorganisms using lignocellulosic biomass-derived hydrolysate.

Table 2. Different oleaginous strains are grown on lignocellulosic biomass hydrolysates as a substrate.

Microorganism	**Substrate**	**Biomass (g/L)**	**Lipid (%)**	**References**
Cryptococcus curvatus ATCC 20509	Wheat Straw	17.2	33.5	[66]
Yarrowia lipolytica ATCC 20460		7.8	4.6	
Rhodotorula glutinis ATCC 204091		13.8	25	
Mortierella isabellina ATCC 42613	Switch Grass	12.55	35.6	[47]
Trichosporon coremiiforme CH005	Corn Cob	20.4	37.8	[67]
Trichosporon fermentans CICC 1368	Rice Straw	28.1	40.1	[68]
Y. lipolytica Po1g	Defatted Rice Bran	10.75	48	[55]
Y. lipolytica Po1g	Sugarcane Bagasse	11.42	58.5	[69]
Y. lipolytica Y203A	Sugarcane Bagasse	14.87	67	[70]
Rhodosporidium glutinis	Glycerol	30	53	[71]
Rhodosporidium toruloides Y4	Corn Stover	15.2	36.4	[72]
Rhodosporidium opacus	*Pinus taeda*	-	27	[73]
R. toruloides NCYC 1576	Wood wastes	7.1	39	[56]
R. toruloides	Sugarcane bagasse	23.5	52.5	[65]

Microorganism	Substrate	Biomass (g/L)	Lipid (%)	References
R. toruloides-1588	Hardwood sawdust	21	43	[74]

2.5.2. Effect of carbon to nitrogen ratio

The optimum carbon to nitrogen (C/N) ratio is an essential factor for achieving the maximum lipid titer in oleaginous yeasts. According to several studies, the C/N ratio of 20 – 120 has been reported to vary the lipid titer in oleaginous microorganisms [42, 75]. Hence, it is essential to optimize the C/N ratio for the desired microorganism [76]. One of the known factors to enhance lipid accumulation is the nitrogen limitation condition [77]. In general, nitrogen starvation leads to the inhibition of the isocitrate dehydrogenase (ICDH) enzyme (Krebs cycle), resulting in citrate accumulation in mitochondria, which then ultimately take part in fatty acid synthesis (FAS) and lipid accumulation [36]. For instance, Munch et al., [78] reported that *Rhodosporidium* sp. was able to accumulate 63.66% of lipid and 10.96 g/L of biomass under excess carbon with nitrogen limitation. When these similar conditions were applied to culture the *Y. lipolytica*, the lipid, and biomass were recorded at 28.81% and 8.61 g/L, respectively [78, 79]. Likewise, when the C/N ratio was increased from 5 to 200, the lipid accumulation in *R. toruloides* increased from 16.3 to 48.2% (*w/w*) [80]. Similarly, *Lipomyces starkeyi* was reported to accumulate maximum lipid (55%) when cultured in a C/N ratio of 72. The lipid concentration was three times higher than the C/N ratio of 24 and 48 [81]. Saini et al., [74] observed a two-fold increase in lipid accumulation in *R. toruloides*-1588 when a C/N ratio of 70 was maintained during wood hydrolysate fermentation.

2.5.3. Effect of inhibitors

The pre-treatment is the process of treating lignocellulosic biomass to enhance the enzymatic accessibility to polymeric sugars such as cellulose and hemicellulose [22]. However, pre-treatment of biomass also produces toxic compounds due to the breakdown of lignin and sugars into several derivatives such as furfural, vanillin, vanillic acid, acetic acid, 5-hydroxymethylfurfural (5-HMF), and levulinic acid. Figure 4 demonstrates the potential effects of inhibitors on microorganisms. These compounds can negatively interfere with the microbial growth and lipid accumulation

ability [29, 30]. For instance, acetic acid decreases the intracellular pH causing an increase in energy demand and reducing nutrient transfer ultimately leading to cell growth inhibition [82, 83]. *Rhodosporidium* sp. is reported to tolerate a high concentration of acetic acid than other species. For example, *R. toruloides* NCYC 1576 grew in the presence of 1.68% of the acetic acid [56] while *Y. lipolytica* Y203A was reported to grow in 0.53% of the acetic acid [70]. Likewise, furfural tends to inhibit cellular growth, and fatty acid synthesis as well as cause oxidative stress and reduce the activity of various enzymes. For instance, 0.05% of furfural led to cell growth arrest in *R. toruloides* [84]. Alternatively, physical [85], chemical [86], and biological detoxification [87] methods can be employed to remove furfural and improve microbial growth and lipid yield. For instance, the physical method generally involves the utilization of vacuum evaporation, precipitation and adsorption techniques. Moreover, most of these methods hold several disadvantages, such as high energy input requirements and generates additional toxic by-products [85]. The chemical method involves the use of chemicals such as alkali (e.g., overliming), which has been widely used as a method for the detoxification of lignocellulosic-derived hydrolysates [88]. On the other hand, the biological method involves the usage of microorganisms to digest the lignin and its derivatives and release the sugars with less or no toxic products. For instance, *Issatchenkia occidentalis* CCTCC M 206097 was able to reduce furfural (62%), ferulic acid (73.33%), 5-HMF (85%), and syringaldehyde (66.67%) in sugarcane bagasse hydrolysate [87]. However, the major disadvantage associated with the biological method is the longer time requirement as well as carbohydrate reduction. Nevertheless, these detoxification methods can remove the inhibitors from the hydrolysate but the addition of an extra step at a large scale might increase the final biofuel production cost, which is already a major constraint in the biorefinery industry. Thus, a microorganism capable of not only consuming multiple sugars but also tolerating inhibitors and accumulating high lipid titer is highly desirable.

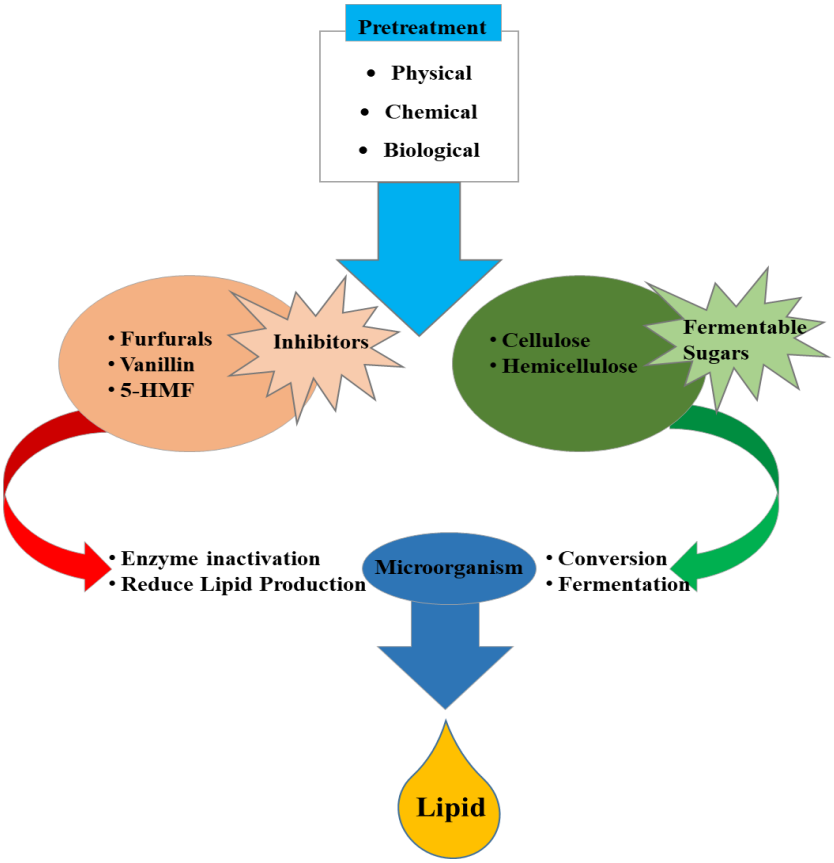

Figure 4. An illustration of the effects of inhibitors on lipid production.

2.5.4. Role of genetic engineering in lipid accumulation

An interest in isolating the desirable strains comes along with the targets to improve lipid titer, carbon consumption, microbial growth rate and availability of low-cost carbon sources. Figure 5 displays the potential targets of genetic engineering in a microorganism.

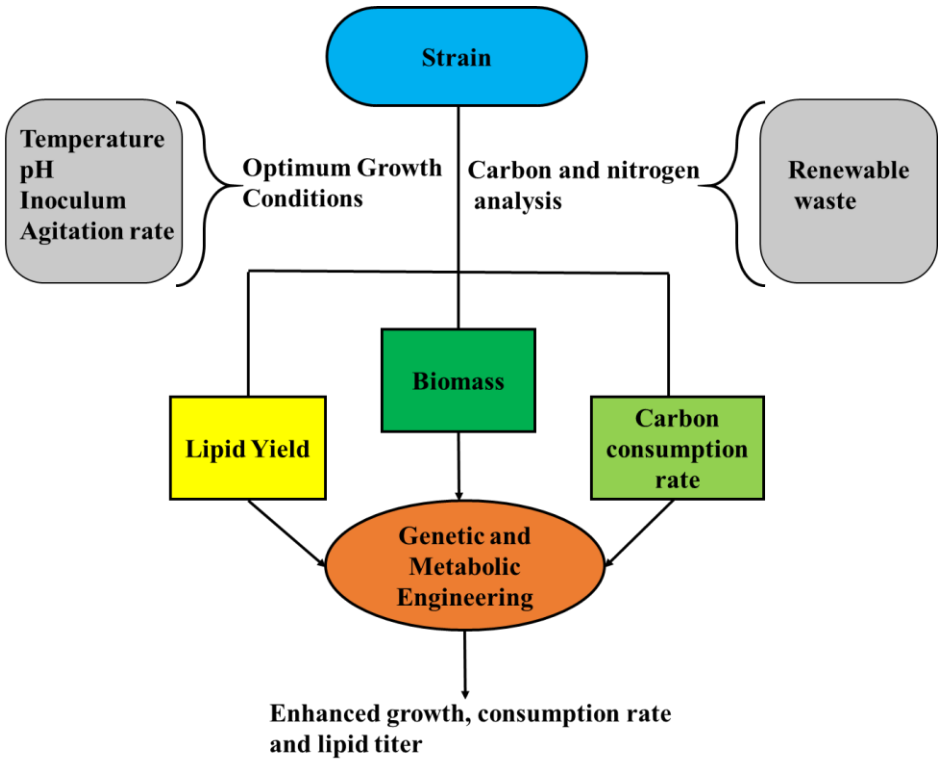

Figure 5. Different targets for genetic engineering in a microorganism.

Genetic engineering allows the microorganisms to increase their growth rate, and product titer as well as increases their ability to consume a wide variety of substrates, which were previously not possible. Thus, changes in gene expressions such as the addition and removal of enzymes or inhibition of side product pathways could vary the product yield. It has been employed to increase the consumption of both hexose and pentose sugars as well as increase tolerance to the inhibitors. Gene sequencing, transcriptomic analysis, RNA sequencing assisted annotation, and proteomic analysis provides integrated information, which eventually leads to the understanding of the molecular mechanism involved in lipid metabolism and its regulation in *Rhodosporidium* sp. [39, 40, 89, 90]. Table 3 illustrates the possible target genes/enzymes overexpressed and their effect on lipid yield in *R. toruloides*. It has been reported that *Rhodosporidium* sp. shares several features, for instance, nitrogen metabolism-induced lipogenesis with other well-studied yeasts, such as *Y. lipolytica*. However, the difference in pathways was also reported. For instance, the sole supplier of NADPH in *Y. lipolytica* is assumed to be the oxidative pentose phosphate pathway [37]; while, the NADPH supply in *Rhodosporidium* sp. comes from cytosolic NADH-dependent isocitrate dehydrogenase [39]. Another example of the difference between these two strains is peroxisome

biogenesis 10 (*pex10*). It is involved in the catabolism of fatty acids into smaller components and requires peroxisome formation [90]. Inhibiting *pex10* reduces the fatty acid breakdown into acetyl CoA resulting in increased lipid accumulation in *Y. lipolytica* while its deletion in *R. toruloides* resulted in a lipid accumulation reduction by 16.9% [90]. These studies allow interpretation of the lipid metabolism in *Rhodosporidium* sp.

Table 3. List of overexpressed genes in *R. toruloides* and their outcomes.

Target Gene	**Target Enzyme**	**Microorganism**	**Outcome**	**Reference**
ACC1	Acetyl-CoA carboxylase	*R. toruloides* IFO0880	Overexpression of these enzymes resulted in the production of 16.4 g/L and 9.5 g/L of lipid using 70 g/L of glucose and xylose, respectively	[38]
DGA1	Diacylglycerol acyltransferase			
ELO1	Elongase-1	*R. toruloides*	Overexpression of *ELO*1 and deletion of *FAD*2 leads to an increase in 23% of oleic content	[91]
FAD2	Delta-12 fatty acid desaturase			
FAD2	Delta-12 fatty acid desaturase	*R. toruloides* NP11	Overexpression enhances the linoleic acid fivefold and reached 1.3 g/L	[92]
KCS	3-ketoacyl-CoA synthases	*R. toruloides* CECT 13085	Strain is then able to produce very-long-chain fatty acids (VLCFAs) such as erucic acid (5.8 g/L) and nervonic acid (7.9 g/L)	[93]

Target Gene	Target Enzyme	Microorganism	Outcome	Reference
FAC	Fatty acyl-CoA reductase	*R. toruloides* CECT 13085	Overexpression of *FAC* leads to the production of ~8 g/L of C16-C18 fatty alcohol	[94]
*KU*70	Nonhomologous end joining protein	*R. toruloides* ATCC 10657	*KU*70 deficient strains were reported to have improved gene deletion frequency	[95]
ME	Malic enzyme	*R. toruloides* IFO0880	Overexpression increased lipid production to 89.4 g/L	[90]
SCD	Stearoyl-CoA desaturase			
BIS	Bisabolene synthase	*R. toruloides* IFO0880	This resulted in the production of 680 mg/L of bisabolene in a high-gravity fed-batch reactor	[96]
PTA	Phosphotransacetylase	*R. toruloides* NP11	The engineered strain was demonstrated to increase cell mass by 8.5% (i.e., 12.8 g/L) and lipid productivity by 64.4% (i.e., 1.20 g/L/d) than that of the parent strain.	[97]

Metabolic engineering approaches aimed at improving lipid production using various genetic engineering tools such as CRISPER-Cas9, *Agrobacterium tumefaciens* mediated transformation, lithium acetate mediated chemical transformation or electroporation to overexpress the genes in *R. toruloides*. For instance, Zhang, et al. [38] were able to increase lipid production by a factor of

1.95 (16 g/L of total lipids via flask fermentation using synthetic media), when diacylglycerol acyltransferase (DGA1) and acetyl-CoA carboxylase (ACC1) were overexpressed in *R. toruloides*. The author further engineered the *R. toruloides* by overexpressing stearoyl-CoA desaturase (SCD) and reported an additional 1.42-fold increase in lipid titers in fed-batch conditions [90]. In addition to the lipid production pathway, studies on increasing the tolerance to inhibitors are also available. For instance, Bonturi, et al. [98] reported not only the increase in lipid accumulation but also the enhanced endurance to 5-hydroxymethyl furfural and furfural in *R. toruloides*. Similarly, enzymes responsible for fatty acid production were also overexpressed or silenced to vary the fatty acid compositions of microbial lipids. For instance, Fillet, et al. [91] knockout the ∆12 desaturase (*FAD2*) gene and overexpressed elongase (*ELO1*) gene in *R. toruloides* resulting in a 23% increase in oleic acid content. In general, a high level of oleic acid leads to an increase in oxidative and thermal stability of microbial oil [12]. Hence, making them applicable for the development of hydraulic fluids, oils for electrical transformers, and biolubricants. Likewise, essential fatty acids, as well as long chain fatty acids, were also overexpressed in *R. toruloides.* These compounds have high value in the food and pharmaceutical industries. For instance, ∆12-fatty acid desaturase was overexpressed in *R. toruloides* resulting in a five-fold increase in linoleic acid production [92]. Fillet et al. [93] produced very long chain fatty acid-rich oils by introducing the 3-ketoacyl-CoA synthases (*KCS*) gene into the *R. toruloides* CECT 13085 genome. The author reported the VLCFAs production with a relative abundance of 27% (i.e., 5.8 g/L of erucic acid and 7.9 g/L of nervonic acid) in recombinant *R. toruloides*. Recently, research on the conversion of lipids into drop-in fuels has increased. For instance, Yaegashi et al. [96] were able to transform the *R. toruloides* with the bisabolene synthase (*BIS*) gene. The authors achieved 680 mg/L of bisabolene production in engineered *R. toruloides* grown in alkaline pre-treated corn stover hydrolysates using fed-batch fermentation. The study highlighted the compatibility of *R. toruloides* to accumulate drop-in biofuels. The importance, need and difference between drop-in fuels and conventional biofuels are discussed in detail in the following section.

2.6. Drop-in fuels: need and importance

The significant barrier associated with using various biofuels is the requirement of blending with conventional fuels. Blending can vary from 5% to 20% depending on the requirement and fuel properties. Blending biofuels with petroleum not only changes their properties but also increases their compatibility with existing motor engines [99, 100]. For instance, biodiesel and bioethanol

contain oxygen molecules, hence making them difficult to use in existing engines. The presence of oxygen molecules has a drastic effect on biofuel combustion properties. They can react with pipeline and refinery metallurgy to form gum acids and various other impurities, ultimately leading to their poor storage stability [101]. The oxygen molecule also enhances the biofuel affinity to water and reduces the biofuel's energy density, thus, it is essential to use oxygen-free biofuels. In general, different methods are available to remove the oxygen molecules such as hydrodeoxygenation (HDO), decarboxylation, and decarbonylation. HDO is highly efficient in removing oxygen in the form of water, but the primary issue is the use of a high amount of hydrogen (H_2) gas [102]. Recently, it has been tackled through a process that includes HDO and hydro-upgrading resulting in a reduction in H_2 consumption [103]. Decarboxylation and decarbonylation remove oxygen as CO_2 and CO, respectively, but these compounds are known to enhance global warming. Hence, the need to find an alternative led to the production of drop-in biofuels. Figure 6 demonstrates the difference between drop-in oil from other types of biofuels as well as conventional fuels.

Drop-in fuels are hydrocarbon chain produced from renewable substrates through variety of thermal, chemical and biological processes. In general, it has a high energy density and has the potential to be used directly as an alternative for diesel, gasoline, and jet fuel such as alkanes. They have advantages over petroleum such as it does not contain sulfur and polyaromatic hydrocarbon; it does not generate particulate matter and carbon monoxides upon combustion [104]. Considering the increasing demand for drop-in fuels, several strains have been exploited as per their potential for lipid accumulation. In general, known precursors for the production of alkanes or alkenes are medium or short-chain fatty acids [35]. Microbial synthesis of alkanes involves the reduction of fatty acyl-ACP/CoA into aldehyde using ACR (fatty acyl-CoA reductase), followed by the action of aldehyde deformulating oxygenase (ADO) or aldehyde decarbonylase (AD) resulting in the conversion of a fatty aldehyde into fatty alkanes [35]. Thus, overexpressing ACR1 and ADO resulted in 17 mg/L alkane production in *Y. lipolytica* [105]. Similarly, the cytosolic expression of carboxylic acid reductase and ADO yielded 23 mg/L of alkanes in *Y. lipolytica* [105]. AD from *Arabidopsis* CER1 and *Drosophila melanogaster* has been expressed in *Saccharomyces cerevisiae* to convert aldehydes into fatty alkanes [106]. Additionally, medium-chain fatty acids (MCFA) are also known as valuable components for biokerosene production. In general, kerosene is composed of saturated MCFA. Overexpressing diglyceride acyltransferase from *Elaeis guineensus* in *Y.*

lipolytica resulted in an increase of MCFA (C14) by 45% relative abundance in total lipids [107]. On the other hand, the study of drop-in fuels production in *Rhodosporidium* sp. has also made progress during the last few years. For instance, Yaegashi et al. [96] were able to produce bisabolene in *R. toruloides* using corn stover hydrolysate as a substrate. It is proven to be more stable than other engineered strains such as *S. cerevisiae* [96]. The stability of engineered strain is an essential industrial phenotype and a critical factor for the economical large-scale production of desirable products.

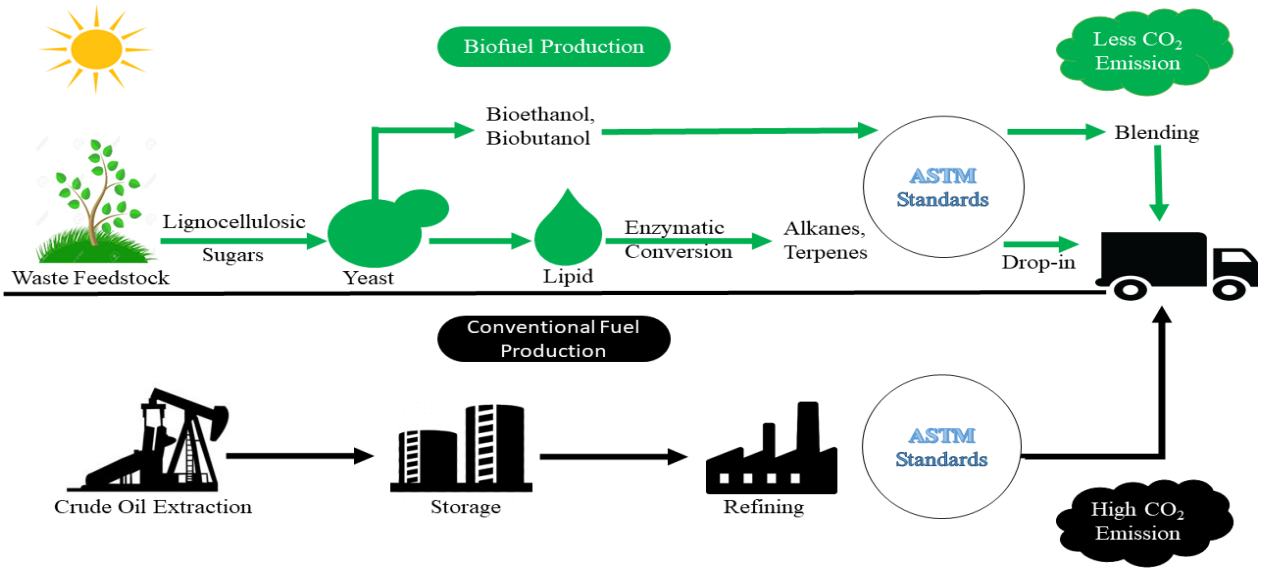

Figure 6. Comparison between conventional oil and biofuel production, utilization and their effect on the environment.

2.7. Challenges and scope

With continuing debates on "food vs fuel" and concerns over the sustainability of fuel crops, utilization of sustainable and renewable feedstocks such as lignocellulosic material seems to be the superlative opportunity for the economical production of biofuels. In this sense, sugar-rich hydrolysate derived from forestry biomass could be used as a substrate for microbial-based lipid production. Besides, the presence of inhibitors in the hydrolysates could decrease microbial growth as well as product yield. Hence, it is important to study the hydrolysate as growth media and explore the microbial biomass yield, product yield, sugar consumption rate and inhibitor tolerance. The C/N ratio optimization, various fermentation conditions as well as supplementation with other carbohydrates will be necessary to achieve the maximum lipid titer. Additionally, life cycle

assessment (LCA) is also one of the important factors used to identify and analyze the potential impact of the lipid production process on the environment as well as on the living systems. It allows foreseeing the effect of the chemicals used, energy utilized, gases and by-products produced if scaled up to a pilot plant.

Nonetheless, the final cost of biofuel production seems to exceed the market cost of fuels ($3/gallon), which are the key challenges in scaling up the projects [10]. To confront this problem, co-production of high-value compounds such as phenolic derivatives, essential fatty acids, enzymes or green chemical products such as furans, and lignin fractions could be an alternative option. However, it will require extra chemicals, maintenance or downstream cost hence raising the LCA methodology challenges. For instance, Cai et al. [108] performed the LCA study of co-produced adipic acid and succinic acid during renewable biodiesel production. During this condition, additional chemicals and energy would be needed to produce the by-products. Each product conversion is entirely dependent on the energy applied, the substrate used, and the chemical provided in the operation of each unit. Nevertheless, the market of co-products for biorefineries requires further studies and exploration to conclude. Furthermore, as the technology matures, LCA results, product yield, and economic analysis will be important for further biorefinery development and expansion. Hence, this is the time to re-contemplate and modify the current strategies for renewable biorefinery development.

The following options could be considered for further analysis:

- Scale-up of lignocellulosic biomass relies on its type, complexity and pre-treatment. Hence, a deeper understanding of the type of pre-treatment effect on biomass complexity and the employment of a cost-effective strategy is required.
- Implementation of zero waste production and proper handling of waste produced after pre-treatment of biomass.
- Detailed evaluation of different types of feedstocks and their potential effect on scale-up. Modification in techno-economic software to extrapolate the economic performance among different feedstocks.
- Life cycle assessment to foresee the potential impact of lipid production steps on the environment.

Nevertheless, microbial-based lipids as a feedstock for large-scale biofuel production are still under research. The high lipid yield using a cost-effective substrate is the major factor in producing advanced biofuels. Therefore, further research in the optimization of microbial growth conditions, process development and media optimization are essential to obtain a high lipid titer, thus, making drop-in fuels production feasible. In addition, obtaining value-added products such as aromatic compounds could complement biofuel production in terms of economic viability. In a nutshell, the production of microbial lipids using forestry-based biomass has the potential to further develop the biorefinery industries while endorsing a green and sustainable environment.

CHAPTER 3: RESEARCH PROBLEMS, HYPOTHESES, OBJECTIVES AND ORIGINALITY

3.1. Problems

The following problems need to be resolved to increase lipid production using *Rhodosporidium toruloides* and to make the biorefinery processes more sustainable:

3.1.1. Utilization of hydrolysate derived from forestry residues as a substrate for microbial lipid production

Lignocellulosic biomass has been used as a feedstock for biofuel production, which could provide aid to eradicate or reduce the energy impact on the environment and climate. In general, lignocellulosic biomass is complex between cellulose, hemicellulose and lignin. Hence, pre-treatment and enzymatic saccharification are required to break the complex link and depolymerize the polymeric sugars into monomeric forms such as glucose, xylose, fructose, and arabinose. The sugar-rich hydrolysate could be a potential feedstock for the microorganism to grow and accumulate lipids. However, microorganism tends to consume the six-carbon sugars such as glucose than five-carbon sugars. Since the hydrolysate produced from lignocellulosic biomass consists of both six and five-carbon sugars, hence it will be necessary to develop a strain capable of consuming both sugars from a biorefinery point of view.

3.1.2. Tolerance to toxic compounds present in hydrolysates

In general, pre-treatment of lignocellulosic biomass also produces toxic compounds such as furfural, 5-hydroxymethyl furfural, vanillic acid, syringaldehyde and organic acids. These compounds negatively interfere with the catalytical activities of enzymes, decrease pH, and increase osmotic pressure and reactive oxygen species resulting in decreased microbial growth rate and lipid titer. Hence, several detoxification methods such as physical, chemical and biological methods have been employed. Although, the detoxified hydrolysate would help enhance microbial growth as well as lipid accumulation, however, the additional detoxification step could increase the final production cost of the biofuels, which is already the biggest constraint of the biorefinery industry. Therefore, a strain capable of tolerating high concentrations of inhibitors and accumulating high lipid titers is highly desirable.

3.1.3. Improving lipid accumulation

Rhodosporidium toruloides are known to accumulate lipids (triacylglycerol) under nutrient limitation conditions. It can divert the excess carbon into triacylglycerol production during nitrogen limitation conditions. Hence, it is important to optimize the carbon to nitrogen ratio to increase lipid accumulation. In addition, several inducers, different types of fermentation and carbohydrates have also been employed to increase lipid accumulation. Hence, it is important to develop conditions that not only result in enhanced lipid titer but are also easy to scale up as well as economically viable.

3.1.4. Identifying the potential impact of microbial-based lipid production from wood hydrolysate on the environmental ecosystem

In general, microbial-based biofuel production requires multiple steps including pre-treatment, saccharification, fermentation, harvesting, lipid extraction and transesterification. Each step varies in terms of chemical used, the energy required, water used, waste streams produced and gases released. These chemicals and their by-products could increase toxicity and leave a long-lasting effect on human and animal ecosystems. Hence, it is important to foresee the effect of lipid production steps on the environment if scaled up from laboratory scale to pilot scale.

3.2. Hypotheses

The following hypotheses are proposed for the current research work:

1. Forestry biomass can produce sugar-rich (pentoses and hexoses sugars) through pre-treatment and saccharification. Thus, *forestry residues (hardwood and softwood sawdust) could be a sustainable and renewable feedstock for oleaginous yeast such as Rhodosporidium toruloides-1588 to grow and accumulate lipids.*
2. The yeast, *R. toruloides*-1588 generally accumulates lipids under nitrogen limitation or carbon excess conditions. In addition, fed-batch fermentation, and supplementation with other carbohydrates, salts or inducers can affect the lipid titer in *R. toruloides.* Thus, *carbon-nitrogen ratio maintenance and co-fermentation strategies could increase the lipid titer in R. toruloides-1588.*
3. *R. toruloides* is known to tolerate microbial growth inhibitor compounds such as furfural present in the wood hydrolysate. However, the research about its ability to use furfural as an energy source is limited. Hence, *R. toruloides-1588 can thrive on furfural and consume it as a carbon source and transform it into non-toxic compound such as furfuryl alcohol and 2-furoic acid.*
4. Depending on the process, chemicals, energy and time required for the conversion of substrates into the product, it can impact the environment by releasing greenhouse gases and toxic by-products. Hence, *Life cycle impact assessment could help identify hotspots in-terms of environmental, ecosystem and resource damage during lipid accumulation process*.

3.3. Objectives

The following objectives were performed based on the aforementioned problems and hypotheses:

1. Study of liquid hydrolysate produced from forestry biomass as culture media to accumulate lipids in *Rhodosporidium toruloides*-1588.
2. Evaluate the effect of carbon to nitrogen ratio maintenance in wood hydrolysate using fed-batch conditions to obtain maximum lipid accumulation in *R. toruloides*-1588.
3. Developing the co-fermentation strategy of hydrolysate and crude glycerol to increase the lipid accumulation in *R. toruloides*-1588.
4. Study the lipid accumulation in a bench-scale bioreactor using optimized conditions.
5. Furfural tolerance and identification of furfural degrading enzymes in *R. toruloides*-1588.
6. Life cycle assessment of lipid production using wood hydrolysate to identify the hotspots and potential impact on the environment.

3.4. Originality

"Increase of lipid accumulation in *Rhodosporidium toruloides*-1588 using a biorefinery concept for the conversion of renewable and sustainable substrates"

CHAPTER 4: HYDROLYSATE DERIVED FROM FORESTRY RESIDUES AS A SUBSTRATE FOR LIPID ACCUMULATION USING *RHODOSPORIDIUM TORULOIDES*-1588

The Chapter 4 involves the screening of hydrolysate produced from two different sawdusts (hardwood and softwood) as a culture media for *R. toruloides*-1588 to grow and accumulate lipids. In addition, carbon consumption, inhibitor tolerance and effect of nitrogen source was also evaluated in this study.

4.1. Evaluating the potential of *Rhodosporidium toruloides*-1588 for high lipid production using undetoxified wood hydrolysate as a carbon source

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Abstract

The study aims to explore microbial lipid production using abundant and low-cost lignocellulosic biomass derived from forestry residues. Sugar-rich undetoxified hydrolysate was prepared using hardwood and softwood sawdust and used for lipid production as a carbon source from an oleaginous yeast, *Rhodosporidium toruloides*-1588. The maximum biomass obtained was 17.09 and 19.56 g/L in hardwood and softwood hydrolysate, respectively. Sugar consumption in both hydrolysates was >95%, with a maximum lipid accumulation of 36.68% at 104 h and 35.24% at 96 h. Moreover, *R. toruloides*-1588 exhibited tolerance to several toxic compounds such as phenols, organic acids and furans present in hydrolysates. The lipid characterization showed several monosaturated and polyunsaturated fatty acids, making it a potential feedstock for biofuels and oleochemical production. This study confirms the credibility of *R. toruloides*-1588 as a suitable lipid producer using hydrolysates from forestry residues as a substrate. Additionally, lipids obtained from *R. toruloides*-1588 could be a potential feedstock for advanced biofuel production as well as for food and pharmaceutical applications.

Keywords: *Rhodosporidium toruloides*; microbial lipid; lignocellulosic biomass; forestry residue; undetoxified hydrolysate

Introduction

A microorganism tends to accumulate lipids in the form of triglyceride, under nutrient-stress conditions, either during nitrogen limitation or excess of carbon [109]. The microorganism that can store more than 25% lipid (*w/w*) of their total dry cell weight (DCW) is termed oleaginous strains. For instance, *Lipomyces starkeyi*, *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Cryptococcus curvatus*, among others. Their capability to thrive on a wide variety of inexpensive substrates renders them an outstanding alternative to conventional oil sources [5]. Microbial lipids have applications in several industries including biofuels, food, pharmaceutical, and chemical industries. For example, docosahexaenoic acid and eicosapentaenoic acid provide aid in the cardiovascular system, eye functioning, and hormonal balance. Similarly, gamma-linolenic acid is known to have anti-cancer properties. Lipids can be transformed into a broad range of biofuels through biochemical or chemical conversions such as biodiesel (mono-alkyl esters), fatty alkanes, and fatty aldehydes [42]. The critical challenges in lipid production are the availability of substrates, process cost and should comply with food security. Over the past decades, biorefinery

has moved towards the usage of lignocellulosic-based biomass as an alternative substrate for microbial-based lipid production [5].

Lignocellulosic biomass is known as an inexpensive and abundant source of carbon. It generally requires a pre-treatment to interrupt the linkage between lignin, hemicellulose, and cellulose followed by the action of cellulolytic enzymes to convert polymeric sugars into monomeric form. Sources of lignocellulosic biomass include but are not limited to the forestry, agricultural, and municipal sectors. Canada possesses 10% of the world's forest and represents 58% of total feedstock, while annual agriculture and municipal feedstock account for 39% and 3%, respectively [8]. Hence, this could be a renewable option to utilize sugar-rich hydrolysate derived from forestry biomass to produce microbial lipids. Over the past decades, a huge number of strains have been exploited for lipid production using biomass hydrolysate. However, the foremost challenge is the consumption of sugars in the presence of inhibitors that are generally released into hydrolysate after the lignocellulose pre-treatment. These compounds generally hinder the use of substrates by interfering in the metabolic pathways of microorganisms [28]. Hence, it is very important to use a robust strain capable of consuming a wide variety of carbon sources and having high inhibitor tolerances. A recent study has shown that random mutagenesis or genetic engineering could increase lipid production, carbon consumption or inhibitor tolerance. For instance, Guo et al. [110] described the surge in lipid production by 43.6% in *Rhodosporidium toruloides* AS 2.1389, when the strain was irradiated with ultraviolet rays.

R. toruloides, a basidiomycetes yeast has been widely studied over the past years and reported to co-metabolize pentose and hexose sugars. *R. toruloides* was found to yield 100 g/L biomass and accumulate 50 – 70% lipid of its total DCW [5]. It is also known as red yeast because of the production of carotenoids, such as astaxanthin, β-carotene, and torularhodin. These are known as high-value compounds for the pharma and food industries [2]. Consequently, research on *R. toruloides* strain has been carried out to study, explore, and gather knowledge on multiple aspects of its genome. Nevertheless, the research on *R. toruloides* has begun to exploit the fields such as inhibitor tolerance, genetic and metabolic engineering, growth and lipid production behaviour in various carbon sources such as sucrose, xylose, crude glycerol, and wood hydrolysates [5, 111].

The present study aims to evaluate the lipid production ability of *Rhodosporidium toruloides*-1588 in undetoxified hydrolysate derived from forestry biomass. Its lipid production ability, fatty acids

methyl esters (FAMEs), biomass, sugar consumption, and inhibitory effect were studied and evaluated. In addition, the effect of the addition of nitrogen source on *R. toruloides* growth, lipid titer and different types of polyunsaturated fatty acids were also analyzed.

Materials and methods

Enzyme Production and Extraction

Aspergillus niger (NRRL-2001) was acquired from the Agricultural Research Service (NRRL) Culture Collection (USA). *A. niger* was maintained on a potato dextrose agar (PDA) plate and incubated at 30 ℃, till black spores appeared. Spores were then harvested, and spore count was determined with a hemocytometer followed by aliquot preparation of spores in 1% of Tween-80 and stored at -20℃. *A. niger* was cultivated in apple pomace (AP) supplemented with 1% rice husk at 75% moisture content, as performed by Dhillon et al. [112].

Enzyme activity

One gram of fermented sample was collected at the end of fermentation and dissolved in 15 ml of 50 mM citrate buffer (pH 4.8). The solution was kept in a shaking condition for 30 minutes at 200 rpm, followed by separation of enzyme solution from biomass using a centrifuge at 9000 *x* g, 4℃ for 15 min (Sorvall RC-5 Plus), followed by the supernatant analysis of enzyme activity.

The total cellulase activity was measured using the standard filter paper assay method. Briefly, the reaction mixture for the filter paper assay contained 500 µL enzyme solution, 1 mL citrate buffer (50mM and pH 4.8) and the filter paper strip of Whatman no.1. The two different enzyme dilutions were prepared i.e., one dilution should release a little lower and the other dilution a little higher than 2.0 mg of glucose. The samples were incubated for 60 minutes at 50 °C followed by 3.0 mL of 3,5-dinitrosalicyclic acid (DNS) addition in solution. The reaction mixture was boiled for 10 minutes and then cooled down by keeping tubes in ice. The amount of sugar released was calculated using a UV- visible spectrophotometer (Cary-50, Varian Inc., Palo Alto, California, USA) at 540 nm.

Pretreatment and enzymatic saccharification

Hardwood (Maple wood) and softwood (Fir wood) sawdust were obtained from Quebec's Forestry Industry Council. Biomass was dried at 60±1°C for 12 h. Sawdust was further milled using a

planetary ball mill (Retsch Corporation; PM100) at 500 rpm for 15 minutes. The resulting milled wood biomass was separated using a sieve with a final size of 300 µm - 1 mm [113]. Afterward, the wood sawdust was used for the pre-treatment and enzymatic saccharification.

Both sawdusts were pretreated using dilute alkali (1% NaOH). The solid to liquid ratio was kept at 1:10 (w/v). The slurry was then autoclaved at 121˚C/15 psi for 30 minutes. Afterward, the slurry was centrifuged (7000 *x* g/15 min) and the solid fraction was washed using tap water and dried at 45±1ºC for 30 minutes. The enzymatic saccharification was performed in a Multitron® shaker at 40±1°C for 24 hours with a crude enzyme extract loading rate of 12 IU/ gram of dry substrate with a substrate loading rate of 10% (*w/v*) with respect to the volume of slurry at pH 4.8, using a citrate buffer.

Microorganism maintenance and inoculum preparation conditions

R. toruloides-1588 were obtained from the NRRL Culture Collection of the USA. *R. toruloides*-1588 was maintained in Yeast Malt (YM) agar plates as described by Osorio-Gonzalez et al. [111]. Yeast was pre-cultured for three generations in YM media i.e., yeast extract and malt extract (3.0g/L each), peptone (5.0g/L), and glucose (10.0 g/L). This media composition was also used as a synthetic media in further experiments. The inoculum was prepared in three different steps with incubation conditions of 25℃, 180 rpm for 18 hours: 1) a 2 mL YM broth was inoculated with a single colony picked from a fresh plate and incubated; 2) Inoculum was then transferred (1:100 *v/v*) to 5 mL YM broth and incubated and; 3) Final inoculum was transferred (1:100 *v/v*) to 50 mL YM broth and incubated at 25℃ and 180 rpm.

Lipid production

For lipid production, yeast was cultured in 50 mL of undetoxified hardwood and softwood hydrolysate. Two sets of the experiment were run, one with the addition of 1 g/L ammonium sulfate and another without any addition and the pH was adjusted to 6.0. The hydrolysates were inoculated using third-generation inoculum with an optical density (OD) at 600 nm of 0.3 and incubated at 25°C and 180 rpm. The samples were collected at regular intervals of time throughout the fermentation process. All the experiments were performed in duplicates. The lipid yield, content and concentration were evaluated using Ma et al. [114] equations as follows:

$$\text{Lipid Content} = \frac{Lipid\ weight\ (g)}{Biomass\ weight\ (g)} \quad \text{Eq. 1}$$

$$\text{Lipid Concentration} = \frac{Lipid\ weight\ (g)}{Culture\ volume\ (L)} \quad \text{Eq. 2}$$

$$\text{Lipid accumulation (\%)} = \frac{Lipid\ concentration\ (g/L)}{Biomass\ concentration\ (g/L)} * 100 \quad \text{Eq. 3}$$

Lipid extraction

Lipid extraction was conducted, as performed by Osorio-Gonzalez et al. [111]. Briefly, cells were separated from the fermented broth using centrifugation (15 000 *x* g for 3 min), and dried at 60℃, until constant dry weight. The cell lysis was performed by boiling them in the presence of 1M/L HCl for 1 hour followed by 3.75 mL chloroform: methanol ratio (2:1 v/v) addition in the samples. The mixtures were then mixed for 15 minutes using a vortex mixer and 1.25 mL of chloroform was added, then the sample was vortexed again for 1 minute, lastly, 1.25 mL of 1M NaCl was added to the mixture and centrifuged (7000 *x* g for 15 minutes).

Analytical methods

Cell growth analysis

Cell growth was measured in grams of DCW. The yeast cells were separated from the fermented broth using centrifugation (15000 *x* g for 2 min), followed by cell washing with phosphate-buffered saline (PBS, pH – 7.0), and drying at 60°C until a constant weight was achieved and expressed in g/L.

Inhibitors and sugar analysis

Sugars and inhibitors analysis was performed, as explained by Osorio-Gonzalez et al. [111]. Briefly, total reducing sugar was analyzed using the DNS method, followed by sugar quantification in UV–visible spectrophotometer (Cary-50, Varian Inc., Palo Alto, California, USA) at 540 nm. The composition of sugar and inhibitors was quantified using a liquid chromatography-mass spectrophotometer (Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer). All compounds were quantified and determined using standards obtained from Sigma-Aldrich (USA).

Fatty acid composition (FAME)

The FAME was quantified using the conditions reported by Osorio-Gonzalez et al. [111]. Crude lipid was esterified in sulfuric acid and methanol at 100°C for 20 minutes. Later, the samples were cooled at room temperature and FAMEs were extracted using hexane. FAME composition was quantified using gas chromatography (Agilent 7890B), with the specification provided by Osorio-Gonzalez et al. [111].

The cetane number (CN) was calculated using equation 4 provided by Fei et al [22].

$$CN = 62.2 + 0.0074X_a + 0.115X_b + 0.177X_c - 0.103X_d - 0.279X_e - 0.366X_f \quad \text{Equation 4}$$

where values of X's are the weight percentage of fatty acids, X_a is methyl myristate; X_b is palmitic acid; X_c is stearic acid; X_d is oleic acid; X_e is linoleic acid and X_f is linolenic acid.

Statistical analysis

The statistical analysis of the data was conducted using Minitab® 17.1.0 (Minitab Inc., Pennsylvania, USA). Parameter effects such as biomass production and lipid accumulation were evaluated by one-way ANOVA (Analysis of Variance). Statistical significance in the mean values of various measured parameters was calculated and compared with the Fisher test (95% level of confidence).

Results and discussion

Wood hydrolysate and its composition

The hydrolysate was prepared from sawdust residues of the Maple tree (hardwood) and Fir tree (softwood). In general, softwood contains 40% cellulose, 25-30% hemicellulose, 25-30% lignin and <5% extractives, while hardwood contains 35% cellulose, 25% hemicellulose, 20% lignin and < 5% extractives. The total reducing sugar of hardwood and softwood hydrolysate was obtained as 46.01 and 45.25 g/L after alkaline pre-treatment and enzymatic saccharification (12 International enzymes units/gram of dry substrate). The sugars and inhibitors compositions are shown in Table 4. The presence of low amounts of organic acids, phenolic compounds and furfurals in hydrolysates could be because alkali solution is known to dissolve 60 – 80% of lignin and phenolic compounds [115].

Table 4. Sugars and inhibitors composition detected in hardwood and softwood hydrolysates

Components	Hardwood Hydrolysate (g/L)	Softwood hydrolysate (g/L)
Glucose	12.64 ± 1.52	13.27 ± 1.23
Xylose	10.16 ± 0.95	13 ± 0.89
Galactose	12.96 ± 0.81	13.66 ± 1.12
Fructose	3.24 ± 0.21	3.30 ± 0.37
Trehalose	0.03 ± 0.00	2.4 ± 0.07
Furfural	0.067 ± 0.01	0.057 ± 0.01
5-hydroxymehtyl furfural	0.025 ± 0.002	0.035 ± 0.001
Vanillic acid	0.034 ± 0.003	0.042 ± 0.001
Vanillin	0.007 ± 0.0005	0.01 ± 0.002
Levulinic acid	0.007 ± 0.0003	0.007 ± 0.0006

Biomass growth and sugar consumption

Biomass growth profiles in synthetic media (SM), hardwood (HW) and softwood (SW) hydrolysates are presented in Figure 7. The cells continued to be in the exponential phase until 72 hours followed by a stationary phase in hardwood hydrolysate while the double exponential phases (i.e., 18 h and 72 h) were observed in softwood hydrolysate. In general, microorganisms demonstrate diauxic growth when subjected to two different carbon sources in the culture media. Specifically in *Rhodosporidium* sp., once the glucose is consumed from the media, it causes the yeast to consume another available carbon source resulting in the second exponential phase for a shorter duration [111]. The maximum cell mass observed was 5.2 and 6 g/L in hardwood and softwood, respectively. In synthetic media, cells continue to be in the exponential phase until 72 hours followed by the stationary phase. *R. toruloides*-1588 reached maximum biomass of 9 g/L in synthetic media.

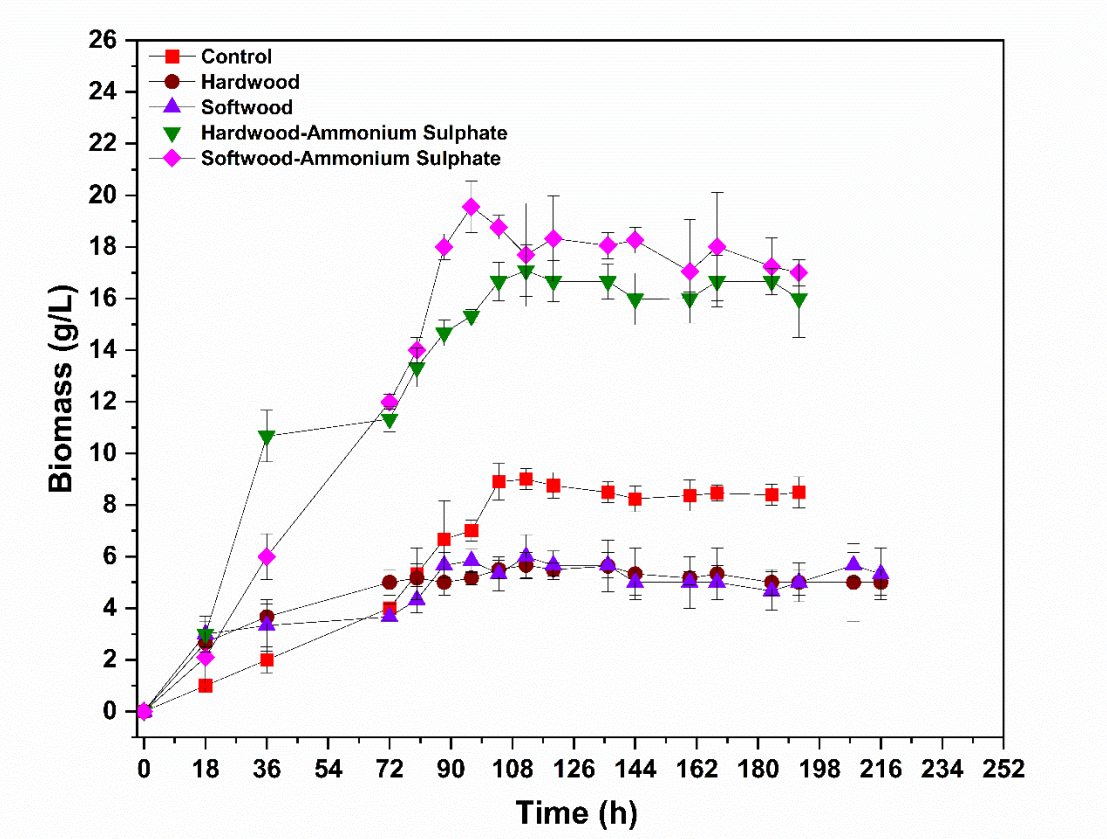

Figure 7. Growth profile of *Rhodosporidium toruloides*-1588 in different media; where: control is yeast grown in synthetic media.

Another possible reason for lower biomass could be a very high C/N (carbon to nitrogen) ratio or a very low amount of nitrogen availability in the hydrolysate. This is because the carbon causes osmotic shock when present in large amounts in the media due to a decrease in Krebs cycle activity, reduced energy production and intracellular increase of critic acid [96]. Total nitrogen in hardwood and softwood hydrolysate was observed to be 85 and 77 mg/L, respectively, and which remained unused throughout the batch experiment (C/N >200). It has already been established that lipid accumulation occurs at C/N ratio 20 and which continues to vary till C/N ratio 120. However, at a very high C/N ratio, lipid and biomass accumulation tends to decrease too [42, 75]. Hence, another experiment was performed with the supplementation of ammonium sulfate (1g/L) in both the hydrolysates. Ammonium sulfate has 0.21% nitrogen content in it [116], resulting in a change of the C/N ratio to 87.6. The addition of ammonium sulfate has not only affected biomass growth but also led to near-complete consumption of sugars (>95%) in both the hydrolysates. This could be due to the availability and use of nitrogen sources at the beginning of fermentation, which allowed

the yeast to utilize the sugar in the hydrolysate. Nitrogen is a known activator of isocitrate dehydrogenase (ICDH). In general, ICDH catalyzes the isocitrate conversion into α-ketoglutarate in the Krebs cycle and releases the nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) as a by-product, which further takes part in the electron transport chain for energy production and microbial reproduction. On the other hand, the limitation of nitrogen results in the inactivation of ICDH which further leads to citrate accumulation in mitochondria and ultimately converts into lipids through a series of conversions [36]. The maximum biomass achieved was 17.09 and 19.56 g/L in hardwood and softwood hydrolysates, respectively (Figure 7). This result is promising as the yeast was able to thrive in undetoxified wood hydrolysate without any supplementation of micronutrients such as zinc sulfate, magnesium sulfate, ferric chloride, and manganese sulfate, which can be seen in several published articles [56, 65, 69, 117]. Lower biomass in the hydrolysate could be due to the presence of inhibitors in the hydrolysates. Even the effect of inhibitors can also be seen on the sugar consumption profile of *R. toruloides*.

On the other hand, in hydrolysate, 26% and 31% consumption of sugars were observed in hardwood and softwood, respectively, while 98% of the sugar has been consumed in control. Synthetic media has only glucose as a carbon source hence resulting in rapid consumption of glucose within 72 hours of fermentation, while hydrolysate has both hexose, pentose and inhibitors which might have hindered the sugar consumption as well as biomass growth. In addition, more than 70 - 80% of glucose consumption was seen while only ~10 and ~14 % of consumption was seen in the case of xylose in hardwood and softwood hydrolysates, respectively, as shown in Figure 8. This could be the reason for the double exponential phase of *R. toruloides*-1588 in softwood hydrolysate (Figure 7). This result states that the difference in consumption of C6 and C5 sugars is due to metabolic limitations. Similarly, when *R. toruloides*-1588 were grown in C5 and C6 hydrolysate separately, <50% and >90% of xylose and glucose consumption were seen, respectively [111]. The diauxic growth can also be seen in other blends of carbon sources such as glucose and glycerol. For instance, Bommareddy, et al. [118] cultivated the *R. toruloides* in mixed media of glucose (10g/L) and glycerol (40g/L). The authors observed the consumption of glycerol (~37%) only after the glucose was consumed (>90%) resulting in a double exponential phase at 7h and 30h. On the other hand, the difference in sugar consumption in hardwood and softwood hydrolysate could be due to the differences in furfural concentrations i.e., 57 mg/L in softwood

while, 66 mg/L in hardwood. Glucose can still be consumed by yeast but increasing the furfural concentration has affected xylose consumption. Based on the above results, the lower the furfural concentration, the higher will be the sugar consumption. Similarly, Zhao et al. [65] demonstrated that the increase in furfural concentration to 1.0 g/L, glucose consumption was seen while xylose utilization by *R. toruloides* AS 2.1389 was inhibited, hence leading to 60% lower biomass production. Nevertheless, several processes and engineering methodologies have been developed to further increase the C5 sugar consumption in *R. toruloides*. For instance, phenotypic heterogeneity, epigenetic inheritance, evolutionary engineering, random mutagenesis, and site-directed mutagenesis. So far, to improve the *Rhodosporidium* strains, different engineering strategies are being employed by overexpressing enzymes such as malic acid, acetyl-CoA citrate, ATP-citrate lyase, and diglyceride acyltransferase. Nevertheless, research and literature on pentose consumption in *Rhodosporidium* sp. is limited or null, hence require further studies and strategies to further increase the sugar consumption as well as lipid accumulation.

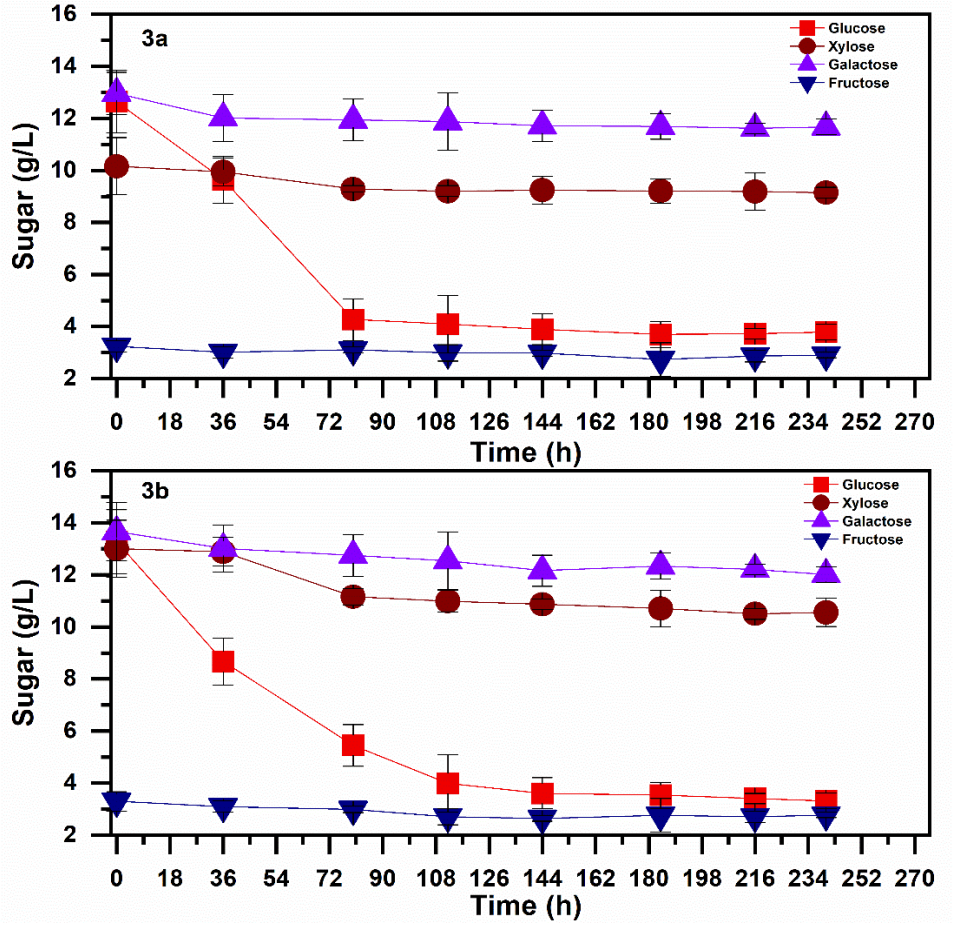

Figure 8. Sugar reduction profile during yeast fermentation: (a) consumption profile in hardwood hydrolysate; (b) consumption profile in softwood hydrolysate.

Lipid accumulation and FAME composition

The lipid accumulation in hydrolysates (hardwood and softwood) without ammonium sulphate addition was 0.25 g lipid/g biomass, and 0.22 g lipid/g biomass, respectively. This lipid accumulation was lower than obtained using synthetic media (0.27 g lipid/g biomass). Nevertheless, we observed a different time to reach the maximum lipid accumulation (104 h, 96 h, and 118 h, respectively), that from the process point of view is a promising result, due to the short production process required less energy. The above fact can contribute to increasing the profitability of the entire process because in both the hydrolysates, the maximum lipid accumulation time was earlier than in control.

On the other hand, the addition of ammonium sulfate (1 g/L) had a significant effect (p-value ≤0.05) on the lipid accumulation in *R. toruloides*-1588 and resulted in a ~30% increase in lipid content i.e., 0.37g lipid/g biomass and 0.35g lipid/g biomass accumulation in hardwood and softwood hydrolysate, respectively. In general, under stress conditions, *Rhodosporidium toruloides* tend to accumulate lipids in the early stages of their growth [111]. The addition of ammonium sulfate as a nitrogen source in the hydrolysate increased lipid accumulation (Supplementary Figure 1). There is no denying the fact that nutrient imbalance in media triggers lipid accumulation in oleaginous yeast. When key nutrient runs out such as nitrogen, excess carbon then converted into storage lipids. Therefore, the initial C/N ratio in media has a key role in lipid accumulation. For instance, Papanikolaou et al. [119] reported the effect of the initial C/N ratio on *Mortierella isabelline* and *Cunninghamella echimulata*. The authors reported an increase in lipid accumulation from 36% to 47% in *Cunninghamella echimulata* and 50% to 56% in Mortierella isabelline when the C/N ratio was changed from 83.5 to 133.5. The authors stated that lipid productivity could vary depending on the initial C/N ratio of media and the type of oleaginous strain. Similarly, in the present study, the change of C/N ratio from ~300 to 83.5 resulted in high biomass as well as lipid accumulation in *Rhodosporidium toruloides*-1588. However, the lipid production is still low i.e., 6 – 7 g/L hence further exploration is needed to improve the lipid production. In addition, with the addition of nitrogen sources, the maximum lipid accumulation time did not change which is quite important for a biorefinery industry. As in the biological

process, fast microbial growth, as well as accumulation of products at the early phase experiment, could help decrease the total production time which ultimately helps in reducing the energy consumption and production cost. Similarly, Osorio-Gonzalez et al. [111] reported the maximum lipid accumulation time of different strains of *R. toruloides* was 112 – 120h when cultivated in C5 and C6 hydrolysates. Zhao et al. [65] reported 120 h for maximum lipid accumulation when *R. toruloides* was cultivated in detoxified sugarcane bagasse hydrolysate, while the present study demonstrated lower than 100 h as a higher lipid accumulation time. Figure 9 shows the accumulation of lipids in *R. toruloides*-1588 in hardwood and softwood hydrolysate.

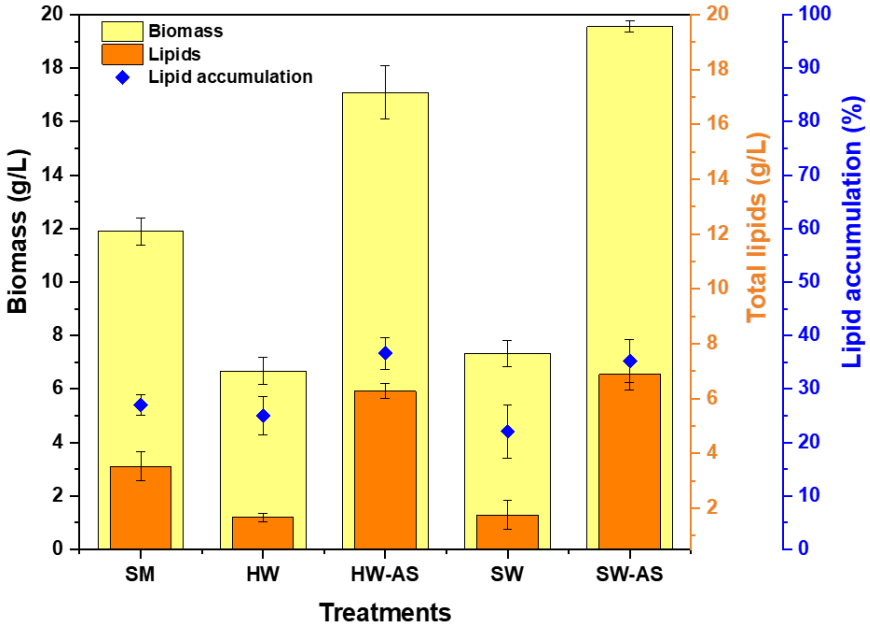

Figure 9. Lipid accumulation profile in synthetic media (SM); hardwood hydrolysate (HW), hardwood hydrolysate-ammonium sulfate (HW-AS); softwood hydrolysate (SW) and softwood hydrolysate-ammonium sulfate (SW-AS).

The microbial lipid composition is generally comprised of carbon atoms varying from 10 to 24. The fatty acid distribution can vary depending on the type of strain, culture media and growth conditions [2]. The fatty acid distribution of lipids was comprised of palmitic, linoleate, oleic, linoleic, and stearic acids, in order of their abundance. Figure 10 represents the fatty acid distribution profile in hardwood, and softwood hydrolysate, as well as in synthetic media. Although, the fatty acid composition pattern was the same in the all culture media (oleic, palmitic,

and stearic acid), we observed a peak abundance decrease in synthetic media compared with the obtained abundance in both wood hydrolysates. Wood hydrolysate is complex media with a wide amount and presence of compounds that can contribute to the variation in fatty acid composition by increasing stress on the yeast. Similarly, Osorio-Gonzalez et al. [111] stated that concentration and fatty acid composition are highly dependent on substrate source and media composition. Likewise, Zhao et al. [65] reported a maximum peak abundance of 49%, 6%, and 35% of oleic, stearic and palmitic acid in a batch experiment using detoxified sugarcane bagasse derived hydrolysate, while Fei et al. [22] reported 45.2 % of oleic acid, 10% of stearic acid and 25% of palmitic acid in fed-batch conditions using corn stover hydrolysate. The fatty acid compositions of microbial lipids have similarities with plant oils in terms of carbon chains while percentage distribution can vary within the different genera of plants, hence can replace the vegetable oils for biofuel production. For instance, the general fatty acids composition of cocoa butter is 37% oleic acid, 23% palmitic acid and 32% stearic acid. Hence could be an alternative to cocoa butter [120]. Thus, it can be speculated that; (a) lipids formed in *R. toruloides*-1588 in undetoxified hydrolysate could be a promising feedstock in various industrial applications; (b) fatty acid distribution can vary depending on media composition and growth conditions, (c) lipid composition is also dependent on genetic makeup and genera of microorganism.

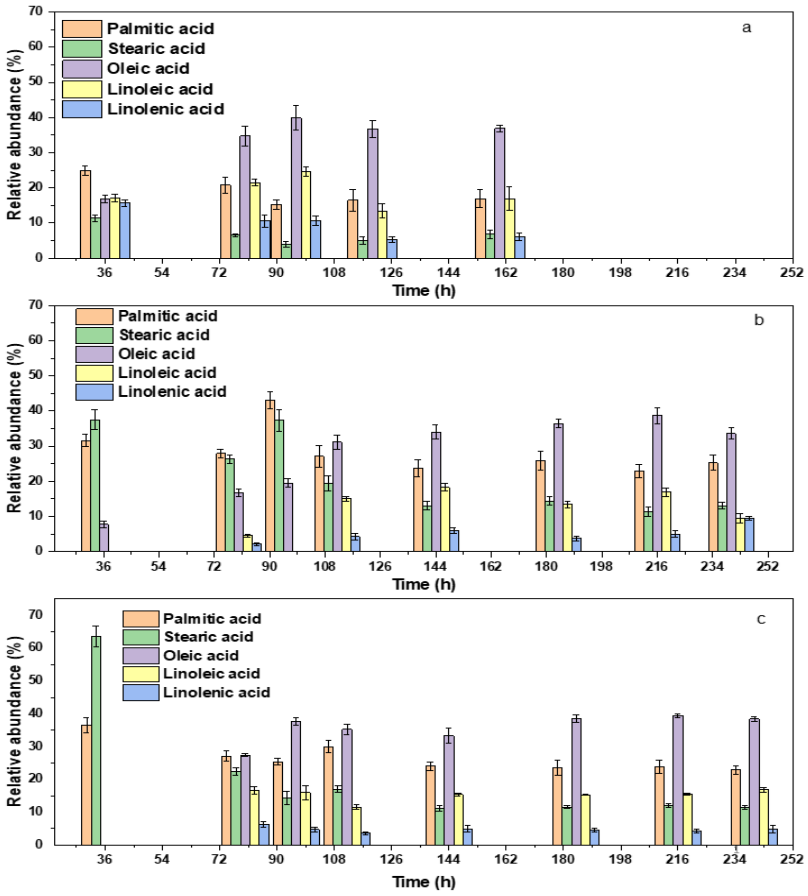

Figure 10. Fatty acid composition of lipid from different growth conditions: a) synthetic media; b) hardwood hydrolysate; c) softwood hydrolysate

The cetane number of fatty acids were calculated. According to the Equation provided by Fei et al. [22], the cetane number (CN) was found to be 49.8, 56.32, 57.12 in synthetic media, hardwood and softwood hydrolysate, respectively. This result is in accordance with the result reported by Fei et al. [22] with a value of 57.7 of the fatty acid produced using lignocellulosic biomass in R. toruloides DSMZ 4444 (Deutsche Sammlung von Mikroorganismen und Zellkulturen). The acceptable value as per the European (EN 14214) and American Society for Testing and Materials (ASTM D 6751) is 50 [22].

Consequently, regardless of the low concentration, polyunsaturated fatty acids, such as docosapentanoic acid or linoleate, are important due to their high commercial value in the chemical, food, and pharmaceutical industries. Table 5 shows the polyunsaturated fatty acid produced in *R. toruloides*-1588. In general, a low C/N ratio lead to an increase in unsaturated fatty

acid production [116]. On the other hand, microbial cells also tend to produce high amounts of unsaturated fatty acids in the presence of inhibitors [111] which could be the possible reason for high polyunsaturated fatty acid production in hydrolysate than in synthetic media grown yeast. In other words, undetoxified and detoxified hydrolysates also affect lipid compositions, for instance, the unsaturated fatty acid proportion has been observed as slightly lower in detoxified hydrolysate [111]. Conversely, inhibitors can cause total or partial growth inhibition of microorganisms. Likewise, these can interfere with carbon consumption, thus ultimately influencing lipid accumulation.

Table 5. The relative percentage of polyunsaturated fatty acids detected in lipids under different media conditions.

Polyunsaturated fatty acids (%)	**SM**	**HW**	**HW-AS**	**SW**	**SW-AS**
Alpha linolenic acid	5.21	9.48	2.73	6.29	3.74
Stearidonic acid	1.26	ND	0.68	0.22	0.43
Eicosatrienoic acid	0.71	3.86	ND	2.82	3.76
Docosapentaenoic acid	5.31	5.9	ND	1.98	1.12
Linoleic acid	0.67	4.51	10.05	16.65	9.86
Trans-linoleic acid	ND	0.49	1.12	0.06	0.62
Gamma linoleic acid	ND	0.22	0.29	0.07	0.15
Eicosadienoic acid	0.18	1.06	0.17	0.46	0.36
Dihomogamma linolenic	ND	0.10	0.06	0.09	0.11
Elaidic acid	ND	0.88	1.66	1.09	0.71
Erucic acid	0.72	ND	0.03	0.54	0.24
Eicosenoate	ND	0.93	ND	0.83	ND

SM: synthetic media; HW and HW-AS: hardwood hydrolysate without and with ammonium sulfate; SW and SW-AS: softwood hydrolysate without and with ammonium sulfate; ND: Not Detected

Effects of Inhibitors

The tolerance and effect of inhibitory compounds on *R. toruloides*-1588 were evaluated throughout the fermentation process. According to obtained results in both hydrolysates, most of the reduction in inhibitors have been seen in 80 to 100 h of the fermentation process as shown in Figure 11. The total reduction of syringaldehyde, vanillin, 5-hydroxymethyl furfural (5-HMF), and furfural

occurred until 80 hours. Furthermore, 32% of the vanillic acid reduction was observed until 72 hours. Approximately 20 to 26% of the inhibitor concentration was reduced in control treatment until 200 hours of fermentation as shown in Figure 11. The degradation in controls could be due to different fermentation conditions like salt, pH, fermentation time, aeration or temperature [111]. It has not been established yet that *R. toruloides* can degrade or metabolize inhibitors, but they are known as inhibitor tolerant species because of overexpression of certain types of genes [98]. For instance, NADPH production was increased due to enhanced expression of ribulose 5-phosphate epimerase (RPE1) and phosphogluconate dehydrogenase (GND1) enzymes in *R. toruloide*s when exposed to inhibitors [98]. The knockout of GND1 in Saccharomyces cerevisiae leads to increased sensitivity towards 5-HMF and furfural [121]. Likewise, aldehyde dehydrogenase 4 (ALD4) is another enzyme that was reported to increase about 3-fold in *R. toruloides*, which involves the conversion of furfural and 5-HMF into furan-2,5-methanol and 2-furan methanol, respectively [122]. Qi et al. [123] reported that if gene expression increases double-fold, it would be considered significant for *R. toruloides* strains. The author demonstrated the change in expression of 39 different genes generally involves in DNA repair, mitogen-activated protein kinase (MAPK) signalling and glycolysis pathway, in the plasma mutated strain gro*wn* on lignocellulosic hydrolysate [123]. The microorganisms capable of degrading furfural and 5-HMF were found to have *hmf* clusters [124]. Various other genes were also found responsible for furaldehyde degradation present in the vicinity of *hmf* are *mfc*, *adh*, *hyd* and *hmf* genes. Likewise, *R. toruloides* have also been found to have aldehyde dehydrogenase (*adh* gene) involved in central metabolism and lipid accumulation but its involvement in furfural degradation is yet to discover [39]. These genes could be used as a marker for the tolerance to inhibitors and screening of the tolerant oleaginous strains. Redox potential has also been found to play a crucial role in enhancing the furfural tolerance in yeast. Recently, Li et al. [125] studied the furfural degradation using *Saccharomyces cerevisiae*, when grown in synthetic media (i.e. yeast extract (3 g/L); glucose (100 g/L); peptone (4 g/L) and furfural (4 g/L)) by controlling the extracellular redox potential (ORP) i.e., by adjusting the fermenter aeration. The author reported the maximum biomass growth and furfural degradation was observed at -100 mV ORP while no aeration condition resulted in a prolonged lag phase, lowest biomass and prolonged furfural degradation time [125]. Hence, it can be suggested the furfural degradation can be due to oxidation-reduction reaction. Although ORP

was not maintained or recorded in the present study, aeration was applied to the culture, hence this could be another reason for inhibitors degradation by *R. toruloides*-1588.

Inhibitors generally interfere with the normal working of the cellular systems such as the reduction in transport of substrate through the plasma membrane, oxidative stress, and variation in the electrochemical gradient of mitochondria, ultimately leading to either reduced growth or growth inhibition, reduced sugar consumption, and variation in bioproduct formation. Hence, it is important to develop or screen the microorganism that could not only tolerate high inhibitor concentrations but can also produce a high concentration of the desired product. The effect of an inhibitor can also be seen on the FAMEs in the present study. The presence of docosapentaenoic acid and linoleate was detected in a sample collected from hydrolysate in the early exponential phase. Furfural is also reported to affect fatty acid distribution, especially on unsaturated fatty acids [65]. Unsaturated fatty acids are not only required for biofuel but also have high commercial value in nutraceuticals, and chemical industries.

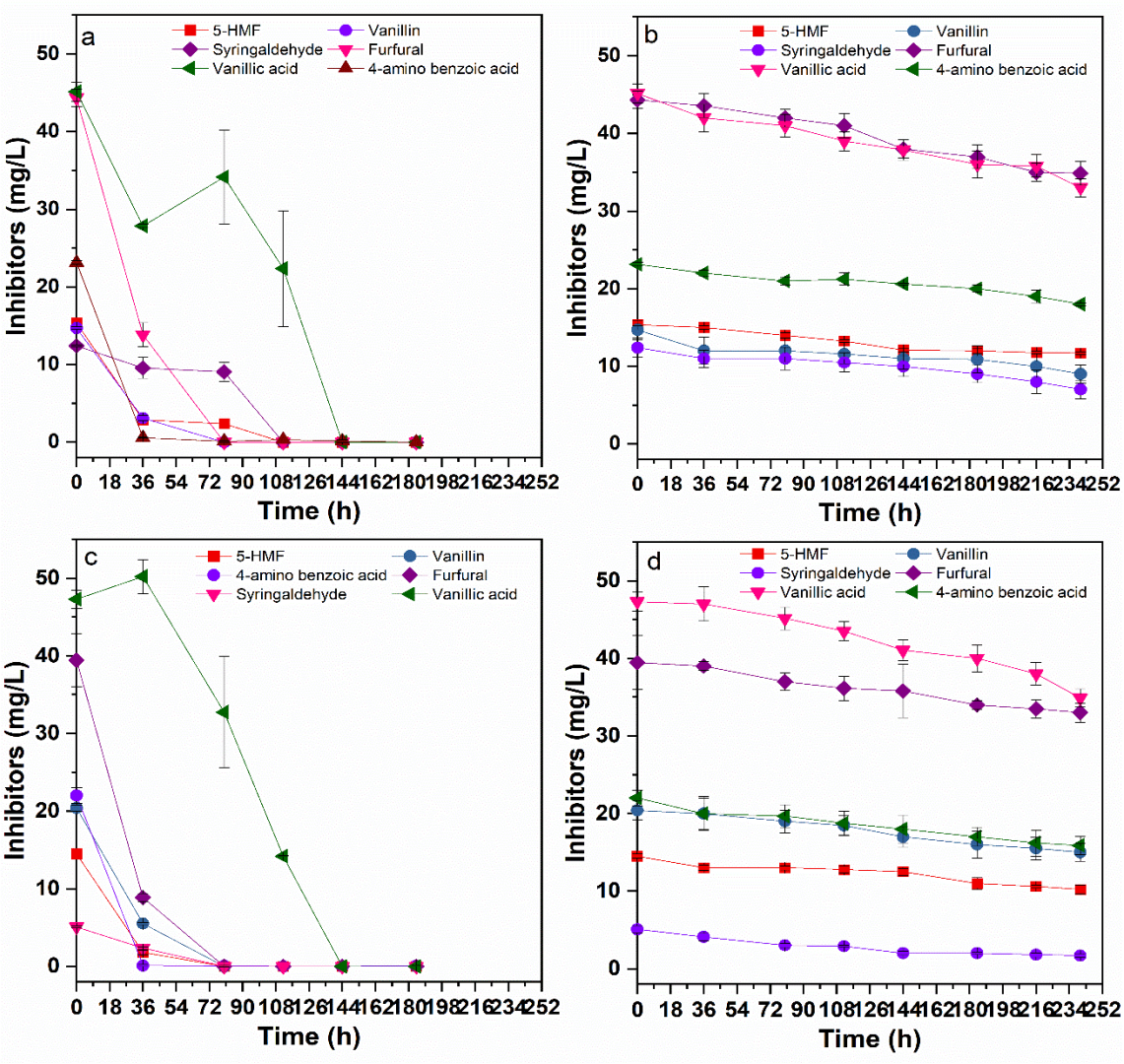

Figure 11. Inhibitor reduction profile in hardwood (HW) and softwood (SW) hydrolysate along with their controls (media without any inoculation): a) HW hydrolysate; b) HW control; c) SW hydrolysate; d) SW control

So far, many inhibitory mechanisms of the toxic compounds have been studied and well-explored [28, 56]. Over the past decade, several microorganisms have been identified as capable of degrading the inhibitors. However, these microorganisms would also metabolize monomeric sugars hence compromising further fermentation procedures. Hence, a microorganism capable of biological detoxification of wood hydrolysate in addition to lipid accumulation could be an effective method. Nevertheless, it is advisable to perform further studies aiming at the optimization of the C/N ratio, detailed study of inhibitors degradation, and random or directed mutations, to

improve the fermentation process that could ultimately lead to high cell mass and lipid titer. Likewise, this study can be a baseline to develop novel detoxification processes or pre-treatment strategies to obtain the maximum fermentable sugars as a feedstock for microbial lipid production.

Conclusion

Forestry biomass as a raw material to obtain liquid hydrolysates to produce biofuel feedstock was investigated in the present study. *Rhodosporidium toruloides*-1588 exhibited a high amount of cell biomass (17.09 g/L and 19.55 g/L) and lipid content of 36.68 and 35.24% when grown in hardwood and softwood hydrolysate, respectively. A high abundance of oleic (38.69%), stearic (37.4%) and palmitic acid (43.06%) in lipids makes it viable feedstock for advanced biofuel production. Additionally, the evaluated strain showed tolerance to different inhibitory compounds such as furfural (67 mg/L), 5-HMF (25 mg/L), vanillic acid (34 mg/L), and vanillin (7 mg/L), which can further be explored by subjecting the yeast to high inhibitor concentrations and studying their effect on lipid accumulation and fatty acids composition. This study highlights the significance of *R. toruloides*-1588 as a lipid producer using undetoxified forestry-based wood hydrolysates while setting up the baseline to further explore the stress resistance profile of oleaginous yeast, advanced biofuel production (such as drop-in biofuels) and its improvement using evolutionary engineering.

Supplementary data for this work can be found in Annex A

CHAPTER 5: STRATEGIES TO ENHANCE THE LIPID ACCUMULATION IN *RHODOSPORIDIUM TORULOIDES*-1588

From the previous chapter, hardwood hydrolysate resulted in maximum lipid accumulation which was then used as a culture media and different strategies were employed to increase the lipid accumulation in *R. toruloides*-1588 such as trace metal salt supplementation, carbon to nitrogen ratio and co-fermentation of hydrolysate and crude glycerol. The best strategy was then scale-up to the bench-scale fermentation

5.1. Evaluating the effect of trace metal salts on lipid production ability of *Rhodosporidium toruloides*-1588 using wood hydrolysate as a carbon source

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Abstract

Rhodosporidium toruloides have been considered as a potential feedstock for lipid accumulation. Several studies have been conducted to enhance lipid productivity, however, studies on the effects of trace metals on *R. toruloides*-1588 are limited. In this sense, the present study investigates the effect of five trace metal salts on the lipid accumulation of *R. toruloides*-1588. Trace metal salts such as iron and manganese resulted in the highest cell mass production of 17.5 and 17 g/L, respectively, while magnesium and copper lead to the maximum lipid yield of 40 and 36.7% (w/w), respectively. The maximum lipid accumulation was observed in magnesium-supplemented media and was twice the amount observed in control hydrolysate. Additionally, supplementation of trace metal salts led to the maximum production of linoleic acid of 31.27% along with the production of several long-chain unsaturated fatty acids such as docosahexaenoic acid and eicosatrienoic acid, which will strengthen the potential of *R. toruloides*-1588 as a feedstock not only for biofuel industries but also for pharmaceutical as well as food industries.

Keywords: Lignocellulosic biomass; oleaginous yeast; biodiesel feedstock; long chain fatty acids; linoleic acid.

Introduction

In response to the increasing fuel demand, significant efforts have been made to produce biofuel feedstocks from renewable and sustainable sources. Microbial lipids have been considered as a suitable feedstock for the biofuel production [5]. Microorganisms capable of accumulating more than 20% of their dry cell weight (DCW) into lipids are known as oleaginous microorganisms such as *Yarrowia lipolytica*, *Mortierella ramanniana*, *Rhodococcus opacus*, *Chlorella minutissima*, *Cryptococcus curvatus*, *Rhodosporidium toruloides* and many more. Recently, *Rhodosporidium toruloides*, a haploid yeast with bipolar mating behavior, has been considered a workhorse for the bioprocess industry due to its ability to accumulate 50-60% of lipids per cell dry weight, can thrive on multiple substrates, and tolerate toxic compounds such as furans, phenols, and organic acids [12, 126].

Lipid production at the pilot scale is still limited due to its high production costs. Hence, numerous waste residues have been explored as potential feedstock for lipid production. Out of several waste residues, sugar-rich hydrolysate derived from renewable lignocellulosic biomass has been identified as a potential feedstock for microbial-based lipid production due to its availability in

form of forestry residues, agricultural residues and industrial wastes [10]. Additionally, to further increase the cell mass and lipid productivity as well as to decrease the lipid production cost, different types of fermentations such as batch, fed-batch and continuous fermentation have been studied. Similarly, various culture conditions with different supplementation such as carbohydrates, lipid inducers, salts and carbon to nitrogen ratio optimization have also been performed to increase the lipid productivity in the microorganisms [74, 111, 127]. Likewise, trace metals have also been commonly employed in growth mediums. Common trace metal salts such as zinc sulfate, copper sulfate, ferric chloride, manganese sulfate and magnesium sulfate are being used as micronutrients in complex media such as wood hydrolysate [55, 56, 65, 69, 128]. These micronutrients are known to boost microbial growth and serve as co-factors for several enzymes, ultimately affecting their catalytic activity. For instance, magnesium is a co-factor for malate dehydrogenase, which is involved in the conversion of malate to oxaloacetate resulting in the production of nicotinamide adenine dinucleotide hydrogen (NADH), which further affects the lipid accumulation [129]. Likewise, magnesium is also known as a cofactor for ATP citrate lyase (ACL), an essential enzyme responsible for the lipid synthesis step by converting citrate into acetyl-CoA [130]. Similarly, metals like zinc, manganese and copper are known to promote microbial growth by increasing the activity of growth promoters such as nucleic acid binding proteins, ATP metabolism and several kinases. Furthermore, these metals may vary the fatty acid composition of lipids by altering the catalytical activity of elongase or desaturase enzymes. However, the specific effect of trace metals on fatty acid profile-producing enzymes is still limited and has not been evaluated precisely.

In this sense, the present investigation involves the study of the effects of trace metals such as zinc, copper, iron, magnesium, and manganese on *R. toruloides*-1588 growth, sugar consumption and lipids accumulation ability. In addition, the fatty acid composition of the accumulated lipids was also evaluated to identify the trace metal effect. This study will set a baseline for the scope of trace metals and their role to increase lipid accumulation or to target the production of a specific fatty acid by *R. toruloides*-1588.

Material and Methods

Hardwood sawdust pre-treatment and saccharification

The hardwood sawdust of maple trees was obtained from Quebec Forest Industry Council. Briefly, the dried sawdust was milled using ball milling (Retsch Corporation) at 500 rpm/ 15 min and sieved through the 300 μm to 1 mm sieve and used for the hydrolysate production [131]. The biomass was pretreated using 1% sodium hydroxide with a ratio of 1:10 (*w/v*) at 121℃/15 psi for 30 min. The pretreated biomass was separated from the solution using centrifugation (7000 *x* g/15 min) and washed with tap water. Following, the enzymatic saccharification was performed using Viscozyme-L® and Cellic-Ctec2® enzymes (Sigma Aldrich, USA). The mixture consists of 10% pretreated biomass, 40 FPU/mL of enzyme solution and 50% citrate buffer (pH - 4.8) [74]. The saccharification was performed at 40℃ for 24 h. After the enzymatic reaction, the hydrolysate was separated by centrifugation (7000 *x* g/20 min) and used as a culture media for lipid production.

Microorganism maintenance and inoculum conditions

Rhodosporidium toruloides-1588 was acquired from NRRL culture collection, USA. The strain was maintained and grown in yeast-malt (YM) agar plates and the inoculum was prepared in YM broth [132]. Briefly, a single colony of yeast from YM plates was inoculated in 2 mL YM broth followed by inoculation (1:100 *v/v*) in 5 mL YM media for 18 h. Finally, the yeast was transferred (1:100 *v/v*) in 50 mL YM broth and incubated at 30℃, 180 rpm and used to inoculate the wood hydrolysate.

Lipid production

The lipid production was performed in a 250 mL Erlenmeyer flask using 50 mL wood hydrolysate with a total reducing sugar concentration of 50 g/L. Six different treatments were used containing the trace metals, individually and together. Individual treatments were designed for each trace metal as follows: zinc sulfate (20 ppm), ferric chloride (150 ppm), magnesium sulfate (1500 ppm) [133], copper sulfate (6 ppm), and manganese sulfate (10 ppm) [134]. To study the conjunct effect of trace metals, all the trace metals were added together in the hydrolysate with the concentration mentioned above. Wood hydrolysate with no supplementation was used as a control. All hydrolysates were inoculated with the final 0.3 optical density (OD_{600nm}) of inoculum yeast and incubated at 30℃ and 180 rpm for 120 h. The experiment was performed in duplicates and error bar were calculated by dividing the standard deviation with square root of measurements used to calculate mean.

Lipid extraction

The lipid extraction was performed as described by Osorio-González, et al. [111]. Briefly, the yeast cells were separated using a centrifuge (13000 *x* g for 3 min) and washed with phosphate buffer saline (pH 7.0) twice. The cells were first dried at 60℃ and then were digested at 100℃ for 1h using 1M/L of hydrochloric acid (HCl). Following this step, the lipid was extracted using a chloroform: methanol ratio of 2:1 (*v/v*) using vigorous vortexing for 15min. The sample was mixed with 1 M sodium chloride (NaCl) and centrifuged at 7000 *x* g for 15 min. The organic layer was separated and dried at 60℃ and the changes in tube weights were recorded in grams.

The following equations were used in the study to calculate different parameters:

$$\text{Cell mass yield } (Y_{X/S,}\ g/g) = \frac{Biomass\ (\frac{g}{L})}{Sugars\ consumed\ (\frac{g}{L})} \quad \text{Eq. 1}$$

$$\text{Volumetric cell mass production } (g/\ (L*h)) = \frac{Biomass\ (\frac{g}{L})}{Time\ of\ maximum\ biomass\ production\ (h)} \quad \text{Eq. 2}$$

$$\text{Lipid yield } (Y_{P/S,}\ g/g) = \frac{Lipids\ (\frac{g}{L})}{Sugars\ consumed\ (\frac{g}{L})} \quad \text{Eq. 3}$$

$$\text{Specific lipid accumulation } (mg/\ (g\ DW*h)) = \frac{Lipids\ (\frac{g}{L})}{Biomass\ (\frac{g}{L}) * Time\ (h)} * 1000 \quad \text{Eq. 4}$$

Analytical techniques

Yeast growth analysis

The yeast cells were centrifuged at 13000 *x* g for 3 min and washed with phosphate buffer saline (pH 7.0). The cell biomass was dried at 60℃ until the constant weight and reported in g/L [11].

Sugar analysis

The sugar analysis was performed using HPLC (Orbitrap Elite, Thermo Fisher Scientific Inc., USA) as described by Saini, et al. [74]. Briefly, a C18 column with 200 Å pore size, 150 µm internal diameter and 20 cm length was used. The mobile phase used for the process was acetonitrile + 0.1% formic acid and 0.1% formic acid + water, and the flow rate of 1.2 µL/min.

Standards (e.g., glucose and xylose) were purchased from Sigma, USA and used to prepare the standard curve and sample quantification.

Fatty acid analysis

The fatty acid analysis was performed as described by Osorio-González, et al. [11]. Briefly, extracted lipids were transesterified using methanol: sulfuric (85:15 v/v) at 100°C for 20 min. Fatty acids were extracted using hexane and quantified using gas chromatography (GC-Agilent 7890B). Briefly, GC was equipped with flame ionization detector, DB-23 column with the dimension of 60m x 250 μm and thickness of 0.25 μm.

Statistical analysis

The statistical analysis of biomass production and lipid accumulation was performed using a one-way analysis of variance in OriginPro®2021 software. Statistical significance of the parameters such as cell mass and lipid concentration were compared using a 95% level of confidence Fisher test.

Results and discussion

Effect of trace metals on biomass production and sugar consumption

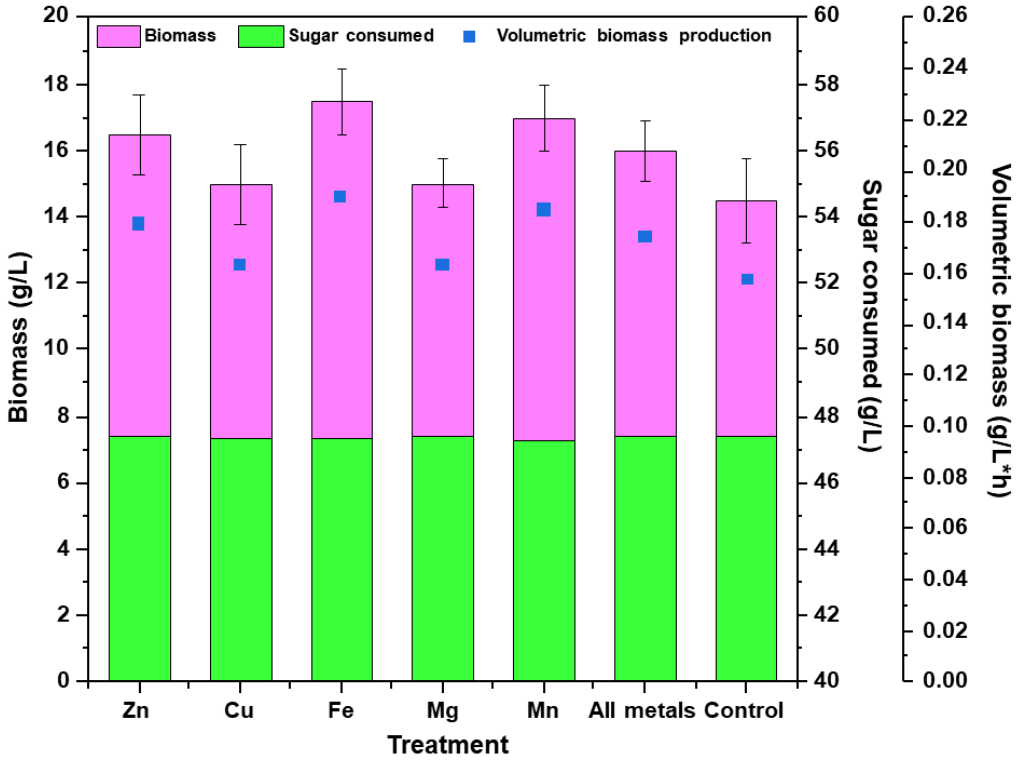

Figure 12. Maximum biomass production, volumetric biomass productivity and sugar consumed in different tested conditions.

The wood hydrolysate consists of 40 ± 1.9 g/L of glucose, 2.5 ± 1.6 g/L of xylose, 60 ± 0.47 mg/L of total nitrogen, 30 ± 0.63 mg/L of furfural and 29.1 ± 0.12 mg/L of 5-hydroxymethyl furfural. Figure 12 shows the cell mass production, sugar consumed and rate of biomass production. The maximum yeast biomass of 17.5 g/L and volumetric cell mass production of 0.19 g/(L*h) was observed in iron-supplemented hydrolysate, followed by 17 g/L in manganese, 16.5 g/L in zinc and 15 g/L in both copper and magnesium supplemented hydrolysate. The lowest cell mass production (14.5 g/L) was observed in control hydrolysate (hydrolysate with no supplementation). Moreover, the addition of trace metal salts increased cell mass of *R. toruloides*-1588 by 0.8 to 1.5-fold compared to the cell mass in the control hydrolysate. More than 95% of total sugars were consumed in all the tested conditions. Moreover, no significant differences were observed in the sugar consumption-ability of *R. toruloides*-1588. This could be because trace metals were used in ppm concentration in the hydrolysate, however, it specifically affected the growth rate of *R. toruloides*-1588, based on type of trace metals. For instance, iron serves as a cofactor for the superoxide dismutase enzyme, therefore increasing its catalytical activity to remove the reactive

oxygen species, which ultimately helps increase the cell mass [135]. Likewise, zinc-supplemented media resulted in higher cell mass (16.5 g/L) than magnesium-supplemented media (15 g/L), this is so, because zinc have higher affinity towards kinases enzymes. These enzymes are responsible for transferring the phosphate among enzymes leading to the activation of series of enzymes involved in yeast growth [136].

Nevertheless, changing the concentration of trace metals might affect the microbial growth. For instance, Mirzaie, et al. [137] reported the 2.76 g/L biomass of *Rhodosporidium diobovatum* grow in glucose media supplemented with 0.5 g/L of magnesium sulfate. Similarly, Antonopoulou, et al. [128] reported the 17.5 g/L of maximum biomass of *Trichosporon fermentans* grown in 40 g/L of glucose supplemented with 60 ppm of manganese sulfate, 1500 ppm of magnesium sulfate and 150 ppm of calcium sulfate. Likewise, increase in biomass production of *Scenedesmus* sp. was observed when grown in increasing magnesium concentrations from 0 to 200 μM. However, reduction in cell mass concentration was reported in media containing more than 2000 μM of magnesium [138]. Hence, microbial growth will increase with the increase in trace metal salts, however, after certain concentration, metals could inhibit the microbial enzymes resulting in decrease in enzymatic activity, ultimately leading towards lower cell growth and increased cell death. Hence, further optimization would be required for each trace metals salts and then optimum concentration can be used to achieve maximum cell mass productivity of *R. toruloides*-1588.

Effect on lipid accumulation

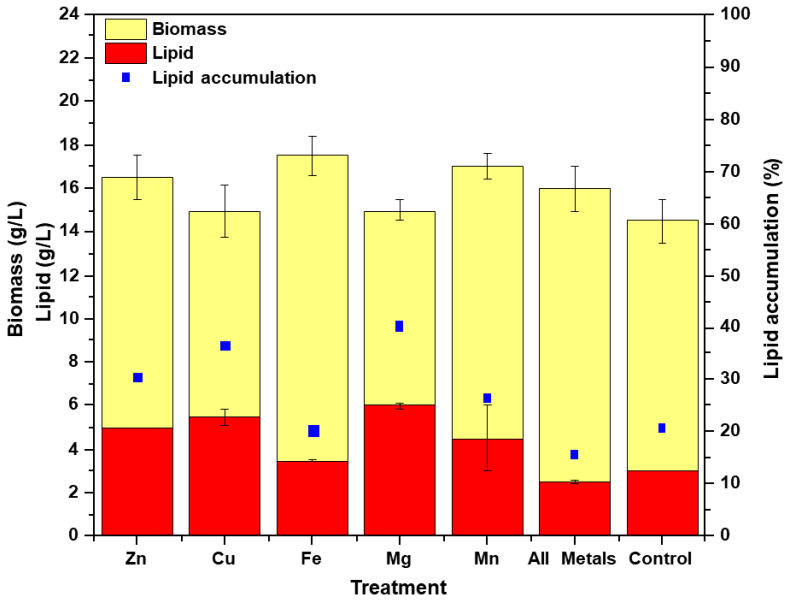

Figure 13. Accumulated lipids by *R. toruloides*-1588 using wood hydrolysate supplemented with different trace metals.

Figure 13 illustrates the lipid accumulation by *R. toruloides*-1588. The maximum lipid accumulation of 40% (*w/w*) was observed in hydrolysate supplemented with magnesium and was double the amount observed in control hydrolysate (hydrolysate without any supplementation), followed by 36.7% in copper-supplemented, 30% in zinc-supplemented, 26% in manganese-supplemented and 20% in the iron-supplemented hydrolysate. The lowest lipid accumulation of 15.62% (*w/w*) was observed in media containing all the trace metals. Furthermore, the maximum specific lipid production rate was observed as 4.3 mg of lipids/g of dry cell weight per hour in magnesium-supplemented media as shown in Figure 14. Interestingly, the addition of iron resulted in high biomass yield but lower lipid yield of 0.07 g/g of substrate consumed which is ~2 times lower lipid yield than magnesium-supplemented media. Similarly, yeast grown in copper-supplemented media resulted in higher lipid yield (0.12 g/g) as well as specific lipid production rate (3.98 mg/ (g dry weight*h)) despite the lower biomass (15 g/L) than observed in iron-supplemented media (17.5 g/L). This points to the fact the addition of trace metal salts resulting in high biomass does not necessarily lead to high lipid accumulation.

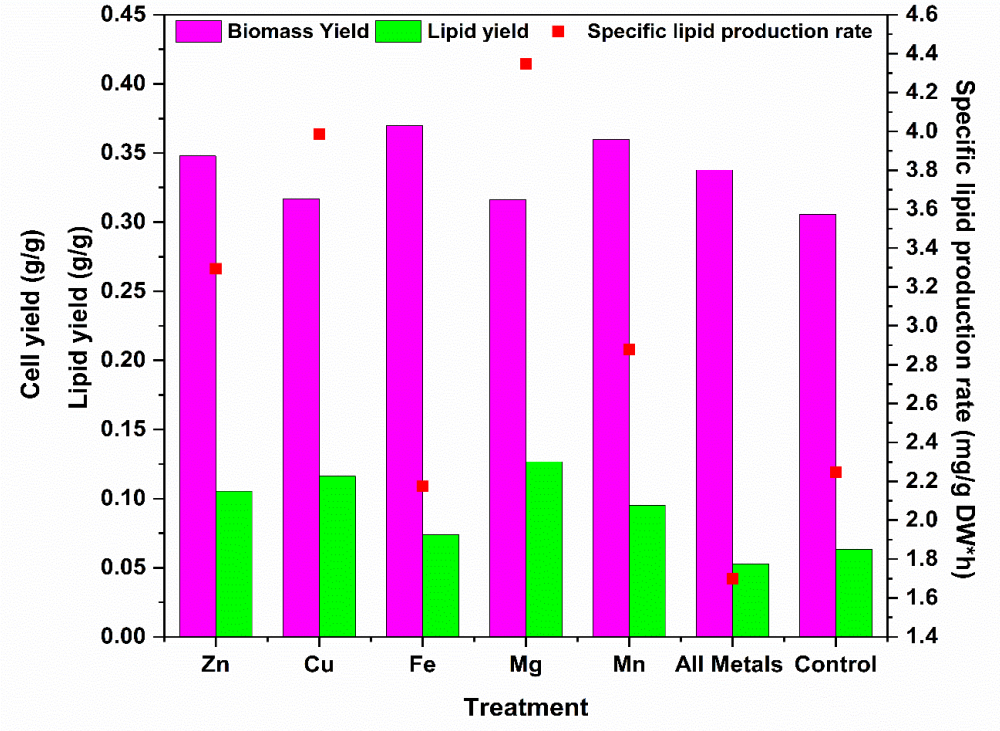

Figure 14. Illustration of maximum biomass yield (g/g), lipid yield (g/g) and specific lipid production rate (mg/g of dry biomass weight*h).

The highest lipid productivity (4.3 mg/(g*h)) in magnesium-supplemented hydrolysate implies the fact that magnesium might have influenced the lipid accumulation by altering the enzymatic activities involved in the lipid production pathway. This is so, because magnesium is known to act as a cofactor for malate dehydrogenase, which in turn provide the electron carriers for the lipid accumulation pathway [129]. Hence the addition of magnesium might have increased its catalytic activity resulting in enhanced production of NADH, ultimately increasing the lipid accumulation. Similarly, magnesium is also known to act as a cofactor of the ATP citrate lyase (ACL) [130]. ATP citrate lyase involves the conversion of citrate into acetyl-CoA, which leads to the formation of a fatty acid complex eventually accumulating the lipids. This step is also known as the rate-limiting step in the lipid accumulation [3]. Hence, increasing the activity of ACL will increase the acetyl-CoA production, finally leading to enhanced lipid accumulation in microorganisms. On the other hand, the synergistic effect of trace metals resulted in the lowest lipid production rate of 0.6 mg/g of substrate per hour was observed (Figure 14). This could be because multiple metals might have affected the enzymes involved in lipid accumulation through allosteric competition. For instance, manganese or iron and copper or zinc is a cofactors of superoxide dismutase depending on the protein folding, which involves eliminating the reactive oxygen species hence promoting the cell growth [5, 135]. Likewise, magnesium binding sites in β-galactosidase, alkaline phosphatase or various kinase enzymes are mostly vulnerable to zinc due to their similar di-valency and difference in free energy [136]. Hence, hydrolysate supplemented with all the metal salts resulted in the competitive inhibition for enzyme binding sites causing variation in microbial growth and lipid accumulation.

The present study involves the utilization of undetoxified wood hydrolysate while several articles have reported similar lipid yields using detoxified wood hydrolysate as substrate. For instance, Matsakas, et al. [56] reported 39% of lipid accumulation in *R. toruloides* using detoxified wood hydrolysates. Similarly, Zhao, et al. [65] utilized the detoxified sugarcane bagasse-derived hydrolysate as a substrate and reported the 52.5% (*w/w*) lipid in *R. toruloides* AS 2.1389. Likewise, Yu, et al. [66] observed 24.6% lipid accumulation when *R. toruloides* grew in the detoxified wheat straw hydrolysate. On the other hand, Huang, et al. [139] reported 23.5% (*w/w*) lipid accumulation

in *Trichosporon cutaneum* grown in detoxified corn stover hydrolysate supplemented with magnesium sulphate, ammonium sulphate and potassium dibasic salts. Likewise, Xu, et al. [53] reported 70% of lipid accumulation in *R. toruloides* AS2.1389 grown crude glycerol and inorganic salts.

Nevertheless, the type of carbohydrate, as well as supplementation, could increase the lipid accumulation at the laboratory scale but it will also impact the production cost at the industrial scale which is the biggest challenge in biorefinery industries. In this study, the addition of trace metals in undetoxified wood hydrolysate resulted in a two-fold increase in lipid accumulation to the amount observed in control hydrolysate, so these trace metal salts can also directly impact lipid titer and its production rate.

Fatty acid composition

Table 6. Effect of trace metal salts on fatty acid compositions shown in relative percentage

Unsaturated fatty acid (%)	**Zn^{2+}**	**Fe^{2+}**	**Cu^{2+}**	**Mg^{2+}**	**Mn^{2+}**	**All Metals**	**Control**
Palmitoleate	0.32	0.39	0.4	0.38	0.4	0.5	0.41
Hexadecadienoic acid	0.02	0.02	ND	0.02	0.02	ND	0.09
Hexadecatrienoic acid	0.07	ND	0.09	ND	ND	ND	ND
Elaidic acid	0.99	0.9	1.51	0.83	0.99	1.05	1.66
Oleic acid	34.76	39.68	37.97	40.06	41.8	41.71	40.74
cis-Vaccenic acid	0.08	0.05	0.18	0.08	0.11	0.56	0.33
Linolelaidic	0.23	0.22	0.22	0.21	0.23	0.2	0.03
Linoleic acid	31.27	30.64	29.56	0.16	30.69	26.93	28.05
Gamma-linolenic acid	0.22	0.2	0.12	0.01	0.03	0.11	0.07
Octadecatrinoic acid	ND	ND	0.15	0.14	0.11	2.59	0.14
Linolenate	6.14	5.37	2.93	4.85	3.2	0.58	2.73
Eicosenoate	0.06	0.11	0.1	0.1	0.11	0.14	0.13
Eicosadienoic acid	0.15	0.1	0.15	0.12	0.19	0.15	0.17

Unsaturated fatty acid (%)	Zn^{2+}	Fe^{2+}	Cu^{2+}	Mg^{2+}	Mn^{2+}	All Metals	Control
Dihomo-gamma-linolenic acid	0.13	0.12	0.13	0.11	0.12	0.1	0.13
Eicosatrienoic acid	ND	ND	ND	0.03	ND	ND	ND
Eicosatetraenoic acid	0.5	0.23	0.16	0.04	0.03	0.25	0.17
Eicosapentaenoic acid (EPA)	0.1	0.04	0.07	0.05	0.06	0.06	0.04
Erucic acid	ND	ND	0.05	ND	0.03	0.04	0.03
Docosadienoate	0.38	0.44	0.5	0.05	0.04	0.65	0.31
Nervonic acid	0.08	0.06	ND	0.04	0.03	ND	0.05
Docosahexaenoic acid	ND	ND	0.05	ND	ND	ND	ND

ND: not detected

Table 6 displays the fatty acid composition of lipids obtained from *R. toruloides*-1588 in relative percentage. Fatty acids, such as oleic acid (34.76 – 41.71%), linoleic acid (26.93 – 31.27%), and linoleate (0.58 – 6.14%) were found in abundance. The maximum linoleic acid (omega 6) was observed to be 31.27 %, which is five to six times higher than the reported value in the literature [111, 127, 140, 141]. On the other hand, several unsaturated fatty acids including essential fatty acids, such as octadecatrienoic acid, eicosatrienoic acid, gamma-linolenic acid and docosahexaenoic acids have also been observed. These are high-value compounds, for instance, docosahexaenoic acid provides aid in the cardiovascular system and eye functioning. Similarly, gamma-linolenic acid has anti-tumour properties [5]. Thus, the production of essential polyunsaturated fatty acids could expand the application of *R. toruloides*-1588 lipids in the food as well as pharmaceutical industries. Moreover, the addition of trace metal salts resulted in the production of specific fatty acids in high concentrations. For instance, eicosatrienoic acid (0.03%) was observed only in media supplemented with magnesium while the addition of copper resulted in the production of docosahexaenoic acid (DHA) (0.05%). Similarly, zinc led to the highest production of linoleic acid (31.27%). In general, the production of fatty acids is mediated by desaturase and elongase enzymes [142]. For instance, δ4-desaturase involves in conversion of ω-3 eicosapentaenoic acid into DHA. Similarly, eicosatrienoic acid production requires the activation

of δ9-desaturase. Hence, the presence of DHA in copper supplemented media and eicosatrienoic acid in magnesium supplemented media points to the fact that copper might have affected the catalytic activity of the δ4-desaturase enzyme and magnesium on δ9-desaturase activity (Supplementary Fig. 1).

This is a well-known fact that the enzyme's activity varies in the presence of several inducers and cofactors. In addition, *R. toruloides* is also known to accumulate carotenoids thus the trace metal salts could also affect the activity of enzymes involved in carotenoid production. Moreover, interaction studies between fatty acids and carotenoids producing enzymes with trace metals are still limited and have hardly been reported, especially in the case of the eukaryotic system such as *R. toruloides*-1588. Hence, it would be advisable to further explore the effect of trace metal salts on long-chain unsaturated fatty acids production and increase the specific fatty acids accumulation and carotenoids. For instance, the microorganisms can be subjected to specific metal salts using minimal media will lead to the increased catalytic activity of the desired enzyme eventually varying the lipid accumulation, fatty acid profile and carotenoids.

Nevertheless, the major concern of microbial-based biofuel production is the high production cost associated with parameters such as enzymatic saccharification, low biomass yield, low lipid titer and pre-treatment requirement of lignocellulosic biomass. Thus, it is imperative to explore further techniques to enhance microbial-based lipid production as well as to decrease the biofuel production cost. For instance, simultaneous saccharification and fermentation (SSF) can be performed. Using the SSF technique, polymeric sugars can be converted into the monomeric form using enzymes following which lipid-producing microorganisms will then convert those carbohydrates into lipids. Similarly, the co-culture technique can also be developed such that a microorganism can convert the polymeric sugars of lignocellulosic biomass to the monomeric form while the other microorganism then converts the sugars into lipids, thus, eliminating the need for enzymes and ultimately affecting the biofuel production cost.

Conclusion

The addition of trace metal salts resulted in enhanced lipid accumulation of 40% *w/w* as well as also led to the production of essential long chain fatty acids such as docosahexaenoic acid, eicosatrienoic acid, hexadecatrienoic acid and linoleic acid. It symbolizes the fact that fatty acids produced from *R. toruloides*-1588 can also be utilized in food and pharmaceutical industries along with biofuel industries. Moreover, the present investigation suggests that trace metals salts are

useful in increasing lipid accumulation as well as targeting the specific desaturase or elongase enzyme to produce desired fatty acids which is necessary to decrease the final production cost of biofuel.

Supplementary data for this work can be found in ANNEX B

5.2. Effect of creating a fed-batch-like condition using carbon to nitrogen ratios on lipid accumulation in *Rhodosporidium toruloides*-1588

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Abstract

Utilizing the undetoxified wood hydrolysate to accumulate maximum lipids in *Rhodosporidium toruloides* under optimum conditions has been regarded as a renewable and cost-effective strategy. The current investigation aims to identify the best carbon to nitrogen (C/N 20, 70, and 120) ratio for maximum lipid accumulation in *R. toruloides*-1588 using wood hydrolysate. Additionally, a fed-batch-like condition was employed, where C/N ratios were maintained during the fermentation which inherently decreases in batch fermentation. The C/N ratio of 70 has been identified as the best condition with 3 times higher lipid accumulation (43% *w/w*) than the control. Additionally, more than 95% and 70% of glucose and xylose consumption were observed, respectively. Moreover, a 50% increase in polyunsaturated fatty acids compared to the control media reinforced the potential of *R. toruloides*-1588 to thrive on undetoxified hydrolysate, high lipid productivity (3.8 mg/g of dry weight per hour) and produce high-value monosaturated and polyunsaturated fatty acids.

Keywords: *Rhodosporidium toruloides*; microbial lipids; lignocellulosic biomass; carbon to nitrogen ratio; polyunsaturated fatty acids

Introduction

The urge to develop the bioeconomy has increased the interest in producing materials, chemicals and fuel feedstocks from sustainable and renewable sources having a low environmental impact. Over the past years, oleochemical production from microorganisms has drawn a lot of attention such as fatty acids, surfactants, lubricants and plastics [143]. Microbial lipids are one of the known sources of fatty acids. Meanwhile, the fatty acids methyl esters and fatty alcohol derived from microbial lipids have been considered as potential feedstock for oleochemicals as well as for biofuel production [5]. In general, microorganisms capable of accumulating >20% lipid in form of triacylglycerides (TAG) are known as oleaginous microorganisms. These microorganisms accumulate lipids under nutrient stress conditions whereas the carbon-to-nitrogen ratio plays a key role in promoting lipid accumulation [28].

Among several oleaginous microorganisms, *R. toruloides* have been exploited over the past decade and are considered the workhorse for biotechnological applications [12]. *R. toruloides* is known to achieve 100 g/L of biomass and 60-70% lipids of its dry cell weight. In addition to lipid accumulation, *R. toruloides* is a natural producer of carotenoids such as β-carotene or lycopene

[144]. It is also known to thrive on multiple substrates such as glucose, xylose, crude glycerol and hydrolysate derived from lignocellulosic biomass. *R. toruloides* has also demonstrated tolerance toward several toxic compounds such as furans, organic acids and phenols [98, 145]. The ability to accumulate high lipid and co-products using low-cost renewable substrate can be an important economic consideration when producing biofuel on a larger scale. Lignocellulosic biomass has been identified as a renewable, cost-effective and sugar-rich source (C5 and C6) for microbial lipid accumulation. Lignocellulosic biomass is a complex of cellulose, hemicellulose and lignin which requires a pre-treatment to break down the complex linkage allowing the cellulolytic enzymes to convert the polymeric sugars into the monomeric form [146]. In terms of lignocellulosic biomass, Canada is the world's second-largest country with 35% of its land covered with forests [8]. Hence, this could be a sustainable and renewable option for microbial lipid production.

In general, factors such as type of fermentation, carbon to nitrogen (C/N) ratio, media composition and inducers are known to affect the lipid accumulation in microorganisms [12]. Hence, finding the best condition to achieve high lipid titer is essential for large-scale biofuel production. Over the past decade, several articles have been published to achieve high lipid accumulation through different types of fermentation and C/N ratio optimization [57, 116, 143]. For instance, Boviatsi, et al. [147] observed a 30% increase in lipid content in *R. toruloides* cultured in fed-batch fermentation than in batch fermentation using molasses as culture media. Likewise, several reports on fed-batch fermentation have been published aiming to maintain initial carbon concentration during fermentation that may increase the accumulation of lipids in oleaginous microorganisms [148, 149]. On the other hand, C/N ratio optimization has also been known to play an important role in enhancing lipid accumulation [28]. It has been estimated that lipid accumulation is stimulated at a C/N ratio >20 and decreases at C/N > 140 [81, 116, 143, 150, 151]. However, most of the literature report has maintained the C/N ratio at the initial stage of the fermentation, which decreases during the batch fermentation, while the study on C/N ratio maintenance during the fermentation is limited [116, 143]. In this sense, maintaining only the C/N ratio through the fermentation would create the fed-batch-like condition, which might lead to enhanced lipid accumulation in oleaginous microorganisms. Contrary to fed-batch fermentation, the present study involves the addition of carbon or nitrogen to maintain the C/N ratio of hydrolysate during the fermentation could be called pseudo-fed-batch fermentation.

The present investigation aims to evaluate the effect of maintaining C/N ratios during wood hydrolysate fermentation. The further aim is to find the optimum condition to achieve maximum lipid accumulation in *R. toruloides*-1588 using undetoxified hydrolysates. This study will also evaluate the effects of maintaining the C/N ratio on lipid accumulation, fatty acid profile, sugar consumption and biomass production of *R. toruloides*-1588.

Materials and methods

Lignocellulosic biomass pre-treatment and enzymatic saccharification

Hardwood sawdust derived from maple trees was kindly donated by Quebec's Forest Industry Council. The sawdust was dried at 50±1℃ for 12-24 h. The sawdust was milled to fine particles using a planetary ball mill (Retsch Corporation; PM 100) at 500 rpm for 15 min. The resulting biomass was sieved through a 300µm - 1mm sieve and subjected to pre-treatment and enzymatic saccharification. The hardwood sawdust was pre-treated using a dilute alkaline solution (1% NaOH). The solid to liquid alkali solution ratio was kept at 1:10 (*w/v*). The slurry was autoclaved at 121℃/15 psi for 30 minutes. The solid fraction was separated using centrifugation (7000 *x*g/15 min) and washed with tap water. The pretreated biomass was dried at 45℃ for 30 min [132].

The enzymatic saccharification was performed using Cellic-Ctec (cellulase blend) and Viscozyme-L procured from Sigma Aldrich. The reaction mixture was prepared with 10% pre-treated biomass (*w/v*), equal ratios of enzymes to obtain the activity of 40 FPU/mL, and 50% citrate buffer (*v/v*) of pH 4.8. The reaction was performed in a Multitron shaker at 40℃ for 24 hours. After the reaction time, the reaction mixture was centrifuged, and hydrolysate was kept at -20℃ until further use.

Yeast growth and inoculum preparation

R. toruloides-1588 was procured from NRRL culture collection, USA. The yeast was grown and maintained in Yeast-Malt (YM) agar plates consisting of malt and yeast extract (3.0 g/L each), peptone (5 g/L), glucose (10 g/L) and bacteriological agar (1.5%).

To prepare the inoculum, yeast was pre-cultured three times in YM media. Briefly, a single yeast colony was picked and used to inoculate 2 mL YM broth and incubated at 25℃, 180 rpm for 18 hours. After the incubation time, the inoculum was transferred to 5 mL of YM broth with a ratio of 1:100 (*v/v*) and incubated. Lastly, 50 mL YM media was inoculated with the ratio of 1:100 *v/v* and incubated at 25℃ and 180 rpm.

Lipid production media and treatment conditions

Table 7. Different media compositions used for lipid production in *R. toruloides*-1588

Treatment	**Total Sugar (g/L)**	**Ammonium Sulphate (g/L)**	**Conditions**
C/N 20	50	4.6	C/N ratio was maintained during the fermentation
C/N 70	50	1.33	
C/N 120	50	0.79	
C/N 20-C	50	4.6	C/N ratio was maintained before yeast inoculation only i.e., control
C/N 70-C	50	1.33	
C/N 120-C	50	0.79	
Control	50	0	Yeast inoculation
NC (Negative control)	50	0	No inoculation

The wood hydrolysate was used for lipid production in *R. toruloides*-1588 and was carried out in a 250 mL Erlenmeyer flask. Table 7 shows the different media compositions used in this investigation. The initial total reducing sugar concentration was kept as 50g/L in hydrolysates. Three different carbon to nitrogen ratios were studied i.e., C/N 20, C/N 70 and C/N 120. Ammonium sulphate was used as a nitrogen source to maintain the carbon to nitrogen ratio in the hydrolysates. In general, ammonium sulphate contains 21% of nitrogen and sugars contain 40% of carbon. In treatments, the C/N ratio was maintained at different time points during the yeast fermentation i.e., zero hours, 36h, 72h, 96h and 140h. The C/N ratio was maintained using either ammonium sulphate in case of high C/N ratio or using hydrolysate in case of lower C/N ratio to achieve the desired ratio. In their respective controls, nitrogen source was added in hydrolysates to achieve the desired C/N ratio before the yeast inoculation only. In control, no nitrogen source was introduced in the hydrolysate and was inoculated with yeast, while in the negative control, no inoculation was performed in the hydrolysate.

For the lipid production, the yeast from the third-generation inoculum was centrifuged (5000 x g for 3 min) and washed with phosphate buffer saline (PBS – pH 7.0) and then used to inoculate the

hydrolysates with an optical density (OD_{600nm}) of 0.3. The hydrolysates were incubated at 25℃ and 180 rpm. The experiments were performed in duplicates.

Lipid extraction

Lipid extraction was performed as per Saini, et al. [132]. The yeast cells were separated from the broth by centrifuging the media at 15,000 *x* g for 3 min. The cells were washed with PBS and dried at 60℃ until the constant weight. The cells were lysed by boiling in presence of 1M/L HCl for 1 hour. After an hour, 3.75 mL of chloroform: methanol ratio (2:1*v/v*) were added and vortexed for 15 min, followed by the addition of 1.25 mL of chloroform. The sample was vortexed again for 1 min. Lastly, 1M NaCl (1.25 mL) was added to the solution and centrifuged (7000 x g for 15 min. The aqueous layer was removed, and the organic layer was dried at 60℃ until the constant weight.

Analytical methods

Cell growth analysis

The grown yeast was centrifuged at 15000 x g for 3 min and washed with PBS, pH-7.0. The cells were dried at 60℃ until the constant weight. The dry cell weight (DCW) was measured in g/L.

Inhibitors, sugar, and nitrogen analysis

Inhibitors and sugar analysis were performed using HPLC-MS (Orbitrap Elite, Thermo Fisher Scientific Inc). Briefly, the C18 column was used with 20 cm length, 200 Angstrom pore size, and 150 µm of internal diameter. The mobile phase includes 0.1% formic acid + water and acetonitrile + 0.1% formic acid and the flow rate was kept at 1.2 µL/min. The sample injection volume used was 1 µL. A positive ionization mode was used for mass detection. On the other hand, total nitrogen was analyzed using the Total Nitrogen Reagent Set (Product #: 2714100; HACH, Canada). The kit uses the Persulfate digestion method with a detection range of 10 – 150 mg/L.

Fatty acid determination (FAME)

The fatty acid was quantified according to the method described by Osorio-González, et al. [113]. Briefly, lipid extract was esterified by boiling in presence of methanol: sulfuric acid (85%:15%) for 20 minutes. Following, the sample was cooled and 500 µL of hexane was added to extract the FAME in hexane. The fatty acid composition was quantified in gas chromatography (Agilent 7890B).

Statistical analysis

The statistical analysis was conducted using OriginPro software. The parameters such as lipid accumulation, nitrogen consumption and biomass production were evaluated by one-way ANOVA (analysis of variance). The significance value was calculated using the Fisher test at a 95% level of confidence. Pearson correlation was used to find the correlation value (r) between different lipid accumulated, biomass produced, and nitrogen consumed.

Results and discussion

Effect of carbon/nitrogen ratio on biomass production and sugar utilization on *Rhodosporidium toruloides*-1588

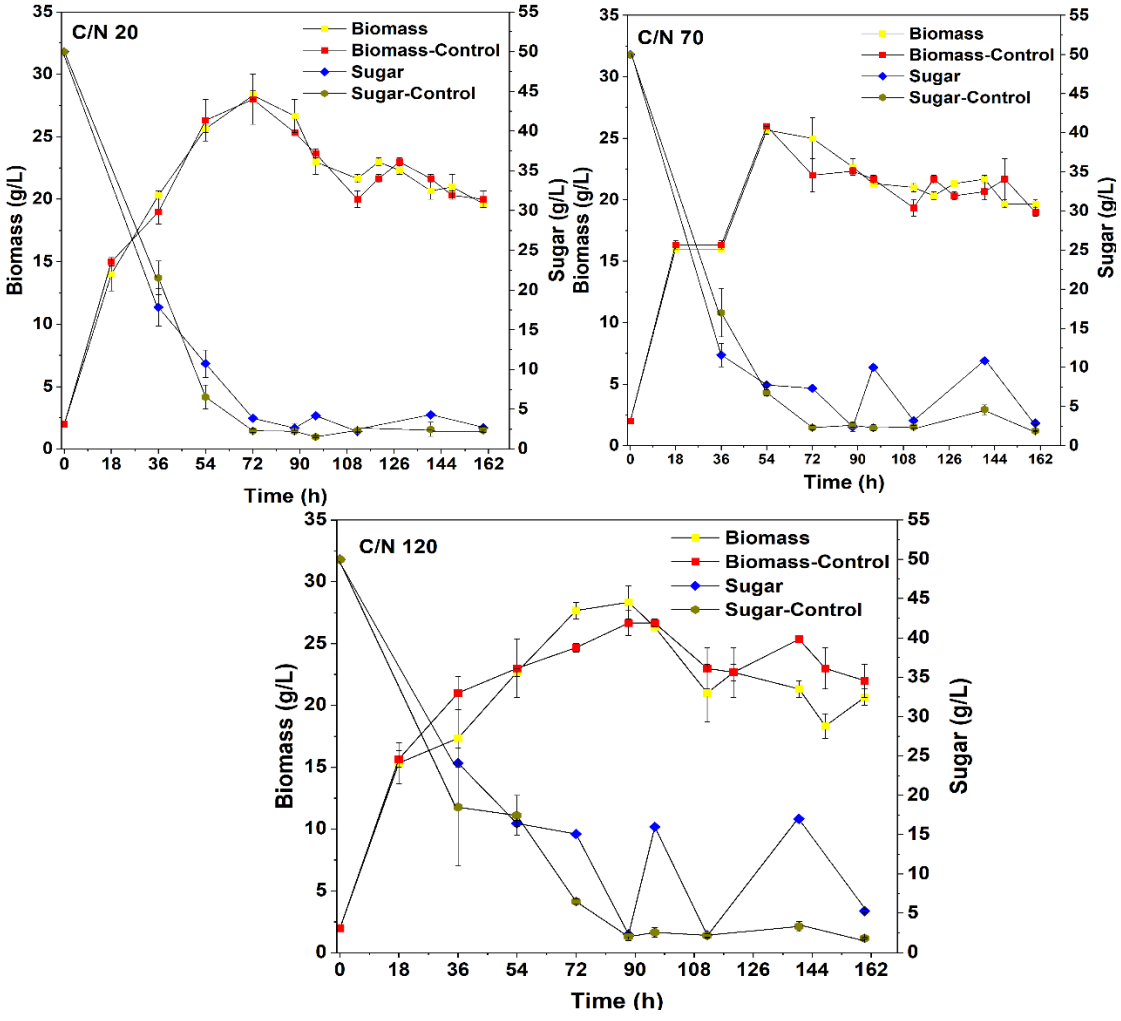

Figure 15. Biomass production and total sugar reduction profile of *R. toruloides*-1588 in different C/N ratios; whereas: control is the media with no C/N ratio maintenance.

The hydrolysate consists of glucose (36 ± 2.3 g/L), xylose (2.1 ± 0.12 g/L), total nitrogen (50 ± 0.37 mg/L), furfural (30 ± 2.1 mg/L) and 5-hydroxymethyl furfural (35.1 ± 1 mg/L). Figure 15 displays the biomass production and sugar profile of *R. toruloides*-1588 in different media conditions. As shown in the figure, C/N ratio 20 led to the production of maximum biomass of 26.66g/L at 88 hours while 21g/L biomass was achieved at 120 hours cultivated in C/N ratios 70 and 120. Moreover, maintenance of the C/N ratio did not change the biomass significantly (p-value = 0.72). In general, 90% of the sugar concentration was reduced after 72 hours of fermentation resulting in yeast entering the stationary phase. Likewise, total nitrogen concentration was reduced to 50 – 60mg/L after 72 hours of fermentation, which led to a change in the C/N ratio of the media. Hence, to maintain the C/N ratio of hydrolysate, the amount of carbon or nitrogen was decided based on the C/N ratio left in the hydrolysate. For instance, in treatment C/N 120, after 100 hours, the total nitrogen concentration left was 53 mg/L and the total sugar concentration left was 3g/L which gave a C/N ratio of 22. Hence, to increase the C/N to 120, hydrolysate was added resulting in an increase of the total sugar concentration to 16 g/L which in turn increased the C/N ratio to 120 and can be easily seen as a spike in Figure 1. The maximum increase was seen in C/N ratio 120 followed by C/N ratio 70, while in C/N 20 only 3-4 g/L of sugar was required to achieve the desired C/N ratio, which was done by adding hydrolysate (Figure 1). However, at 36 h of fermentation, nitrogen was added to the media when *R. toruloides*-1588 was in an exponential phase, which resulted in a high rate of sugar consumption concerning the nitrogen consumption rate leading to the increase in the C/N ratio in the hydrolysates. For instance, at 36h, total carbon and nitrogen left in the media were 6.26 and 0.121g/L, respectively, increasing C/N from 20 to 52. Hence, to decrease the C/N ratio, ammonium sulphate was added to C/N 20 and 120.

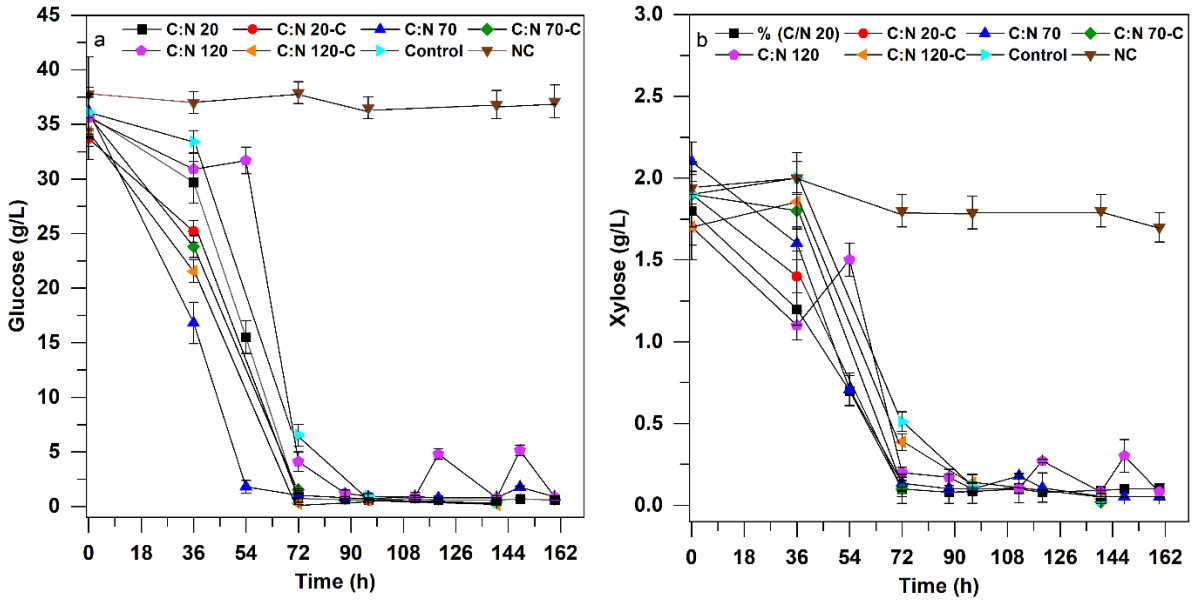

Figure 16. Illustration of C6 and C5 sugar profile in hydrolysate: a) glucose consumption profile and b) xylose consumption profile; where C/N 20, 70, 120-C is the ratio maintained before inoculation; C is control (hydrolysate with yeast inoculation only) and NC is negative control (hydrolysate without inoculation)

Figure 16 displays the glucose and xylose consumption profile in different C/N ratios. As shown in Figure 16, more than 90% of glucose present in the hydrolysate was consumed in 72 h, while xylose was consumed approximately 75 - 80% in 72 h in comparison to its control. There is no denying the fact that glucose is the preferred carbon source for microbial growth. For instance, in the present study, 40% of glucose has been consumed till 36h of fermentation, whereas only 25% of xylose consumption was observed at the same time. In general, upon being subjected to multiple sugars, microorganism tends to metabolize sugars sequentially starting with glucose because glucose has been known to suppress the conversion of other sugars through allosteric competition or catabolic repression [152]. Generally, the wood hydrolysate consists of different C6 and C5 sugars, while the more challenging part is the maximum consumption of these C5 and C6 sugars by the microorganisms. Several oleaginous strains have been grown in media containing different sugars to evaluate their consumption potential. For instance, Wiebe, et al. [153] reported the 95% consumption of glucose and 50% of xylose in *R. toruloides* when grown separately in glucose and xylose media. On the other hand, Ong, et al. [154] observed only 28% consumption of xylose by *Yarrowia lipolytica* cultured in sugarcane bagasse hydrolysate, which further decreased when the

concentration of xylose was increased. Similarly, Karnaouri, et al. [142] observed the increase in doubling time of *Crypthecodinium cohnii* when grown to different types of sugars. The authors reported the doubling time of *C. cohnii* as 8.4 h, 14.9 h and 39.4 h for glucose, xylose and arabinose-containing media, respectively. Additionally, the maximum sugar consumption was observed in glucose (86%) followed by arabinose (46%) and xylose (23%) media.

Interestingly, *R. toruloides*-1588 was not only able to grow but also consumed more than 70% of xylose. It can be further increased by subjecting the yeast to increasing the concentration of C5 sugars. For instance, yeast can be first grown in glucose and then could be employed in xylose-containing media hence affecting the biomass as well as lipid accumulation in yeast. Hence, it is highly desirable to have microorganisms able to consume multiple sugars which would ultimately aid in the circular economy and economical biofuel production.

Effect of carbon/nitrogen ratio and nitrogen consumption on lipid accumulation in *R. toruloides*-1588

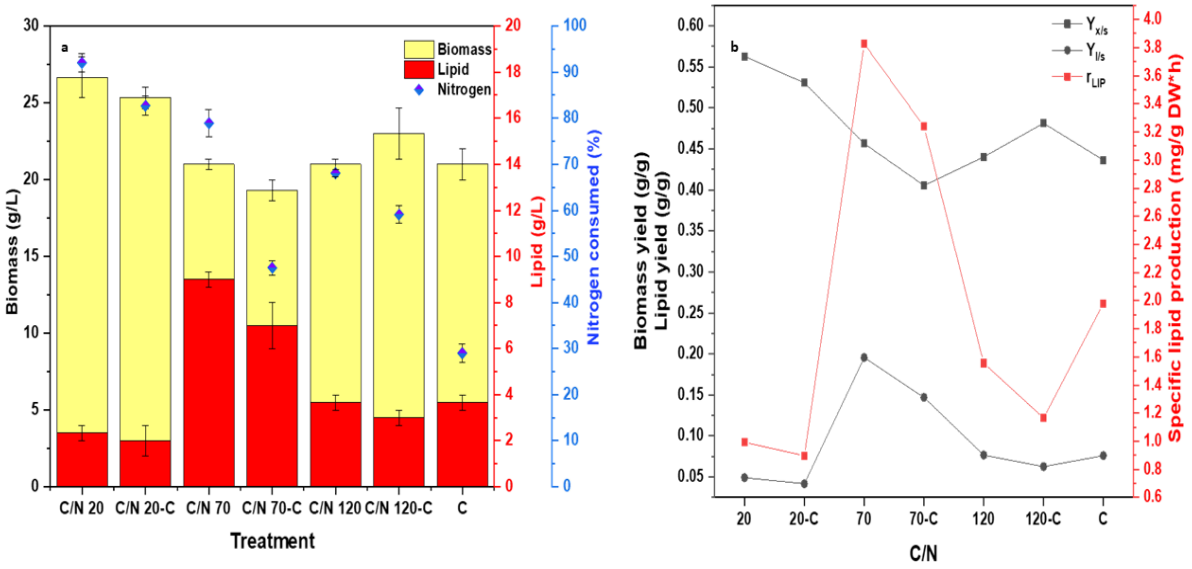

Figure 17. Demonstration of the effect of C/N ratios on different parameters: a) lipid accumulation, nitrogen consumption and corresponding biomass concentration; b) biomass yield ($Y_{X/S}$), lipid yield ($Y_{L/S}$) and specific lipid production (r_{LIP}); whereas C is control hydrolysate.

The maximum lipid accumulation was observed at 88h for C/N ratio 20 and control, while, at 112h for C/N ratio 70 and 120. The maximum lipid, biomass and nitrogen consumption is shown in

Figure 17a. C/N 70 was found to be the best condition (p-value < 0.05) to achieve maximum lipid accumulation as 0.43g of lipid per gram of dry biomass, followed by its control, 0.36 g of lipid per gram of dry biomass. On the other hand, despite having maximum biomass in C/N 20, the maximum lipid accumulation was observed as 8% (*w/w*). Moreover, a negative Pearson correlation (r = -0.25) was observed between biomass and lipid obtained in different C/N ratios. In other words, lipid accumulation was decreased with an increase in biomass when subjected to different C/N ratios. Also, the C/N ratio maintenance in the hydrolysate led to the increased consumption of nitrogen for their controls. Moreover, maintenance of C/N ratios had a significant effect (p-value <0.05) on nitrogen consumption in the hydrolysate. For instance, maximum nitrogen consumption was observed in a C/N ratio of 20 to 90% which was 10% higher than its control (Figure 17a). Similarly, in the C/N ratio 70, nitrogen consumption increased from 47.5% to 78.9% which is ~35% higher than its control. As the C/N ratio was maintained over time, a culture eventually triggered the metabolic shift towards lipid accumulation and nitrogen was consumed much faster than its control resulting in extensive accumulation of lipid in yeasts. Additionally, an increase in nitrogen consumption in different C/N ratios led to an increase in biomass but a decrease in lipid accumulation (Figure 17). Pearson correlation between nitrogen consumed vs biomass produced was slightly positively correlated (r = 0.168) while, between nitrogen consumed vs lipid accumulated was found to be negatively correlated (r = -0.33). Consequently, high nitrogen concentration such as in C/N 20 sustain the cell reproduction leading to high dry cell weight and did not encounter the nitrogen limitation resulting in lower lipid accumulation. In other words, the nitrogen deficiency could have negative consequences on biomass production and substrate consumption which ultimately affect the lipid yield. Hence, an optimum C/N ratio is required for the maintenance of biomass production as well as enhanced lipid accumulation. Figure 17b displays the biomass yield, lipid yield and lipid productivity in different C/N ratios. As expected, lipid yield (g/g) and specific lipid productivity (mg/g DW/h) increased while biomass yield decreases with the increase in C/N ratio. Moreover, the maximum lipid was observed after 80h where nitrogen concentration was reduced to 50 mg/L. This points to the fact that carbon shift towards lipid accumulation triggered only after total nitrogen availability drop below a critical level. The maximum lipid production was 3.9mg/g of dry weight per hour in C/N 70, which is 4 times higher than C/N 20, while maximum biomass yield on the substrate was observed in C/N 20 as 0.56g/g. Similarly, Calvey, et al. [81] reported the maximum lipid content of 55% and

productivity of 0.06g/L/h in *Lipomyces starkeyi* cultured at C/N ratio 72, which was 3 times higher than cultured in C/N ratio 24. Also, the optimum C/N ratio to achieve maximum lipid accumulation varies with the strains and media conditions. For instance, Kommoji, et al. [134] reported the C/N ratio of 30 as the optimum condition to achieve maximum lipid accumulation (49%) in *Y. lipolytica* MTCC 9515. Similarly, Lopes, et al. [143] observed the maximum lipid accumulation of 52% (*w/*w) at C/N 100 in glucose media while 60% (*w/w*) at C/N 120 in xylose media in *R. toruloides* CCT 0783. Hence, it is highly desirable to find the optimum C/N ratio for the desired strain to achieve maximum lipid accumulation. On the other hand, the type of fermentation also plays an important role. For instance, in the present study, C/N ratio maintenance created the fed-batch-like condition which led to the maximum lipid accumulation of 43% (*w/w*) in C/N ratio 70. There are several articles published on *Rhodosporidium* sp. cultivated under different conditions. For instance, Boviatsi, et al. [147] achieved 61% (*w/w*) of lipid accumulation in *R. toruloides* Y-27012 cultivated using molasses under fed-batch fermentation. Similarly, Lopes, et al. [143] reported 60% (*w/w*) lipid accumulation in *R. toruloides* grown in xylose-containing media using continuous fermentation. On the other hand, Huang et al. (2016) observed 50% lipid accumulation in *R. toruloides* AS 2.1389, when grown at flask level using acetic acid (20 g/L) and glucose (50 g/L) containing media. Hence, the type of fermentation and media could change the lipid titer. Although in this study, fermentation was performed in the flask, however, the maintenance of the C/N ratio led to an increase in lipid accumulation. Moreover, yeast cultivated under C/N 20 did not encounter the nitrogen limitation condition resulting in lower lipid production. This result demonstrates that consideration of the initial C/N ratio to achieve maximum lipid accumulation might not be sufficient. Cells enter in lipid accumulation only after the depletion of nitrogen sources. Thus, high lipid accumulation could be achieved by maintaining the C/N ratio only by adding the carbon source using two-stage fermentations or pseudo-fed-batch fermentation. In this type of fermentation, cell division and lipid accumulation phase can be temporally separated i.e., lipid accumulation can be increased once nitrogen fell below critical concentration followed by adding only carbon to maintain the C/N ratio, which would result in diverting the extra carbon towards the lipid accumulation.

Effect of carbon/nitrogen ratio on fatty acid profile

Table 8. Effect of different C/N ratios on fatty acids compositions

Fatty acids (%)	C/N-20	C/N-20 Control	C/N-70	C/N-70 Control	C/N-120	C/N-120 Control	Control
Saturated							
Myristic acid	0.49	0.382	0.502	0.43	0.789	0.554	0.888
Palmitic acid	15.21	14.273	15.91	15.411	18.968	19.117	23.883
Stearic acid	9.728	8.21	9.99	8.869	15.623	13.414	14.166
Lignoceric acid	1.69	1.42	1.58	1.69	1.59	2.11	2.21
Heptadecanoic acid	0.266	0.257	0.365	0.403	0.411	0.397	0.585
Unsaturated							
Palmitoleic acid	0.706	0.646	0.569	0.516	0.508	0.5	0.428
Oleic acid	45.325	45.184	42.93	44.81	44.71	43.692	47.362
Linoleic acid	22.39	24.201	24.336	23.511	15.109	15.991	7.989
Trans-linoleic acid	0.061	0.07	0.062	0.156	0.085	0.104	0.078
Alpha linoleic acid	2.43	3.039	3.422	3.872	1.893	2.934	0.847
Eicosadienoic acid	0.083	0.084	0.078	0.09	0	0.054	0.059
Eicosenoate	0.176	0.182	0.142	0.176	0.144	0.121	0.128

Table 8 summarizes the fatty acid profile in relative percentage obtained from *R. toruloides*-1588 grown in different C/N ratios. The palmitic acids (14 – 23%), stearic acids (10 – 17%), and oleic acids (43 – 47%) were found to be in abundance in all the treatments. The abundance of fatty acids are in agreement with other articles published on oleaginous yeasts [66, 81, 111, 132, 155]. However, the major difference is the relatively higher production of omega-6 fatty acid i.e., linoleic acid (C18:3). In the present investigation, the maximum linoleic acid is found to be 24.34% which is 4-5 times higher than reported amounts in similar species [127, 140, 141, 143, 156].

Generally, fatty acid production depends on media, growth conditions, type of strain and stress conditions. Hydrolysate is complex media containing different types of sugars, phenols, furans, and organic acids, which exhibits the stress conditions on microorganisms resulting in enhanced accumulation of polyunsaturated fatty acids. For instance, Zhao, et al. [65] reported 9.62% linoleic acid accumulation in *R. toruloides* AS 2.1389 cultured in sugarcane bagasse. The author also reported a decrease in saturated fatty acids due to the presence of furfural. Similarly, in the present study, 20 – 30% of stearic acid and palmitic acid content was reduced in comparison to control while ~50% increase in linoleic and alpha-linolenic acid was observed. It seems that the presence of inhibitors affects the growth and lipid composition of yeast. Fed-batch fermentation has also been found to affect the fatty acid profile of microorganisms. For instance, Karnaouri, et al. [142] achieved 0.17g of docosahexaenoic acid (DHA) per gram of *C. cohnii* under fed-batch fermentation. The authors observed maximum DHA production after the nitrogen limitation in media. In general, sufficient nitrogen led to the production of saturated fatty acids while nitrogen limitation can lead to unsaturated fatty acids production, as seen in the present study. These results are in accordance with Karnaouri, et al. [142], in which an increase in unsaturated fatty acids was seen in microalgae upon nitrogen limitation In other words, upon subjected to excess nitrogen conditions, biomass production is higher while lipid accumulation is limited during the exponential growth phase. Once nitrogen is limited in the media, the cell division is halted due to insufficient nitrogen for *de-novo* nucleotide and protein synthesis [157]. Hence, the supplied carbon during fed-batch fermentation could be converted into storage lipids rich in unsaturated fatty acids. This could be the reason for higher unsaturated fatty acids production due to C/N ratio maintenance causing the fed-batch-like condition or pseudo-fed-batch fermentation than control.

Nevertheless, the corresponding metabolic conversion and effect on desaturase and elongase should be further studied and analyzed. Moreover, the fatty acid profile of *R. toruloides*-1588 lies in range with plant oils such as soyabean oil, palm oil or corn oil, which reflects its candidacy as a feedstock for biofuel production. In general, palm oil primarily composed of myristic acid (0.7%), stearic acid (6.6%), oleic acid (46.1%) and linoleic acid (8.6%). Similarly, soyabean oil contains palmitic acid (12%), oleic acid (51%) and linoleic acid (49%) (Lakshmidevi et al. 2021). These values are in contrast with the fatty acids compositions observed in present study (Table 8). Additionally, the presence of several polyunsaturated fatty acids including linoleic acid,

eicosadienoic acid and heptadecanoic acid, could expand its application from biofuel to pharmaceutical and food industries.

Inhibitor reduction profile

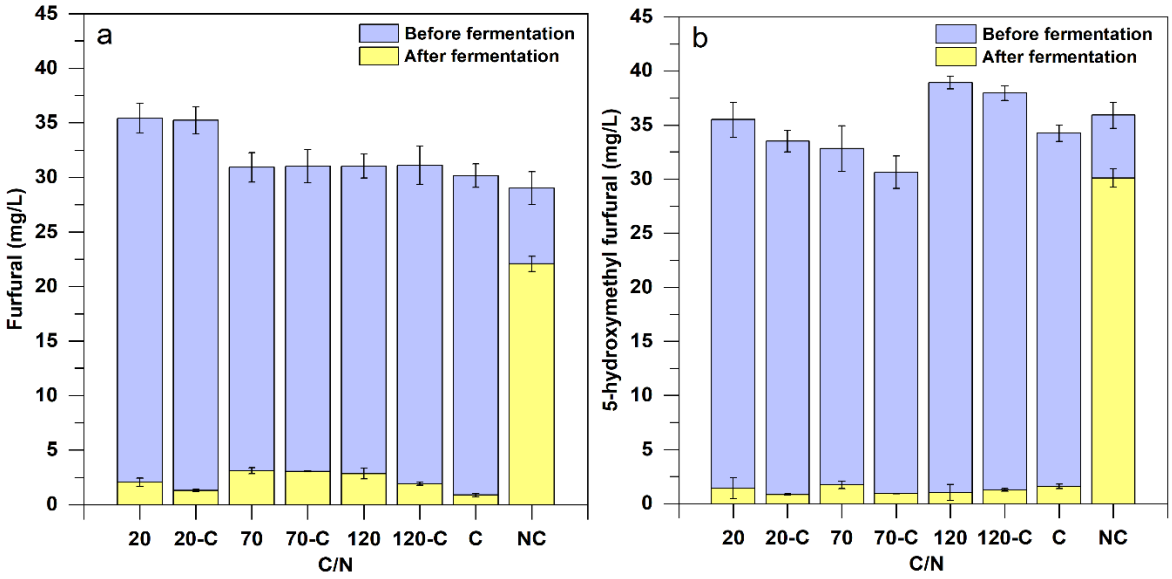

Figure 18. Inhibitors reduction profile during hydrolysate fermentation; a) furfural profile and b) 5-hydroxymethyl furfural profile where C/N 20, 70, 120-C is the ratio maintained before inoculation; C is control i.e., hydrolysate with inoculation and NC is negative control i.e., hydrolysate without inoculation.

Figure 18 illustrates the furfural and 5-hydroxy methyl furfural (5-HMF) reduction profiles present in hydrolysate. In all the treatments, 85 – 90% reduction in furfural and 5-HMF was recorded. However, in negative control i.e., hydrolysate without any yeast inoculation, 16 – 20% reduction was seen in furfural and 5-HMF (studies on inhibitor degradation by *R. toruloides* are under progress and communicated as a separate manuscript). The reduction in controls could be due to long fermentation time, media pH, temperature or salts [111]. For instance, Bonturi, et al. [98] observed the overexpression of NADPH production and different enzymes such as phosphogluconate dehydrogenase (GND1) in *R. toruloides* when subjected to inhibitors. However, knockout of GND1 resulted in enhanced sensitivity against furfural and 5-HMF [121]. Similarly, expression of aldehyde dehydrogenase 4 (ALD4) has been reported to increase in *R. toruloides* which has been known to convert furfural into furan 2,5-methanol [122]. Several gene clusters such as *hmf, adh, mfc* and *hyd* are responsible for metabolizing furfural and 5-HMF. Additionally,

R. toruloides have aldehyde dehydrogenase (*adh* gene) involved in lipid accumulation and central metabolism [158], but its involvement in furfural degradation requires further investigation.

Nevertheless, other options such as inhibitor recovery or hydrolysate detoxification are also available. However, the detoxification step would increase the final cost of biofuel production which will further increase the challenge of economical biofuel production. Therefore, being able to thrive in undetoxified hydrolysate as well as accumulating high lipid titer would be a cost-effective strategy for large-scale biofuel production. Moreover, further tolerance study would be needed by subjecting the *R. toruloides*-1588 to increasing concentrations of inhibitors.

So far, nitrogen limitation has been commonly used to increase lipid accumulation in several oleaginous organisms. Additionally, it has been previously demonstrated that how C/N ratios influence lipid production under dynamic batch conditions. On the other hand, fed-batch fermentation has also been explored to achieve maximum biomass and high lipid yield, which is crucial because titer and production rate plays an important role in the final product price. Several studies have been carried out on fed-batch fermentation to achieve high biomass and high lipid titer [57, 148, 149]. However, none of them mentioned the C/N ratio maintenance in fed-batch fermentation. Conventionally, fed-batch fermentation involves the feeding of sugars resulting in altering the C/N ratio of media. Hence, the change in the C/N ratio may alter the ability of oleaginous microorganisms to achieve maximum lipid titer. In other words, optimum nitrogen concentration is also important to achieve maximum lipid accumulation. For instance, high nitrogen concentration generally promotes cell growth and increases the growth phase resulting in low lipid production as reported in the present investigation. On the other hand, upon limitation of nitrogen, cell division is halted resulted in the diversion of carbon toward lipid production. Hence, the combination of the C/N ratio with fed-batch fermentation will not only provide the necessary carbon for metabolism during the exponential growth phase but also divert the excess carbon toward lipid accumulation after the late exponential phase resulting in maximum sugar consumption as well as high lipid titer. Considering, the result obtained due to C/N ratio maintenance reveals a possibility of achieving high xylose (70%) and glucose (>90%) consumption as well as high lipid titer in *R. toruloides*-1588 using undetoxified wood hydrolysate. Moreover, unlike fed-batch fermentation, the present process feathers the maintenance of the C/N

ratio, which can be economical on a large production scale, however, will require further investigation and techno-economic studies.

Conclusion

The results demonstrated that *R. toruloides*-1588 can efficiently utilize xylose (70%) and glucose (>90%) generated from pre-treated hardwood residues and accumulate 43% (*w/w*) of lipid per DCW. C/N ratio 70 is the best condition with specific lipid production of 3.8 mg/g dry weight per hour with 25 % (relative weight percentage) linoleic acids. It symbolizes that the production of PUFA from oleaginous yeast utilizing waste biomass residues is an attractive option than PUFA extraction from fish oil. Moreover, the current study suggests that maintenance of the C/N ratio would create a fed-batch-like condition resulting in high lipid accumulation which is necessary to reduce the final product price of biofuel.

5.3. A co-fermentation strategy with wood hydrolysate and crude glycerol to enhance the lipid accumulation in *Rhodosporidium toruloides*-1588

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Abstract

Wood hydrolysate has been regarded as sustainable and renewable substrate to produce microbial lipids, a potential feedstock for the biodiesel industry. Moreover, the major by-product of biofuel industries is crude glycerol but its implementation as a carbon source is still constrained due to the presence of impurities resulting in low biomass production and low lipid titer. Thus, this study investigates the effect of different carbon ratios of hydrolysate and crude glycerol on *R. toruloides*-1588. Hydrolysate to crude glycerol ratio of 60:40 resulted in maximum lipid accumulation of 49% (*w/w*), more than 90% of sugars and glycerol consumption. Further, scale-up to bench-scale fermenter resulted in 12% higher lipid accumulation (56.3% *w/w*, 0.15 g/L h) in 50% less time than flask fermentation. Hence, the ability of *R. toruloides*-1588 to flourish on different carbohydrates and accumulate high lipid content will be beneficial for the further development of biorefinery industries.

Keywords: *Rhodosporidium toruloides*; wood hydrolysate; crude glycerol; fatty acid composition; microbial lipids

Introduction

Liquid hydrolysate derived from lignocellulosic biomass has been regarded as a potential renewable source for biorefinery industries. The obtained hydrolysates can be converted either chemically or biochemically into biofuels. Nevertheless, over the past decade, research on biochemical-based conversion has exponentially increased [21]. Particularly, microbial-based conversion into lipids has been widely accepted and deemed as a potential feedstock for biofuel and oleochemical industries [3]. In general, microorganisms able to accumulate more than 20% of lipids per dry cell weight are known as oleaginous microorganisms and among the most important are yeast (*Rhodosporidium toruloides*, *Yarrowia lipolytica*), algae (*Chlorella vulgaris*, *Chlorella emersonii*), bacteria (*Rhodococcus jostii*, *Rhodococcus opacus*) and fungus (*Mortierella ramanniana*, *Mycena vinacea*) [4]. Out of these, *R. toruloides* have been recently considered a potential workhorse for biorefinery industries due to its ability to thrive on multiple carbon sources such as glycerol, xylose, glucose, galactose and arabinose with lipid accumulation up to 50-70%, and tolerance to toxic compounds such as vanillic acid, 5-hydroxymethyl furfural, levulinic acid, syringaldehyde and furfural [5, 144, 159].

Nonetheless, the microbial-based conversion of lignocellulosic hydrolysates into biofuels is still limited to laboratory scale because of the presence of inhibitors, low biomass production, low lipid productivity and low pentose consumption [10]. Hence, immense research on increasing lipid accumulation has been performed, such as growth inducers, carbon-nitrogen ratio optimization, media detoxification, and genetic engineering [28, 74, 160]. Similarly, the utilization of renewable co-substrates could be another option to increase biomass as well as lipid productivity. For instance, crude glycerol is a major by-product of the biodiesel industries that can be employed as a potential organic substrate [161]. Its usage may help in decreasing the biodiesel production cost and accomplish the concept of a zero-waste production process. Several investigations have been performed on using crude glycerol as media for lipid accumulation [59-61], but its implementation on large scale is limited due to its inhibitory effect on microorganism, caused mainly by the presence of high methanol, metals or soap content, resulting in lower biomass production and lower lipid accumulation [162]. Hence, it would be beneficial to develop the strategy to reuse the crude glycerol or mix it with another renewable source such as wood hydrolysate in different ratios to attain maximum lipid yield while supporting the concept of a circular economy.

In this sense, the present investigation aims to evaluate the influence of co-fermentation of undetoxified wood hydrolysate and crude glycerol on biomass profile, lipid titer and fatty acid profile of *R. toruloides*-1588. Different treatments were employed containing varying carbon ratios of hydrolysate and crude glycerol. Additionally, the carbon ratio of hydrolysate to crude glycerol leading to maximum lipid accumulation was scaled up to a lab-scale bioreactor and its effect on carbohydrate consumption, biomass growth, and lipid yield in *R. toruloides*-1588 was evaluated.

Materials and methods

Crude glycerol, sawdust, pre-treatment and saccharification

The crude glycerol was collected from BIOLIQ and stored at room temperature. Sawdust from maple trees was obtained from Quebec Forestry Council and dried at 50°C. It was processed and stored as described in Saini, et al. [74]. Briefly, the dried sawdust was thermally pretreated at 121°C/15 psi using 1% (*w/v)* sodium hydroxide (1:10 *w/v*) for 20 min. The solid fraction was cooled at room temperature, washed with tap water, and removed using centrifugation (8000 *x* g/ 10 min). Following this step, Viscozyme-L and Cellic-Ctec enzymes were used to perform the saccharification of pretreated biomass (Sigma-Aldrich, USA). The mixture for saccharification

consisting of 10% biomass and enzyme with 40 FPU/mL activity was mixed in citrate buffer of pH 4.8 and incubated at 40 ℃ for 24 h. Later, biomass solids were separated using centrifugation (7000 *x* g/ 10 min) and the liquid hydrolysate was utilized as a culture medium for lipid production experiments.

***Rhodosporidium toruloides*-1588 maintenance and inoculum conditions**

R. toruloides-1588 was purchased from NRRL culture collection USA and maintained as explained by Osorio-González, et al. [159]. Briefly, it was maintained and grown in yeast malt (YM) agar plates containing of peptone (5 g/L), yeast extract (3 g/L), glucose (10 g/L), malt extract (3 g/L) and 1.5% (*w/v*) of agar.

For the inoculum preparation, a single colony of yeast was picked from plate and mixed in 2 mL of YM media and incubated for 18 h at 30℃, 200 rpm. Following, 5 mL of media was inoculated and incubated again using previous conditions. Finally, YM media of 50 mL was inoculated and used for inoculating the different culture media.

Lipid accumulation in shaking-flask conditions

The yeast grown in 50 mL YM medium was separated using centrifuge (6000 *x* g/ 5 min). The cell pellets were washed twice using phosphate buffer saline (PBS) to remove the residual glucose and other nutrients present in YM medium. Finally, it was used to inoculate the different media compositions with a final optical density (OD_{600nm}) of 0.3 and incubated at 30℃, 200 rpm.

Table 9. The carbohydrates percentage ratio of hydrolysate and crude glycerol

Treatment	Hydrolysate (%)	Crude glycerol (%)
T1	100	0
T2	90	10
T3	80	20
T4	70	30
T5	60	40
T6	50	50
T7	0	100

Different media compositions and their effect on yeast were performed using 250 mL Erlenmeyer flask containing 50 mL culture media. Table 9 shows the different ratios of wood hydrolysate and crude glycerol used in this investigation. The amount of carbon required to maintain the 50 g/L of sugars in different treatments was estimated based on the carbon content in sugars and glycerol. The total carbon to nitrogen ratio of 70 was kept using ammonium sulphate. Negative control i.e., media without inoculation was also kept. The experiment was performed in duplicates and the samples were collected during the fermentation for biomass, sugar, glycerol, lipid accumulation and fatty acid analysis.

Lipid accumulation at bench-scale fermenter

The best media composition from the flask experiment was scaled-up to a lab-scale fermenter of 3.5 L (Labfors-4, Infors USA) with a 70% of working volume. Bioreactor was equipped with a radial flow Rushton turbine and was operated at 30℃, pH 6.0, 35% minimum dissolved oxygen, 1vvm and up to 500 rpm. Foaming was controlled using 0.05% *v/v* of antifoam 204® and pH was maintained using 1N hydrochloric acid and 1N sodium hydroxide. Dissolved oxygen and pH were monitored using Mettler Toledo® and Hamilton EasyFerm® probes, respectively. All the probes were calibrated and the fermenter was filled with desired media and sterilized at 121 ℃/15 min. Eventually, it was cooled and left for oxygen probe polarization overnight. Later, the saturated oxygen probe was maintained as 100% oxygen and the medium was inoculated with a inoculum seed grown in the same media composition as of bioreactor with a final OD_{600nm} of 0.1.

Lipid extraction

Lipids were extracted as explained by Osorio-González, et al. [159]. The cells from the broth were separated using a centrifuge at 13000 *x* g/ 3 min. The cell pellets were washed twice using PBS followed by centrifugation and dried. The dried cells were mixed with 1 M HCl and boiled for 1 hour and then vortexed for 15 min in presence of chloroform: methanol ratio of 2:1. Finally, 1N of sodium chloride (NaCl) was added in the solution and then centrifuged at 7000 *x* g for 10 min. The organic layer was separated, dried and the weight was calculated.

The following equations are used in present study:

$$\text{Lipid content (\%)} = \frac{Lipid\ (\frac{g}{L})}{Biomass\left(\frac{g}{L}\right)} * 100 \qquad \text{Equation 1}$$

Volumetric biomass production (g/L·h) = $\frac{Biomass\ (\frac{g}{L})}{Time\ of\ maximum\ biomass\ production\ (h)}$ Equation 2

Volumetric lipid production (g/L·h) = $\frac{Lipid\ (\frac{g}{L})}{Time\ of\ maximum\ lipid\ production\ (h)}$ Equation 3

Analytical techniques

Biomass growth analysis

The fermented samples were separated from broth using centrifugation at 13000 *x* g for 3 min. The pellets were re-dissolved in PBS and centrifuged again. Finally, the pellets were dried at 60℃ and the weight was measured.

Glycerol consumption analysis

Glycerol analysis was performed using UV-Vis spectrophotometric method as described [163, 164]. Briefly, reaction buffer consisting of 4.0 M ammonium acetate and 1.6 M acetic acid (1:1 ratio) was used. the solutions of acetylacetone (0.2M) and sodium periodate (10 mM) were prepared in a reaction buffer. The test samples were diluted using a working solvent consisting of 95% ethanol to water in equal ratio. The samples mixed with 1.2 mL of acetylacetone and sodium periodate solution were incubated for 1 min at 70 ℃. The samples were cooled immediately by putting the samples tubes in ice. Following this step, absorbance was recorded at 410 nm. Glycerol concentration in the samples was calculated in g/L using a standard curve.

Sugar analysis

Sugar concentration was analysed as described by Saini, et al. [132]. The samples were processed in Liquid Chromatography-Mass Spectroscopy (Thermo TSQ Quantum Access MS) and standards were acquired from Sigma Aldrich (Missouri, USA).

Fatty acid composition

The fatty acids were quantified using Gas Chromatography (GC) as described by Osorio-González, et al. [11]. Briefly, extracted lipids were dissolved in methanol to sulfuric acid ratio of 85:15 and boiled for 20 min. Soon after, the samples were cooled, and the fatty acids esters were extracted using hexane.

Statistical analysis

The analysis was performed using OriginPro®2022 software. ANOVA was used to evaluate the significance of biomass production, lipid accumulation and sugar consumption at 95% confidence level using Fischer test. The correlation value was calculated between lipid, biomass and glycerol consumed using Pearson correlation.

Results and discussion

Effect of different carbon ratios on biomass production

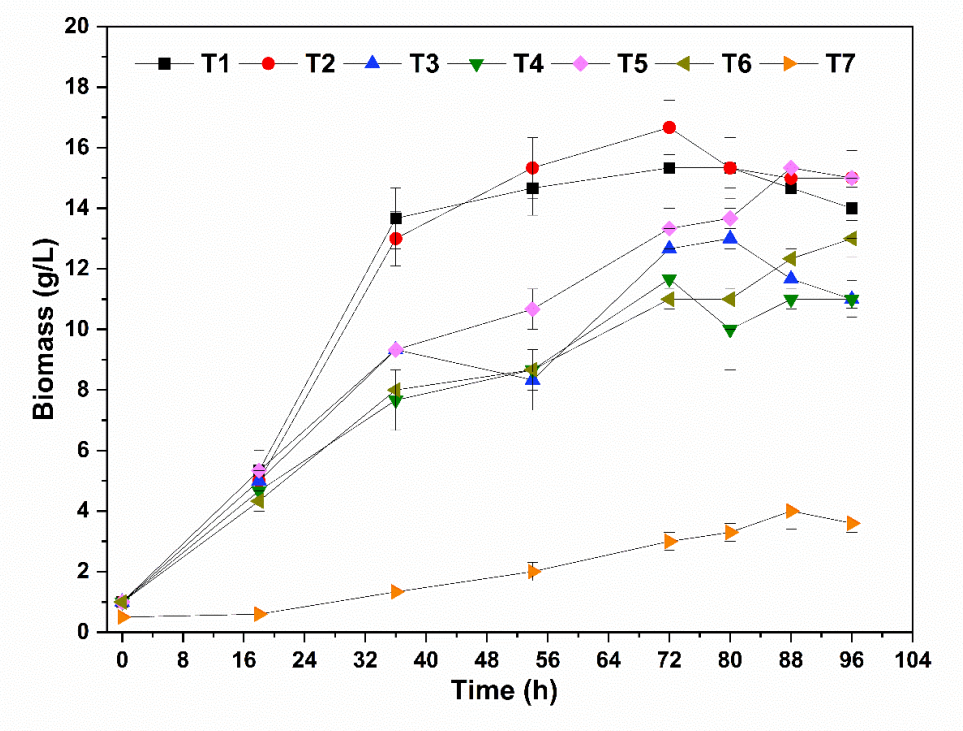

Figure 19. *R. toruloides*-1588 growth in different carbon ratios of hydrolysate: crude glycerol; where, T1- 100:0; T2- 90:10; T3- 80:20; T4- 70:30; T5- 60:40; T6- 50:50 and T7- 0:100.

Figure 19 displays the biomass production profile using different carbon ratios of hydrolysate and crude glycerol. The maximum biomass of 16.6 g/L can be observed in T2 (hydrolysate: crude

glycerol; 90:10), followed by 15.3 g/L in the media containing only hydrolysate (T1 100:0) at 72h. Subsequently, T5 (60:40) with 13.3 g/L, T3 (80:20) with 12.67 g/L and T4 (70:30) with 11.67 g/L of biomass at 72 h. Moreover, the lowest biomass of 4 g/L was seen in 100% crude glycerol media (T7). Additionally, a diauxic growth was seen in media containing both crude glycerol and hydrolysate. This is because glucose is the preferred carbon source by microorganisms resulting in repression of conversion of other carbon sources also known as catabolite repression. Similar findings were reported by Bommareddy, et al. [118], where the authors reported a double exponential phase in *R. toruloides* DSMZ-4444 grown in glycerol (40 g/L) and glucose (10 g/L) media. Similarly, Osorio-González, et al. [111] reported in the diauxic growth of *R. toruloides*-1588 grown in C6 and C5 hydrolysate. In addition, no lag phase was observed in hydrolysate as well as the mixture of hydrolysate and crude glycerol media, while the longest lag phase was observed in crude glycerol media.

The above tendency could be due to the presence of toxic compounds, such as soap content, methanol and metals or due to substrate inhibition resulting in a decrease in biomass production. In addition, crude glycerol with high methanol content alters the membrane fluidity of microorganisms resulting in decreased growth rate and increased cell death [165]. For example, Yang, et al. [166] reported reduction in biomass production of *R. toruloides* Y4 when 4 g/L of methanol were added to a pure glycerol culture medium. Likewise, more than 50 g/L of glycerol could inhibit microbial growth due to substrate inhibition resulting in a significant reduction in the enzymatic hydrolysis rate [167]. For instance, Signori, et al. [62] reported the growth inhibition in *Cryptococcus curvatus* DSM 70022, *Lipomyces starkeyi* DSM 70295 and *R. toruloides* DSM 4444, when the crude glycerol concentration was increased from 50 g/L to 100 g/L. Similarly, a high concentration of metals could also lead to low biomass production. For instance, Kumar, et al. [59] reported a decline of almost 50% in biomass growth when *Yarrowia lipolytica* SKY7 was grown in crude glycerol than in purified glycerol. The authors concluded that the low biomass in crude glycerol was because of the high potassium concentration (6.32 g/L), which provokes a hyperosmotic shock, hindering microbial growth [59]. Thus, carbohydrate concentration, as well as impurities play a essential role in microbial growth.

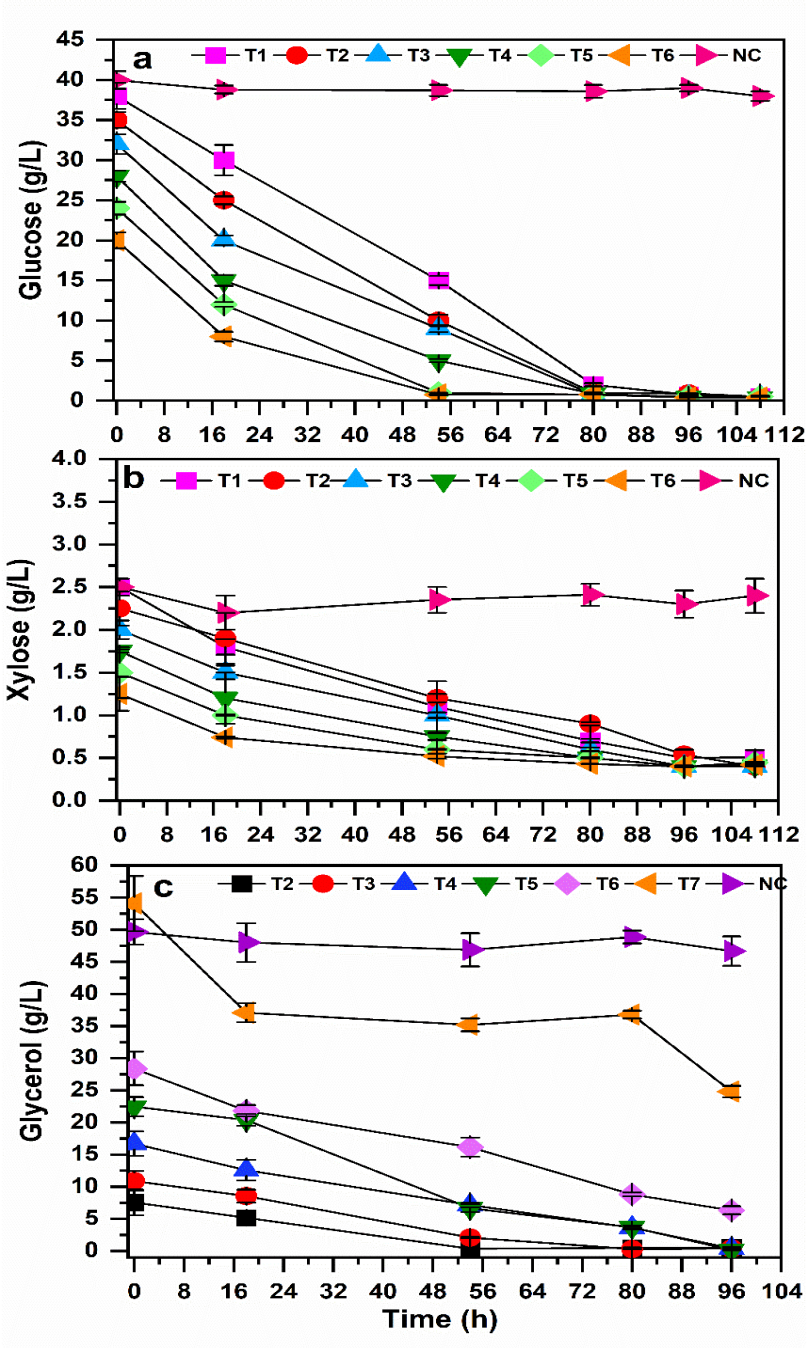

Figure 20. Profiles of the glucose (a), xylose (b), and glycerol (c) consumption profile in different carbon ratios of hydrolysate and crude glycerol; where, T1- 100:0; T2- 90:10; T3- 80:20; T4- 70:30; T5- 60:40; T6- 50:50; T7- 0:100 and NC- negative control.

Figure 20 displays the sugars and glycerol consumption profile where more than 95% glucose consumption can be seen within the first 72 hours of fermentation (20a), while 50-80% of xylose

consumption has been observed (20b). In general, glucose is known as a preferred carbon source while the consumption of xylose is limited among several microorganisms due to the lower energy production when consumed via the pentose phosphate pathway. For instance, Jagtap and Rao [168] reported 80% consumption of xylose by *R. toruloides* IFO0880 grown in 70 g/L xylose media, while Wiebe, et al. [153] observed 50% of xylose consumption, when *R. toruloides* CBS14 was grown in xylose-based media. Similarly, Osorio-González, et al. [111] reported the ~50% consumption of xylose when *R. toruloides* 7191 was grown in 50 g/L xylose-based hydrolysate. Moreover, several proteomic, as well as genetic engineering methods, have been employed to increase xylose consumption [169, 170]. Nevertheless, further optimization can be performed for the desired microorganism to find the optimum xylose concentration with more than 90% consumption rate.

On the other hand, variation in glycerol consumption among different media compositions was observed and shown in Figure 20c. For instance, more than 90% of glycerol consumption was seen in T1 to 5, followed by 77% glycerol consumption in T6 and 50% in T7. Increasing the glycerol concentration resulted in a reduction in glycerol consumption due to substrate inhibition which further led to a decline in yeast growth (Figure 19). The present results can be corroborated with studies performed by Chmielarz, et al. [171], where *Rhodosporidium toruloides* CBS14 consumed 52% of glycerol, grown in 50 g/L crude glycerol. Likewise, Pereira, et al. [172] reported a 95% consumption of 10.4 g/L of crude glycerol by *Rhodotorula mucilaginosa* CCT 7688, while Prabhu, et al. [173] observed the 95% glycerol consumption by *Yarrowia lipolytica* grown in media containing 20g/L pure glycerol and 20 g/L of xylose.

Effect on lipid accumulation profile

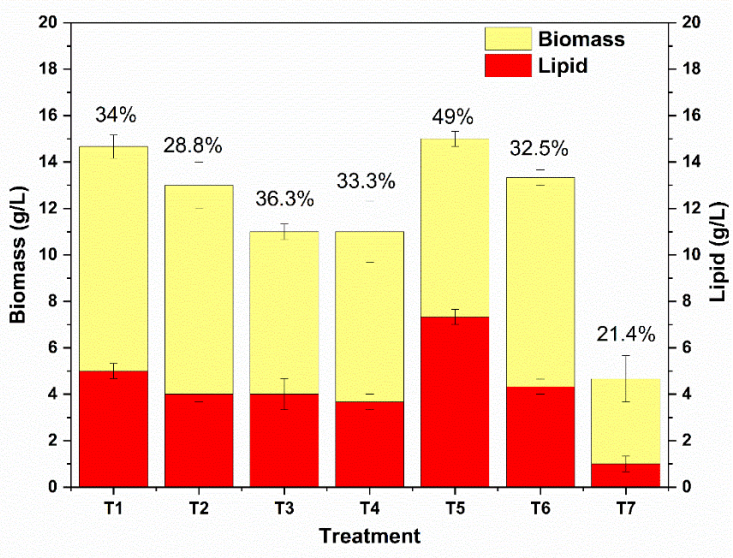

Figure 21. Shows the maximum lipid accumulation in *R. toruloides*-1588 using different treatments.

Figure 21 displays the maximum lipid accumulated in different tested conditions. The maximum lipid accumulation observed was 49% (*w/w*) in hydrolysate: crude glycerol ratio of 60:40, while the lowest lipid accumulation of 21.4% (*w/w*) was observed in crude glycerol media. In addition, media containing only wood hydrolysate (T1) resulted in 1.5 times higher lipid than media containing crude glycerol (T7). Biomass growth and lipid accumulation are negatively correlated with increasing concentration of glycerol concentration (R^2: 0.59; and 0.31, respectively) (Supplementary Figure 3). However, maximum biomass production, as well as lipid accumulation at 40% glycerol, states that, up to a certain threshold value of glycerol, biomass and lipid accumulation can be increased. Further increase in the concentration of glycerol may inhibit lipid as well as biomass production. This could be due to the varying carbon flux when grown in different carbon sources. For instance, glycerol is generally channelled through the second part of glycolysis via dihydroxyacetone into glyceraldehyde 3-phosphate ultimately leading to high adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) production, while most of the carbon from glucose generally channelled through the pentose phosphate pathway which might lead to loss of carbon in form of carbon dioxide [118]. Hence, by mixing glycerol and glucose, the yeast would have enough carbon from glucose to grow while carbon from glycerol can be efficiently converted into lipids. For instance, Bommareddy, et al. [118] predicted using *in-silico* modelling that most of the carbon source from glucose was wasted as carbon dioxide and

yeast grown in glucose media will not able to reach 45% (*w/w*) of lipid content. The authors also reported that using glycerol as a culture media, more than 55% of lipid accumulation can be achieved, where the lowest carbon dioxide production was observed (30.7 mol/mol triacylglycerol). This indicates that glycerol could be a desirable substrate in terms of efficient and maximum carbon conversion into lipids. As this can also be observed in the present case, where crude glycerol media (T7) resulted in a lipid content of 21.3% (*w/w*) despite the lowest biomass (5 g/L).

Based on the above results, the hydrolysate to crude glycerol ratio of 60:40 (T5) was scaled-up to a bench-scale bioreactor, as this ratio resulted in maximum lipid accumulation in flask fermentation.

Bioreactor studies

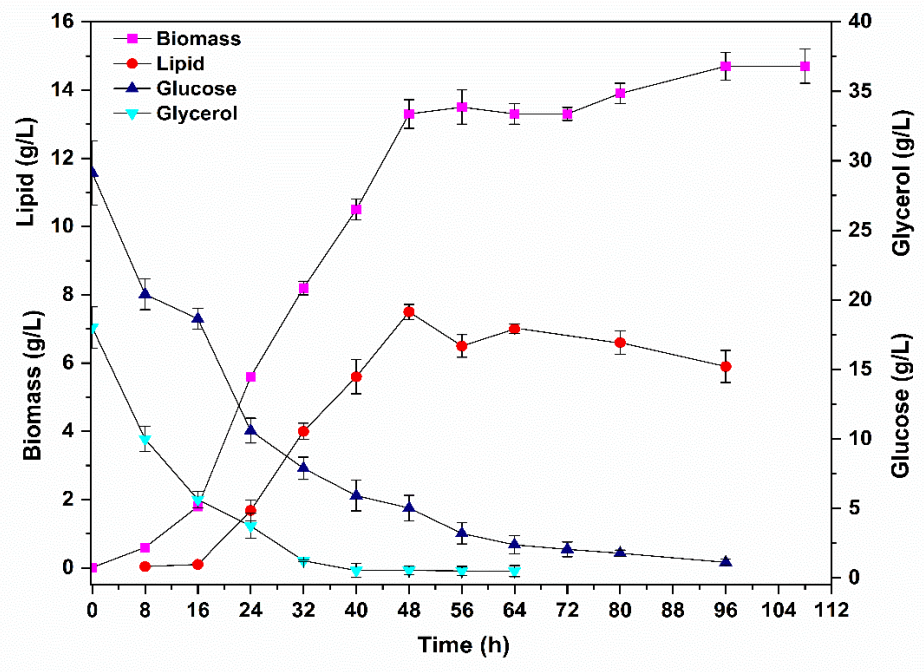

Figure 22. Displays the effect of batch fermentation on biomass production, lipid accumulation and carbohydrates consumption by *R. toruloides*-1588.

Figure 22 illustrates the biomass growth, lipid profile and carbohydrates consumption by *R. toruloides*-1588 in wood hydrolysate: crude glycerol (60:40) culture media. The biomass production rate was 0.28 g/(L·h), while the lipid production rate was 0.15 g/(L·h). Additionally, more than 90% of glycerol was consumed within 40 h of fermentation, while 70 % of glucose

consumption was observed. This explains the fact that yeast can consume glycerol faster than glucose. This is because, microorganisms tend to divert the excess of glycerol towards lipid accumulation while glucose towards biomass production [118]. The maximum lipid accumulation was 56.3 % (*w/w*), which is 12 % higher than observed in the flask. This could be due to the enhanced supply of oxygen in the bioreactor. In addition, oxygen supply has also decreased the maximum biomass production time in the fermenter to 48 h than in the flask (96 h). With the cultivation conditions tested in this study, the authors achieve a decrease of 50 % of the total fermentation time to reach the maximum biomass production as well as the lipid accumulation, which is highly beneficial for the process, as the maximum production in a short time will save energy and lower the total production cost. In terms of lipid productivity, the obtained results in this study are similar (0.17 g/(L·h)) to those reported in the literature utilizing glycerol and glucose as a culture media [174]. Moreover, to further increase the biomass and lipid accumulation, studies to optimize the oxygen saturation will be required. For instance, Bommareddy, et al. [118] reported a rise in lipid productivity (0.07 to 0.15 g/(L·h)) when oxygen was controlled in media containing glucose, similarly, 0.12 g/(L·h) lipid productivity was increased under oxygen-controlled conditions in glucose + glycerol media. Likewise, Zhang, et al. [60] reported a 2.8 folds increase in biomass concentration of *Trichosporon oleaginosus* when dissolved oxygen concentration was increased from 10% to 60%.

In general, oxygen concentration has been known as one of the critical parameters for fatty acids and lipid accumulation. Microorganisms tend to accumulate higher lipids under anoxic conditions. In aerobic conditions, glucose is converted into adenosine triphosphate (ATP) via oxidative phosphorylation. However, oxygen limitation leads to lesser production of ATP and yeasts recover the NAD^+ required for substrate breakdown by switching to anaerobic fermentation. This further enhances NADH production, which acts as an electron acceptor during lipid accumulation. Meanwhile, lower ATP results in a lower growth rate and the carbon then can be diverted towards lipid production. This is so, because the ATP requirement for protein and DNA synthesis is higher than for lipid accumulation [81]. Thus, variation in oxygen concentration can lead to variation in glucose oxidation as well as lipid accumulation.

The major obstacle in large-scale biofuel production is lower lipid and biomass productivity. High lipid concentration in a short time could be economically viable. In the present study, scale-up

from flask to bench-scale fermenter not only increased the lipid titer but also resulted in lowering the time by 50% for maximum lipid accumulation. Hence, to further maximize the biomass as well as lipid productivity, dissolved oxygen levels should be optimized.

Fatty acid profile

Table 10. Effect of different carbon ratios on the fatty acid composition of lipid.

Fatty acid (%)	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Palmitic acid	34.51 ± 0.12	34.46 ± 1.49	39.30 ± 0.42	38.92 ± 0.77	33.83 ± 2.47	29.32 ± 0.75	32.04 ± 1.05
Stearic acid	24.41 ± 0.14	25.52 ± 2.58	22.43 ± 0.69	31.80 ± 1.36	32.48 ± 0.19	37.66 ± 0.38	46.94 ± 2.44
Oleic acid	32.22 ± 0.53	34.24 ± 0.54	30.37 ± 1.02	22.55 ± 2.48	28.11 ± 6.16	24.12 ± 0.88	15.31 ± 3.42
Linoleic acid	0.27 ± 0.06	0.94 ± 0.29	0.33 ± 0.12	0.02 ± 0.02	0.06	0.12 ± 0.02	1.7 ± 0.81
Myristic acid	1.45 ± 0.09	2.09 ± 0.61	1.51 ± 0.02	2.13 ± 0.16	1.81 ± 0.29	1.72 ± 0.06	1.25 ± 0.06
Heptadecanoic acid	0.67 ± 0.03	0.85 ± 0.13	0.71 ± 0.01	0.74 ± 0.05	0.59 ± 0.01	0.54 ± 0.01	0.85 ± 0.1
Elaidic acid	1.18 ± 0.16	0.86 ± 0.11	0.78 ± 0.03	1.09 ± 0.22	8.94	0.64 ± 0.14	0.72 ± 0.11
Capric acid	0.07 ± 0.01	0.05 ± 0.01	0.06	0.13 ± 0.03	0.1 ± 0.02	0.11 ± 0.01	0.03
Pentadecylic acid	0.22 ± 0.03	0.21 ± 0.03	0.22	0.21 ± 0.02	0.18	0.16 ± 0.01	0.15 ± 0.02
Docosapentaenoic acid	3.64 ± 0.29	3.72 ± 0.10	3.79 ± 0.01	4.26 ± 0.19	3.74 ± 0.1	3.86 ± 0.02	5.62 ± 0.47
Arachidic acid	0.59 ± 0.03	0.61 ± 0.06	0.45 ± 0.01	0.75 ± 0.03	0.86 ± 0.01	1.24 ± 0.15	1.07 ± 0.08
Methyl 18-methylicosanoate	0.66 ± 0.05	0.71	0.77 ± 0.01	0.81 ± 0.04	0.63	0.66 ± 0.02	0.89 ± 0.06

Table 10 illustrates the fatty acid profile of lipids in relative percentage during different carbon ratios. The fatty acids found in abundance are palmitic acid (29.32 - 39.30%), stearic acid (22.43 - 46.94%) and oleic acid (15.31 - 34.24%) in all the media conditions and are in accordance with previously published studies [74, 81, 111, 134]. In addition, fatty acids such as capric acid, short chain fatty acid, have been detected which have a significant advantage in alkane production. However, long-chain fatty acids are beneficial for biodiesel production as the chain length enhances factors such as viscosity, combustion temperature, melting point and cetane number [61]. In addition, essential unsaturated fatty acids, as well as branched-chain fatty acids such as linoleic acid, docosapentaenoic acid and methyl 18-methylicosanoate, have been detected. These compounds have high value in the pharmaceutical and food industries such as in flavors, pheromones, and cosmetic additives [175].

In general, the fatty acid composition is affected by media composition and different stress conditions [74, 176]. For instance, increasing the glycerol concentration led to an increase in stearic acid concentration from 22.41 to 46.54%. Similarly, the lowest amount of oleic acid (15.31%) was observed in crude glycerol media, while a change in carbohydrate ratio resulted in maximum palmitic acid production in hydrolysate to crude glycerol ratio of 80:20 (39.30%). Hence, depending on the type of substrate as well as media conditions, a high level of saturated or unsaturated fatty acids production can be targeted. For instance, Saini, et al. [74] reported an increase in linoleic acid to 24.34% in *R. toruloides*-1588, when C/N 70 was maintained in wood hydrolysate throughout the fermentation. Similarly, Chmielarz, et al. [171] observed increased production of oleic acid from 39.6% to 46% in *R. toruloides* CBS 14, when crude glycerol concentration was raised from 50 to 60 g/L. Tchakouteu, et al. [177] reported a decrease in palmitic acid from 23 to 21.8% in *R. toruloides* NRRL 27012 grown in increasing concentration of glycerol from 30 to 50 g/L. Hence, based on the requirement, the desired carbon ratio can be chosen to increase the production of a specific fatty acid. The fatty acids extracted from *R. toruloides*-1588 lies within the range of plant-based fatty acids, therefore, can be employed as a potential feedstock for biorefinery industry. For instance, rapeseed oil is composed of 18% linoleic acid, 51% oleic acid and 5% palmitic acid [178], while palm oil consists of 46.1% oleic acid and 8.6% linoleic acid [81].

Limitation and scope

Scaling up of fermentation from flask to bench-scale bioreactor increased lipid accumulation, and carbohydrate consumption, and decreased the time of maximum lipid accumulation while biomass concentration did not change. Hence, dissolved oxygen optimization would be required to increase the biomass production rate and high lipid accumulation rate. In addition, biomass concentration can also be increased using modified fermentation techniques such as a two-stage fermentation process. For instance, fermentation can be initiated with media containing hydrolysate and crude glycerol mixture and when the yeast will reach the mid-exponential phase, the media can be supplemented with components such as potassium sulfate (K_2SO_4) or dipotassium hydrogen phosphate (K_2HPO_4). The potassium ions will act as an activator of various growth-promoting enzymes while sulfur will be essential for sulphur-containing amino acids and acyl-S-CoA, ultimately affecting microbial growth [166]. Also, these compounds can inhibit growth if increased to a certain concentration. For instance, Yang, et al. [166] observed a reduction of 10% in biomass of *R. toruloides* Y4, when the K_2HPO_4 concentration was increased from 1 g/L to 2 g/L. Hence, optimization will be required based on microorganisms and their growth pattern.

Based on the above investigation, it is certain that *R. toruloides*-1588 can prosper on multiple carbon sources such as glucose, xylose and crude glycerol, which is highly beneficial in terms of industrial-scale fermentation. This investigation will also help in setting up the circular bioeconomy in biofuel industries, i.e., by recycling the crude glycerol and mixing it with hydrolysate to achieve maximum lipid accumulation. In addition, replacing a portion of carbon of hydrolysate with the carbon from crude glycerol might be more economical than using 100 % hydrolysate. For instance, due to the substantial production of crude glycerol from biodiesel industries, the price of 80% purified crude glycerol has been lowered to 0.1 US$/kg [162]. Hence, crude glycerol has become competitive with other carbon sources due to its low price and could serve as an economical alternative route for enhanced microbial growth and high lipid accumulation. Nonetheless, the major limitation is low biomass concentration thus, further investigation will be required to increase the biomass production rate and lipid titer. In addition, techno-economic and life cycle assessment will also be needed to evaluate the process feasibility and economic viability of biodiesel production using microbial lipids at an industrial scale.

Conclusion

This work reveals the ability of *R. toruloides*-1588 to thrive on mixed carbon sources. The hydrolysate to crude glycerol ratio of 60:40 resulted in maximum lipid accumulation of 56.3% *w/w* as well as more than 95% consumption of carbon sources. Based on the above investigation, dissolved oxygen, maximum substrate consumption and short fermentation time were found to be crucial factors to increase the efficiency of microbial-based lipid production. Moreover, recycling biodiesel-derived crude glycerol is imperative to develop a circular bioeconomy, and zero-waste production and will require techno-economic and life cycle assessment to evaluate the process and economic feasibility.

Supplementary data for this work can be found in Annex C.

CHAPTER 6: FURFURAL TOLERANCE AND IDENTIFICATION OF FURFURAL DEGRADING ENZYMES

Based on the previous studies, more than 90-95% of furfural reduction was observed in the hydrolysates. Since, hydrolysate is complex media thus it was not certain that the *R. toruloides*-1588 can degrade furfural into non-toxic products and use it as an energy source. Therefore, Chapter 6 explores the ability of *R. toruloides*-1588 to thrive on 2 g/L of furfural and its conversion into toxic products as well as presence of enzymes required for furfural degradation.

6.1. Investigating the ability of *Rhodosporidium toruloides*-1588 to use furfural as a carbon source and its degradation: An enzyme identification study

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Abstract

Lignocellulosic biomass has been regarded as a sustainable feedstock for the biorefinery industry. In general, pre-treatment and saccharification are required to breakdown the complex link between lignin, cellulose and hemicellulose, thus releasing the fermentable sugars such as glucose, xylose or arabinose. However, microbial growth inhibitors such as furans, phenolic compounds and organic acids are also produced during pre-treatment of lignocellulosic biomass. Among all, furfural is specifically notorious for negatively affecting microbial growth and their product accumulation ability. In this sense, the present study aims to identify the ability of *Rhodosporidium toruloides*-1588 to use furfural as a carbon source and to accumulate lipids and carotenoids. *R. toruloides*-1588 was able to grow in 2 g/L of furfural and degrade it into non-toxic products. Upon proteomic evaluation of *R. toruloides*-1588, furfural degrading enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase were identified to be responsible of furfural degradation into furfuryl alcohol and 2-furoic acid. The maximum furfural degradation rate was observed as 25 mg/L*h in minimal media containing glucose and furfural, while 15 mg/L*h in minimal media containing furfural. Additionally, the role of carotenoid accumulation and its impact during furfural exposure has been identified. The study shows the capacity of *R. toruloides*–1588 to use furfural as an energy source and the presence of potential furfural degrading enzymes. This research paves the way for in-situ detoxification of hydrolysates as a major step towards the efficient utilization of lignocellulosic biomass and decrease the biofuel production cost.

Keywords: *Rhodosporidium toruloides*; furfural degradation; aldehyde dehydrogenase; furfuryl alcohol; 2-furoic acid

Introduction

Lignocellulosic biomass has been regarded as a renewable and sustainable source of fermentable sugars to produce microbial- based biofuels, oleochemicals and extractives [179]. However, lignocellulose is a complex matrix composed of lignin, cellulose and hemicellulose hence require pre-treatment and enzymatic saccharification to release the fermentable sugars [10]. Among the most used processes are alkaline, steam explosion, ionic liquid, ammonia fiber explosion and acid-based pre-treatment [180]. Nevertheless, these methods inevitably lead to the formation of toxic compounds such as furans, phenolic compounds and organic acids [113]. These toxic compounds generally hinder the microbial ability to grow and produce secondary metabolites as value-added

products. For instance, furfural, a hydrolytic derivative of glucose, is one of the most toxic compounds which causes detrimental effects at a molecular level. It tends to inhibit cellular growth, and fatty acid synthesis as well as cause oxidative stress and reduces the activities of various enzymes [181].

Furfural higher than 1g/L has been reported to inhibit the microbial growth causing growth arrest and ultimately leading to the cell death [182-184]. Alternatively, physical, chemical, and physiochemical detoxification methods can remove furfural resulting in improved microbial growth and product yield. For instance, Covarrubias-García, et al. [185] employed the reduced graphene oxide-magnetite nanoparticles to adsorb the furfural present in wood hydrolysate. The authors reported increase in biomass of *R. toruloides*-1588 by 1.5 times grown in detoxified wood hydrolysate than in undetoxified wood hydrolysate. Similarly, other detoxification methods such as vacuum membrane distillation [28], hydrophobic polymers and resin-based adsorption [186] are available. However, addition of detoxification step could increase the final biofuel production cost, which is already a major hurdle for the biorefinery industries. Hence, strain capable of tolerating high furfural concentration (>1 g/L) as well as accumulate biofuel feedstock is highly desirable.

Recently, *Rhodosporidium toruloides*-1588 has been reported to not only able to accumulate lipids and carotenoids, but also to thrive in undetoxified wood hydrolysate [5, 11, 12, 111, 132]. Microbial lipids have been considered a potential feedstock for biofuel as well as oleochemical production [74]. Hence, studying the ability of *R. toruloides*-1588 to accumulate lipids as well asto tolerate inhibitors could be a cost-effective strategy to produce biofuels. Moreover, most of these studies have been conducted using complex media such as wood hydrolysate which have different type of sugars (pentoses and hexose sugars) that could impact the inhibitor tolerance ability and its consumption profile. Nevertheless, it has not been established yet that *R. toruloides*-1588 can use furfural as a carbon source and contain enzymes responsible for furfural degradation. Thus, the ability of *R. toruloides*-1588 to thrive on furfural was evaluated. *R. toruloides*-1588 was grown in minimal media with furfural as the sole carbon source and its effects on yeast growth, lipid accumulation and carotenoid accumulation were studied. The furfural metabolized products such as furfuryl alcohol and 2-furoic acid were also determined. Additionally, the proteome of *R. toruloides*-1588 was used

to identify the furfural degrading enzymes such as aldehyde dehydrogenase and alcohol dehydrogenase. This study provided the firsthand insight into the ability of *R. toruloides*-1588 to use furfural as a carbon source and determine its potential as a biofuel feedstock for future biorefinery applications.

Material and Methods

Yeast maintenance

Rhodosporidium toruloides-1588 was acquired from the Agricultural Research Service culture collection (NRRL, USA). The yeast was maintained and grown in yeast-malt extract (YM) agar plates as described by Saini, et al. [74]. Briefly, YM plates were prepared by mixing 2.1% (*w/v)* of Difco™ YM broth powder (Fischer Scientific, Canada) and 1.5% (*w/v*) agar. The solution was mixed and sterilized at 120℃/ 15psi for 15 min and poured in petri plates. The yeast was streaked and grown at 30±1℃ until the single colonies appeared.

Furfural degradation experiment

Minimal media was prepared using Yeast Nitrogen Base (YNB) without amino acids (Sigma Aldrich, USA). Briefly, a 10X stock solution was prepared to contain 6.7 g of YNB in 100 mL of distilled water. The pH was maintained at 6.0 using potassium phosphate buffer (0.1 M and 8.0 pH). The solution was slightly warmed to completely solubilize the particles and sterilized using 0.22 µm filters. The stock solution was diluted at 1:10 *v/v* using sterile distilled water containing the desired concentration of carbohydrates.

To prepare the inoculum, a single colony of yeast from a Petri plate was inoculated in 2 mL of YM broth and incubated for 18h at 30±1℃ and 200 rpm. As follow-up to this step, 5 mL of minimal media containing 10 g/L glucose was inoculated (10% *v/v)* and incubated for 24 h at 30±1℃ and 200 rpm. Finally, 50 mL of minimal media was inoculated (10% *v/v*) using previously grown culture and incubated at 30±1℃ and 200 rpm and used as inoculum (10:100 *v/v*) for furfural degradation experiment. Briefly, the inoculum was centrifuged, and the yeast cell pellet was washed with phosphate buffer saline (PBS- pH 7) twice to remove the residual glucose and used to inoculate the different test conditions.

Furfural degradation experiment was performed in triplicates using 250 mL Erlenmeyer flask with 50 mL media at 30±1℃ and 200 rpm for 108 h. Three test conditions were investigated:

i) minimal media containing 10 g/L of glucose, ii) minimal media containing 2 g/L of furfural, and iii) minimal media containing 2 g/L furfural and 10 g/L of glucose. Negative controls for each tested conditions were kept without yeast inoculation. Furfural degradation rate was calculated using equation [11]:

$$\text{Furfural degradation rate (mg/L*h)} = \frac{\textit{Initial furfural concentration}\left(\frac{mg}{L}\right) - \textit{final furfural concentration}\left(\frac{mg}{L}\right)}{\textit{Cultivation time}\,(h)}$$

Whole-cell enzyme extraction and enzyme activity

Samples (5 mL) were collected for each tested condition and centrifuged at 6000 *x* g for 5 min. The cell pellets were washed with potassium phosphate buffer (0.1 M and 7.0 pH). Later, the cell pellets were re-suspended in 5mL of potassium phosphate buffer and subjected to sonication at 20kHz with a pulse mode of 30s on and 30s off for 20 minutes. The total protein concentration was determined spectrophotometrically using a Pierce™ BCA Protein Assay Kit and Bradford dye reagent as a standard at 595nm.

Enzyme activity was performed by measuring a change in the absorbance of nicotinamide adenine dinucleotide phosphate (NADPH) cofactor at 340nm as reported by Lewis Liu, et al. [187]. Briefly, the reaction mixture consisted of 10 mM of furfural, 100 μM of NADPH and performed in 100 mM of potassium phosphate buffer with a pH of 7.2 at 30±1°C for 10 min. One enzyme unit was defined as the amount of enzymes oxidizing 1 μM of NADPH per minute, while a specific enzyme unit was defined as enzymatic units per mg of protein used in the reaction.

Lipids and Carotenoids extraction

Lipid extraction was performed as described by Osorio-González, et al. [159]. Briefly, the cells were pelleted, washed with PBS, and dried at 60±1°C. The dried cells were dissolved in 1M hydrochloric acid (HCl) and digested at 100°C for 1h. The lysed cells were subjected to vortex for 15 min in the presence of chloroform: methanol solution (2:1 *v/v*) to extract the microbial lipids. Finally, 1M sodium chloride solution was added, and the solution was centrifuged at 7000 *x* g for 15 min. The top layer was removed while the organic layer was dried, and the weight was recorded.

For carotenoid extraction, the dried yeast cells were treated with 1M of HCl and centrifuged at 7000 *x* g for 10 min. Carotenoids were extracted from cell pellets using acetone with a solid to liquid ratio of 1:3. The mixture was vortexed for 5 minutes, centrifuged and the supernatant was collected. The process was repeated 3 to 4 times until the cells were completely discolored. The solution was dried, and the weight was recorded.

Analytical techniques

Cell dry weight

About 2 mL of cell growth samples were separated by centrifugation at 13000 *x* g for 3 min, washed twice with PBS and dried at 60±1°C until the constant weight. The biomass was reported in g/L.

Glucose analysis

Glucose was determined using a liquid chromatography system coupled with mass spectrophotometer detector (LC-MS) (Thermo Scientific Liquid TSQ) as described by Saini, et al. [132]. Briefly, a HILIC column (150 mm internal diameter, 5 µm and 4.6 mm length) was used. The mobile phase was acetonitrile: water (89:11 v/v). The glucose standard was purchased from Sigma-Aldrich, USA and used to quantify the concentration in samples.

Furfural, 2-furoic acid and furfuryl alcohol analysis

Furfural and 2-furoic acid were determined using LC-MS with a Betabasic-18 column with 3 µm, 100 mm x 2.1 mm at 30°C. The mobile phased was water: methanol with a ratio of 80: 20 *v/v*. To determine furfuryl alcohol, a Gas Chromatography coupled to a Flame ionization detector (Agilent 7890B) was used with a NUKOL® capillary column (15m *x* 0.25 mm and 0.25 µm). The standards were acquired from Sigma-Aldrich, USA.

Peptide sequence analysis

Samples for peptide analysis were prepared using a mini-S-Trap kit (100-300µg) (Protifi, New York, USA). Briefly, 250 µg of protein was reduced using 30 mM of tris(2-carboxyethyl) phosphine and incubated at 55°C during 15 min and cooled at room temperature. Following, an alkylation step was performed using 25 mM of methyl methane thiosulfonate dissolved in isopropanol and incubated at room temperature for 20 min. Then, an acidification step was

performed using 12% of phosphoric acid at room temperature. The acid treated samples were mixed with the binding buffer. The samples were transferred into S- Trap column and washed using 350 µl of triethylammonium bicarbonate (TEAB) buffer three times. Subsequently, trypsin and 50 mM of TEAB buffer (25:1) was added and incubated at 47℃ for 1.5 h. After incubation, the digested peptide was eluted using TEAB buffer, 0.2% formic acid and 50% acetonitrile in water. Finally, peptides from different eluted condition were pooled and dried at 45℃. The final peptides were resuspended in a solution of 5% acetonitrile. Resulting peptides were analyzed using Mass Spectrometry using an Orbitrap elite mass spectrometer (Thermo Fisher Scientific, USA). The data were processed using Proteome Discoverer V:2.2 and aligned with the protein database of UniProt (www.uniprot.org) [188, 189].

Statistical analysis

The statistical analysis was performed using OriginPro®, 2022 software. Parameters such as furfural degradation, biomass production, furfuryl alcohol and 2-furoic acid were evaluated using One-way-analysis of variance at a 95% confidence interval. Pearson correlation was also performed to find the correlation between biomass *vs* furfural profile as well as its metabolized products.

Results and discussion

Furfural degradation and its by-products production profile

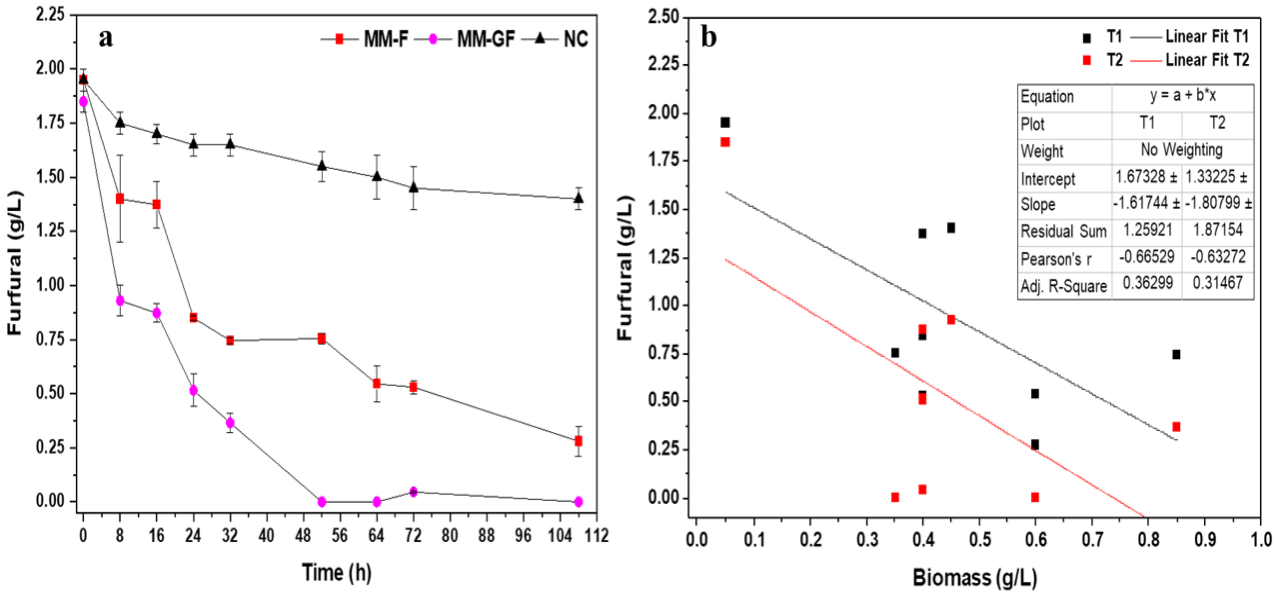

Figure 23. Shows a) Furfural consumption profile by *Rhodosporidium toruloides*-1588 where, MM-F: minimal media-furfural; MM-GF: minimal media-glucose and furfural; NC: negative control and b) correlation between biomass and furfural concentration; where, T1 is minimal media with furfural and T2 is minimal media with glucose and furfural.

Figure 23a shows the furfural degradation profile by *R. toruloides*- 1588 using minimal media. More than 80% of furfural was degraded by 32 h in minimal media containing glucose and furfural while 60% was metabolized by 32 h in minimal media containing only furfural. Additionally, more than 98% of furfural was degraded in minimal media containing furfural and glucose until 56 h. The main reason for the complete degradation of 2 g/L furfural was the presence of glucose. Microorganisms can use glucose to grow and produce energy required to degrade the furfural resulting in its faster degradation than in minimal media containing only furfural. For instance, increased furfural degradation of 25 mg/L*h was observed in minimal media containing glucose and furfural, while 15 mg/L*h in minimal containing furfural. Similar results were reported by Ran, et al. [190], where the authors reported accelerated degradation of furfural by *Amorphotheca resinae* ZN1 grown in the presence of glucose. This suggested that microbial system had a priority for furfural utilization over glucose. On the other hand, the minimal media containing only furfural showed more than 85% of furfural reduction by 96 h. Additionally, biomass production was negatively

correlated with the decreasing furfural concentration (r = -0.66) (Figure 23b). The degradation of furfural by *R. toruloides*-1588 can be confirmed by the presence of furfural metabolized products such as furfuryl alcohol and 2-furoic acid.

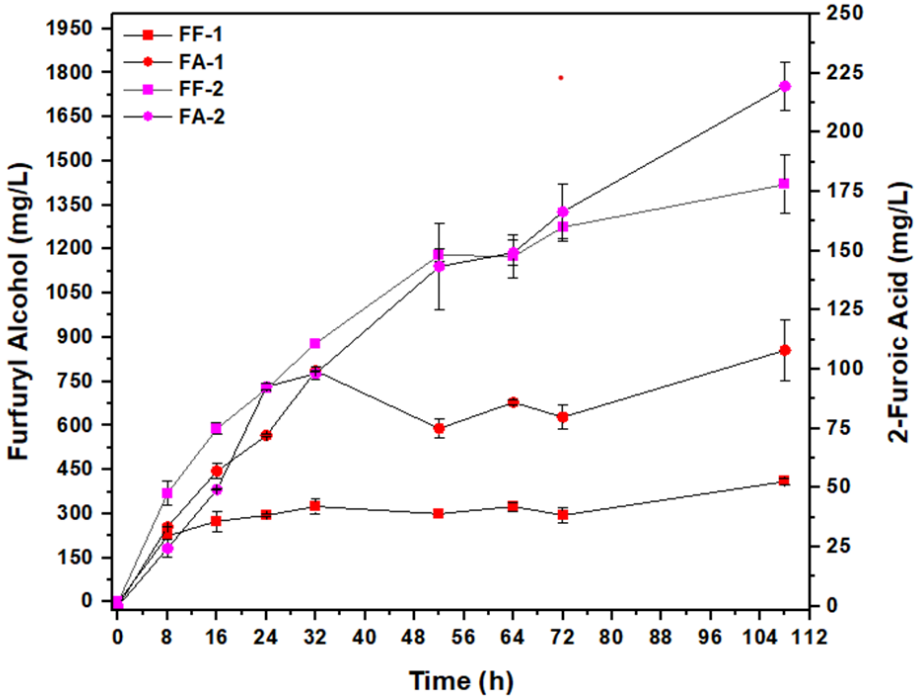

Figure 24. Demonstrate the furfuryl alcohol and 2-furoic acid production profile; where, FF is furfuryl alcohol and FA is 2-furoic acid;1 is minimal media with furfural and 2 is minimal media with glucose and furfural.

Figure 24 displays the furfuryl alcohol and 2-furoic acid production profile in different test conditions. The maximum furfuryl alcohol and 2-furoic acid in minimal media containing furfural and glucose were 1.4 g/L and 0.2 g/L, respectively, while in minimal media containing only furfural was 0.4 g/L and 0.1 g/L, respectively. Production of 2-furoic acid and furfuryl alcohol with respect to biomass production was strongly positively correlated (r = 0.59 and r = 0.47, respectively) (Supplementary Figure 4 (a and b)). Additionally, no furfuryl alcohol and 2-furoic acid, was detected in the control media. In minimal media containing only furfural, *R. toruloides*-1588 thrived on furfural as sole carbon source and might have also consumed metabolized products of furfural for its growth. Thus, low amount of furfuryl alcohol and 2-furoic acid was observed in minimal media containing only furfural in comparison with tested conditions using minimal media containing both glucose and furfural.

In minimal media containing furfural, increase in 2-furoic acid till 32 h was observed following which a sudden decrease in its concentration can be clearly seen (Figure 24). On the other hand, exponential production of 2-furoic acid was detected in minimal media containing both glucose and furfural. The sudden decrease in 2- furoic acid in minimal media containing furfural explained the factthat 2-furoic acid can be potentially transformed into 2-oxoglutaric acid, finally entering Krebs cycle leading to energy production (Supplementary Figure 5) [28, 124]. This can be further corroborated with the present results as concentration of 2-furoic acid was ~3 times lower than the furfuryl alcohol. In addition, oxygen has been reported to play a key role in furfural degradation and its by-product accumulation. For instance, microbes such as *Saccharomyces cerevisiae* or *Escherichia coli* are devoid of a furfural oxidative degradation pathway. However, they can utilize the native oxidoreductase pathways converting furfural into furfuryl alcohol only under anaerobic conditions. In general, microorganisms tend to convert furfural into furfuryl alcohol as it is less toxic than furfural and 2-furoic acid [191]. Following, furfuryl alcohol oxidizes into furfural and then into 2-furoic acid under aerobic conditions [190]. Between these consequent steps furfural as well as 2-furoic acid maintained at low level so that it does not affect the microbial growth and metabolic activities. Hence, this could be another reason for lower concentration of 2-furoic acid than furfuryl alcohol in the minimal media.

Enzyme identification and activity

Table 11. Peptide identification in the samples from furfural and furfural-glucose media using LCMS/MS and corresponding enzyme accession number from UniProt databank.

S. No.	Enzyme	UniProt ID	Molecular Weight (kDa)	Coverage (%)
1	Alcohol dehydrogenase	A0A0K3C8W5	50.2	50
2	Aldehyde dehydrogenase	A0A511KBG5	56.3	40
3	S-(hydroxymethyl)glutathione dehydrogenase	M7XJI6	40.5	25

Table 11 illustrate the identification of digested peptide and their coverage to the corresponding enzyme from the UniProtKB databank. The other identified proteins are provided in Supplementary Table 3. In general, alcohol dehydrogenase and aldehyde dehydrogenase are known to degrade furfural [192]. Several microorganisms have been reported to degrade furfural mostly belonging to Gram negative bacteria (such as *Pseudomonas putida* Fu-1 and *Cupriavidus basilensis*) and fungal strains (such as *Coniochaeta ligniaria* and *Amorphotheca resinae* ZN1) [124]. Moreover, Zhu, et al. [158] reported the multi-omic map of *R. toruloides* NP11 with its complete proteomic profile. The profile includes aldehyde dehydrogenase as well as alcohol dehydrogenase, but no link has been established with furfural degradation by *R. toruloides*–1588. Hence, in the present study, the furfural transformation into 2-furoic acid and furfuryl alcohol and peptide detection confirmed the presence of aldehyde dehydrogenase and alcohol dehydrogenase in *R. toruloides*- 1588. Park, et al. [122] engineered the *Saccharomyces cerevisiae* with aldehyde dehydrogenase gene, which resulted in its increased growth rate and ethanol production in media containing 2 g/L of furfural. Similarly, aldehyde dehydrogenase in *Comamonas testosteroni* has been identified for the oxidation of furans into furanic carboxylic acids [193]. Peng, et al. [194] reported the 95% conversion of furfural into furoic acid using *Escherichia coli* transformed with a horse liver alcohol dehydrogenase gene.

Table 12. Enzyme activity of whole-cell extract from *Rhodosporidium toruloides*-1588 grown in different conditions.

Sample	**Minimal Media**	**Fermentation time (h)**	**Substrate (10 mM)**	**Cofactor (100 µM)**	**Specific activity (IU/mg)**
1	Furfural	16	Furfural	NADPH	93.24
2		96			11.89
3	Glucose	16			1.6
4		96			0.32
5	Glucose and	16			38.26
6	Furfural	96			11.25

Table 12 represents the whole-cell enzyme extract activity using furfural as a substrate. The maximum specific activity was observed at 16 h as 93 IU/mg of protein in yeast cells grown in minimal media containing only furfural while the lowest activity of 1.6 IU/mg of protein was observed in yeast cells grown in minimal media containing only glucose. This led to the fact that dehydrogenase is present in *R. toruloides*-1588 and overexpressed in the presence of furfural. Furthermore, enzyme activity in tested conditions using minimal media containing only glucose decreased to 0.32 IU/mg of protein after 96 h in comparison with the enzyme activity using minimal media containing only furfural, which was decreased to 11.89 IU/mg of protein. Similarly, at 16 h the enzyme activity in minimal media containing both furfural and glucose was 38.26 IU/mg, whereas after 96 h it decreased to 11.25 IU/mg of protein (Table 12). *R. toruloides*-1588 was able to consume glucose in minimal media containing both furfural and glucose, unlike the minimal media containing only furfural, where it had to increase the catalytic activity of dehydrogenase, which resulted in an increase in the activity to metabolize furfural leading to its growth. A similar tendency was reported by Lewis Liu, et al. [187], where the authors reported a specific activity of 86 IU/mg of aldehyde dehydrogenase and 66 IU/mg of alcohol dehydrogenase in recombinant *S. cerevisiae*-50049 grown in presence of 12 mM of furfural. On the other hand, Koopman, et al. [192] reported the activity of aldehyde dehydrogenase in a cell extract from *Pseudomonas putida* and *Cupriavidus basilensis* grown in 10 mM of furfural as 172 and 245 U/g of protein, respectively.

Biomass production and glucose consumption profile

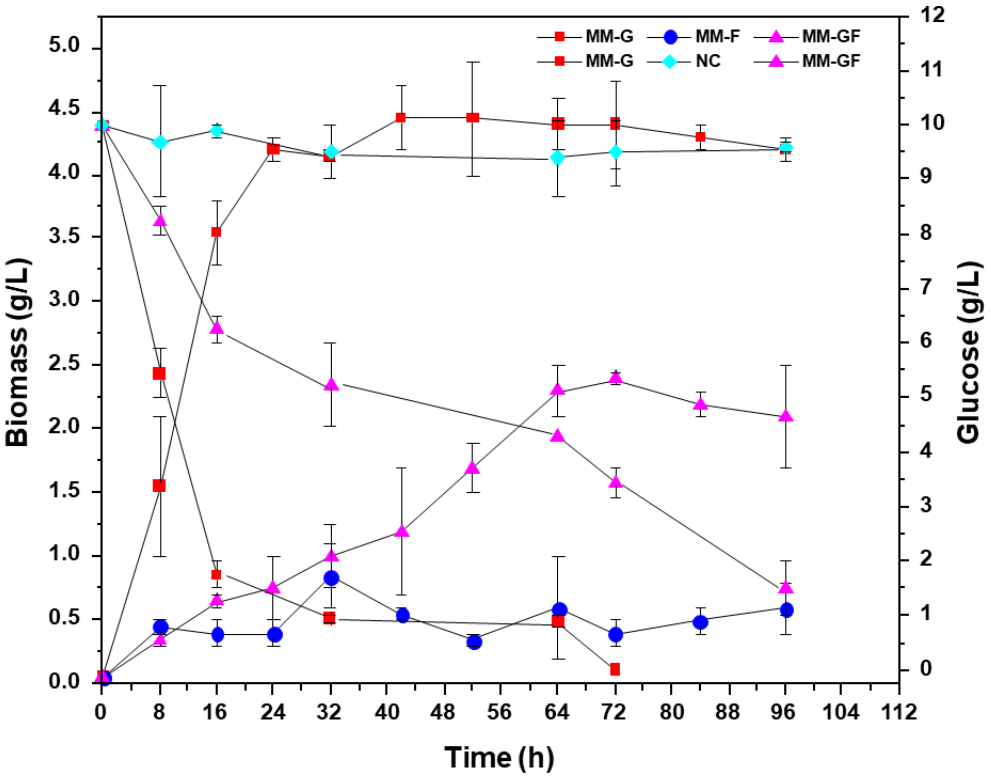

Figure 25. Biomass production and glucose consumption profile in different tested conditions. Where, MM-G: minimal media-glucose; MM-F: minimal media-furfural; MM-GF: minimal media-glucose and furfural; and NC: negative control.

Figure 25 shows the *Rhodosporidium toruloides*-1588 biomass production and glucose consumption profile. The maximum biomass was observed in minimal media containing glucose as 4.45 g/L at 42 h of fermentation, followed by minimal media with glucose and furfural was 2.4 g/L at 72 h and 0.85 g/L at 32 h in minimal media containing only furfural. On the other hand, the glucose consumption profile corresponds to the maximum biomass production profile in minimal media containing glucose and minimal media containing both furfural and glucose. For instance, 90% of glucose was consumed in minimal media containing only glucose within the first 16 h of growth while 80% of glucose was consumed till 64 h of fermentation in media containing furfural and glucose. This could be due to the presence of 2 g/L of furfural resulting in decrease in growth rate of *R. toruloides*-1588 which further led to an extended lag phase in minimal containing both furfural and glucose. This was further related to the maximum biomass peak at 72 h in minimal media containing glucose and furfural as compared to 42 h in minimal media containing only glucose.

Similarly, Sitepu, et al. [195] reported extended lag phase in *Yarrowia lipolytica* and *Lipomyces* sp. grown in media containing 1 g/L of furfural. Chen, et al. [84] reported the complete cell arrest of *R. toruloides* 2.1389 grown in media with 0.5 g/L of furfural while, Zhao, et al. [65] reported 4 g/L of maximum biomass by *R. toruloides* AS 2.1389 grown in media with 2 g/L of furfural, 60 g/L of glucose and 50 g/L of xylose in sugarcane bagasse hydrolysate, respectively. In the present study, despite the lowest biomass in minimal media containing only furfural, the growth of *R. toruloides*-1588 points to tolerance of 2 g/L furfural and growth without the presence of other carbon source such as C5 and C6 carbon sugars. Interestingly, the ability of *R. toruloides*-1588 to grow in minimal media containing only furfural would be an excellent result in terms of process point view, as it can thrive in lignocellulosic biomass-based hydrolysate containing multiple sugars while tolerating the microbial growth inhibitors, such as furfural. Nevertheless, it is necessary to further increase the tolerance of *R. toruloides*-1588 against furfural as well as to decrease the lag phase. For instance, subjecting the strain to the furfural using the adaptive laboratory evolution strategy could result in random mutagenesis ultimately affecting the microbial growth rate. For instance, Liu, et al. [160] subjected the *R. toruloides* Y-1091 strain to increasing concentration of inhibitors (furfural, vanillic acid, 5-hydroxymethyl furfural, syringic acid and syringaldehyde) using wheat straw hydrolysate as culture media. The authors reported the reduction in lag phase by 72 h along with a maximum lipid and carotenoid accumulation of 27.89% (*v/v*) and 14.09 mg/g dry cell weight, respectively. Similarly, *Saccharomyces cerevisiae* was subjected to increasing furfural concentration from 3 to 20 mM. The resulting mutant strain after 300 generations had high ethanol productivity and a shorter lag phase [196]. Hence, this could be a key strategy to decrease the lag phase and increase tolerance against furfural in *R. toruloides*-1588.

Effect on lipids and carotenoids accumulation

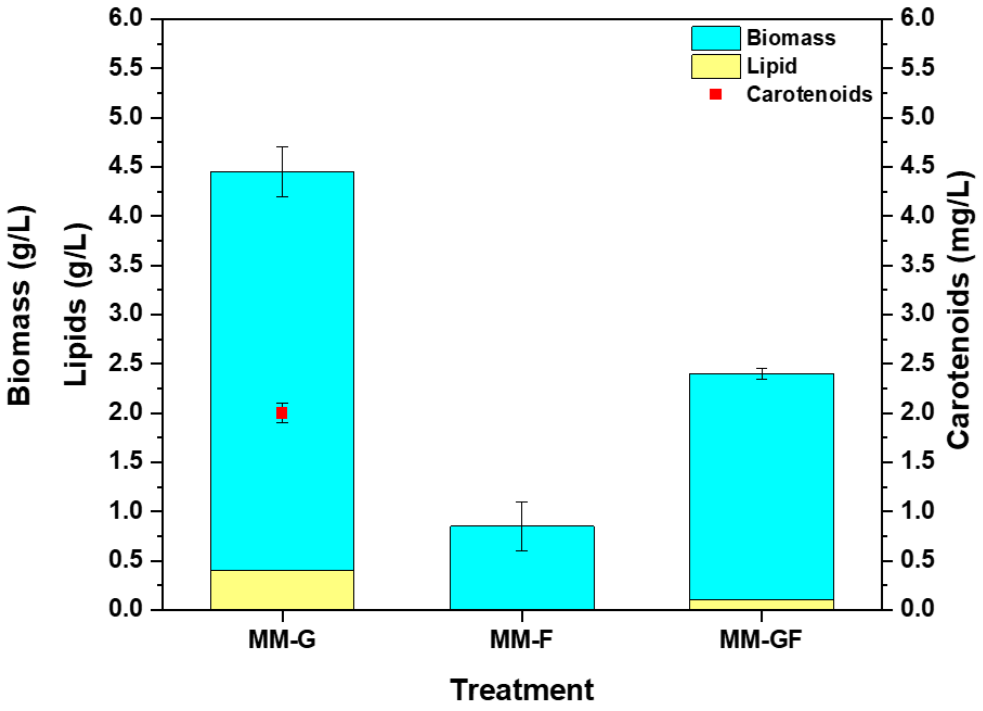

Figure 26. Effect of different media conditions on lipid and carotenoid accumulation in *Rhodosporidium toruloides*-1588; where MM-G: minimal media-glucose; MM-F: minimal media-furfural; MM-GF: minimal media-glucose and furfural.

Figure 26 shows the total lipid, and carotenoid accumulation during different media conditions. Due to the low carbon content in minimal media containing furfural, no microbial lipids and carotenoids was observed, while the maximum lipid and carotenoid of 0.4 g/L and 2 mg/L, respectively were observed in minimal media containing only glucose. It is worth to note thatno carotenoids were detected in minimal media containing furfural.

In general, it is a well-known fact that furfural tends to cause an upsurge of reactive oxygen species resulting in damage of the mitochondria as well as vacuole membranes [197]. For instance, Allen, et al. [198] reported mitochondrial fragmentation and a 50% increase in reactive oxygen species in *S. cerevisiae* exposed to 25 and 50 mM of furfural, which resulted in its inability to grow further. On the other hand, the absence of carotenoids in *R. toruloides*-1588 grown in minimal containing furfural suggests that *R. toruloides*-1588 can use this compound to protect the cellular system from reactive oxygen species while thriving on furfural as a carbon source. Consequently, the absence of carotenoids in media containing

furfural suggest that mevalonate pathway in *R. toruloides*-1588 is highly affected by the presence of furfural. However, to best of our knowledge, research on role of carotenoids in furfural degradation has hardly been reported yet.

It would be beneficial to further investigate the role of carotenoids on inhibitor degradation. For instance, a gene silencing strategy, in which, a key gene related to an enzyme involved in carotenoid production such as the gene for hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl- CoA reductase or phytoene synthase can be knockout from *R. toruloides*-1588. The resulting mutant will be incapable of producing carotenoids and should then be completely exposed to furfural to study its effect. The results will aid in developing a strain capable of tolerating high furfural concentrations. For instance, supplementing the media with antioxidants could reduce the oxidative stress on microorganisms resulting in their increased growth and enhanced product accumulation in the undetoxified wood hydrolysate. The yeast grew in minimal media, consumed furfural as a carbon source, produced biomass, and metabolized furfural into furfuryl alcohol and 2-furoic acid, hence reinforcing the potential of *R. toruloides*-1588 as an inhibitor tolerant strain. Moreover, this would bypass the need for wood hydrolysate detoxification, ultimately aiding in decreasing the biofuel production cost and the development of sustainable and renewable biorefineries.

Conclusion

The results obtained in this study demonstrate the ability of *R. toruloides*-1588 to thrive in furfural media as well as to use it as a carbon source and degrade it into non-toxic products such as furfuryl alcohol and 2-furoic acid. Furfural degrading enzymes i.e., aldehyde dehydrogenase and alcohol dehydrogenase were also identified in *R. toruloides*-1588 with a maximum activity of 93 IU/mg of protein. Additionally, carotenoid accumulation has been found to play a key role in the survival of *R. toruloides*- 1588 when exposed to furfural. This strategy can be further explored to extend the possibility of implementing fermentation at industrial scale to overcome inhibitors while keeping high sugar consumption and high lipid titer. In fact, ability of *R. toruloides*-1588 to grow on inhibitors is imperative to develop sustainable and economical process for biofuel industries.

Supplementary data for this work can be found in Annex D.

CHAPTER 7: LIFE CYCLE ASSESSMENT OF BIODIESEL PRODUCTION USING *RHODOSPORIDIUM TORULOIDES*-1588

Chapter 7 explores the life cycle impact assessment of *R. toruloides*-based lipid accumulation process on environment, resources and human health. It provides the hotspots in biodiesel production process that could negatively impact the environment in-terms of greenhouse gas emissions, toxicity, ecotoxicity and resource depletion.

7.1. Life cycle assessment of *Rhodosporidium toruloides*-1588 based oil production using wood hydrolysate and crude glycerol

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Saini *et al.*, "Life cycle assessment of *Rhodosporidium toruloides*-1588 oil lipid production using wood hydrolysate and crude glycerol". **Under preparation and to be submitted in Journal of Cleaner Production**

Abstract

Rhodosporidium toruloides, an oleaginous yeast, has been reported as a potential feedstock for biodiesel production due to their ability to thrive on hydrolysate derived from lignocellulosic biomass as well as achieve high lipid titer (50-70% *w/w*). In this sense, using the cradle-to-gate approach, the present investigation provides expedient insight into the environmental impacts of *R. toruloides-1588*-based oil production using renewable feedstock such as wood hydrolysate and crude glycerol as a substrate. The normalized life cycle assessment (LCA) results revealed human carcinogenic toxicity as the most impacted category by the lipid extraction stage. In addition, 11 out of 18 midpoint impact categories including global warming potential (93.65 kg CO_2 eq), ozone depletion (1.97E-02 kg CFC11 eq), fossil fuel scarcity (21.59 kg oil eq) and human non-carcinogenic toxicity (74.98 kg 1, 4-dichlorobenzene eq) were found to have a maximum contribution from chloroform used in lipid extraction stage. The environmental performance can be improved by using eco-friendly solvents, increasing lipid recovery and minimizing chloroform usage. In addition, this is the first LCA report for *R. toruloides*-based oil production from wood hydrolysate and crude glycerol, thus, this study can set up a baseline for the further scale of yeast-based oil production.

Keywords: *Rhodosporidium toruloides*; wood hydrolysate; life cycle assessment; oleaginous yeast; wood hydrolysate

Introduction

Renewable biofuel has gained considerable attention due to its ability to replace conventional fuel as well as meet growing energy demand [199]. Numerous countries such as Canada, the USA, France and Europe, have developed policies to reduce fossil fuel usage and promote biofuel production from renewable sources such as energy crops, lignocellulosic biomass as well as industrial and domestic wastes [5]. Under the Clean Fuel Standard (CFS) initiative, Canada aims to increase the biofuel production capacity from 3 to 8.5 billion liters and avoid 30 million tonnes of GHG emissions by 2030 [200]. So far, biofuel such as biodiesel has only been commercialized. Regardless, 85-90% of the current biodiesel-producing industries rely on vegetable oils and cereals because of their facile, cheaper and faster conversion than other sources such as lignocellulosic

biomass [10]. Nonetheless, other alternate substrates should be explored to reduce the pressure on food industries for biodiesel production.

The microbial-based conversion of industrial, agricultural and domestic wastes into biodiesels where lipid-producing microorganisms can be used as feedstock is an upcoming avenue for research. Microorganisms capable of producing more than 20% lipid per cell dry weight are known as the oleaginous microorganisms [5]. Out of these, oleaginous yeast such as *Rhodosporidium toruloides* has gained worldwide interest due to its ability to produce more than 60% lipids, accumulate carotenoids, consume multiple carbohydrates such as glucose, glycerol, galactose, xylose and ability to tolerate microbial growth inhibitors such as organic acids, phenol and furans [12]. In addition, lipids extracted from *R. toruloides* are comparable to vegetable-based oils and the presence of saturated as well as unsaturated fatty acids in lipids makes it versatile feedstock for oleochemicals, nutraceuticals, and transportation fuel production [144].

Meanwhile, numerous efforts have been made for the bioconversion of renewable resources produced from various sectors into biofuels. For instance, lignocellulosic biomass has been widely exploited due to its ability to produce sugar-rich hydrolysate [111, 131, 140, 171, 182, 201-203]. Despite this, most of the studies are limited to laboratory scale due to several hurdles such as high energy and water consumption, low biomass or lipid yield, biomass pretreatment and chemical requirement, greenhouse gases (GHG) emission, toxic byproducts, and waste treatment. Thus, it is important to foresee the potential impacts of microbial-based biodiesel production using lignocellulosic biomass on the environment as well as organisms using life cycle assessment (LCA).

LCA is a standardized tool to evaluate the process feasibility by assessing the environmental impacts associated with the product's life cycle. LCA can cover all the stages starting from raw material, processing, conversion, and disposal. It is a substantial tool to showcase the bioenergy benefits in terms of GHG balance. LCA also provides sensitivity to the change in product concentration as well as uncertainties in environmental and energy performance [204]. Numerous LCA studies are available on the production of biodiesel mostly using microalgae [205-209], while very few are available on oleaginous yeast such as *R. toruloides* [201, 210]. Therefore, it is imperative to perform the LCA studies on *R. toruloides*-based biodiesel production to identify the potential hotspots that could immensely impact the environment. In this sense, this study aims to

evaluate the impact of biodiesel production using *R. toruloides*-1588 grown on renewable substrates. Potential hotspots resulting in high energy, toxicity, eutrophication and water consumption were identified and recommendations to reduce gases emission, as well as energy consumption, are provided. The LCA was carried out at bench scale fermenter to strategize further improvements for large-scale production. Ultimately, the study will promote yeast-based oil production technologies to reduce the usage of resources for numerous purposes such as biofuels, oleochemical and nutraceuticals.

Material and methods

Goal and scope of the study

The goal of the LCA study is to evaluate the environmental impacts associated with oleaginous yeast-based lipid production from the wood hydrolysate. The LCA investigation is primarily based on the laboratory-based experiments carried out by Saini, et al. [131], for biodiesel production. One MJ of energy produced from biodiesel was considered as the functional unit. The LCA was performed for a cradle-to-gate system boundary comprising of five-unit processes, i.e., pretreatment, saccharification, fermentation, lipid extraction and transesterification. Figure 27 displays the system boundary of biodiesel production using sawdust.

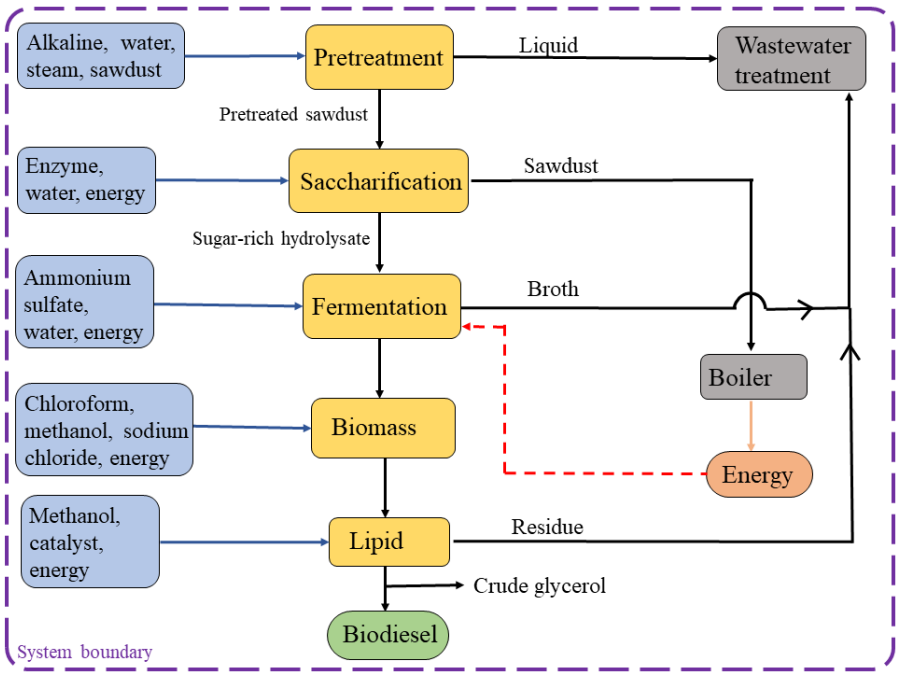

Figure 27. System boundary for *R. toruloides*-based biodiesel production.

Life cycle inventory

The life cycle inventory (LCI) is based on an upscaled fermenter of 1000 L based on assumptions and suggestion provided by Piccinno, et al. [211]. LCI is divided into five stages i.e., pretreatment, saccharification, fermentation, lipid extraction and transesterification (Table 13). Briefly, hardwood sawdust was used as feedstock and pretreated using 1% (*w/w*) alkaline solution (sodium hydroxide) [131]. The pretreatment was followed by enzymatic saccharification using cellic-ctec2, and hydrolysate was separated from lignocellulosic biomass using a centrifuge. The hydrolysate was mixed with crude glycerol (60:40) and subjected to fermentation using *Rhodosporidium toruloides*-1588 and the lipid obtained was 56.3% (*w/w*) [131]. The required energy was calculated using method described by Piccinno, et al. [211].

The lipid extraction and transesterification were performed as described by Sharma, et al. [201]. Briefly, cells were dried at 60°C and mixed with a chloroform-methanol ratio of 2:1 and subjected to Soxhlet extraction. The lipid was treated using hexane and dried using vacuum evaporation and the lipid weight was quantified gravimetrically. Following, the reaction was carried out using a lipid to methanol ratio of 1:10 and zinc oxide as a catalyst. The glycerol and biodiesel were separated using separating funnel. The wastewater treatment unit receives the streams from pretreatment, fermentation, and lipid extraction stages. The lignocellulosic biomass recovered from saccharification stage was sent to boiler. The boiler efficiency was assumed to be 80% and defined as its ability to convert the sawdust heating value into steam heat on basis of its higher heating value [212].

Table 13. Life cycle inventory of biodiesel production using *R. toruloides*-1588.

Process/ Parameters	**Amounts**	**Units**
Pretreatment		
Input		
Hardwood sawdust	75	kg
Sodium hydroxide	7.5	kg
Water	750	L
Electricity	20.19	kWh
Output		

Process/ Parameters	Amounts	Units
Pretreated Biomass	75	kg
Liquor	750	L
Saccharification		
Input		
Pretreated biomass	75	kg
Enzymes	2.25	kg
Citric buffer	375	L
Water	375	L
Energy	1.153	kWh
Output		
Hydrolysate	750	L
Biomass	75	kg
Fermentation		
Input		
Hydrolysate	22.5	kg
Crude glycerol	15	kg
Ammonium sulfate	0.99	kg
Water	750	L
Electricity	150	kWh
Centrifuge	10	kWh
Output		
Biomass	13.5	kg
Supernatant	750	L
Lipid extraction		
Input		
Chloroform	22	kg
Methanol	5.8	kg
Hexane	4.32	kg
Biomass	13.5	kg
Electricity	164.75	kg

Process/ Parameters	Amounts	Units
Output		
Lipids	7.29	kg
Transesterification		
Lipids	7.29	kg
Methanol	3.2	kg
Catalyst (zinc oxide)	3	g
Electricity	17.25	kWh
Output		
Biodiesel	0.13	kg
Crude glycerol	30	g

Life cycle impact assessment

The life cycle impact assessment was performed using SimaPro® v9.1.0 and calculated using ReCiPe 2016 (H) midpoint and endpoint methods. Table 2 displays the list of components from Ecoinvent 3.7 database used for the study. The midpoint impact categories include are ionizing radiation (IR), terrestrial acidification (TA), global warming (GW), land use (LU), ozone formation: human health (OHH) and terrestrial ecosystem (OTE), terrestrial ecotoxicity (TE), marine ecotoxicity (ME), stratospheric ozone depletion (SOD), marine eutrophication (MEu), fossil resource scarcity (FRS), fine particulate matter formation (FPM), human carcinogenic toxicity (HCT), water consumption (WS), human non-carcinogenic toxicity (HNT), freshwater eutrophication (FE), mineral resource scarcity (MRS) and freshwater ecotoxicity (FE). The scores from midpoint categories were summed up and translated to the endpoint categories on resources, ecosystem and human health. The foreground data was derived from laboratory experiments and estimations based on suitable calculation while the background data was derived from the Ecoinvent database v3.7 (Table 14).

Table 14. List of Ecoinvent database used in the study.

Components	Process
Ammonium sulfate	N (RoW)| ammonium sulfate production | Cut-off, U

Citric acid	Citric acid (RNA)| production | Cut-off, U
Electricity	Medium voltage (CA-QC)| electricity voltage transformation from high to medium voltage cut-off, U
Glycerol	Glycerine (RoW)| esterification of soybean oil | Cut-off, U
Hexane	Hexane (RoW)| molecular sieve separation of naphtha | Cut-off, U
Methanol	Methanol | production | Cut-off, U
Sawdust	Measured as dry mass (CA-QC)| suction, sawdust cut-off, U
Sodium hydroxide	Chemical, without water, in 50% solution state (CA-QC)| chlor-alkali electrolysis, membrane cell| cut-off, U
Chloroform	Trichloromethane (RoW)| production | Cut-off, U
Wastewater	Wastewater, average (CA-QC)| treatment of wastewater, average, capacity 1E9l/year | Cut-off, U
Water	Tap water (CA-QC)| tap water production, conventional treatment| cut-off, U
Zinc Oxide	Zinc oxide (RoW)| production | Cut-off, U

Results and discussion

Effect on midpoint categories

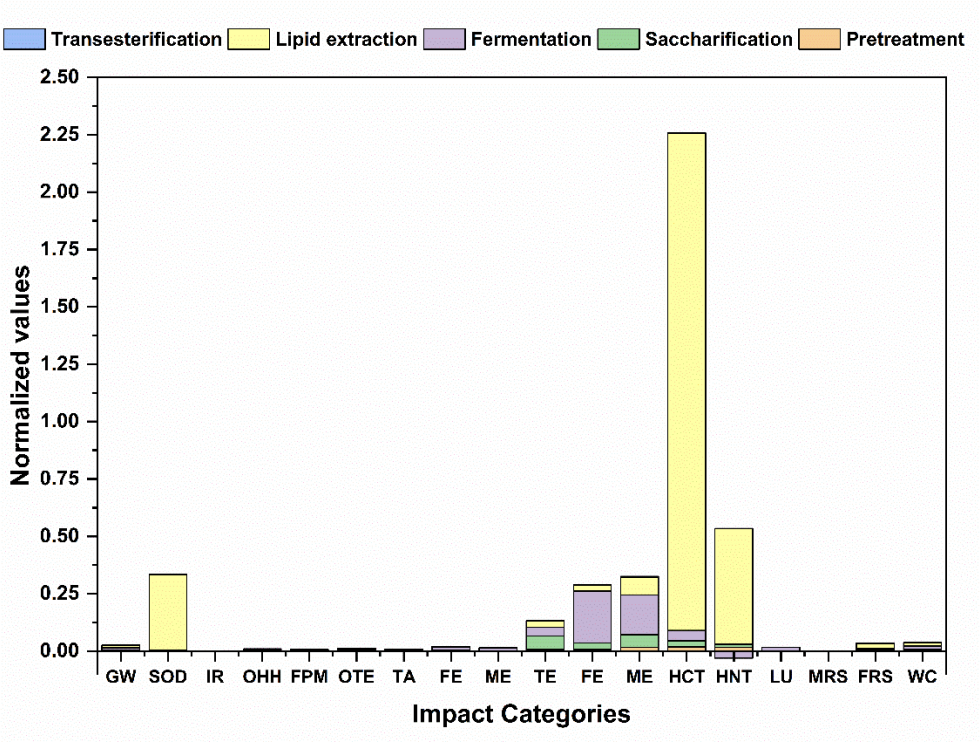

Figure 28. The midpoint normalized data for different biodiesel production stages*;* where, GW: global warming; SOD: stratospheric acid; IR: ionizing radiation; OHH: ozone formation, human health; FPM: fine particular matter formation; OTE: Ozone formation, terrestrial ecosystems; TA: terrestrial acidification; FE: freshwater ecotoxicity; ME: marine ecotoxicity; MEu: marine eutrophication TE: terrestrial ecotoxicity; HCT: human carcinogenic toxicity; HNT: human non-carcinogenic toxicity; LU: land use; MRS: mineral resource scarcity; FRS: fossil fuel scarcity and WC: water consumption.

Figure 28 illustrate the normalized data of biodiesel production stage on midpoint categories. Human carcinogenic toxicity (HCT) was found to be one of the main impacted categories by lipid extraction stage. The main contributor is chloroform (6 kg 1, 4-dichlorobenzene eq) due to its emission during the production and distillation process. It is known to damage several human organs and as well as causes cancer [213]. Also, it has also been found to significantly contribute to ozone depletion. In addition, the lipid extraction stage was energy-intensive process because of electricity requirements for cell drying, grinding, Soxhlet extraction and solvent concentrator. Thus, the solvent could escape during these processes causing harm to the environmental ecosystem. Similarly, the lipid extraction stage was found to be the main contributor to human

non-carcinogenic toxicity (HNT). On the other hand, Barbanera, et al. [214] found enzymatic hydrolysis as the main contributor to the HCT because of corn starch glucose in enzymatic hydrolysis followed by chloroform used in the lipid extraction stage. Alternatively, Huang, et al. [215] replaced the chloroform and hexane with ethyl acetate and reported a decrease in HCT by 90%.

A global warming potential (GWP) of 206.49 kg CO_2 eq. was observed, where lipid extraction and fermentation accounted for around 45.35% and 41.46% of the total GWP, respectively (Figure 29). The total GWP observed is comparable with Sharma, et al. [201] (260.03 kg CO_2 eq.) during lipid production using *Rhodotorula mucilaginosa* IIPL32 and sugarcane-bagasse derived hydrolysate as substrate. The higher GWP could be because of lower lipid yield (25% w/w) and the inclusion of the sugarcane cultivation stage in LCA. On the other hand, sawdust harvesting was not considered in the present investigation and the maximum lipid yield was 56.3% (w/w). Similarly, Lu, et al. [206] emphasized achieving high cell density and high lipid titer in short duration to reduce the overall impact. The authors reported the increase in lipid content by increasing the fermentation time, however, the impact is mainly caused by electricity and nutrients. Moreover, in the present study electricity accounted for around 1.5% of the total GWP. Thus, achieving a high product yield in a short duration will not only aid in decreasing the environmental impact but will also affect the production cost by decreasing the need for nutrients and electricity.

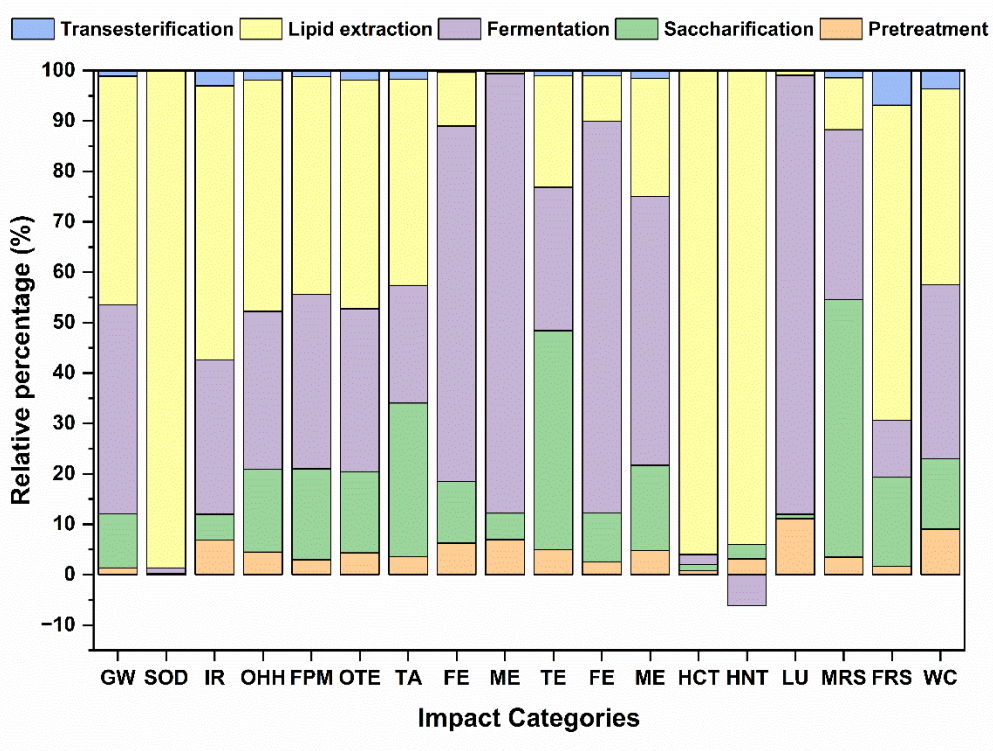

Figure 29. Effect of different stages of biodiesel production on impact categories.

Nevertheless, in the fermentation stage, glycerol contributed more than 95% of GWP. The GWP contribution from glycerol can be further reduced by recycling the crude glycerol produced during the biodiesel production process. For instance, Yang and Rosentrater [216] reported 15% less GWP, when bio-glycerol was used to produce bio-adhesives than petro-glycerol. On the other hand, the high GWP in the lipid extraction stage is attributed to the utilization of a high amount of chloroform which contributed more than 90% (84.77 kg CO_2 eq) to global warming potential. Huang, et al. [217] reported more than 95% contribution when chloroform was used in lipid extraction, rendering this method an environmentally unsustainable scenario. Alternatively, in-situ transesterification method can be used, where lipids present inside the cell can directly be converted into biodiesel. For instance, Huang, et al. [217] observed 10 times lower GHG emissions when in-situ transesterification was used to convert lipids into biodiesel. Likewise, Kumar, et al. [218] extracted lipids using biosurfactant and free-nitrous acid and reported a decrease in GHG emission as well as saved 102 GJ/ tonne of biodiesel energy.

On the other hand, the fermentation stage owing to glycerine use was found to be a major contributor to marine eutrophication (0.06 kg N eq) and freshwater ecotoxicity (0.28 kg 1, 4

dichlorobenzene eq). Eutrophication as well as ecotoxicity owed to the usage and loss of fertilizers required to produce wood. These fertilizers are rich in nitrogen and phosphorus sources and when discharged into water bodies result in increased algal growth. The algal bloom ultimately affects the aquatic biodiversity by depriving them of oxygen [201]. Moreover, eutrophication may vary depending on the type of feedstock. For instance, Bonatsos, et al. [210] utilized the sugarcane and corn as sources of glucose required to produce single-cell oil. The authors reported a 53% lower eutrophication contribution by sugarcane. Similarly, eutrophication contribution within the crops varies such as, sunflower (0.045 kg PO_4 eq/ kg oil), rapeseed (0.018 kg PO_4 eq/ kg oil), palm (0.013 kg PO_4 eq/ kg oil) and soybean (0.005 kg PO_4 eq/ kg oil) [210].

The terrestrial ecotoxicity (TE) was found to be most impacted by the saccharification stage (43 %). Citric acid used for enzymatic saccharification was mainly responsible for TE. Moreover, the impact on TE might vary depending on the type of organic compounds. For instance, Sendão, et al. [219] reported high contribution to TE by urea than citric acid. This is because urea is rich in nitrogen and generally used as fertilizer, however, if used in excess could result in increased ammonia in soil which would ultimately impact the seed germination and plant growth [220]. Similarly, citric acid is reported to affect plant germination by decreasing the pH below 4.0 [221].

Moreover, the lipid extraction stage has also significantly contributed (99%) to stratospheric ozone depletion (SOD). In general, ozone-depleting gases contains chloroform such as, hydrochlorofluorocarbons (HCF), and chlorofluorocarbons (CFC). Therefore, chloroform directly or indirectly when escaped, causes ozone depletion by reacting and converting ozone into an oxygen molecule [201]. Sharma, et al. [201] reported the 2.69E-06 kg CFC11 eq emission while in the study the impact is slightly higher i.e., 1.92E-02 kg CFC11 eq. This is because of the amount of chloroform in this study was increased with respect to the biomass thus ultimately increasing the impact on SOD despite keeping the biomass-to-chloroform ratio same [201].

Lastly, water consumption is impacted equally by the fermentation (34%) and lipid extraction (38%) stages. The high energy requirement is the main contributor (>95%) in both stages. In addition, in the present study, water was not used as a coolant after the pre-treatment of lignocellulosic biomass. However, it could increase water consumption if used as a coolant [222]. Moreover, water consumption is subject to a great deal of uncertainty. For instance, Das and Sarmah [223] reported high energy utilization at a high water-to-biomass ratio. As an alternative,

lower ratios of water to biomass were used resulting in lowering the impact [224, 225] however it could lead to lower sugar yield which will ultimately affect the final product titer as well as the overall environmental impact.

Effect on endpoint categories

Midpoint indicators provide the scores for environmental problems, while endpoint indicators describe the damage while summarizing and aggregating the individual midpoint categories under three damage categories i.e., human health, resource availability and ecosystem quality [226]. These endpoint categories describe the damage caused by a wide range of environmental problems arising due to different production stages. For instance, the human health category defines the loss of life when exposed to chemicals or gases. Similarly, the ecosystem category defines the number of species affected due to the emission of gases, chemicals or by-products from the production system, while the resources category includes the load on its reserves based on their amount and consumption rate required to produce desired products. Figure 30 displays the impact of five stages of biodiesel production on human health (disability-adjusted loss of life year - DALY), ecosystems (species.yr) and resources ($). The lipid extraction stage has a maximum damage contribution of 52% and 65% on human health and resources depletion, while fermentation stage has 66% of damage contribution on ecosystems. The main reason of high contribution from lipid extraction stage is usage of high amount of chloroform as well as high energy requirement.

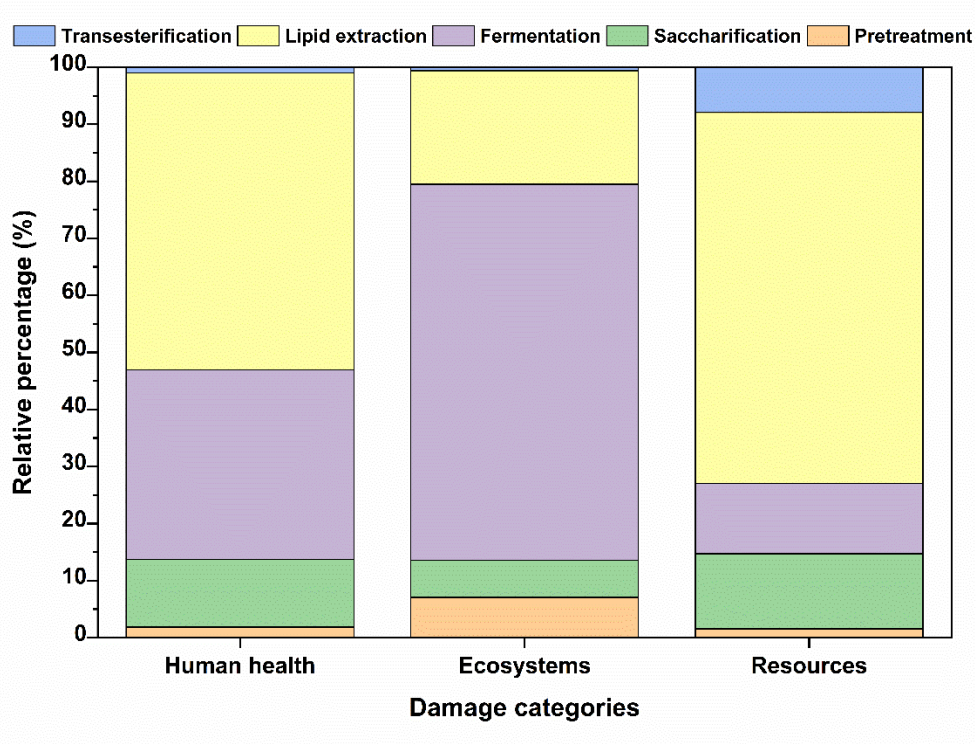

Figure 30. Illustrates the effect of biodiesel production stages on three endpoint categories.

From the environmental point of view, these results indicate that the *R. toruloides*-1588-based biodiesel production from forestry biomass has a further huge scope in terms of decreasing the environmental impact and increasing the lipid titer. The lipid extraction stage is found to be responsible for most of the midpoint and endpoint categories. Thus, several strategies can be incorporated to reduce the environmental impact. For instance, other solvents such as ethyl acetate [215] or dichloromethane [227] can be used in order to increase lipid recovery. Similarly, replacing the lipid extraction stage with in-situ transesterification might help in reducing the extra solvent as well as energy requirement.

Limitations and scope of the study

The present study indicates the sensitivity of high lipid titer as well as high biomass yield in decreasing the overall impact on the environment. The present study is mostly based on lipids as a the main product of *R. toruloides*. However, *R. toruloides* is also known to produce carotenoids such as β-carotene and lycopene [228]. Thus, producing carotenoids as a co-product could help in reducing the global warming potential. On the contrary, it might require extra chemicals for its extraction and purification hence increasing the cost and uncertainty of analysis. For instance, Cai,

et al. [108] reported the LCA of co-products (succinic acid and adipic acid) during biodiesel production. Each co-product is entirely dependent on the substrate used, chemicals added, and energy applied in each unit operation. Therefore, co-products could benefit in reducing the emission depending on the type of conventional petroleum counterparts applied during the process.

Conclusion

High lipid content is found to be crucial in decreasing the impact on the environment. The lipid extraction stage was highly energy intensive as well as found to have impacted several categories such as global warming potential, human carcinogenic toxicity and fossil resource scarcity due to the presence of the high amount of chloroform. Thus, it is imperative to either replace the solvents with other compounds or eliminate the lipid extraction process and directly convert it into biodiesel through in-situ transesterification. Nonetheless, the *R. toruloides*-based biodiesel production is under exploration and requires further optimization to include the co-products such as carotenoids and evaluate their impact on the environment.

Supplementary data for this work can be found in Annex E.

8. CONCLUSIONS

Following conclusions are drawn from the research:

8.1. *Rhodosporidium toruloides*-1588 exhibits the ability to thrive on undetoxified hydrolysate derived from forestry residues (hardwood and softwood sawdust). The maximum lipid accumulation of 36% (*w/w*) along with more than 95% of glucose and 60% of xylose consumption was observed in the hardwood hydrolysate. In addition, the evaluated strain showed tolerance to several growth inhibitors such as furfural, vanillic acid, vanillin, 5-HMF and syringaldehyde. Moreover, several fatty acids including stearic acid, palmitic acid, and oleic acid were observed which as suitable feedstock for biodiesel production. Additionally, omega-3, 6 and 9 fatty acids were also identified and thus expanding the market from biofuel to food and pharmaceutical industries. This signifies that liquid hydrolysate derived from forestry biomass could be used as a potential substrate for yeast-based lipid accumulation.

8.2. Supplementing the hydrolysate with trace metal salts resulted in maximum lipid accumulation up to 40% in *R. toruloides*-1588. In addition, specific trace metal salts also lead to the production of polyunsaturated fatty acids such as docosahexaenoic acid, eicosatrienoic acid, hexadecatrienoic acid and linoleic acid. Thus, this investigation suggests that trace metal salts are useful in increasing lipid accumulation as well as targeting the specific desaturase or elongase enzyme to produce desired fatty acids.

8.3. In the C/N ratio maintenance study, C/N 70 led to the 3 times higher lipid accumulation than control hydrolysate along with specific lipid production of 3.8 mg/g dry weight per hour and 25% (relative weight percentage) of linoleic acid. It symbolizes that the production of PUFA from oleaginous yeast utilizing waste biomass residues is an attractive option for PUFA extraction from fish oil. In addition, more than 90% of carbohydrates (such as glucose and xylose) consumption was observed, which is useful in-terms of low residual sugar release in wastewater treatment plants.

8.4. With the implementation of a co-fermentation strategy using wood hydrolysate and crude glycerol, 49% of lipid accumulation was achieved using the 60:40 carbon ratio of hydrolysate to crude glycerol. More than 95% of carbohydrates such glucose, crude glycerol and xylose consumptions were observed in the media thus reflecting the ability of *R. toruloides*-1588 to consume multiple carbon sources. Furthermore, the scale-up of the bench-scale fermenter resulted

in a 12% increase in lipid accumulation and a 50% reduction in lipid accumulation time than observed in flask fermentation. This could be useful in-terms of reducing the production cost as well as resource consumption during the fermentation process. The recycling of crude glycerol could provide aid in developing a circular bioeconomy and zero-waste production.

8.5. *R. toruloides*-1588 was able to thrive on 2 g/L of furfural and use it as a carbon source. It was able to degrade the furfural into non-toxic compounds such as furfuryl alcohol and 2-furoic acid. Furthermore, furfural degrading enzymes i.e., alcohol dehydrogenase and aldehyde dehydrogenase were also identified with the maximum enzymatic activity of 93 IU/mg of protein. In addition, carotenoids were found to play a key role in yeast growth during furfural exposure. Therefore, the ability of *R. toruloides* to grow on inhibitors is crucial for developing an economical process for biofuel production by removing the media detoxification step.

8.6. In life cycle impact assessment analysis, lipid extraction stage has found to have highest impact on environment including human carcinogenic toxicity, global warming potential, ozone depletion and ecotoxicity. In addition, maximum global warming potential of 206.49 kg CO_2 eq. with 45% and 41% contribution from lipid extraction and fermentation stage were observed, respectively. Chloroform in lipid extraction stage contributed more than 90% impact on HCT (95.9%), resource depletion (65%) and ozone layer depletion (98.6%). Second most contribution was found to be from fermentation stage. It has maximum impact on freshwater eutrophication (70.6%), marine eutrophication (87.2%), freshwater ecotoxicity (87%) and land use (87%). As an alternative, either chloroform can be replaced solvent with high lipid affinity and low environmental impact or *in-situ* transesterification can be performed to convert the lipids into biodiesel without performing the lipid extraction.

8.7. In a nutshell, hydrolysate produced from forestry residue can be a potential culture media for microbial-based lipid accumulation. *R. toruloides*-1588 can thrive on undetoxified hydrolysate, consume >95% glucose, >70% xylose, tolerate inhibitors and accumulate lipids (56.3% *w/w*).

9. FUTURE RECOMMENDATIONS

The following suggestions are based on the findings of the present study:

1. Based on the above results, it was observed that mixing two or more carbohydrates could increase the lipid titer thus, it is essential to identify the carbon flow towards lipids and carotenoid accumulation. The carbon flux analysis will help to identify the carbon flow during different growth conditions.
2. In this dissertation, separate saccharification and fermentation were performed separately, so, further optimization of simultaneous saccharification and fermentation techniques can be employed to reduce the lipid production steps as well as could help in reducing the biofuel production cost.
3. *R. toruloides*-1588 grew and accumulated lipid in media containing 2 g/L of furfural. However, to decrease the lag phase and increase the biomass growth rate, adaptive laboratory evolution can be used to increase the tolerance toward inhibitors as well as decrease the lag phase.
4. High amount of CO_2 release was observed during LCA analysis, thus by developing the co-culturing technique using yeast and algae might result in reducing GHG impact as well as increasing the lipid titer.
5. To study the economic feasibility of lipid accumulation in *R. toruloides*-1588 using forestry-based liquid hydrolysate as culture media.

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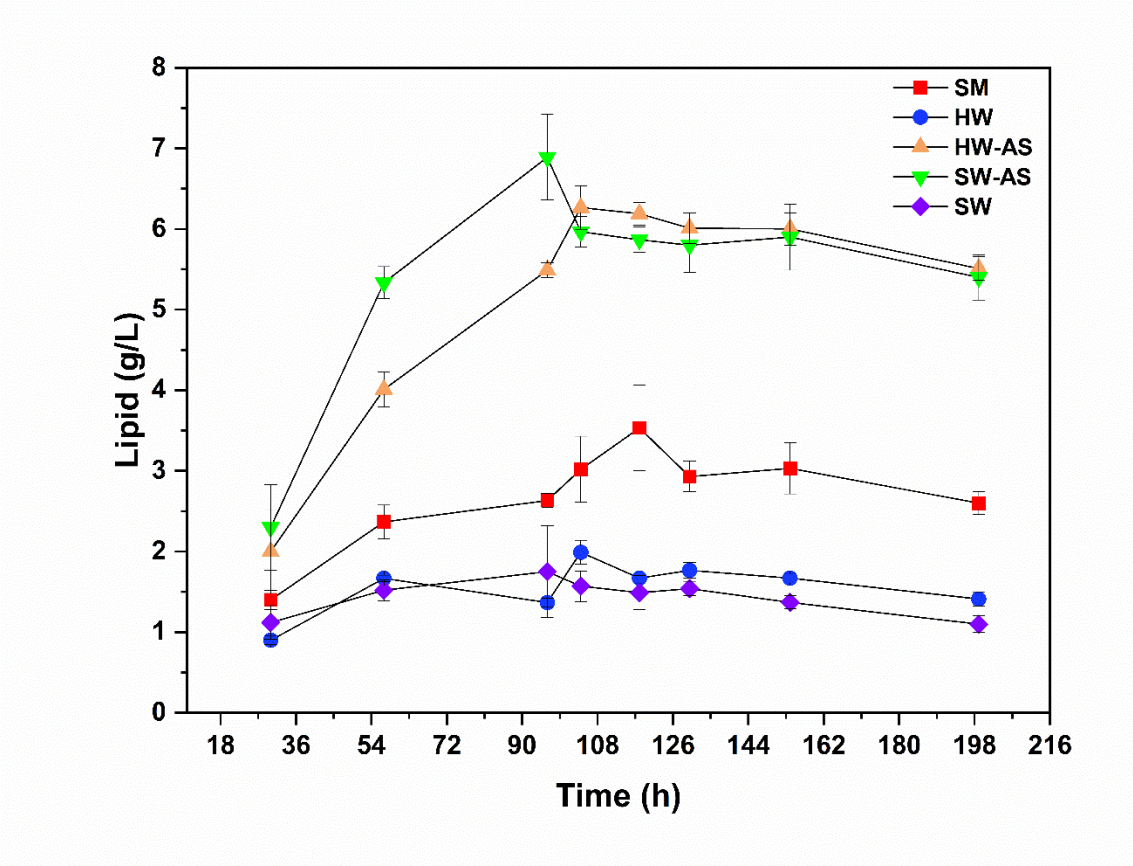
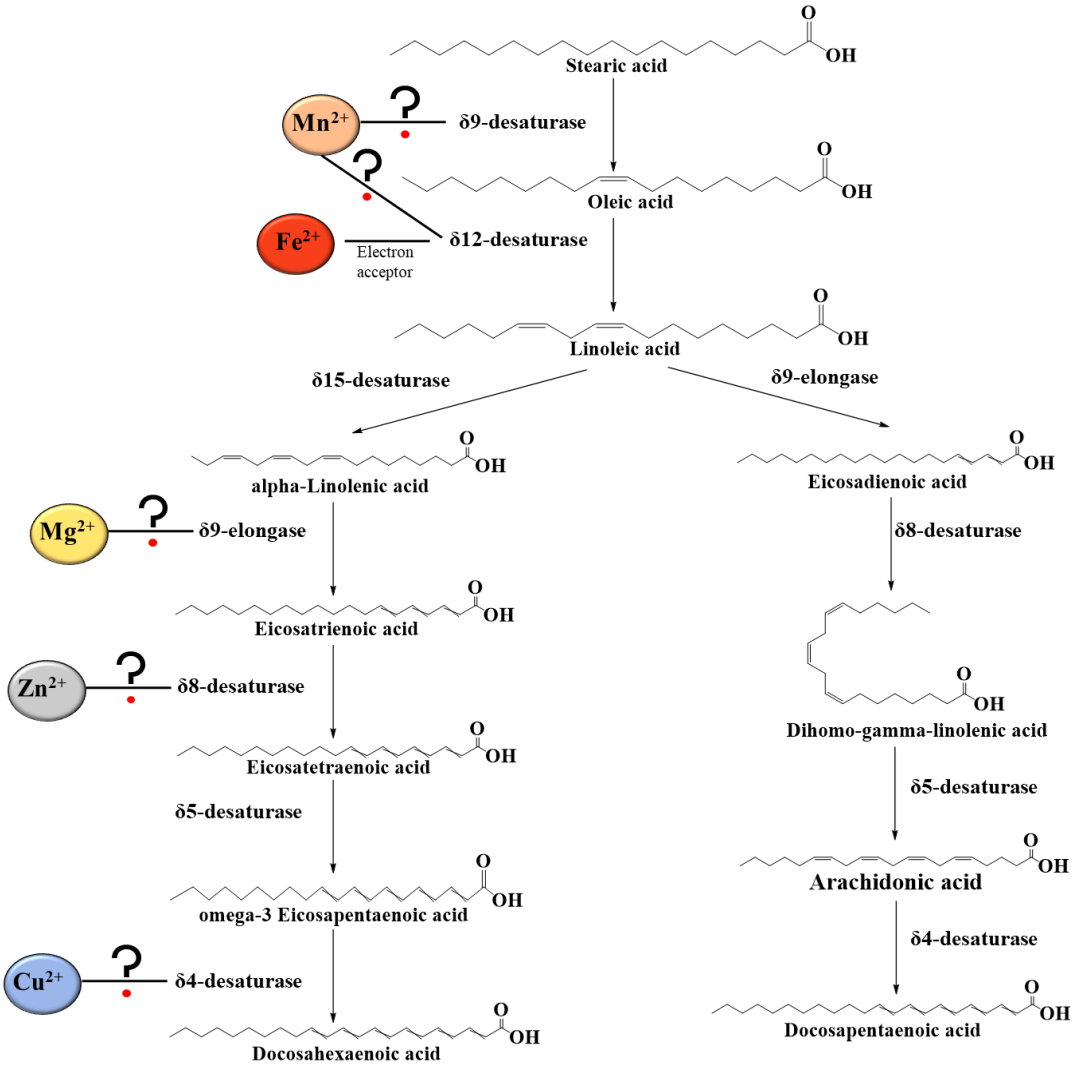
APPENDINCES

APPENDIX A. Supplementary information of Section 4.1



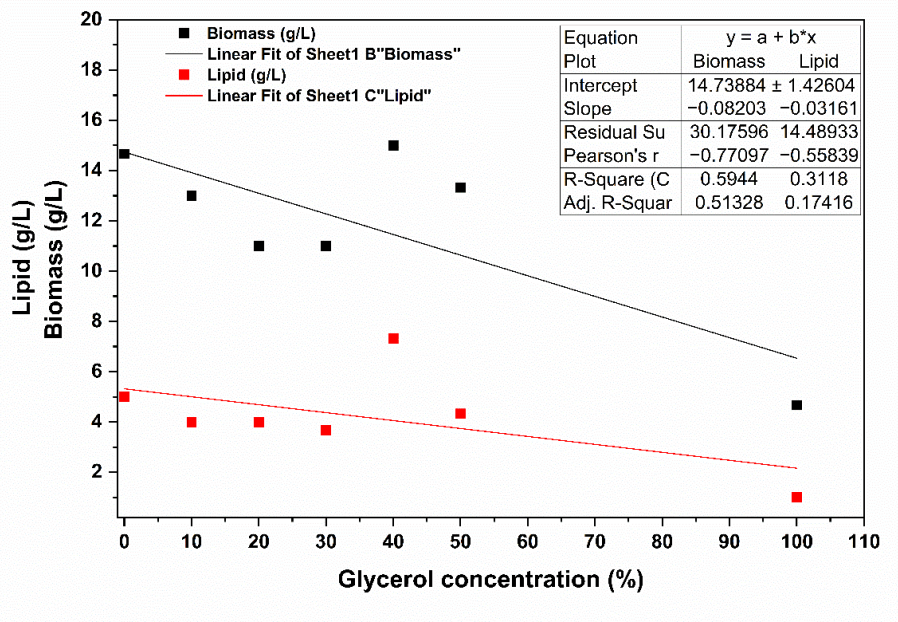
Supplementary Figure 1: The kinetics of lipid production in *Rhodosporidium toruloides*-1588 when grown in different media compositions i.e., synthetic media (SM); hardwood hydrolysate (HW), hardwood hydrolysate + ammonium sulfate (HW-AS); softwood hydrolysate (SW) and softwood hydrolysate + ammonium sulfate (SW-AS).

APPENDIX B. Supplementary information of Section 5.1



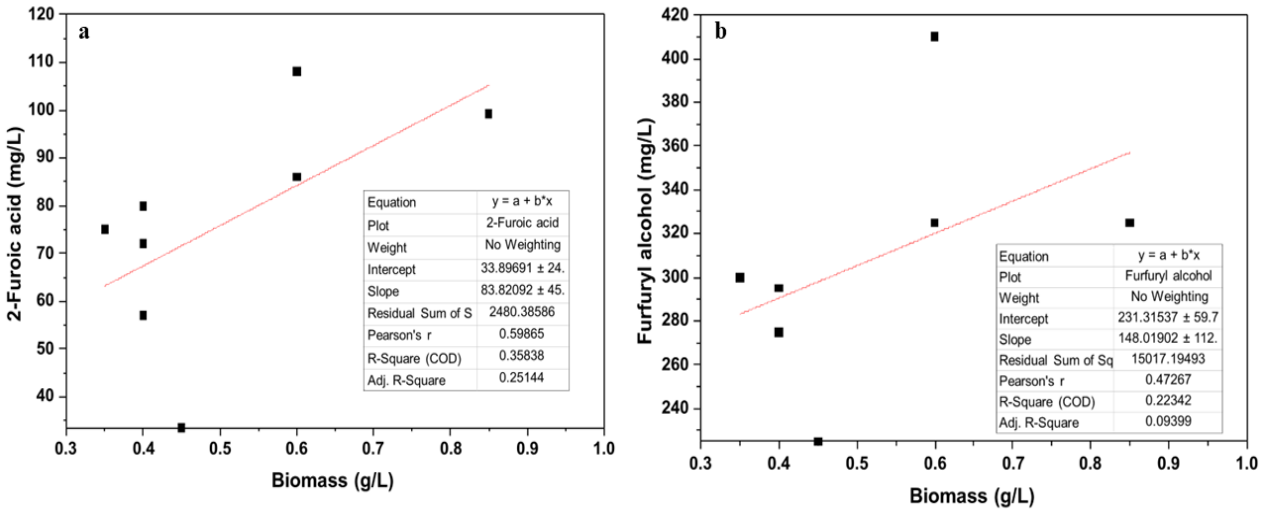
Supplementary Figure 2. Illustration of fatty acid production pathway and potential targets of different trace metals.

APPENDIX C. Supplementary information of Section 5.3

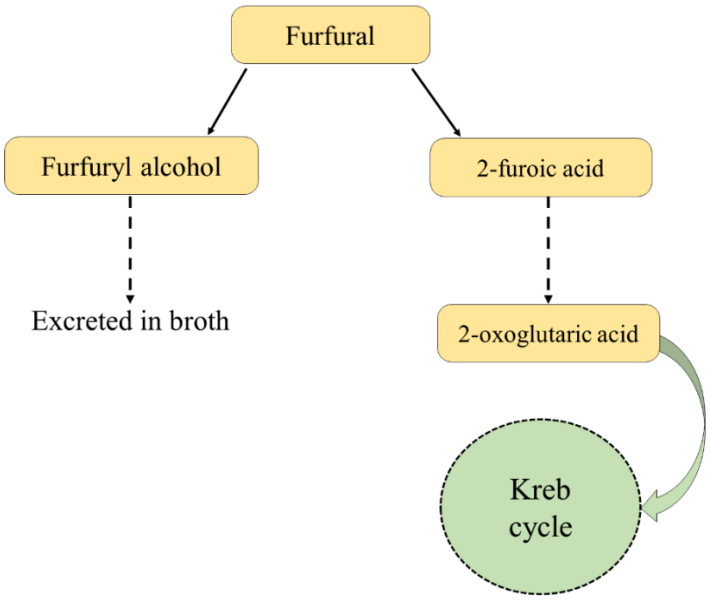


Supplementary Figure 3. Illustrates the correlation profile between glycerol concentration, biomass and lipid accumulated.

APPENDIX D. Supplementary information of Section 6.1



Supplementary Figure 4. Illustration of Pearson correlation between biomass with 2-furoic acid (2a) and furfuryl alcohol (2b) production.



Supplementary Figure 5. Illustration of Furfural degradation pathway

Supplementary Table 1. Peptide profile identified in the samples from glucose, furfural and furfural-glucose media using LCMS/MS and corresponding enzyme accession number from UniProt databank.

S. No.	Enzyme	UniProt ID	Molecular Weight (kDa)	Coverage (%)
1	FGENESH: predicted gene_1.797 protein (Fragment)	A0A0K3CCP2	5.9	42
2	Nucleoside diphosphate kinase	A0A0K3CM94	16.5	31
3	Voltage-dependent ion-selective channel	A0A0K3CMF3	30.6	24
4	ATP synthase subunit alpha	A0A0K3CJN0	56.4	11
5	Elongation factor 1-alpha	A0A0K3C7H5	48.7	10
6	40S ribosomal protein s20	A0A061B3U3	13.7	10
7	Superoxide dismutase	A0A0K3CIK6	23.7	8
8	ADP/ATP translocase	A0A0K3C7I2	33.2	6
9	Citrate synthase	A0A0K3CN89	50.2	5
10	Glyceraldehyde-3-phosphate	A0A0K3CP51	31.3	5
11	Peroxiredoxin	A0A0K3CAW1	24.9	5
12	S-adenosylmethionine synthase	A0A0K3CCG4	41.4	4
13	Inorganic diphosphatase	A0A0K3CIY4	31.9	3
14	Heat shock protein 70 family	A0A0K3C908	70.8	2
15	Amine oxidase	A0A0K3CAM7	87.5	1
16	ATP-citrate synthase	A0A0K3CJ29	125.7	1

APPENDIX E. Supplementary information of Section 7.1

Supplementary Table 2. Impact of parameters used in pretreatment stage on midpoint categories

Impact categories	**Units**	**Sodium hydroxide**	**Sawdust**	**Water**	**Electricity**	**Wastwater**
Global warming	kg CO_2 eq	1.694344	0.576816	0.086578	0.393002	0.065719
Stratospheric ozone depletion	kg CFC11 eq	8.02E-06	4.72E-07	5.44E-08	1.29E-06	9.78E-07
Ionizing radiation	kBq Co-60 eq	0.02695046	0.007955	0.000953	0.031498	0.000377
Ozone formation, Human health	kg NO_x eq	0.00454046	0.003107	0.000207	0.000337	0.000598
Fine particulate matter formation	kg PM2.5 eq	0.00308927	0.00098	0.000188	0.000148	0.000408
Ozone formation, Terrestrial ecosystems	kg NO_x eq	0.00458081	0.003298	0.00021	0.000341	0.000599
Terrestrial acidification	kg SO_2 eq	0.00635499	0.001972	0.000398	0.000476	0.001813
Freshwater eutrophication	kg P eq	8.43E-05	6.14E-05	2.92E-06	4.77E-07	0.000687
Marine eutrophication	kg N eq	0.00014527	1.37E-05	5.47E-07	1.30E-06	0.004377
Terrestrial ecotoxicity	kg 1,4-DCB	4.093067	2.258958	0.126077	0.371238	0.048421
Freshwater ecotoxicity	kg 1,4-DCB	0.00122716	0.000485	8.90E-05	5.70E-05	0.007313
Marine ecotoxicity	kg 1,4-DCB	0.00381907	0.001777	0.000198	0.000293	0.010022
Human carcinogenic toxicity	kg 1,4-DCB	0.01037506	0.001474	0.002446	0.00023	0.035729
Human non-carcinogenic toxicity	kg 1,4-DCB	0.40052598	0.067401	0.030093	0.026492	1.807259
Land use	m2a crop eq	0.06077156	11.16844	0.002588	0.079299	0.001164
Mineral resource scarcity	kg Cu eq	0.00403517	0.000151	0.001208	0.000228	9.61E-05
Fossil resource scarcity	kg oil eq	0.36544901	0.160437	0.021204	0.025167	0.004529
Water consumption	m^3	0.35953671	0.0485	0.760512	0.410587	-0.67123

Supplementary Table 3. Impact of parameters used in enzymatic saccharification stage on midpoint categories

Impact categories	Units	Enzyme	Citric acid	Water	Sodium biocarbonate	Electricity	Electricity: co-generation
Global warming	kg CO_2 eq	9.20115	10.27878	0.086578	2.5269183	0.022443	-0.06437
Stratospheric ozone depletion	kg CFC11 eq	0	3.33E-05	5.44E-08	5.67E-07	7.35E-08	-5.10E-07
Ionizing radiation	kBq Co-60 eq	0	0.042683	0.000953	0.005264692	0.001799	-0.0004
Ozone formation, Human health	kg NO_x eq	0.001637	0.026333	0.000207	0.005866446	1.93E-05	-0.00191
Fine particulate matter formation	kg PM2.5 eq	0.009968	0.013216	0.000188	0.00622717	8.46E-06	-0.00045
Ozone formation, Terrestrial ecosystems	kg NO_x eq	0.002638	0.026881	0.00021	0.005923505	1.95E-05	-0.00192
Terrestrial acidification	kg SO_2 eq	0.034373	0.038891	0.000398	0.022195305	2.72E-05	-0.00143
Freshwater eutrophication	kg P eq	0.000695	0.000727	2.92E-06	0.000208843	2.73E-08	-4.22E-06
Marine eutrophication	kg N eq	0	0.003405	5.47E-07	1.43E-05	7.41E-08	-1.89E-07
Terrestrial ecotoxicity	kg 1,4-DCB	0	58.94779	0.126077	3.2327414	0.0212	-2.08551
Freshwater ecotoxicity	kg 1,4-DCB	0	0.032385	8.90E-05	0.00213305	3.26E-06	-0.00018

Impact categories	Units	Enzyme	Citric acid	Water	Sodium biocarbonate	Electricity	Electricity: co-generation
Marine ecotoxicity	kg 1,4-DCB	0	0.053606	0.000198	0.004529155	1.67E-05	-0.00145
Human carcinogenic toxicity	kg 1,4-DCB	0	0.0616	0.002446	0.011712408	1.31E-05	-0.0004
Human non-carcinogenic toxicity	kg 1,4-DCB	0	2.064939	0.030093	0.35991453	0.001513	-0.32491
Land use	m2a crop eq	0	1.910249	0.002588	0.15600982	0.004529	-1.21905
Mineral resource scarcity	kg Cu eq	0	0.039565	0.001208	0.043957168	1.30E-05	-5.49E-05
Fossil resource scarcity	kg oil eq	2.577762	3.055153	0.021204	0.48486146	0.001437	-0.0164
Water consumption	m^3	0.046917	0.483714	0.760512	0.095597896	0.023448	-0.00074

Supplementary Table 4. Impact of parameters used in yeast cultivation stage on midpoint categories.

Impact categories	Units	Ammonium sulfate	Water	Glycerine	Electricity	Wastwater
Global warming	kg CO_2 eq	1.638728	0.086578	80.70433	3.114427	0.065719
Stratospheric ozone depletion	kg CFC11 eq	4.79E-07	5.44E-08	0.000214	1.02E-05	9.78E-07
Ionizing radiation	kBq Co-60 eq	0.001628	0.000953	0.049336	0.249611	0.000377
Ozone formation, Human health	kg NO_x eq	0.002726	0.000207	0.055151	0.002672	0.000598
Fine particulate matter formation	kg PM2.5 eq	0.002125	0.000188	0.052144	0.001174	0.000408
Ozone formation, Terrestrial ecosystems	kg NO_x eq	0.002837	0.00021	0.061453	0.002705	0.000599
Terrestrial acidification	kg SO_2 eq	0.005172	0.000398	0.061115	0.003773	0.001813
Freshwater eutrophication	kg P eq	3.04E-05	2.92E-06	0.008714	3.78E-06	0.000687
Marine eutrophication	kg N eq	1.46E-06	5.47E-07	0.052633	1.03E-05	0.004377
Terrestrial ecotoxicity	kg 1,4-DCB	0.630217	0.126077	35.65204	2.941957	0.048421
Freshwater ecotoxicity	kg 1,4-DCB	0.000405	8.90E-05	0.269095	0.000452	0.007313
Marine ecotoxicity	kg 1,4-DCB	0.000865	0.000198	0.165558	0.002323	0.010022
Human carcinogenic toxicity	kg 1,4-DCB	0.001626	0.002446	0.081245	0.001822	0.035729
Human non-carcinogenic toxicity	kg 1,4-DCB	0.098969	0.030093	-6.76095	0.209944	1.807259
Land use	m2a crop eq	0.02401	0.002588	87.92814	0.628422	0.001164
Mineral resource scarcity	kg Cu eq	4.53E-05	0.001208	0.052764	0.001807	9.61E-05
Fossil resource scarcity	kg oil eq	0.46209	0.021204	3.20004	0.199442	0.004529
Water consumption	m^3	0.002426	0.760512	0.129049	3.253786	-0.67123

Supplementary Table 5. Impact of parameters used in lipid extraction stage on midpoint categories.

Impact categories	Units	Methanol	Hexane	Trichloromethane	Electricity
Global warming	kg CO_2 eq	3.666969	2.002522	84.77398	3.206886
Stratospheric ozone depletion	kg CFC11 eq	2.00E-06	2.76E-06	0.019716	1.05E-05
Ionizing radiation	kBq Co-60 eq	0.00612	0.077168	0.196066	0.257021
Ozone formation, Human health	kg NO_x eq	0.006003	0.011331	0.070014	0.002751
Fine particulate matter formation	kg PM2.5 eq	0.003163	0.004499	0.061062	0.001209
Ozone formation, Terrestrial ecosystems	kg NO_x eq	0.006508	0.014567	0.071239	0.002786
Terrestrial acidification	kg SO_2 eq	0.008799	0.013333	0.10073	0.003885
Freshwater eutrophication	kg P eq	8.99E-05	2.61E-05	0.001297	3.89E-06
Marine eutrophication	kg N eq	1.43E-05	1.76E-06	0.000337	1.06E-05
Terrestrial ecotoxicity	kg 1,4-DCB	1.738208	3.220655	22.6844	3.029296
Freshwater ecotoxicity	kg 1,4-DCB	0.006762	0.003296	0.021662	0.000465
Marine ecotoxicity	kg 1,4-DCB	0.008414	0.006674	0.061437	0.002392
Human carcinogenic toxicity	kg 1,4-DCB	0.003988	0.003082	5.993548	0.001876
Human non-carcinogenic toxicity	kg 1,4-DCB	0.357778	0.247207	74.16846	0.216177
Land use	m2a crop eq	0.005051	0.019388	0.233252	0.647078
Mineral resource scarcity	kg Cu eq	0.003929	0.000632	0.010651	0.001861
Fossil resource scarcity	kg oil eq	4.280617	3.68422	13.42332	0.205363
Water consumption	m^3	0.024153	0.032674	0.501572	3.350383

Supplementary Table 6. Impact of parameters used in transesterification stage on midpoint categories.

Impact categories	Units	Methanol	Zinc oxide	Electricity
Global warming	kg CO_2 eq	2.023155	0.001967	0.335774
Stratospheric ozone depletion	kg CFC11 eq	1.11E-06	7.44E-10	1.10E-06
Ionizing radiation	kBq Co-60 eq	0.003377	8.19E-06	0.026911
Ozone formation, Human health	kg NO_x eq	0.003312	3.83E-06	0.000288
Fine particulate matter formation	kg PM2.5 eq	0.001745	2.65E-06	0.000127
Ozone formation, Terrestrial ecosystems	kg NO_x eq	0.003591	3.90E-06	0.000292
Terrestrial acidification	kg SO_2 eq	0.004854	4.54E-06	0.000407
Freshwater eutrophication	kg P eq	4.96E-05	4.96E-08	4.08E-07
Marine eutrophication	kg N eq	7.91E-06	3.11E-09	1.11E-06
Terrestrial ecotoxicity	kg 1,4-DCB	0.959011	0.174484	0.31718
Freshwater ecotoxicity	kg 1,4-DCB	0.003731	8.37E-06	4.87E-05
Marine ecotoxicity	kg 1,4-DCB	0.004642	0.000149	0.00025
Human carcinogenic toxicity	kg 1,4-DCB	0.0022	3.77E-06	0.000196
Human non-carcinogenic toxicity	kg 1,4-DCB	0.197395	0.010661	0.022635
Land use	m2a crop eq	0.002787	8.43E-06	0.067752
Mineral resource scarcity	kg Cu eq	0.002168	1.62E-07	0.000195
Fossil resource scarcity	kg oil eq	2.36172	0.000604	0.021502
Water consumption	m^3	0.013326	6.66E-06	0.350799

Supplementary Table 7. Impact of parameters used in pretreatment stage on endpoint categories.

Damage Categories	Units	Sodium hydroxide	Sawdust	Water	Electricity	Wastewater
Human health	DALY	3.70E-06	1.18E-06	1.67E-07	4.66E-07	8.49E-07
Ecosystems	species.yr	8.08E-09	1.02E-07	1.09E-09	2.48E-09	2.80E-10
Resources	USD2013 ($)	0.08945466	0.058378	0.005486	0.010433	0.001308

Supplementary Table 8. Impact of parameters used in pretreatment stage on endpoint categories.

Damage Categories	Units	Enzyme	Citric acid	Water	Sodium bicarbonate	Electricity	Electricity: Co-generation
Human health	DALY	1.49E-05	1.96E-05	1.67E-07	6.59E-06	2.66E-08	-4.18E-07
Ecosystems	species.yr	3.45E-08	6.52E-08	1.09E-09	1.54E-08	1.41E-10	-1.16E-08
Resources	$	0.20973	1.10509	0.0054	0.087855422	0.000596	-0.00728

Supplementary Table 9. Impact of parameters used in pretreatment stage on endpoint categories.

Damage Categories	Units	Ammonium sulfate	Water	Glycerin	Electricity	Wastewater
Human health	DALY	2.89E-06	1.67E-07	0.000107	3.69E-06	8.49E-07
Ecosystems	species.yr	6.30E-09	1.09E-09	1.03E-06	1.96E-08	2.80E-10
Resources	USD ($)	0.131832	0.005486	1.091742	0.082675	0.001308

Supplementary Table 10. Impact of parameters used in lipid extraction stage on endpoint categories.

Damage Categories	Units	Methanol	Hexane	Trichloromethane	Electricity
Human health	DALY	5.54E-06	4.83E-06	0.000165	3.80E-06
Ecosystems	species.yr	1.34E-08	1.09E-08	2.77E-07	2.02E-08
Resources	USD ($)	1.517875	1.62305	3.709207	0.08513

Supplementary Table 11. Impact of parameters used in transesterification stage on endpoint categories.

Damage Categories	Units	Methanol	Zinc oxide	Electricity
Human health	DALY	3.05E-06	5.94E-09	3.98E-07
Ecosystems	species.yr	7.39E-09	9.13E-12	2.12E-09
Resources	USD ($)	0.837448	0.000175	0.008913

Supplementary Table 12. Normalized impact data of different stages of biodiesel production.

Impact categories	Units	Pretreatment	Saccharification	Lipid extraction	Fermentation	Transesterification
Global warming	kg CO_2 eq	0.000352621	0.002760848	0.011725024	0.010718345	0.000295584
Stratospheric ozone depletion	kg CFC11 eq	0.000180594	0.000559452	0.32951147	0.003769404	3.68E-05
Ionizing radiation	kBq Co-60 eq	0.000140884	0.000104626	0.001115662	0.000627961	6.30E-05
Ozone formation, Human health	kg NO_x eq	0.000427152	0.001562832	0.004378782	0.002981736	0.000175148
Fine particulate matter formation	kg PM2.5 eq	0.000188164	0.001140176	0.002734398	0.002191108	7.33E-05
Ozone formation, Terrestrial ecosystems	kg NO_x eq	0.000508314	0.001900273	0.005354112	0.003817371	0.000218807
Terrestrial acidification	kg SO_2 eq	0.000268737	0.00230466	0.003092605	0.0017634	0.000128484
Freshwater eutrophication	kg P eq	0.00128806	0.002509829	0.002182373	0.014535515	7.71E-05
Marine eutrophication	kg N eq	0.000984654	0.000742143	7.90E-05	0.012373692	1.96E-06
Terrestrial ecotoxicity	kg 1,4-DCB	0.006656339	0.058133818	0.029599021	0.038019752	0.001399901
Freshwater ecotoxicity	kg 1,4-DCB	0.007474926	0.028062845	0.026231157	0.22604332	0.003087048

Impact categories	Units	Pretreatment	Saccharification	Lipid extraction	Fermentation	Transesterification
Marine ecotoxicity	kg 1,4-DCB	0.015609082	0.055132512	0.076469871	0.17341732	0.004885558
Human carcinogenic toxicity	kg 1,4-DCB	0.018141676	0.027207234	2.1669004	0.044354996	0.000866625
Human non-carcinogenic toxicity	kg 1,4-DCB	0.015646187	0.014302727	0.50318032	-0.030964515	0.001547929
Land use	m2a crop eq	0.001832586	0.000138401	0.000146573	0.014350661	1.14E-05
Mineral resource scarcity	kg Cu eq	4.76E-08	7.05E-07	1.42E-07	4.66E-07	1.97E-08
Fossil resource scarcity	kg oil eq	0.000588322	0.006246502	0.022025392	0.003965051	0.002431503
Water consumption	m^3	0.003404633	0.005285432	0.01465793	0.013029522	0.001365493