OLEAGINOUS YEAST AS A CELL FACTORY FOR THE SUSTAINABLE BIOFUEL FEEDSTOCK PRODUCTION FROM THE CANADIAN FOREST RESIDUES

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ABSTRACT

With an ever-growing population, global energy demand increases, thereby contributing to the depletion of fossil resources and their limited reserves. Thereby, to lessen the environmental damage caused by fossil fuels, there has been a surge of interest in developing and producing biofuels from renewable feedstocks, such as microbial lipids. Typically, they are derived *via* a biochemical process using liquid hydrolysates obtained from forestry residues as a substrate. However, microbial lipid production using hydrolysates presents numerous challenges, including the need for a strain that can accumulate high lipid titers, consume five-carbon sugars (C5), and tolerate inhibitory compounds (e.g., furans, phenols, and organic acids), among others. Out of several microorganisms, *Rhodosporidium toruloides*, an oleaginous yeast, could be a potential alternative to produce lipids. It is known to accumulate lipids up to 70% of its dry cell weight, use different carbon sources, and tolerate several inhibitory compounds. In this sense, the current thesis explores the ability of *Rhodosporidium toruloides* as a bio-factory to produce microbial lipids using C5 and C6 wood hydrolysates as a culture media. Different *R. toruloides* strains were screened, and *R. toruloides*-1588 was determined to have the highest lipid accumulation of 35%. Following the culture media, carbon to nitrogen ratio, use of lipid inducers, and sugar concentration optimization, the lipid accumulation increased from 35% to 57.14%,with 95% and 80% of glucose and xylose utilization in hydrolysates, respectively. Likewise, palmitic, stearic, and oleic fatty acids were the most prominently on the produced lipids. Finally, *R. toruloides*-1588 demonstrates the capacity to grow, accumulate lipids, and transform furfural into furfuryl alcohol and 2-furoic acid. The strain was also assessed for its ability to tolerate inhibitory compounds, such as 5-hydroxymethyl furfural, vanillin, syringaldehyde, levulinic acid, ferulic acid, acetic acid, vanillic acid, and aminobenzoic acid. With all these findings, this dissertation concludes that *R. toruloides*-1588 is a suitable microorganism to produce microbial lipids, which can serve as a feedstock to manufacture biodiesel or advanced biofuels using undetoxified wood hydrolysates as a renewable and sustainable culture media.

Keywords: *Rhodosporidium toruloides*, Wood hydrolysate, Lipid production, Fatty acids, Sustainability, Biofuels, Fermentation technology, Genetic engineering, Modeling, Scale-up

DEDICATION

This thesis is dedicated to my family for being there and for always supporting me and loving me.

"Fear is the mind-killer.....I will face my fear"
Frank Herbert

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ABBREVIATIONS

Ac-CoA	Acetyl-CoenzymeA
ACL	ATP citrate lyase
ACC1	Acetyl CoA carboxylase
ADP	Adenosine diphosphate
ALD1	Aldehyde dehydrogenase
ALD4	Aldehyde dehydrogenase4
ALD6	Aldehyde dehydrogenase6
AMP	Adenosine monophosphate
AMP-deaminase	Adenosine monophosphate deaminase
ANOVA	Analysis of variance
ARD1	arabitol dehydrogenase
ARTP	atmospheric room temperature plasma
ATP	Adenosine triphosphate
$CaCl_2$	Calcium chloride
C/N	Carbon:Nitrogen
CoA	Coenzyme A
CTM	Citrate malate translocase
DCW	Dry cell weight
DGA	Diglyceride acyltransferase
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
EJ	Exajoules
FAA1	Long-chain acyl-CoA synthetase
FAA2	Long-chain acyl-CoA synthetase
FAA3	Long-chain acyl-CoA synthetase
FAA6	Long-chain acyl-CoA synthetase
FAME's	Fatty acid methyl esters
FAs	Free fatty acids
FAS2	Fatty acid synthase subunit alpha, fungi type
FAO	Food and Agriculture Organization of the United Nations
FBA	Fructose 1,6-P2 aldolase
FID	Flame ionization detector
FTIR	Fourier-Transform Infrared Spectroscopy
GC	Gas chromatography
GDP	Gross domestic product
GHGs	Greenhouse gases
GPD	Glycerol 3-phosphate dehydrogenase
G6PDH	Glucose 6-P-dehydrogenase
HCl	Hydrochloric acid
5-HMF	5-hydroxymethyl furfural
IDH	Isocitrate dehydrogenase enzyme
KGD	α-ketoglutarate dehydrogenase
KH_2PO_4	Monopotassium phosphate
LC-MS	Liquid Chromatography-Mass Spectrometry
MAE	Microwave-assisted extraction

MAP kinase	Mitogen-activated protein
MDH1	L-malate dehydrogenase, mitochondrial
$MgSO_4$ $7H_2O$	Magnesium sulfate heptahydrate
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NADP	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
Na_2HPO_4	Disodium phosphate
$(NH_4)_2SO_4$	Ammonium sulfate
NH_4OH	Ammonium hydroxide
OD	Optical density
PBH	p-hydroxybenzaldehyde
PBS	Phosphate-buffered saline
PDC	Pyruvate decarboxylase
QFIC	Quebec Forest Industry Council
RKI1	Ribose-5-phosphate isomerase
RNA	Ribonucleic acid
RPE1	Ribulose 5-Phosphate Epimerase
SDR	Short-chain dehydrogenase/reductase
SRM	Selected reaction monitoring
TAGs	Triacylglycerols
TCA	Tricarboxylic acid cycle
TGL	Triglyceride lipase
TKL1	Transketolase1
TOR kinase	Target of rapamycin kinase
TPI1	Triosephosphate Isomerase 1
TRS	Total reducing sugars
TT	Thermal treatment
UV–VIS	Ultraviolet-visible
XDH	Xylitol dehydrogenase
XKS1	Xylulokinase
XYL1	Xylose reductase
XYL2	Xylitol dehydrogenase

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CHAPTER ONE: BACKGROUND

1. INTRODUCTION

According to the International Energy Agency (IEA), biofuels are defined as "energy generated from the conversion of biomass into liquid, solid and gaseous products". Now, bioenergy is classified into two main categories: traditional and modern. Traditional bioenergy refers to the traditional use of lignocellulosic biomass (charcoal and wood) for cooking and heating. On the other hand, modern bioenergy refers mainly to technologies used to produce liquid biofuels which contributes five times more to the total energy demand in comparison with the bioenergy produced from wind and solar sources. For instance, liquid biofuels such as bioethanol and biodiesel can be used by the transport sector, in which 90% of its consumption depends on fossil fuels. Because of the above facts, the growing demand for energy, the limited reserves of fossil resources for their production and the damage caused to the environment have aroused the interest to develop and implement new methods and technologies to obtaining biofuels from renewable feedstocks [1], [2]. In this sense, currently, the feedstocks used for biofuel production are highly diverse. For instance, to produce biodiesel, raw and refined vegetable oils, cooking oils, and rendered animal grease has been used. Nevertheless, this feedstock has some restrictions to be used, for example in the case of vegetable oils, the amount of land used for crop cultivation. The aforementioned can have a negative impact on national food security as well as on the supplies chain of the country that uses this feedstock for biofuel production. The previous fact has generated the use of different feedstocks with special characteristics such as all year availability, economically competitive, renewable, and that can be obtained through simple processes [3]. A potential alternative are microbial lipids obtained using renewable substrates such as liquid hydrolysates obtained from lignocellulosic biomass, because they can be produced relatively easily and have begun to show their profitability [4]. Lipids obtained through microorganisms is an independent process in terms that do not depend directly on climatic conditions, does not require large amounts of land, and can use residues from different industries (industrial, agricultural, and foods) as an energy source for its growth [5], [6]. Furthermore, the above action contributes to decreasing environmental pollution caused by agricultural, food, beverage, and forestry industries due to in most cases, companies do not have appropriate infrastructure and technology to handle, process or take advantage of the residues [3], [7]. An excellent alternative for microbial lipid production is *Rhodosporidium toruloides* that during the last two decades has generated a growing interest due to its great potential to accumulate lipids (up to 70%), their capability to utilize five and six-carbon

sugars as a carbon source as well as a high tolerance to microbial inhibitory compounds. The above facts open different areas of opportunity that need priority attention to make the biofuel process suitable. This dissertation drives you trough several chemical, physical, and biological processes and its combination. Firstly, the literature review (Chapter Two) offers a general perspective about the forestry industry in Canada as well as its residues as a potential renewable feedstock to produce wood hydrolysates that can be used as renewable substrate to produce microbial lipids. Furthermore, a wide perspective about the production technologies, advantages, and disadvantages about its use as a culture media to produce microbial lipids using *Rhodosporidium toruloides*. Likewise, the Chapter Four in this dissertation provides key information about the alternative use and exploitation of undesired compounds present in wood hydrolysates that has been reported to be responsible to inhibit the microbial growth during the biochemical process. This information is the great value due to the fact that most of the substrates content these compounds are submitted to different process to remove these undesired compounds, with a consequent economic increase to the microbial lipid production process. In Chapter Five, the dissertation provides an extensive analysis about the effect of different culture conditions to improve five carbon sugars as well as lipid accumulation by *Rhodosporidium toruloides* using undetoxified wood hydrolysates as a culture media. Lastly, the Chapter Six present novel data about the degradation by *Rhodosporidium toruloides* of several compounds that can cause partial or total microbial growth inhibition, decreasing considerably the lipid accumulation process. In summary, this dissertation provides key information on i) impact, consequences, and implications of pretreatments utilized on forestry residues to produce wood hydrolysates, ii) potential use of wood hydrolysates as a culture media for lipid production, and iii) tolerance to inhibitory compounds, and its impact in lipid production using wood hydrolysates as a substrate. In this sense the research in this dissertation contribute to developing new processes with a biorefinery approach as well as to promote the efficient conversion of forestry residues in form of liquid hydrolysate to produce value-added bioproduct and raise a renewable-green biofuel industry.

CHAPTER TWO: LITERATURE REVIEW

2. REVIEW OF LITERATURE

2.1. Biofuels: a general overview

Biofuels can be defined as organic fuel, that can be obtained from some type of biomass or its derivatives in combination with any of the current biotechnological processes to generate thermal energy by combustion [8]. Figure 1 shows the classification of biofuels using biomass as a primary source of feedstock. According to the International Energy Agency in 2019, the biofuels it produces provide 1.5% of the transportation fuel worldwide. This fact has generated an increase in biofuels production around the world during the last decade. Currently, biofuels production is focused on specific parameters such as yield increasing, high-energy content, reduction of greenhouse gases (GHGs), low carbon content, and economic feasibility.

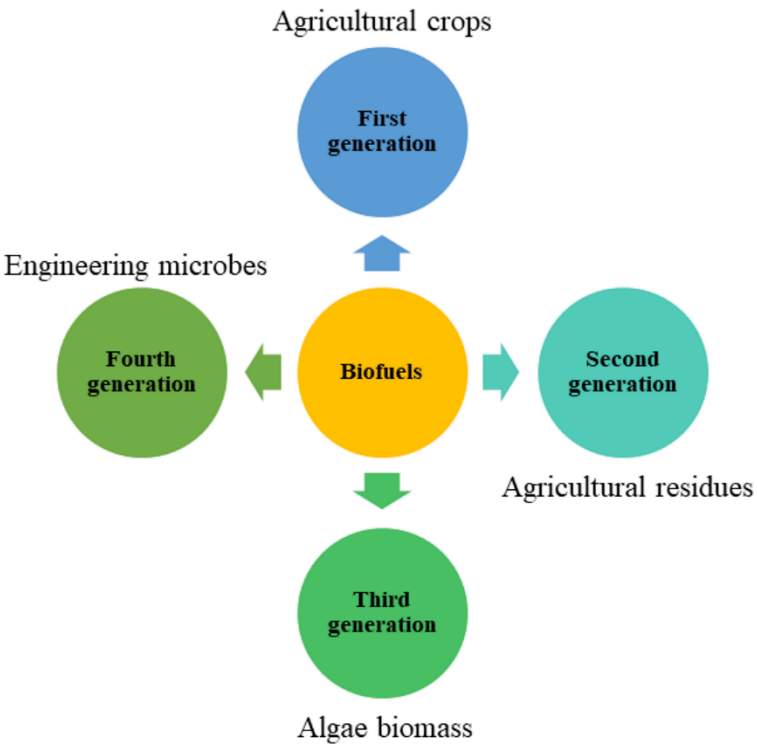

Figure 1.Biofuel classification

In recent years, a great variety of biomass has been used for biofuels production. Biomass is divided mainly into four categories: lignocellulosic (agricultural and cellulosic residues, food wastes, forest residues, urban wood residues), sugar and starch (food residues with residual sugars and starch from maize, rice, wheat, or sugarcane mainly), solid wastes (municipal solid waste, non-recycled paper, furniture and demolition wood wastes), bio-oils (forestry and agricultural

oleaginous crops, waste fats, grease, oils, and algal oil), other wastes (residues from wastewater treatment plants, animal wastes, landfill gas, and biogas) [9]. The annual primary production of this raw material worldwide is approximately 220 billion tons on a dry basis (4500 exajoules (EJ) of solar energy captured), equivalent to 270 EJ of sustainable energy (International Energy Agency, 2019).

However, the best yields in terms of energy have been obtained from lignocellulosic biomass (64% of the total biomass produced), specifically from wood and its residues. Fast-growing tree species such as spruce, fir, willow, eucalyptus, and poplar are commonly used by the forest industry. The average yield of this species is up to 13 tons of biomass in dry weight per hectare/year under controlled conditions. Likewise, residues from the forestry industry such as sawdust, bark, chips, among others, are a potential renewable source for biofuels production [10].

As a response to pollution caused by fossil fuels, biofuels have been strongly investigated in European Union countries such as France, England, and the Netherlands. In the American continent, the main countries in terms of developing processes for biofuels obtention using lignocellulosic biomass are the USA, Brazil, and Canada. Particularly, in Canada, the transformation of lignocellulosic biomass, especially from the forestry industry, represents approximately 6% of the total energy supply [11]. This fact has been generated the interest of Canadian governments (federal and provincial) during the last two decades, to develop and study new processes for biofuel production, especially "advanced biofuels". These "advanced biofuels" differ from the conventional ones because they are being produced it using green conversion technologies and methods and from renewable feedstocks, to minimize the use of land and water, as well as lower greenhouse gas emissions during their combustion [12]. In this sense, Canada and its vast forestry industry represent a great potential for the development and production of conventional or advanced biofuels through lignocellulosic biomass as feedstock.

2.2. Forest and its industry in Canada

Canada has 35% of its land covered with forest, which is equivalent to 3.47 million hectares. The Department of Natural Resources of Canada summarizes that 76.6% of the total forest area is distributed into all provinces, 12.9% in territorial reserves, 6.2% in private areas, 2% in indigenous areas, 1.65% in federal areas, 0.3% in municipalities and 0.4% in other forms. Figure 2 shows the

distribution of predominant tree species in the Canadian forest, with spruce and poplar being the most abundant (Natural Resource Canada, 2019).

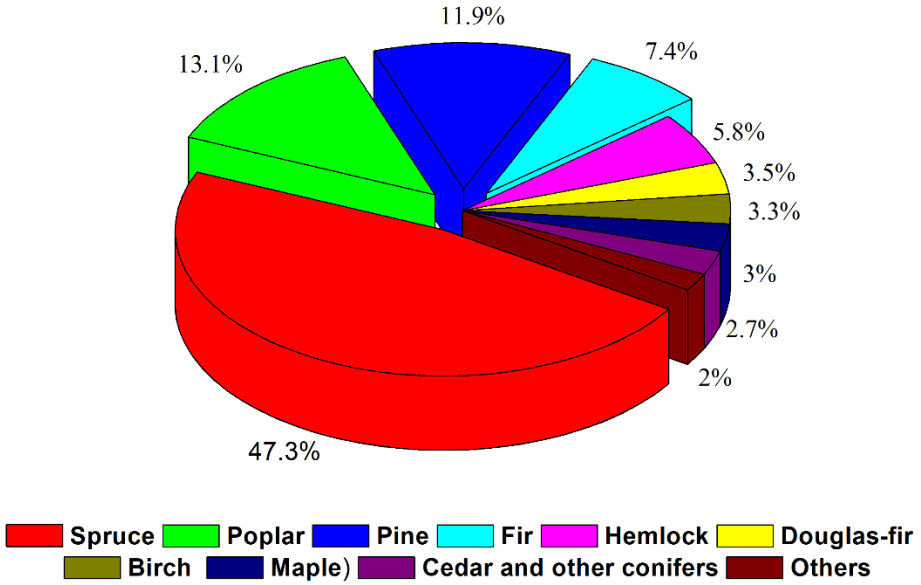

Figure 2.Tree genus distribution in the Canadian forest.

The forestry industry is one of the most important manufacturing sectors in Canada. In 2017, this industry generated more than 209,000 jobs across the country, contributing $24.6 billion, equivalent to 1.6% of total Canada´s gross domestic product (GDP). Furthermore, this industry creates more jobs than other sectors, so it contributes in a better manner to the economic trade due to added-value in all their products (Natural Resource Canada, 2019). According to the Food and Agriculture Organization of the United Nations (FAO), the sawn wood is the principal product of the Canadian forestry industry with a production of 4.50 million cubic meters. Figure 3 shows the main wood products obtained through a minimum transformation process, as well as the residues generated from this industry. In general, the tendency in production and residues during the past four years was constant with an exception in the production of sawn wood process by coniferous species.

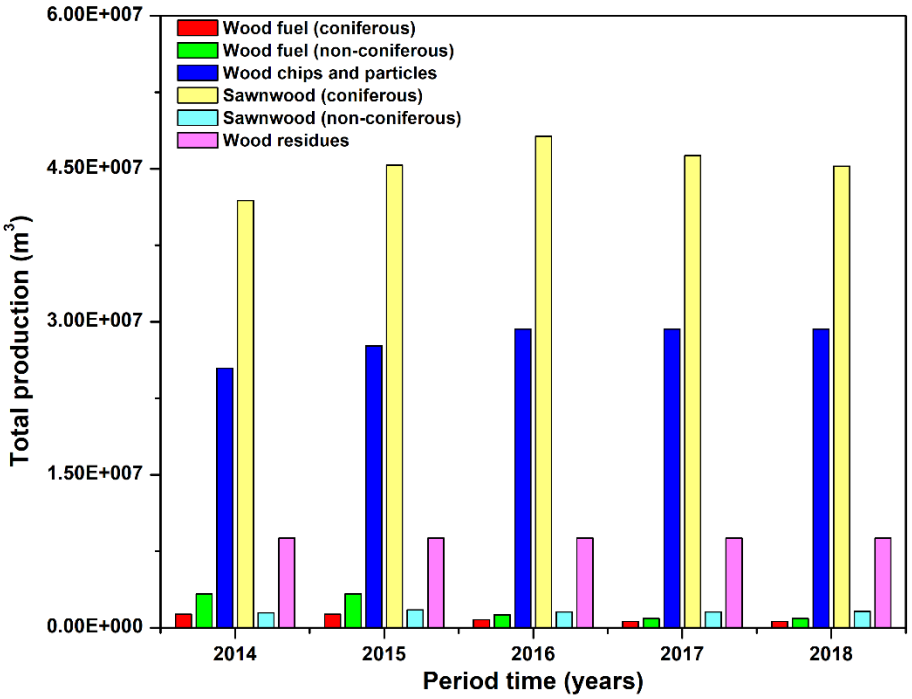

Figure 3.Total production of wood products.

The forestry in Canada shows a big capacity to export a wide range of products to other industrial sectors. Figure 4 shows the six main products that the Canadian forestry export. However, with Canada's commitment to clean technology and the transition to a low-carbon economy, non-traditional forest products, such as advanced by-products (green chemicals and biofuels production), are gaining importance (Natural Resource Canada, 2019). Additionally, the biorefinery concept to lignocellulosic biomass exploitation specifically is focused on forestry residues due to having enormous potential as a feedstock for the obtention of a wide range of products. In this sense, the Canadian government is advocating scientific research to develop green processes and increase the added value to this renewable feedstock and its residues.

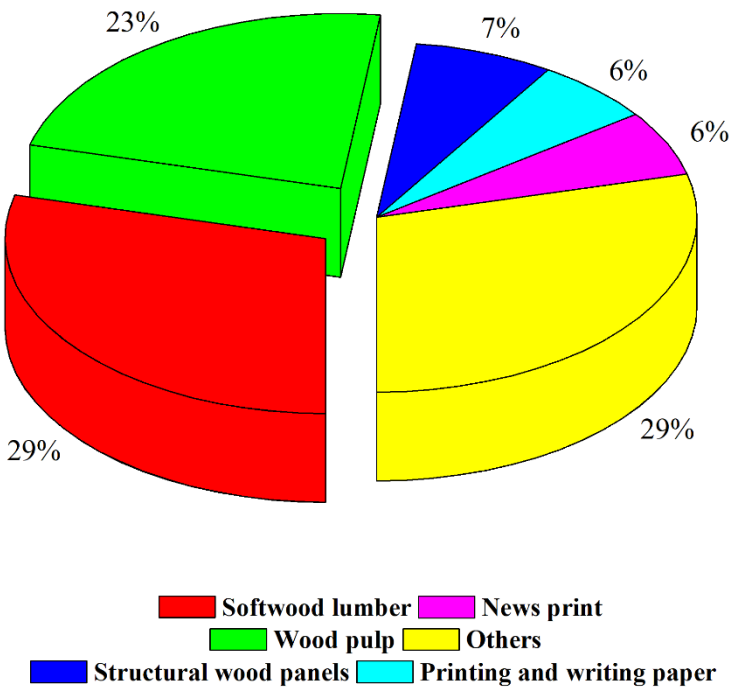

Figure 4.Main forestry products exported by Canada in 2017 (Natural Resource Canada, 2019).

Lignocellulosic residues account for approximately 50% of all biomasses produced on the earth. Within this residue, cellulose and hemicellulose represent 35% and 25% in agricultural residues, and 40% and 20% in forestry residues, respectively. In general, lignocellulosic biomass is composed of three different fractions: cellulose (~30 to 50%), hemicellulose (~20% to 35%) and lignin (~15% to 25%). The main advantage of lignocellulosic biomass is that it can be hydrolyzed and thus obtain different compounds, mainly sugars, that can be used as feedstock to produce several industrial products [6]. Nevertheless, due to the complex structure and rigidity of lignocellulosic biomass, it is necessary to perform a pretreatment to break the lignin fraction and have access to the cellulose and hemicellulose to obtain five and six-carbon sugars. Likewise, the amount and compositions of these three components could vary between lignocellulosic material sources and Likewise, the amount and compositions of these three components can vary according to the lignocellulosic biomass source, increasing the specificity of the used pretreatment [13]. On the other hand, these three components individually have different characteristics, which are described in Table 1.

Table 1.Main characteristics of the lignocellulosic biomass.

Component	Features
Cellulose	Linear polymer linked by β-1,4 glycosidic bonds. Composed mainly of glucose units. Made up of two regions, a crystalline (2/3 of the total cellulose), and amorphous cellulose. Insoluble in water.
Hemicellulose	Linear or branched heterogeneous polymer. Composed of five different sugar units (xylose, arabinose, mannose, glucose, and galactose). Homopolymers or heteropolymers of short chains are linked by β-(1,4) glycosidic or β- (1,3) glycosidic linkages. The classification is determined by the remains of sugars (xylan, mannans or glucans). Hemicellulose features are determined by biomass sources. Structure completely amorphous.
Lignin	It is an insoluble amorphous compound. It is formed by coumaric alcohol, sinapyl alcohol, and coniferyl alcohol units. It gives a very high resistance to the cell wall, provides support, isolation, and protection. Difficult to degrade. Lignin features are different between species, age, and stage of growth.

The disruption of the lignocellulosic biomass matrix (lignin, hemicellulose, and cellulose) is carried out through the use of different pre-treatments. The above generates an opportunity to create a long-term industry based on the use of a biological basis [14]. This suggests a more extensive, efficient, economical, and flexible process development since these processes are the part that generates the most expensive cost in the productions process and most cases determine if the process is economically viable [15].

Pretreatments that are applied to wood biomass and its residues, such as wood chips and sawdust, are carried out before the saccharification of cellulose to obtain fermentable sugars [16]. The effect of these pretreatments on biomass is to break lignin and solubilize hemicellulose fractions, to increase the surface area of accessible cellulose. Another benefit of using a pretreatment is that the hemicellulose can be hydrolyzed into its monomeric forms (D-xylose and D-arabinose) during this process, as well as reducing the crystallinity of cellulose, which represents a great benefit at the time of carrying out its hydrolysis [17]. Currently, the processes that are most used for this purpose, are physical- and thermochemical methods, such as steam explosion, which shows higher proportions of lignin and hemicellulose solubilization, a decrease in toxic compounds released as well as an easy scale-up technology [18]. Chemical, physical, and biological-based pretreatments have been used to produce substrates rich in sugars that can be used in biochemical processes as feedstock. However, during this process, several chemical compounds are generated, which vary in presence and quantity depending on the type and conditions of pretreatment. The chemical pretreatments, using acids (sulfuric, nitric, phosphoric, malic, formic), oxidant agents (hydrogen peroxide), alkaline compounds (sodium hydroxide) or organic solvents (ethyl alcohol, methyl alcohol), generate several compounds (weak acids, furans, phenolic and aldehydes). When using physicochemical and thermochemical pretreatments such as extrusion, ammonia, fibre explosion, ultrasonication, autohydrolysis or wet oxidation, the concentration of the above compounds is lower than in treatments where a chemical catalyst is used. However, with this type of pretreatment, the recovery of carbohydrates is low. The biological treatments are well known because they do not generate inhibitory compounds [1], [17]–[19]. Table 2.2 shows some of the advantages and disadvantages of each type of pretreatment.

In this sense, the use of combined treatments is suggested, as they improved the solubilization of lignin and hemicellulose from lignocellulosic biomass, which improves the rate of saccharification carried out chemically or enzymatically. The importance of a good choice of pre-treatment method lies mainly if it effectively disrupts the structure of the lignocellulosic biomass for the efficient production of fermentable sugars from its two main compounds (cellulose and hemicellulose). In the first instance, it could be assumed that the use of chemical processes is the best pre-treatment for lignocellulosic biomass, since in the same operation the solubilization of lignin and hemicellulose is obtained, as well as the hydrolysis of cellulose. However, this type of pre-

treatment also generates co-products (aromatic compounds and organic acids), which have a toxic effect on microorganisms [20]. These effects will be discussed in the following sections.

Table 2.Advantages and disadvantages of pretreatments methods on lignocellulosic biomass.

Pretreatment	Physical	Chemical	Biological	Physicochemical
Advantages	- It does not use chemical catalysts. - Reduce the size of the raw material. - Alters hemicellulose. - Decreases the degree of polymerization of cellulose. - There is a high recovery of xylose.	- It eliminates hemicellulose and lignin. - Diluted acids eliminate hemicellulose. - Strong acids hydrolyze cellulose. - Acids are accessible and inexpensive. - Increases surface area and makes hydrolysis faster.	- Suitable for the environment. - Low energy consumption. - Profitable - Sustainable. - It does not require chemical catalysts. - It can be used for the hydrolysis of the three compounds (cellulose, hemicellulose, and lignin).	- Low amount of chemicals - Low energy consumption. - Elimination of hemicellulose and lignin. - The addition of acids improves hydrolysis. - Increases the contact area and improves the enzymatic hydrolysis of cellulose. - It is suitable for scaling at an industrial level.
Disadvantages	- It has high operating costs. - Depreciation of equipment. - Lignin is not eliminated - High energy consumption.	- It requires multiple stages at low temperatures. - Extended residence times. - High consumption of water and energy. - Corrosion problems in the equipment. - Formation of toxic compounds-inhibitors. - High environmental impact.	- Slow process - Partial hydrolysis of hemicellulose. - Possibilities of danger to health.	- Degradation products can inhibit other processes. - Need high pressure. - Low performance but high energy consumption. - Possibilities of chemical danger.

2.3. Technologies for inhibitor removal from hydrolysates

To reduce the concentration of inhibitors that are present in the hydrolysates obtained from lignocellulosic biomass, different strategies and methods have been developed. Some of these methods use physical, chemical and biocatalytic principles [21], [22].

2.3.1 Physical Removal

Currently, membrane-based technologies (vacuum membrane distillation, reverse osmosis, and nanofiltration) have gained great importance for the separation of inhibitory compounds present in hydrolysates obtained from lignocellulosic biomass, due to the energy efficiency and simplicity. The efficient removal of inhibitors by these technologies is determined by several factors, such as the temperature of the hydrolysate, the workflow, and the type of compound to be separated. Nevertheless, the use of this type of technology encompasses a higher separation rate and is more selective concerning the compounds and the concentration [23]–[25]. This is mainly due to the exclusion effect of the membrane that is used. For example, the main inhibitors, such as furfural, 5-HMF, and acetic acid have a lower molecular weight (96, 126 y 60 $gmol^{-1}$ respectively) than C-5 and C-6 sugars (150 y 180 $gmol^{-1}$), which is best at the time of carrying out the separation since the loss of sugars is minimized. However, other compounds, such as ferulic acid, vanillic acid, and syringaldehyde have a molecular weight equal to or higher than sugars (194, 168 y 182 $gmol^{-1}$ respectively), thus their separation through this method is very low, and it increases the loss of sugars [23]. Figure 5 shows a scheme for acetic acid and furfural separation from hydrolyzed lignocellulosic biomass using a membrane distillation vacuum. The main limitation of this method is its high selectivity, disregarding inhibitors with different molecular sizes to the target one, which consequently decreases the compound recovery. The above fact increases the operating and energy cost of these methods, compromising their economic feasibility.

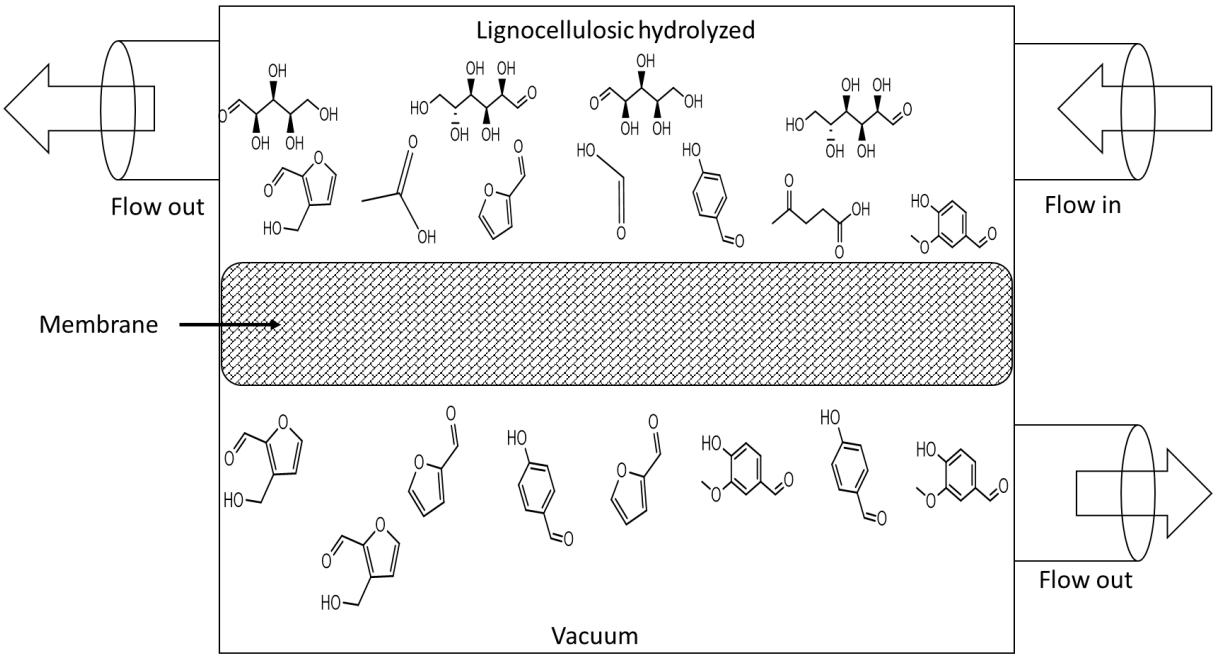

Figure 5.Representation of membrane technology used for removal of inhibitory compounds in a hydrolysate obtained from lignocellulosic biomass.

2.3.2 Chemical removal

Chemical methods include precipitation, ionization, and adsorption of inhibitors. However, some of these methods along with the inhibitor removal affect the fermentable sugars causing a decrease in their content. One of the most used methods for the removal of inhibitors in hydrolysates obtained from lignocellulosic biomass is through adsorption using activated carbon [26]. Figure 6A showed the chemical principle of this method, based on the functional groups present on the carbon surface (carbonyl and carboxyl groups), which can form ionic bonds with the phenolic and furfural compounds present in the hydrolysates. However, this method is a function of several variables, such as pH, temperature, contact time among others [27]. Other materials widely used for the removal of these compounds are hydrophobic resins and polymers, such as IRA-400 (OH), XAD-4, IRA-400 (Cl) y IRA 958 (Cl), due to their high adsorption rate and specificity to furfural, 5-HMF, and phenolic compounds. Figure 6B shows the link sites of the three main compounds with the resin [28]–[30]. The use of these materials is due to their hydrophobic nature since they can extract non-polar compounds (low water-soluble). However, for the adsorption of furfural, it is necessary to use high polar polymeric species to obtain better separation of this compound [27].

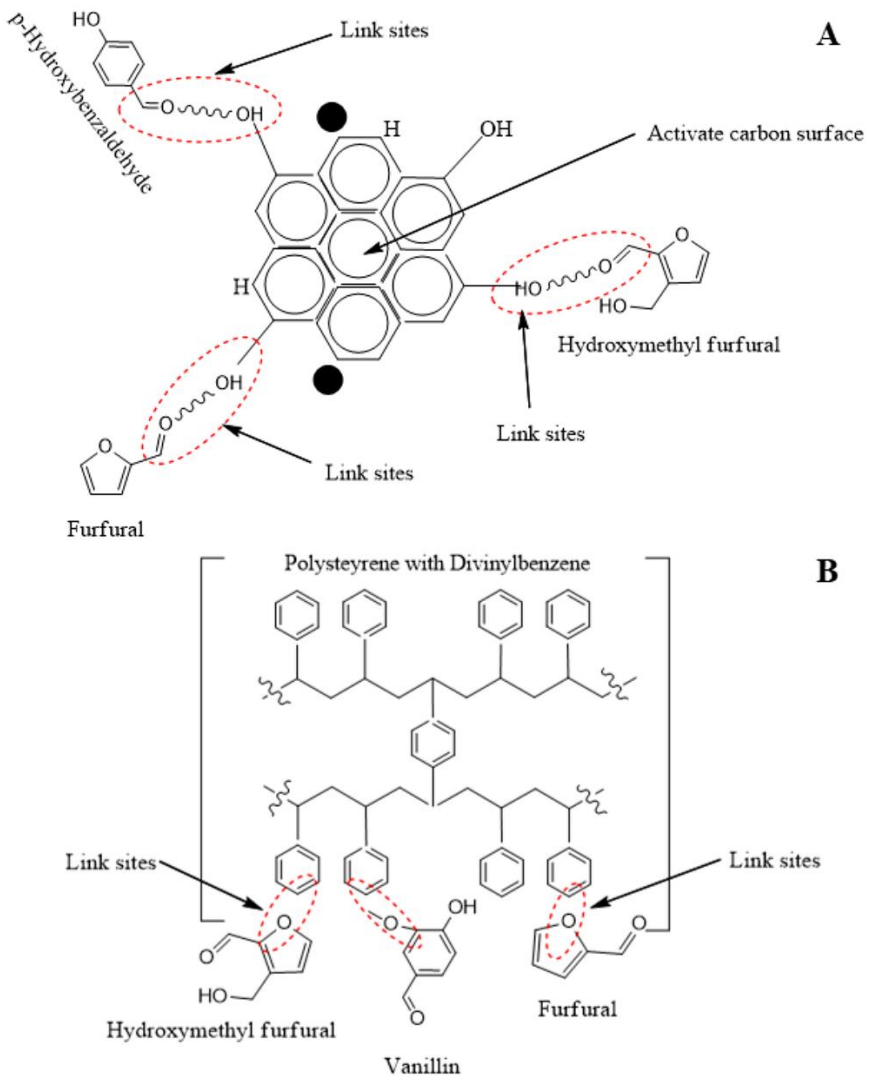

Figure 6.Mechanism of removal of inhibitory compounds in a hydrolysate obtained from lignocellulosic biomass: (A) Active charcoal, (B) Polymers.

From the technological point of view, this type of removal is feasible due to its easy use and low energy consumption. This type of technology does not require a constant energy supply to be able to carry out the removal of the inhibiting compounds, selective materials can be used for each type of compound, the inhibitors can be recovered from the polymeric matrix without modifying its chemical structure, and the material that is used for the removal can be used for several cycles. Due to these characteristics, this technology is a good option for the removal of inhibitor compounds from hydrolysed lignocellulosic biomass.

2.3.3 Biological removal

Different enzymes and microorganisms, which are capable of changing the chemical nature or degrading microbial inhibitory compounds present in lignocellulosic hydrolysates can also be used [31]. Figure 7 shows the transformation or degradation mechanism of furfural. The majority of these enzymes are responsible for the oxidation or reduction of these compounds [32]. Cocktails can be prepared from crude and purified enzymes that improve the catalytic activity, which affects the reduction of the detoxification time. On the other hand, the use of microorganisms to carry out the removal of these compounds is mainly based on their conversion to by-products. Aerobic and anaerobic microorganisms (*Coniochaeta ligniaria, Ureibacillus thermosphaericus, Issatchenkia occidentalis, Enterobacter* sp.) have been used for the removal of furfural and 5-HMF (Figure 8) from hydrolysates obtained from lignocellulosic biomass [33], [34].

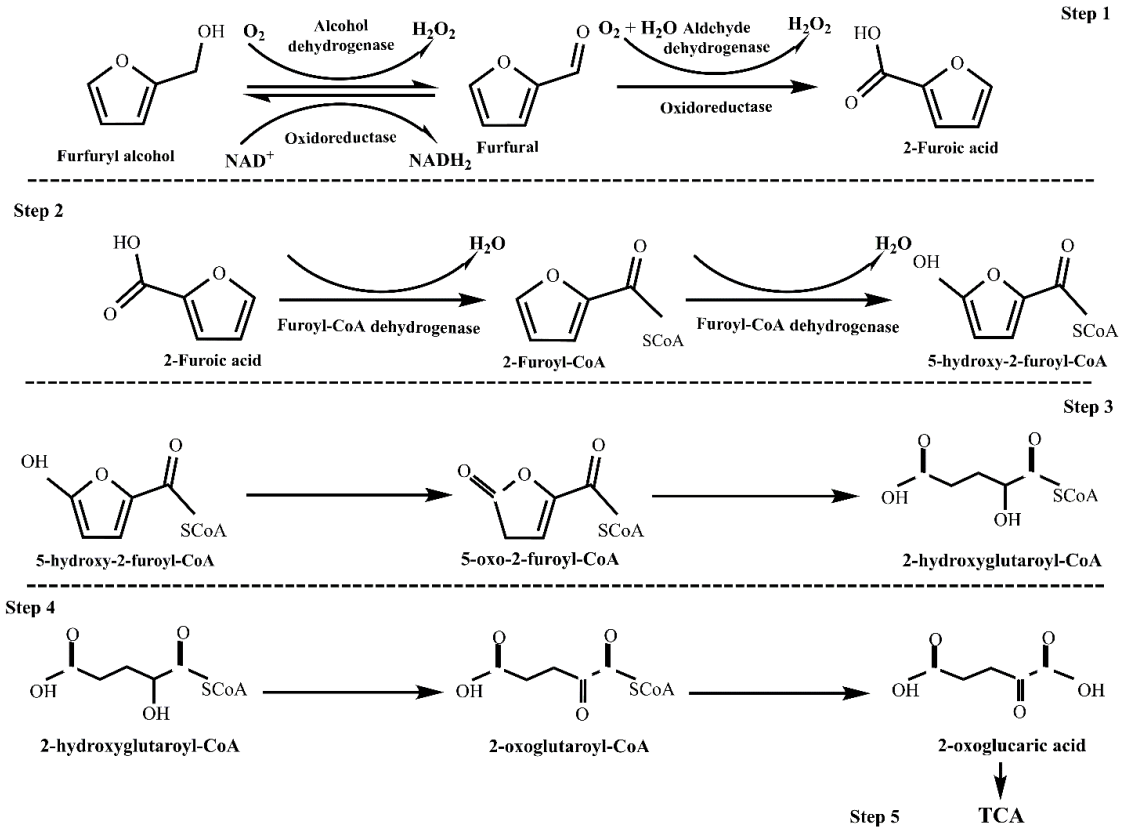

Figure 7.Biological pathway of furfural transformation.

These microorganisms use the metabolic pathway of Trudgill, which consists of various stages of oxidation and reduction catalyzed by enzymes such as aldehyde dehydrogenase, furoyl-CoA synthetase or furoyl-CoA dehydrogenase for the degradation of these compounds. In general, these

reactions constitute the beginning of the degradation of compounds, such as furfural and 5-HMF through biological pathways [34]. Nevertheless, during the degradation process of furfural and 5-HMF into furfuryl alcohol and 5- hydroxymethyl furfuryl alcohol, these microorganisms utilize compounds that are necessary for energy production (NADH or NADPH) [35]. Nevertheless, the degradation of this inhibitor has two major disadvantages, the first is that it does not allow the recovery of compounds that are of industrial interest such as furfural or 5-HMF, and the second is that the temperature must be increased to optimum temperature of microbial or enzymatic activity, increasing the cost of the process due to the requirements of energy.

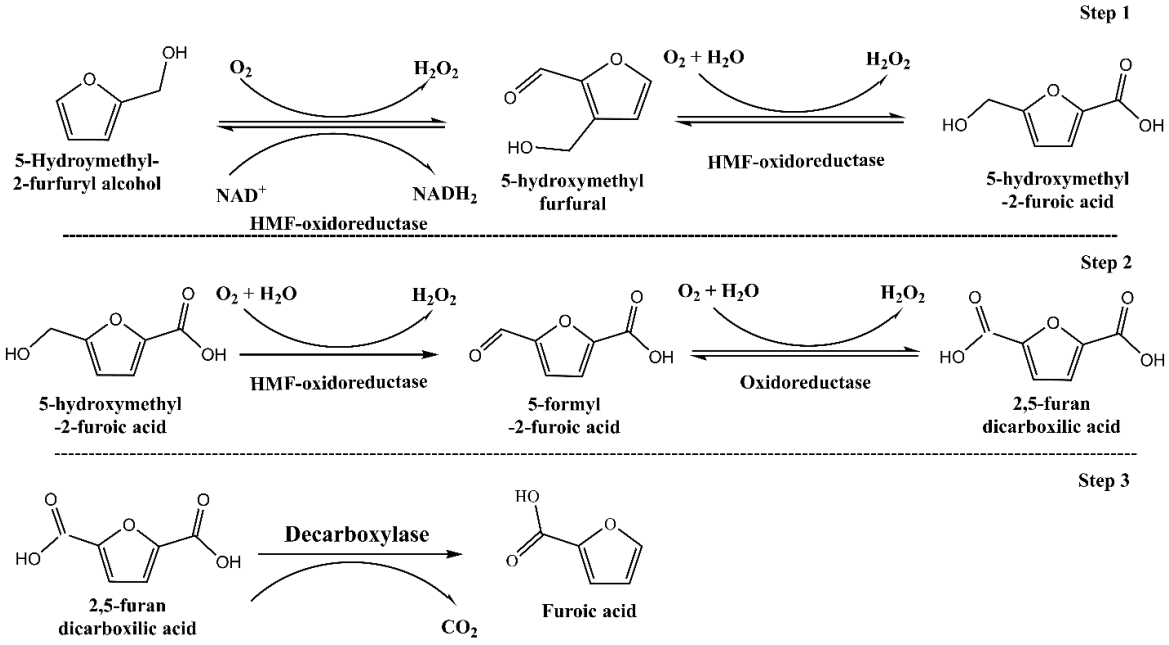

Figure 8.Biological pathway of 5-HMF transformation into furoic acid.

2.4. *Rhodosporidium toruloides*: a brief overview

Rhodosporidium toruloides is a non-pathogenic, aerobic, oleaginous red yeast that has been isolated from a variety of sources, e.g., conifers, soil, wood pulp, dry leaves, among others [36]. It has been reported that this microrganism can accumulate more than 70% in lipids of its dry cell weight [37], [38] this occurs when carbon is in excess during growth, and other key nutrients, such as nitrogen are limited [39]. It has been reported that in a native state, *R. toruloides* produces two main bioproducts: lipids, and carotenoids [40], [41], making it a promising organism to lipid-based chemicals, such as biofuels, lubricants, surfactants, solvents, waxes, creams, and adhesives

production [36], [42], [43]. This oleaginous yeast can grow on a variety of carbon sources, such as sugarcane juice, crude glycerol, lignocellulosic hydrolysates, vegetable market waste, and *Jerusalem artichoke* plants. Due to the ability of *R. toruloides* to use different types of carbon sources, this yeast has potential industrial applications [6], [44]. Figure 8 shows the phylogenetic tree of *Rhodosporidium toruloides* strain.

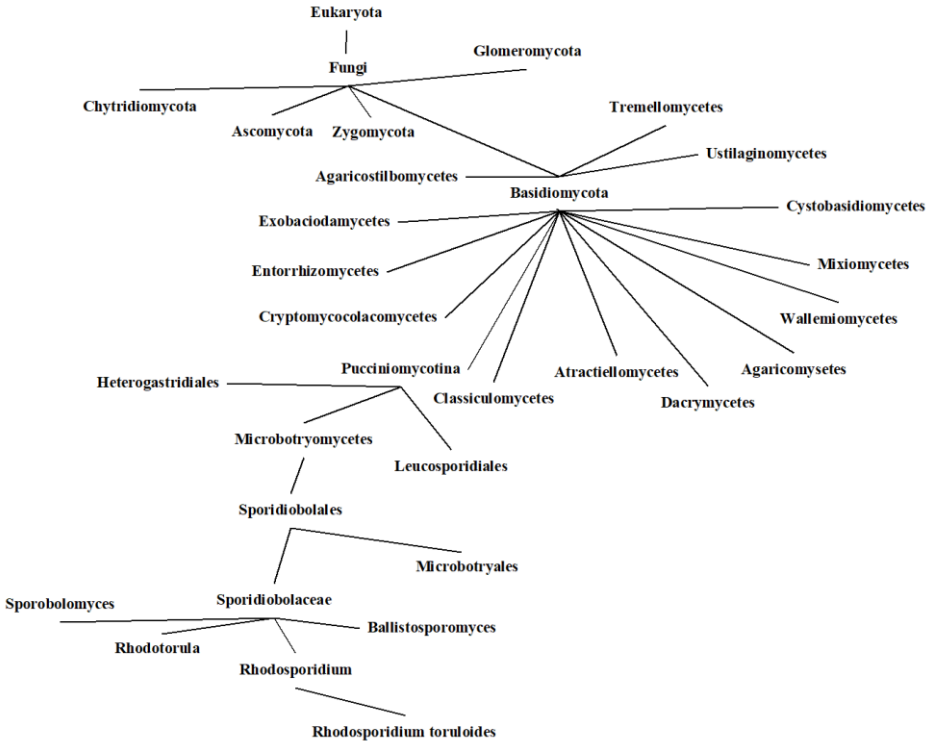

Figure 9.Taxonomic information of Rhodosporidium toruloides. Obtained from the National Center for Biotechnology Information (NCBI, 2019).

2.4.1. Metabolic pathway of lipid production in *Rhodosporidium toruloides*

Rhodosporidium toruloides is a single-cell eukaryotic fungus that shows a heterotrophic behaviour like almost all oleaginous yeast, when is exposed to environmental stress (commonly nitrogen limitation), a change in the metabolic machinery it generated, stopping the synthesis of proteins and nucleic acids, and converting available carbon source into lipids. A general practice to increase lipid accumulation has been the variation in nitrogen ratios on the culture media. In different studies, a maximum lipid accumulation in a range of carbon/nitrogen from 30 to 80 has been reported. However, a key factor to increase the lipid accumulation in *Rhodosporidium toruloides*

lies in the ability to produce Acetyl-CoenzymeA (Ac-CoA), as a principal building precursor. Figure 10 shows four main steps involved in lipid accumulation in *Rhodosporidium toruloides*: i) Ac-CoA and NADPH production, ii) biosynthesis of fatty acyl chains, iii) acyl moieties distribution into polar or neutral lipids groups, and iv) biogenesis of lipid droplets.

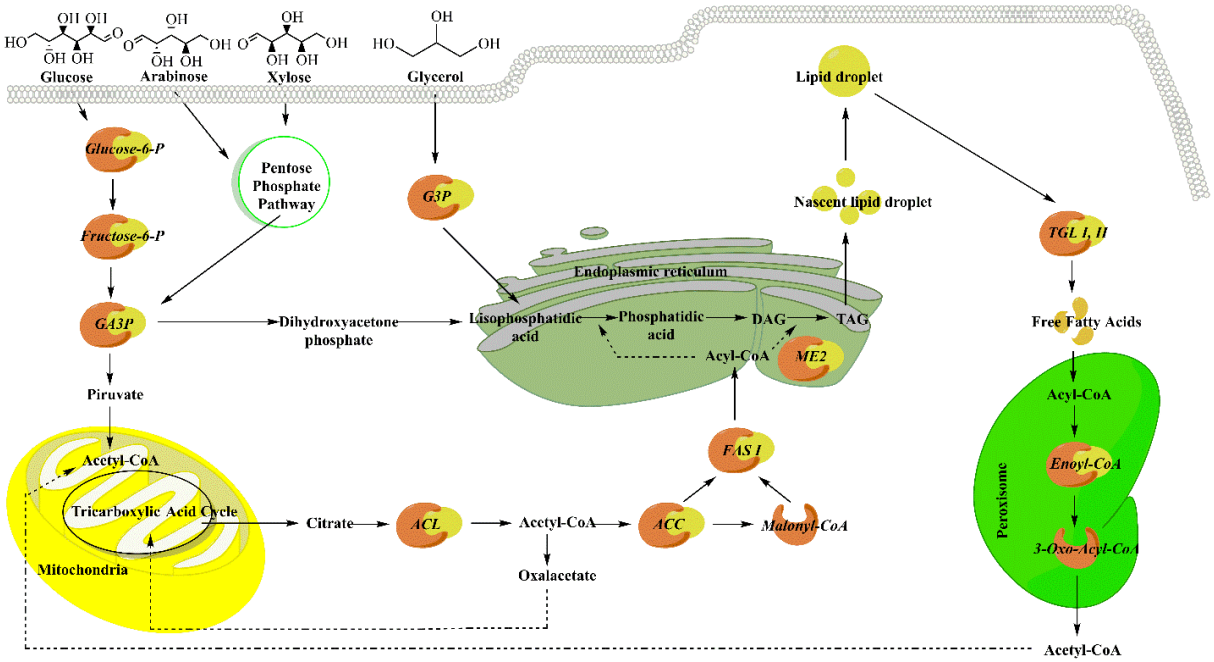

Figure 10.Main steps involved in lipid production in oleaginous yeast Rhodosporidium toruloides.

2.4.2 Feedstock utilized by *Rhodosporidium* genre to produce lipids

The feedstock traditionally used to obtain lipids through oleaginous yeasts is glucose, which is obtained mainly from the hydrolysis of amylaceous raw materials (cereals, corn, or potatoes), some agro-industrial residues such as molasses and whey, as well as waste from industrial processes such as glycerol. However, among all the substrates, glucose has the highest costs ($400/t) decreasing the feasibility of microbial lipid production process at industrial level [45]. Currently, the production of biofuels faces a shortage in the availability of accessible feedstock since it is observed that between 60 and 85% of the total cost of the process is the feedstock, which can be significantly reduced through the use of renewable substrates or different residues. However, carbon sources that are found naturally or that can be obtained from the saccharification of some

of these feedstock's are very diverse (glucose, xylose, arabinose, lactose, galactose) and can be found by themselves or mixed. Sometimes, this is a limitation during the fermentation process because some yeasts cannot metabolize all types of sugars. One of the advantages of *Rhodosporidium* is its ability to grow on a wide range of feedstock. Table 3 shows the biomass and lipid yields by different strains of the *Rhodosporidium* genus using different feedstocks. The use of hydrolysates obtained from lignocellulosic biomass has a great potential to produce biofuels, feedstock to produce it as well as chemicals at industrial level.

Table 3.Biomass and lipid yields obtained by Rhodosporidium strains using different feedstock.

Microorganism	Carbon source	Pretreatment	Hydrolysis	Inhibitors	Biomass (g/L)	Lipids (g/L)	Culture mode	References
R. toruloides	Xylose	NR	NR	NR	21.9	9.5	Batch	[46]
880-ADS	Glucose	NR	NR	NR	26.8	16.4		
(designed)								
R. toruloides	Glucose and	NR	NR	Acetic acid,	14.3	7.9		[47]
DMKU-RE16	Xylose			formic acid,				
				furfural, 5-HMF				
				and vanillin				
R. toruloides	Glycerol	NR	NR	Methanol	21.1	11.2		[48]
ATCC 10788								
R. babjevae		NR	NR	NE	9.9	2.4		[49]
R. diobovatum		NR	NR	NE	14.1	7.1		
R. toruloides AS		NR	NR	Methanol	24.9	12.2		[50]
2.1389								
R. toruloides AS	Acetic acid	NR	NR	NE	6.7	3.3		[51]
2.1390								
R. toruloides	Sugarcane	Hydro thermic	Acid	Furfural and	8.5	4.5		[52]
(adapted strain)	bagasse		(H_2SO_4)	phenol				
				compounds				

Microorganism	Carbon source	Pretreatment	Hydrolysis	Inhibitors	Biomass (g/L)	Lipids (g/L)	Culture mode	References
R. toruloides NCYC 1576	Wood wastes	Hot water	Acid (H_2SO_4)	Acetic acid, furfural, and phenol compounds	7.1	2.8		[53]
R. diobovatum	Pinewood pyrolysates	Thermal	Acid (H_2SO_4)	Phenolics, aldehydes and aromatics compounds	10.5	6		[54]
R. toruloides DSM 4444	Glucose	NR	NR	NR	36.2	23.6		[55]
R. kratochvilovae SY89		NR	NR	NR	15.3	8.6		[56]
R. toruloides AS2.1389	Glycerol	NR	NR	NR	26.5	10		[57]
R. toruloides ATCC 10788		NR	NR	NR	27.4	18.6		[58]
R. kratochvilovae HIMPA1	Aqueous extract of hemp seed	Boiled	Sonication	5-HMF and furfural	15.1	8.3		[59]

Microorganism	Carbon source	Pretreatment	Hydrolysis	Inhibitors	Biomass (g/L)	Lipids (g/L)	Culture mode	References
	Aqueous extract of Cassia fistula	Boiled	NR	NR	8.9	4.7		[60]
R. toruloides 880-ADS (designed)	Glucose	NR	NR	NR	118.4	89.4	Fed-batch	[46]
R. toruloides A29		NR	NR	NR	23.3	12.5		[61]
R. azoricum		NR	NR	NR	79	32		[62]

NR: Not reported

2.4.3. Main cultivation parameters with influence on lipid accumulation by *Rhodosporidium toruloides*

***2.4.3.1. C/N ratio*:** This parameter plays a key role in the efficient use of the carbon source for its conversion into fatty acids by the genus *Rhodosporidium* since this ratio affects the production of biomass and the accumulation of lipids within the cell. Several studies have shown that the use of carbon: nitrogen ratios in a range of 65 to 90 allows this microorganism to reach its maximum performance and the maximum lipid accumulation [63]. This is mainly due to the effect of the repression that glucose can cause when it is in high proportion in the media. This causes an osmotic shock since the activity of the Krebs cycle decreases due to the intracellular increase of citric acid. Additionally, the assimilation of other types of sugars such as xylose present in the hydrolysates obtained from lignocellulosic biomass decreases. In this sense, cellodextrin transporters expressed in *S. cerevisiae* have been evaluated in order to reduce this repression by intracellular hydrolysis of cellodextrin, giving way to better utilization of xylose present in this type of hydrolysates [64]. Capusoni et al. [62] analyzed the effect of two initial concentrations of C/N (75.3 and 20.3) in a strain of *R. azoricum* on biomass and lipids production. They observed that for high C/N ratio, the strain depleted the nitrogen source in 40 hours. On the contrary, for low C/N ratio, after 65 hours of fermentation there was a presence of carbon source. This was reflected in a decrease in lipid accumulation (47%) for the initial low C/N. This suggested that the limitation of nitrogen with respect to the carbon source in this strain negatively affected lipid production. This is due to the effect that the limitation of nitrogen in the medium can decrease the activity of some key enzymes such as fructose 1,6-P2 aldolase (FBA), which carries out the catalysis of dihydroxyacetone phosphate, a precursor of glycerol-3-phosphate which is indispensable for triglyceride synthesis. However, the activity of glucose 6-P-dehydrogenase (G6PDH) was favoured by the limitation of nitrogen in the medium, because the pentose phosphate route is the main source of NADP. The latter is a key intermediary for the functioning of a malic enzyme, which plays a key role in the production of lipids. Some studies in *Y. lipolytica* have shown that the path of the pentose phosphate is the main source of NADPH to carry out the overproduction of lipids in this type of yeast [65]. This leads to a more rigorous approach to this parameter, because, the type of carbohydrate (carbon source), the C/N ratio and the fermentation system (batch, fed-batch and continuous) have an adverse effect during lipid accumulation. In this sense, the best results for *Rhodosporidium* strains have been obtained using glucose as the sole carbon source and at C/N

rates between 65 and 80, where the lipid content in batch systems is up to 50% DW and in fed-batch up to 61% DW. On the other hand, when mixtures of carbohydrates such as glucose, xylose, and arabinose are used, the lipid content can be reduced by almost the half of only glucose (31% DW) using high C/N rates (65 and 80). However, if a lower C/N (40) rate is used with this type of mixtures, the lipid content increases up to 58% DW.

***2.4.3.2. Osmotic stress*:** One of the important parameters that govern the growth and production of lignocellulosic hydrolysates, is the presence of compounds that can cause osmotic stress to yeast cells. The foregoing statement implies an important challenge during formulation from culture media for the competitive production of lipids. In this regard, the presence of high concentrations of salts in the fermentation broth causes damage to the cell wall of the yeast due to high osmolarity. Several studies have shown that osmotic stress largely affects cellular metabolism, since it directly controls the transcription stages of cells, such as a polysomic association of mRNA and translation inhibition. This parameter is critical when selecting or optimizing the composition of the fermentation broth. Likewise, [66], analyzed the production of lipids in *R. toruloides* under conditions of osmotic stress by the addition of sodium chloride (NaCl). The results showed a direct correlation on the culture time. The interaction between NaCl and the content of glucose affected the production of lipids. When the concentration of NaCl was increased up to 10% v/v in the media, it adversely affected the production of lipids using low glucose concentrations (5 g/L). However, this effect is reversible when the glucose content is increased since the negative effect of NaCl is compensated by lipid production. This behaviour suggests that when the initial glucose content is maintained in concentrations higher than NaCl, it promotes a proportional production of lipids concerning the NaCl that is added to the media.

***2.4.3.3 pH*:** Although it has been demonstrated that the strains of the genus *Rhodosporidium* tolerate a wide range of pH values (2.2-7.5), it has been found that this parameter has a significant effect at low (pH=4), intermediate (pH=5.5) and high (pH=7) for the accumulation of biomass (13.5, 16 and 10 g/L respectively), lipid yield (4, 6.4 and 2.5 g/L respectively) and lipid content for its biomass (32%, 38% and 27% respectively) [56]. Likewise, Yaegashi et al. [67], found that the pH of the culture medium was an important factor for the efficient use of fermentable sugars in the conversion of lignocellulosic hydrolysates to terpenes by a genetically modified strain of *R. toruloides*. In this study, they reported the maximum production of terpene by this strain at low

pH (3.4), which suggested that the strain was susceptible to produce organic acids natively. However, when the pH decreased (lower than 2.5), the use of sugars by the strain was largely inhibited, which suggested that for efficient use of sugars, it was necessary to maintain pH within the range from 2.5 to 3.4. However, pH values can change depending on the substrate utilized as well as culture conditions, for example, accumulate of lipids using wood hydrolysates as a substrate, a pH of 6 has been reported to be the optimal [48], [66], [67]. Likewise, when the values of pH lower than 5 affects the production of biomass and the yield of lipids because its variation influences the dissociation of some toxic compounds (acetic, formic and levulinic acids) formed during the pretreatment, disrupting the cell membrane, and inhibiting the microbial growth.

***2.4.3.4 Nitrogen source*:** Naturally, oleaginous microorganisms do not accumulate lipids in substantial quantities. However, if a substrate is limited (mainly nitrogen source), microorganisms use the carbon source to form lipids as a protective reaction [68]. The effect of limiting the nitrogen source in the culture medium triggers a reaction that allows the increase of the AMP-deaminase enzyme, which decreases the AMP levels in the cell, with the consequent decrease in the enzyme malate dehydrogenase that is present in oleaginous yeast. Due to these factors, a balance during the transformation of oxalacetate into citrate by the enzyme aconitase is necessary to be later transferred to the cytoplasm by the citrate malate translocase (CTM enzyme). Once in the cytoplasm, the ATP citrate lyase (ACL) converts the citrate into oxaloacetate and acetyl Co-A (the first step in the synthesis of fatty acids). However, to preserve a continuous flow of citrate, a recycling of the oxaloacetate is necessary, which is carried out by the enzymes malate dehydrogenase and citrate synthase [69]. However, it is very difficult to reach the lipid content by using natural substrates with low nitrogen content. Wu et al. [70], [71] limited the phosphorus and sulfur content in the culture medium to produce lipids using a strain of *R. toruloides* Y4, obtaining yields of 62% and 58%, respectively. Few studies are focused on the effect that the nitrogen source causes on the growth and development of the basic cell cycle. This effect has been studied under the limitation of nutrients in the environment since the depletion of nitrogen is signalled through the TOR and MAP kinase routes through the detection of nutrients in the environment. Relevant results have been obtained in genetically modified strains, despite the limitation of nitrogen, to continue with their capacity of a growth rate compared to the native strains. These results suggest that the growth process is adaptive and can separate the cell division of the availability of nutrients present in the environment [71]. This suggests that the limitation of the nitrogen source in process

for lipid production can be equally efficient and economical in terms of materials with high nitrogen content.

***2.4.3.5 Oxygen*:** This parameter is of importance when carrying out the fermentation of sugars into lipids. It has been demonstrated that at high oxygen rates (100 to 225 rpm in the flask) during fermentation, lipid yields increase from 9% to 46.3% [56], [72]. This is because the restriction of oxygen during fermentation affects the yeast growth due to pyruvate decarboxylase (PDC) enzyme is largely limited by the lack of oxygen. The previous phenomenon is reflected in the decrease in acetyl CoA, which is produced through the conversion of citrate via ATP citrate lyase. Additionally, the low oxygen concentration also leads to a decrease in the cellular biomass yield with the consequent decrease in lipid accumulation [73]. All of the aforementioned subjects suggest that oleaginous yeast needs substantial amounts of oxygen to generate energy and to be able to carry out a better lipid biosynthesis. Past research suggests that maintaining a dissolved oxygen content in a range from 40 to 50% generates a high-density culture (107 g/L dry biomass) and high lipid content (67.5% w/w) [74]. It is also necessary to maintain good control of the amount of oxygen at the time of carrying out the fermentation since the presence or absence of this element affects the fatty acid's composition. Another key factor in the efficient use of sugars is the presence of inhibitors in the hydrolysate, which causes a partial or total catabolic suppression of carbon source, and in consequence a decrease in cell growth and lipid production [75].

2.4.4. Inhibitors effect in lipid production

The pretreatment of lignocellulose biomass generates various products during its degradation, which include acetic acid, formic acid, levulinic acid, vanillin, vanillic acid, syringaldehyde, hydroxybenzaldehyde, furfural, and 5-hydroxymethyl furfural as shown in Figure 11. These compounds are present in different concentrations in the hydrolysates (34-180 mg/L, 43-250 mg/L, 0.48-41 mg/L, 1.7-6.7 mg/L, 1.5-4.4 mg/L, 0.40-220 mg/L and 0.89-44 mg/L, respectively), these concentrations are function of the type of pretreatment [47], [52], [75], [76]. Almost all of these compounds affect the metabolism of the microorganisms during fermentation, negatively impacting the product yield [18], [47].

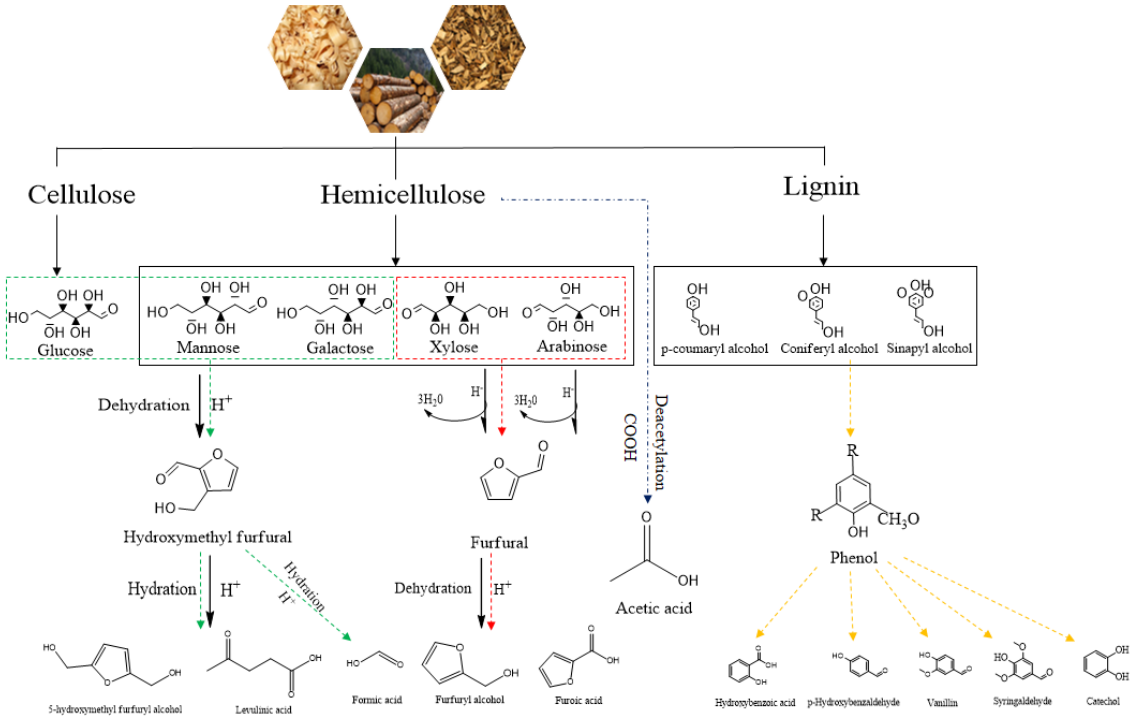

Figure 11.Main inhibitory compounds generated during the hydrolysis of wood biomass.

Table 4 shows the effect of inhibitors on biomass accumulation and lipid production in three different oleaginous yeast. This effect must be studied in-depth, in order to obtain data for the development of new detoxification strategies, because the concentration in the culture medium varies greatly depending on the feedstock [75]–[78].

Table 4.Biomass and lipid yields obtained by Rhodosporidium strains using different feedstock.

Microorganism	Feedstock	Inhibitors (g/L)	Cell mass (g/L)	Lipids (g/L)	Reference
R. toruloides AS 2.1389	Sugarcane bagasse hydrolyzate	Formic acid= 2 Acetic acid=5 Furfural= 0.5 Vanillin= 1.5 Sodium salt= 2	23.5	12.3	[78]
R. toruloides DMKU-RE16	Glucose and Xylose	Acetic acid= 3 Formic acid= 5 Furfural= 2 5-HMF= 4 Vanillin= 2.5	14.3	7.9	[47]
R. toruloides NCYC 1576	Wood wastes	Acetic acid= 16.8 Furfural= 0.04 Phenol compounds= 0.05	7.1	2.8	[53]
Y. lipolytica YB-420	Switchgrass hydrolysate	Acetic acid= 5.4 Furfural= 15.9 HMF= 4.4	13.4	5.1	[79]
Y. lipolytica Y203A	Sugarcane Bagasse Hydrolysates	Acetate= 5.3 Furfural= 0.6	14.87	10.01	[80]
Y. lipolytica W29	Agave bagasse	Furfurals= 3.5 Acetic acid= 3.5 Formic acid= 0.6	16.5	11.1	[81]
C. curvatus ATCC 20509	Sweet sorghum bagasse	HMF= 1.5 Levulinic acid= 5	10.8	4.32	[82]

	Formic acid= 0.7 Furfural= 0.5			
Straw hydrolysate	Furfural= 3 PHB= 1 Vanillin= 1.5 Syringaldehyde= 2	10.7	3.21	[83]

***2.4.4.1. Organic acids*:** The formation of these compounds occurs at the expense of the sugars and, therefore, it is desirable to operate the pretreatments under the conditions which minimize the formation of these acids. They are formed by the breakdown of acetyl groups (hemicellulose hydrolysis) and their effect on microorganisms is the inhibition of its growth. This is due to the damage they cause to the plasma membrane of the yeast after its diffusion through it. The inhibitory effect of these compounds is reflected in the higher demand for ATP and the deficient production of ATP due to the uncoupling of the respiratory chain and the oxidative phosphorylation of ADP. This leads to a higher glycolytic activity (42 moles of NADPH and 21 moles of ATP) generating ATP at the expense of the biomass formation [84]. This is possible due to the fat-soluble nature of weak acids that are not dissociated. Its inhibitory effect varies, depending on the parameters and conditions used during the fermentation, such as low pH. Further, this compound diffuses into the cytoplasm of the cell, inducing a decrease in intracellular pH that causes a decrease in the transfer of nutrients and an increase in the needs of ATP, which when exceeded beyond certain values, causes the total inhibition of cell growth [85]. In other yeasts such as *Cryptococcus curvatus*, *Yarrowia lipolytica*, *Trichosporon cutaneus*, these compounds decrease and inhibit the activity of the malic enzyme, necessary to carry out the production of NADPH (nicotinamide adenine dinucleotide phosphate oxidase) in the cell [86]. An advantage of some *Rhodosporidium* genus strains is their tolerance to high concentrations of organic acids compounds such as levulinic acid and their ability to degraded during fermentation.

***2.4.4.2. Phenolic compounds*:** These compounds are diverse and are formed by the degradation or hydrolysis of lignin. The most inhibitory compounds are vanillin, hydroxybenzoic acid,

syringaldehyde, and catechol [85]. Their effect on lipid accumulation has not been studied. Some authors theorise that the main effect of phenolic compounds is the rupture of the cell membrane, allowing the free entry of compounds to the cell, causing a decrease in cell growth and sugar assimilation. Additionally, they can cause a change in the electrochemical gradient in the mitochondrial membrane by transferring protons through it [87]. The inhibition effect of these compounds is related to their molecular weight. In this sense, the phenolic compounds with low molecular weight, such as syringaldehyde and catechol (138.12 and 110.1 g/mol, respectively), have a high degree of inhibition compared to the effect of vanillin, hydroxybenzoic acid or syringic acid (152.15, 182.17 and 198.7 g/mol, respectively) [88].

***2.4.4.3. Furans*:** These compounds are formed due to dehydration of pentoses, and hexoses present in the lignocellulosic biomass. They are highly toxic compounds that affect the yield and production of cellular biomass and the specific growth rate. Furans inhibit the production of precursor compounds for the synthesis of fatty acids [89]. Additionally, they inhibit the enzymes alcohol dehydrogenase, aldehyde dehydrogenase, pyruvate dehydrogenase, hexokinase, and glyceraldehyde 3-phosphate dehydrogenase, which interact directly in the process of obtaining acetyl CoA from pyruvate. Therefore, the direct formation of acetyl CoA through pyruvate dehydrogenase and so that the acetyl-CoA synthase is inhibited [83]. The inhibitory effect of 5-HMF is caused by the assimilation of amino acids (cysteine and methionine), which directly affects the mechanism of cellular replication of microorganisms [90]. Chen et al. [23] analyzed the effect of acetic acid, formic acid, vanillin, p-hydroxybenzaldehyde (PBH) and furfural on the production of lipids in *R. toruloides* Y4 using glucose as carbon source. The strain can growth in presence of a maximum concentration of 5 g/L of acetic acid. In the presence of formic acid (3 g/L) showed a slight growth but without lipid production, and with levulinic acid (10 g/L) the sugar consumption decrease, without stopping growing. In the presence of furfural, the strain showed strong inhibition of cell growth and production of lipids at the concentration of 0.5 g/L. 5-HMF weakly inhibited the cell growth and lipid production. However, the increase of its concentration in the medium inhibited the strain. Finally, for the phenolic compounds (vanillin, 1 g/L and, p-hydroxybenzaldehyde), complete inhibition of the strain (growth and lipid production), was observed when the compound concentration increased in the medium. Hu et al. [75], and Zhao et al. [78] found similar compounds in corn stover and sugarcane bagasse as well as the inhibition of

R. toruloides 2.1389. In general, these compounds have a negative effect on the growth and production of lipids on strains of the genus *Rhodosporidium* sp.

2.4.5. Challenges in lipid production in *Rhodosporidium* genus

In the last decades, the production of lipids using lignocellulosic hydrolysates has become an attractive alternative for obtaining biofuels, chemical commodities, or precursors for the production of different compounds. In general, the accumulation of lipids in oleaginous yeast is carried out through two routes (de novo and ex novo), under limited nitrogen conditions in the culture media [2], [61], [91]. The production of lipids by native strains of the genus *Rhodosporidium* using hydrolysates obtained from lignocellulosic biomass as a substrate is limited because of the presence of toxic compounds that greatly affect the development and viability of the microorganisms [92]. Due to this reason, the use of this type of strain faces challenges such as adaptation to these inhibitors, fermentation processes optimization for efficient use of the different carbon sources and the development of efficient and low-cost technologies and implementation to detoxify the hydrolysates [30]. In recent years, few studies have been performed to understand tolerance to this compounds, as well as how to increase the yeast tolerance to them [52], [75], [92]. *R. toruloides* have been genetically modified to improve fatty acid biosynthesis, at the level key genes of its metabolic pathway. However, most of these works are focused only on the increase of lipid yield [37], [43], [46], [93] and few are focused on other strategies, such as the inhibitor effect with respect to microbial growth and lipid accumulation. In this regard, synthetic biology and bioengineering are essential tools to improve the production and adaptation capacities that *R. toruloides* presents [44]. For example, Bonturi et al. [52] modified *R. toruloides* to increase lipid production and obtain greater tolerance to furfural and 5-hydroxy-methyl-furfural (5-HMF) using a hydrolyzed from cane bagasse as carbon source. In this sense, the Ribulose 5-Phosphate Epimerase (RPE1) and Transketolase1 (TKL1) genes were induced, 5.8-fold and 1.5-fold respectively, after 72 hours of fermentation. These two genes participate in the non-oxidative phase of the pentose phosphate pathway and the generation of the main precursor of glycerol 3-phosphate (GA3P). However, when the modified strain was grown in xylose as the sole carbon source, the expression of TKL1 gene increased the yield of lipids in the yeast. Likewise, the expression of the genes Aldehyde dehydrogenase4 (ALD4) and Aldehyde dehydrogenase6 (ALD6) was 2.8-fold and 3.5-fold at 48 h, respectively, both genes are involved in the oxidative stress of the cell and

the ability to transform furfural to 2-furan methanol and 5-HMF to furan-2,5-dimethanol. These results are promising in terms of tolerance of the strain to toxic compounds such as furfural and 5-HMF. Qi et al. [92] reported that the expression of a gene on *R. toruloides* species is ≥ 2-fold is considered a competitive strain in terms of tolerance to these inhibitors. Similarly, these three genes can be used as reference markers for other types of oleaginous yeasts in which it has not been investigated for tolerance to inhibitory compounds present in hydrolysates obtained from lignocellulosic biomass.

CHAPTER THREE: PROBLEMS, HYPOTHESIS, OBJECTIVES AND ORIGINALITY

3.1 PROBLEMS

The literature on the production of lipids and drop-in biofuels suggested the need for further research in making the biorefining processes more sustainable and economically feasible. The need for resolving the following technical problems was deduced:

3.1.1 Increase the use of hydrolysates obtained from wood biomass and its residues as a feedstock for biofuel production.

In general, lignocellulosic biomass is composed of three different fractions: cellulose (~30 to 50%), hemicellulose (~20% to 35%) and lignin (~15% to 25%). The main advantage of lignocellulosic biomass is that it can be hydrolyzed and thus achieve different compounds, mainly sugars, that serve as feedstock for obtaining different industrial products. One of the main problems for the biofuel industry to be economically feasible is the feedstock cost and availability. Nowadays, an alternative carbon source for biofuel production is a liquid hydrolysate of lignocellulosic biomass from different sources, such as agricultural and cellulosic residues, food wastes, forest residues, urban wood residues, among others. These types of hydrolysates have high sugar content: six-carbon sugars (glucose and galactose, mainly) and five-carbon sugars (xylose and arabinose, mainly) that microorganisms can be used as a carbon source. In this sense, interest has been increased in hydrolysates obtained from wood biomass or their residues as a carbon source for biofuel production through fermentation processes.

3.1.2 Increase tolerance to toxic compounds present in hydrolysates obtained from wood biomass.

Pretreatments that are applied to wood biomass and wastes, such as wood chips and sawdust, are carried out before carrying out the saccharification of cellulose to obtain fermentable sugars. The main reason for the pretreatments before carrying out the saccharification of cellulose is due to the need to eliminate the barriers that make the biomass recalcitrant. The main effect of these pretreatments on biomass is to solubilize lignin and hemicellulose, to increase the surface area of accessible cellulose. Derived from this process, different chemical compounds are generated, which vary in presence and quantity depending on the type of pre-treatment and the conditions that are used. These compounds such as furfurals, organic acids, and phenolic compounds, cause

partial or total inhibition in microorganisms used for biofuel production. In this sense, it is necessary to develop strains to increase tolerance to this inhibitor compound through adaptation, modification, or molecular engineering methods.

3.1.3 Improving utilization of five-carbons sugars to increase lipid production.

Generally, microorganisms consume in high proportion six-carbon sugars (C6) in comparison with five-carbon sugars (C5) consume. Nevertheless, some microorganisms with specific characteristics can use C5 sugars at the same proportion as C6 sugars. *R. toruloides* genre is capable to use both types of sugars as a carbon source. However, in a native state, the consumption of five-carbon sugars by this yeast is limited to approximately 50%. In this sense, it is necessary to develop different strategies to increase the consumption of C5 sugars, which are present in wood hydrolysates used as a substrate for biofuels production.

3.2. HYPOTHESIS

The current research work comprises the following hypotheses:

Hypothesis 1: Hydrolysate of forestry residues as a renewable feedstock for biofuel feedstock and drop-in fuel production, via fermentation using an oleaginous yeast, can have a profound impact on the economic feasibility of the bioproduction process. E***ffects of different pretreatments and their impact on lipids and drop-in biofuel production using forestry residues as feedstock.***

Hypothesis 2: The oleaginous yeast *Rhodosporidium toruloides* has been identified as a natural overproducer of lipids, precursors to biofuel production. Moreover, the strain can grow in C6 and C5 sugars, as well as excellent tolerance to inhibitor compounds present in wood hydrolysate. ***Hence, wood hydrolysates obtained from forestry residues can be used as a substrate for lipid and drop-in biofuel production using an oleaginous yeast Rhodosporidium toruloides***.

Hypothesis 3: In its native form, *Rhodosporidium toruloides* can degrade growth inhibitory compounds present in liquid hydrolysates obtained from lignocellulosic biomass sources such forestry residues. Nevertheless, the research about inhibitor degradation is limited complex substrates. ***Thus, R. toruloides-1588 can degrade microbial inhibitory compounds derivatives from lignocellulosic biomass***.

3.3. OBJECTIVES

The general objective of the current study is to implement the concept of biorefinery, to produce feedstock that can be used for biofuel production using the oleaginous yeast *Rhodosporidium toruloides*. This biorefining process enables the use of wood hydrolysate obtained from forestry residues as a carbon source to improve its valorization and achieve a circular bioeconomy using a renewable feedstock.

The following specific objectives will be carried out, based on the problems reported in the literature and the hypotheses mentioned:

Objective 1: Study the production of green extraction of high-value chemicals using forestry residues as a feedstock

Objective 2: Study of C5 and C6 undetoxified wood hydrolysates as a culture media to produce microbial lipids using the oleaginous yeast *Rhodosporidium toruloides*.

Objective 3: Compare the lipid accumulation on five *R. toruloides* strains using C5 and C6 wood hydrolysates as a culture media

Objective 4: Optimize the culture conditions to improve lipid accumulation by *R. toruloides*-1588 using undetoxified C5 and C6 wood hydrolysates as a culture media.

Objective 5: Compare the catabolite repression effect on *R. toruloides*-1588 using C5 and C6 undetoxified wood hydrolysate as a culture media.

Objective 6: Study of lipid accumulation by *R. toruloides*-1588 in bioreactor using wood hydrolysate as a culture media.

Objective 7: Study of microbial inhibitory compounds degradation by *Rhodosporidium toruloides*-1588

Objective 8: Study of furfural degradation by *R. toruloides*-1588.

3.4 ORIGINALITY

"Increase five-carbon sugar utilization through the optimization of cultivation parameters to enhance the lipid accumulation in *Rhodosporidium toruloides*-1588 using undetoxified wood hydrolysates as culture media."

CHAPTER FOUR: *FORESTRY RESIDUES AS FEEDSTOCK TO PRODUCE GREEN CHEMICAL COMPOUNDS*

4.1 Data set of green extraction of valuable chemicals from lignocellulosic biomass using microwave method

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C. S. Osorio-González, K. Hegde, S. K. Brar, A. Kermanshahipour, and A. Avalos-Ramírez, "Data set of green extraction of valuable chemicals from lignocellulosic biomass using microwave method," *Data in Brief*, vol. 26, p. 104347, Oct. 2019, doi: 10.1016/j.dib.2019.104347.

Abstract

Lignocellulosic biomass is a promising alternative for the replacement of limited fossil resources to produce various chemical compounds, such as 5-hydroxymethylfurfural, furfural, vanillin, vanillic acid, ferulic acid, syringaldehyde, and 4-aminobenzoic acid. However, the complex biomass structure is a limitation to making effective use of this naturally found feedstock. This research presents a data set of different compounds obtained directly from forest residues, with special emphasis on achieving effective utilization of the biomass. The extraction method and the catalyst are considered as the two main factors in this valorization process.

Keywords: Forestry residues, Organic acids, Furans, Phenolic compounds, Microwave extraction

Specifications Table

Subject area	**Chemistry**
More specific subject area	Biomass, bioproducts, biorefining, forestry residues.
Type of data	Tables, figures, and text file.
How data was acquired	-Planetary ball mill (PM100; Retsch Corporation). -Microwave-assisted extraction (MAE) instrument (Perkin-Elmer Anton Paar Multi-Wave Sample Prep System, USA). -Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer (Thermo Scientific, Mississauga, Canada).
Data format	Raw and Analyzed data set
Experimental factors	-Data set for different compounds were obtained from forestry residues using microwave-assisted extraction. -The compound analysis was carried out directly from the liquid hydrolysate.
Experimental features	-Forestry residues from hardwood and softwood were used for products obtention. -Experiments in batches were performed to evaluate the reaction time, temperature, and catalyst concentration effect. -Data was analyzed using a response surface model (Box-Behnken) in a Minitab® Software version 17.
Data source location	Quebec City, Quebec province, Canada
Data accessibility	Data are available in article

Value of the data

- Lignocellulosic biomass was used as a feedstock to obtain the data of valuable chemical compounds. Therefore, the data might contain valuable information in a sustainable frame to approach and suggest the exploitation of this renewable feedstock.
- Data from alkaline-microwave assisted extraction shown that is an efficient method to obtain valuable chemical compounds from lignocellulosic biomass. Additionally, the data can be used as a base to develop scale-up experiments.
- Data can offer an overview of the importance and application of these valuable compounds in the market and their use in different industries.

Data

Figures 12 and 13 show desirability plots for the optimal conditions to increase the concentration of different compounds from hardwood and softwood sawdust (respectively). Figure 14 from the data showed that vanillic acid, vanillin, and ferulic acid its present in high concentration in softwood sawdust compare with hardwood sawdust, where 4-aminobenzoic acid and syringaldehyde was found in highest concentrations. On another hand, 5-HMF and furfural did not show a significative difference (p-value=0.32) in both feedstocks. Finally, data that are shown in the table with characteristics of each of the compounds can suggest their possible uses in the industry as precursors for other compounds (Table 5).

Experimental design, materials, and methods

Hydrophilic compounds derived from sawdust of hardwood and softwood were obtained after processing in microwave-assisted extraction in the presence of an alkaline catalyst (NaOH). Compounds were extracted separately for each type of wood. A response surface methodology (Box-Behnken) was used to validate the effect of three different factors: temperature (65, 80 and 95°C), time (15, 30, and 45 minutes) and NaOH concentration (1, 4, and 7% v/v) on levulinic acid, 5-hydroxymethylfurfural, furfural, 4-aminobenzoic acid, vanillic acid, vanillin, syringaldehyde and ferulic acid concentration.

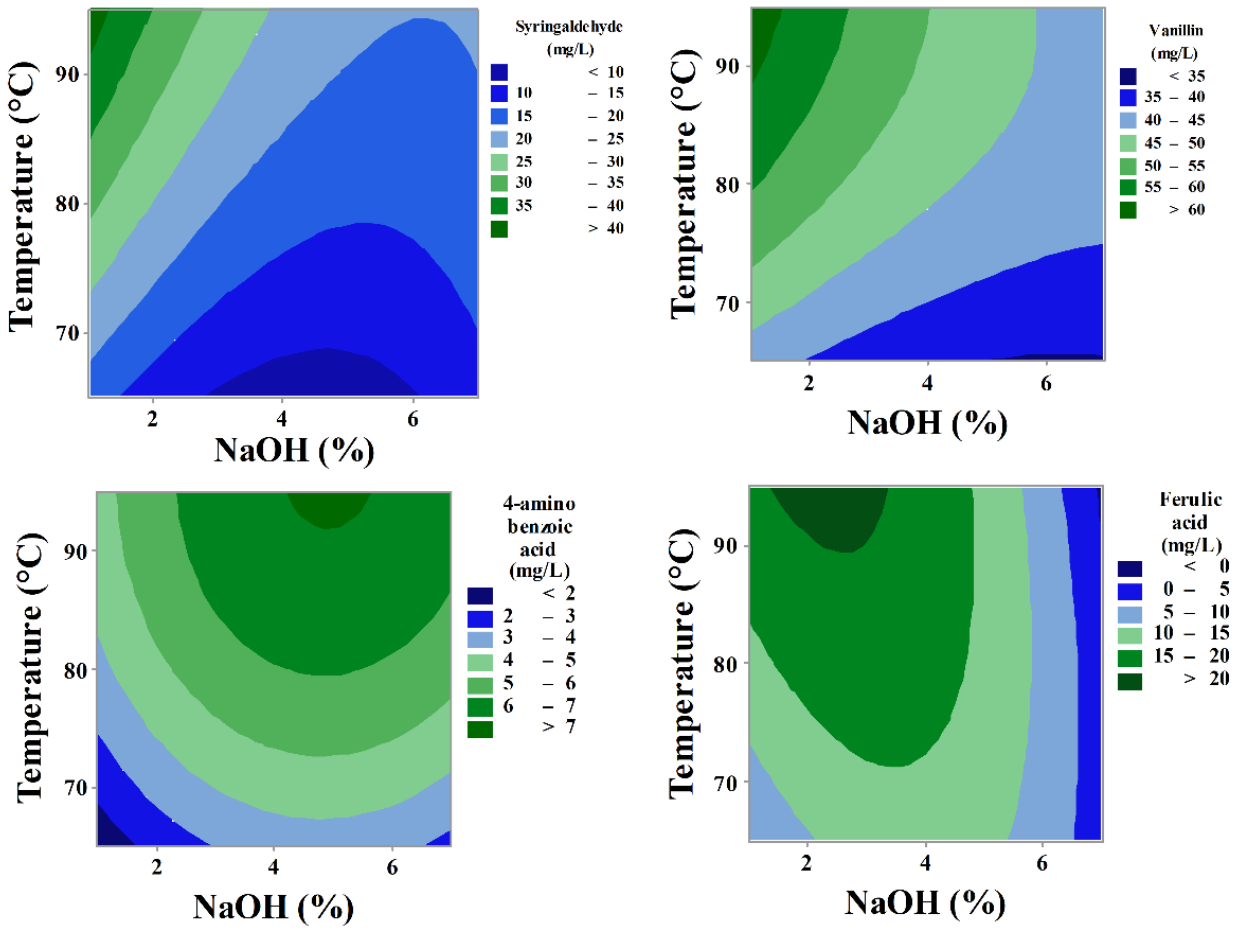

Figure 12.Desirability plots for different compounds obtained from hardwood sawdust.

Sawdust softwood (35%White Spruce and 65% Fir) and hardwood (100% Maple wood) was kindly provided from Quebec Forest Industry Council (QFIC). Both materials were dried at 60°C for 12 hours and ground in a planetary ball mill (PM100; Retsch Corporation) for 15 min at 500 rpm. The resulting milled feedstock was fractioned in a range of 1 mme300 mm. After that, samples were uniformly mixed and used for the preparation of test samples. Sawdust samples (200 mg) for both kinds of wood were treated with MAE at 1000W of power. After MAE, samples were centrifuged at 9000g for 15 min at room temperature and the supernatant and solids were keep separately in sterile tubes to 4°C for further experiments. To determine furfural, 5-HFM, levulinic acid, vanillin, vanillic acid, ferulic acid, syringaldehyde, and 4-aminobenzoic acid a Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer (LC-MS) equipped with a BetaBasic-18 100 mm x 2.1 mm; 3 mm column heated at 30°C, was used. Water: methanol (80:20 v/v) was

used as a sample diluent and water: acetic acid (0.1%), methanol: acetic acid (0.05%), in a ratio of 82.5:17.5 (respectively) as mobile phase with a flow of 0.3 mL/min, and 20 mL as total injection volume. The mass detector was operated in a mode SRM of a positive detection. Finally, 20 mL/mL of phenylethanol-D5 was used as an internal standard. All green chemical compounds were determined and quantified by comparing peak areas and the retention times with their similar standard compound provided from Sigma-Aldrich, USA. All the experiments were carried out in duplicates and the average and standard deviation for data set were calculated.

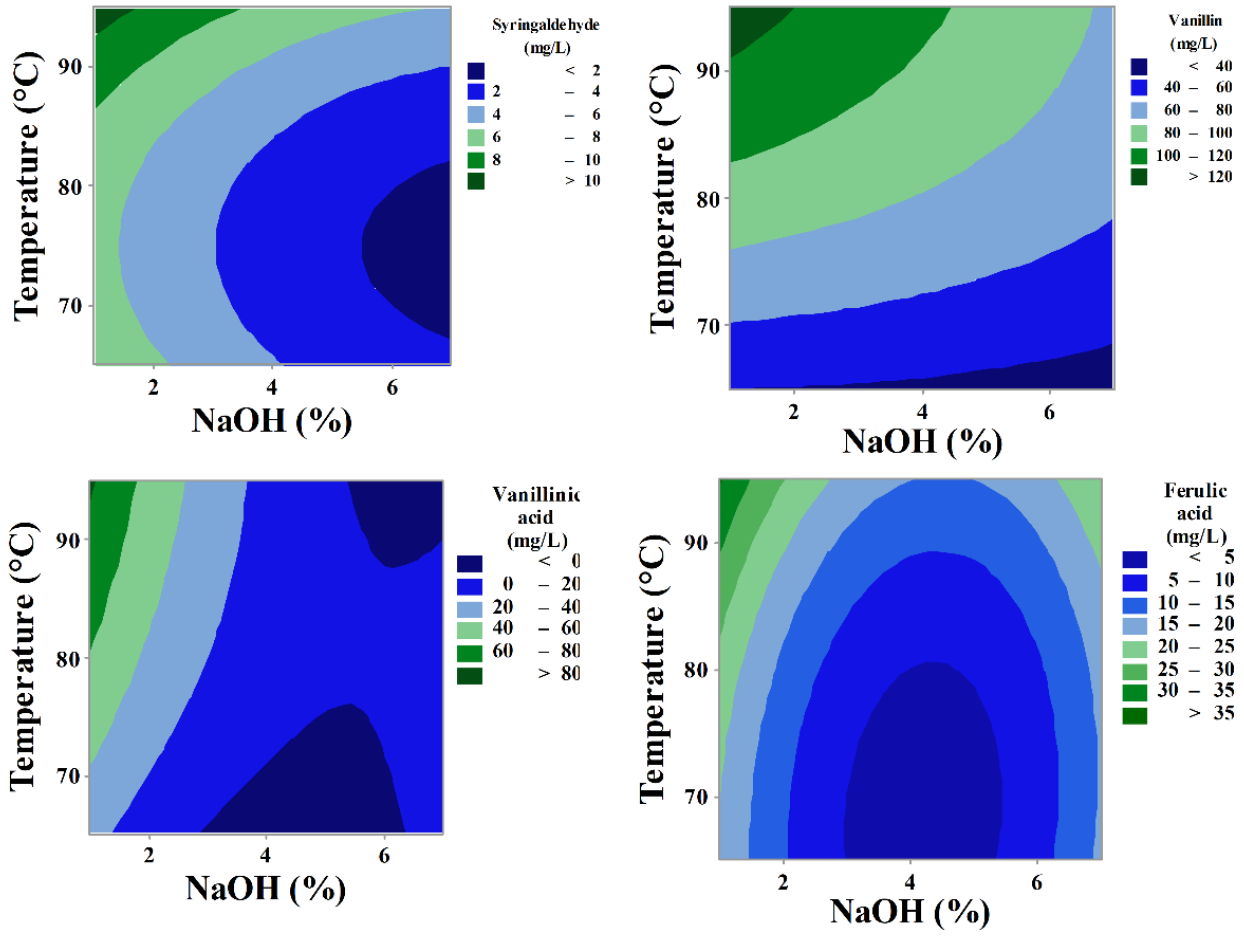

Figure 13.Desirability plots for different compounds obtained from softwood sawdust.

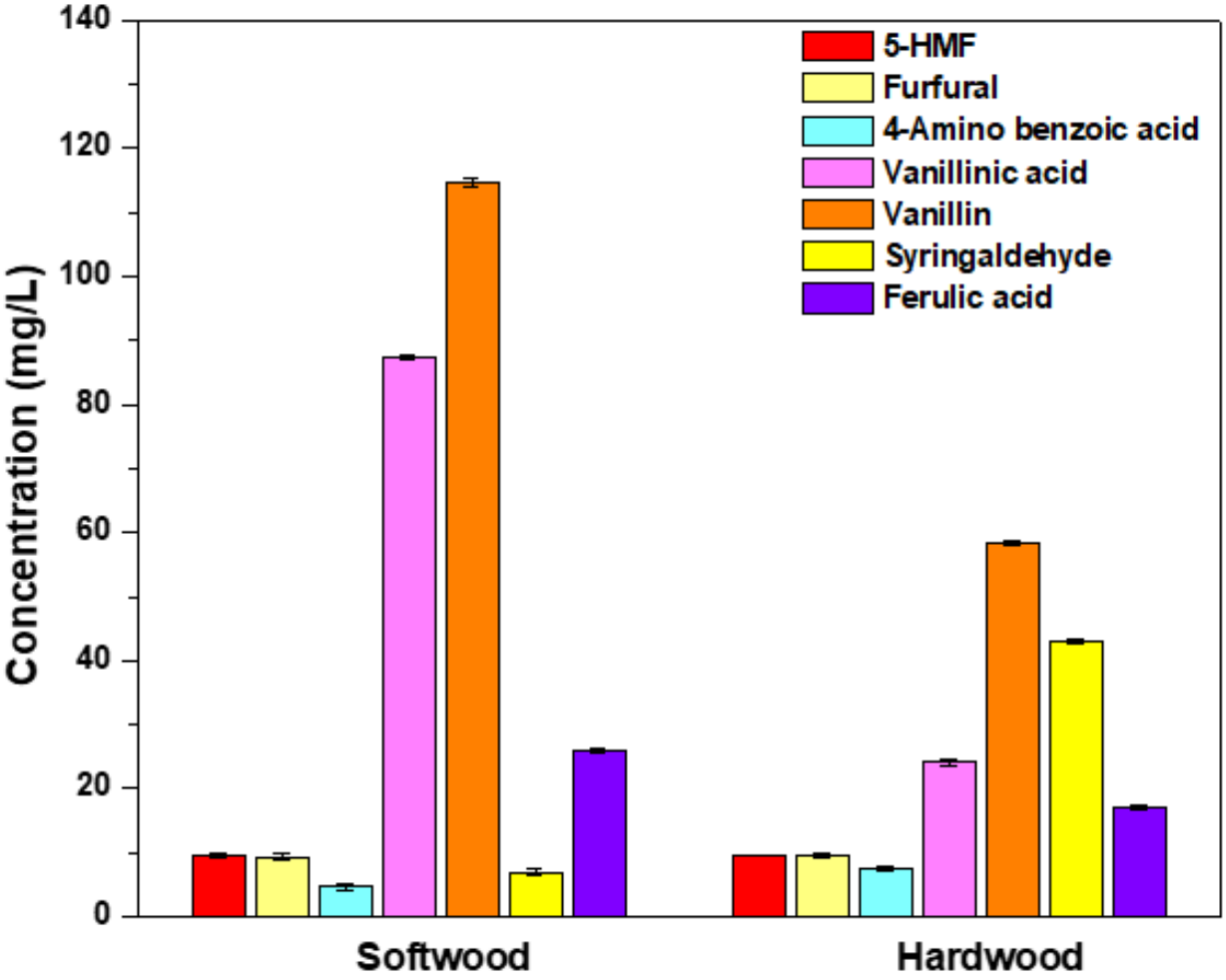

Figure 14.Total concentration of green chemical compounds.

Table 5.Industrial uses as a precursor compound.

Compound	Characteristics	Industrial use	Reference
5-HMF	This compound is considered as a source of energy and a green chemical platform molecule with high added values. Its global consumption has been estimated at 100 tons per year. The most common production route for obtaining this compound is through the dehydration of C6 sugars, especially fructose.	Production of polymers and polyurethane foams, biofuels, polyethylene plastics, and food additives.	[94], [95]
Furfural	Furfural production from lignocellulosic biomass is due to the dehydration of C5 sugars. This chemical compound is considered within of 30 building block chemicals by the US Department of Energy. Its annual production is approximately 200,000 tons per year.	Precursor molecule for the furfural alcohol. Used as a chemical additive in the preparation of adhesives, fungicides, and flavorings. Additionally, also has a wide implementation in the petrochemical industry.	[96], [97]
4-Aminobenzoic acid	These compounds come from lignin fraction in the biomass. Its biological activity has turned it into a compound of high expectations in different industries, the reason why its demand has been increasing during the last decade.	Main applications are in the biomedical industry as an anti-inflammatory drug, analgesic, and antipyretic. On another hand, it is used in chemical, food, and agricultural industries as an intermediate, additive, or inhibitor compound.	[98]
Vanillin	The demand for this compound it is around 20,000 ton per year. This compound can be obtained from guaiacol	A most common use for this compound is In the food industry as a fragrance and	[99], [100]

	oxidation group present in lignin fraction of biomass. Same previous compounds, vanillin its considerer as a top-priority renewable building block.	flavoring ingredient. Also, used in the chemical industry as a building block, mainly in polymer production.	
Vanillic acid	Obtained by the oxidation of two different compounds, guaiacol and vanillin, derived from lignin. Global annual production of the compound is about 18.9 megatons.	Widely used in the food and pharmaceutical industries as an antimicrobial, antibacterial, and antioxidant agent. In recent years, the interest for this compound has been increased, due to its capability to decrease oxidative stress on cardiovascular system following pathogenesis.	[101], [102]
Syringaldehyde	Obtained directly from the oxidation of syringol group, a key component of lignin, as well as from oxidation of vanillin. The market price of this compound is reported to be about 22 USD per kilogram.	The compound is widely used in the food and cosmetic industries as a flavor and fragrance agent. However, has been used as a precursor in the chemical industry for second-generation products. Also used in the pharmaceutical industry as an antibacterial compound.	[103], [104]
Ferulic acid	This compound can be obtained from two different sources: guaiacol oxidation group or 5-HMF dehydration group. The global market is valued in USD $ 53 million.	It is used in food, pharmaceutical and personal care industry as an antioxidant as well as a photoprotective compound for other anti-oxidant such as ascorbic acid.	[105]–[107]

	Additionally, in recent years it has been used in polymer production.

CrediT author statement

Carlos S. Osorio-González.: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft. **Krishnamoorthy Hegde**.: Conceptualization, Methodology, Supervision, Writing – Review & Editing. **Satinder Kaur Brar**.: Funding acquisition, Supervision, Writing-Review & Editing. **Azadeh Kermanshahipour**.: Writing- Reviewing and Editing. **Antonio Avalos-Ramírez**.: Writing- Reviewing and Editing, Supervision.

4.2 Pulsed-ozonolysis assisted oxidative treatment of forestry biomass for lignin fractionation.

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C. S. Osorio-González, K. Hegde, S. K. Brar, P. Vezina, D. Gilbert, and A. Avalos-Ramírez, "Pulsed-ozonolysis assisted oxidative treatment of forestry biomass for lignin fractionation," *Bioresource Technology*, vol. 313, p. 123638, Oct. 2020, doi: 10.1016/j.biortech.2020.123638.

Abstract

Lignocellulosic biomass has been used to produce biomolecules of industrial interest through thermochemical, biological, and chemical transformation. However, few works have been developed over lignin fractionation to obtain monolignols with commercial potentials, such as sinapyl, coniferyl, and p-coumaryl alcohols. This study is focused on developing a thermochemical method to delignify biomass. Additionally, an oxidative treatment with ozone was studied to increase the release of monolignol compounds. The results showed that with 30 sec of ozonation in liquid samples from softwood sawdust a total concentration of 368.50±0.73 mg/kg of monolignols was released after microwave-assisted extraction (256.5±0.51 mg/kg of sinapyl alcohol and 112±0.22 mg/kg of coniferyl alcohol) and 629.20±0.21 mg/kg was released after thermal treatment (453.70±0.15 mg/kg of sinapyl alcohol and 175.5±0.06 mg/kg of coniferyl alcohol). For p-coumaryl alcohol, 16.32 mg/kg was obtained only in hardwood samples. The results of the present study showed that ozonolysis improves monolignols release from forestry residues.

Keywords: Coniferyl alcohol, lignin, monolignols, ozonation, sinapyl alcohol

Introduction

The forestry industry produces a wide variety of products such as softwood lumber, structural wood panels, wood pulp, among others. Nevertheless, a study by [108] suggests that 20% of the total roundwood harvested are waste residues. Currently, this type of waste valorization is majorly focused on conversion into electricity and heat. For instance, in Canada, 80% of sawmills residues are processed into wood pellets [11]. For the last two decades, scientific research to develop processes for better use of forestry residues has substantially increased, mainly motivated by the impetus on lignin valorization which represents 15 to 30% of lignocellulosic biomass. It has been reported that different compounds that structure lignin are linked by different types of bonds, which are divided into three main groups, carbon-carbon, ester, and aryl ether [109], [110]. Due to the complex three-dimensional structure of lignin, it has a high degree of resistance to depolymerization [111]. Its usage has been limited as a source of heat, emulsifier, and feedstock for polymers and resins production. However, very few research has been focused on their exploitation to obtain high-value commercial compounds, such as sinapyl alcohol, m-coumaric acid, p-coumaric acid, p-coumaryl alcohol, and coniferyl alcohol [112]. Extensive knowledge of

the advantages and disadvantages of different pre-treatments used to remove or decrease the amount of lignin from lignocellulosic biomass has been previously reported [113]–[115]. Many methods are commonly used to delignify lignocellulosic biomass and increase cellulose and hemicellulose exposure from the lignin structure as much as possible [116], [117].

However, to obtain monolignol compounds, it is preferred to use a combination of physicochemical methods with non-destructive conditions. For instance, an alternative option is a microwave-assisted extraction or thermochemical processes in addition to an oxidative treatment, such as ozonation to release monolignol products [118]. Generally, the use of ozone in biomass treatment is related to oxidation reactions in aromatic groups as 4-O-5 (diaryl ether), resulting in an increase in lignin oxidation, provoking an increase in monolignols production. The process is based on oxygen electrons attraction from lignin structure by ozone. However, this reaction is highly dependent on the lignin source (softwood or hardwood) and their specific composition [119]. Hence, ozone is a promising oxidative agent to increase the monolignol production. Moreover, the ozone treatment has many advantages compared with other types of pretreatment methods, such as high lignin removal with minimum degradation of carbohydrate coming from cellulose and hemicellulose. One of the main advantages of a technology point of view is that ozone can be produced in-situ (i.e., the same place where feedstock is produced). Due to different equipment for ozone production are portable and can carry where the forestry residues are produced. This fact contributes to decreasing total process cost, and environmental pollution, as well as promotes its scale-up [120].

Different lignin monomers have been used as precursors or feedstock to produce resins (epoxies and phenolics), copolymers and polymers (polyamides, polyester, and polyurethanes). For instance, Kaneko et al. [121] synthesized a biopolyester with high biological compatibility through melt condensation of p-coumaric acid, acetic acid, and sodium acetate. Matsusaki et al. [122] studied the copolymerization of p-coumaric acid and lactic acid. The authors observed that when the amount of p-coumaric acid decreased in the mixture, the copolymer showed specific characteristics such as high solubility, photoreactivity, and high biodegradability, features that improve its use in medicine as drug delivery, graft bones, or tissue platform, among others. Some of the other commercial applications of monolignols include several other industrial commodities, such as polyurethane resins, antifungal or antimicrobial agents, foams, polyols, medical biosensors as well as cosmetic products [123].

The present study evaluates and compares the fractionation of monolignol using two different physicochemical methods in combination with an oxidative treatment. The main objective of this work was to obtain monolignols from two forestry residues, namely, hardwood and softwood sawdust. Furthermore, the expected results of the present study serve as a baseline to develop a sustainable platform to produce high value-added products using forestry residues as a renewable feedstock through low-cost technologies and environmentally friendly methods.

Material and methods

Forestry residues as feedstock

Two kinds of residual sawdust (softwood and hardwood) were kindly provided by the Quebec Forest Industry Council (QFIC). Softwood sawdust was a mixture of two different tree species samples: white spruce (*Picea glauca*) and fir (*Abies* spp), and hardwood sawdust were from maple (Acer sp). Feedstock preparation was performed according to Osorio-González et al. [124].

Microwave-assisted extraction (MAE)

In order to perform a good degree of delignification, parameters for microwave-assisted extraction were selected according to results obtained in our previous work [124]. In brief, they used lignocellulosic biomass to obtain hydrophilic compounds (furans, organic acids, and phenolic compounds) using alkaline microwave-assisted extraction. The observed results show that using high temperature and high concentration of sodium hydroxide an overoxidation of the lignin fraction was performed, resulting in an increase of different secondary phenolic compounds such as vanillin, vanillic acid, syringaldehyde, and 4-aminobenzoic acid. In this sense, hardwood, and softwood samples (200 mg/microwave vessel) were treated separately using microwave-assisted extraction (MAE) at 80°C for 30 min, and 1000 Watts of power using a Perkin-Elmer Anton Paar Multi-Wave Sample Prep System (Liu et al., 2018). in a solid/liquid ratio of 1:25. 5 mL of 4% of sodium hydroxide solution was used as a delignifying agent. After treatment, samples were centrifuged at 9000 x g for 15 min at room temperature and supernatant and solids were stored separately in sterile tubes at 4°C for further experiments. To get enough number of samples to perform ozonolysis treatment six microwave-assisted extraction runs were performed.

Thermal treatment (TT)

Thermal treatment was performed through autoclave (20 min at 120°C, and 15 psi of pressure) using 4 g each of the samples separately in a solid/liquid ratio of 1:25. Sodium hydroxide solution (4%) was used as a delignifying agent. After treatment, samples were centrifuged at 9000 x g for 15 min at room temperature and supernatant and solids were stored separately in sterile tubes at 4°C for further experiments.

Ozonolysis treatment

After the first pretreatment (MAE or TT), two fractions were obtained (liquid and solid) and they were treated separately with ozone, to evaluate the effect of ozonation treatment on the release of monolignols. Ozone treatment in liquid and solid samples (5 mL and 4 g, respectively) was performed by direct ozone exposure with different ozonation times (30, 45, 60, 120 and 240 sec) using an Ozonair EMO3-131® with a maximum flow of 47.19 L/s and a minimum rate of conversion of 0.02 ppm of ozone.

Analytical methods

Quantification of lignin derivates by Liquid Chromatography-Mass Spectrometry (LC-MS)

To quantify the monolignols, a Liquid Chromatography-Mass Spectrometry (Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer) equipped with a HILICpak VG-50 2D column (5 μm, 150mm ID, and 4.6mm df) heated at 30ºC was used. Acetonitrile: water (80:20 v/v) was used as a sample diluent. A solution of acetic acid in water (0.1% v/v), and an acetic acid in methanol (0.05% v/v), in a ratio of 82.5:17.5 (respectively) was used as a mobile phase. The mass detector was operated in a mode SRM of a positive detection. Finally, 20 μL/mL of glycerol was used as an internal standard. All compounds were identified and quantified by comparing peak areas and retention times with standard compounds of coniferyl alcohol, coumaryl alcohol, sinapyl alcohol, p-coumaric acid and m-coumaric acid (Sigma-Aldrich, USA). All the experiments were carried out in duplicates and the average and standard deviation for data set were calculated.

Fourier-Transform Infrared Spectroscopy (FTIR) analysis

To achieve Fourier-Transform Infrared Spectroscopy (FTIR) analysis, samples were frozen in a freeze-drying system (Dura Freeze Dryer, Kinetics) at -20˚C for 24 h. The lyophilized samples were analyzed by FTIR using a Cary 670 FTIR Spectrometer. The spectra were obtained at a resolution of 4 cm-1, in the range of 4000-400 cm-1. Spectra was constructed with 16 replicates per sample.

Statistical analysis

Statistical analysis was conducted using Minitab® 17.1.0 (LEAD Technologies, USA). Monolignols yield was evaluated by One-Way ANOVA. The statistical significance in mean values of the different measured parameters was calculated and compared with the Fisher test at the 95% level of confidence.

Results and discussion

MAE and MAE + ozone effect in monolignols release

In order to release monolignols from lignocellulosic biomass, microwave-assisted extraction (MAE) and MAE+ozone as an oxidative treatment to improve the release of these compounds were performed. Table 6 shows a comparison in monolignol fractionation for MAE and TT samples (single and treated with ozone). After MAE pretreatment in liquid hardwood samples, the highest concentration for monolignols released was observed for coniferyl alcohol (76.05 mg/kg). On the other hand, the samples were treated with ozone resulted in 96% of degradation of coniferyl alcohol and a total degradation for sinapyl and p-coumaryl alcohol. For softwood samples, the same trend was observed in MAE+ozone, with a decrease in the concentration of coniferyl alcohol by 15% (112 mg/kg). For both sawdust, the highest monolignol fraction achieved using MAE was coniferyl alcohol, followed by sinapyl and p-coumaryl alcohols. However, when the samples were treated with ozone, 14 times increase in sinapyl alcohol was observed while a 15% decrease in coniferyl alcohol and the total degradation of p-coumaryl alcohol was observed.

Table 6.Monolignols obtained in liquid samples using Microwave-assisted extraction (MAE) and thermal treatment (TT) + ozonolysis.

Sawdust	Treatment	Alcohol concentration (mg/kg)			
		Sinapyl	**Coniferyl**	**p-Coumaryl**	**Total**
Softwood	MAE	17.87±0.03	131.72±0.26	5.55±0.01	155.14±0.30
	MAE+Ozone	256.5±0.51	112±0.22	nd	368.50±0.73
	TT	nd	328.50±0.12	nd	328.50±0.12
	TT+Ozone	453.70±0.15	175.5±0.06	nd	629.20±0.21
Hardwood	MAE	15.87±0.03	76.05±0.15	9.52±0.02	101.44±0.20
	MAE+Ozone	nd	2.85±0.01	nd	2.85±0.01
	TT	433.00±0.23	418.20±0.50	2.1±0.00	853.30±0.73
	TT+Ozone	5.80±0.09	406.5±0.48	16.32±0.05	428.62±0.62

nd: not determined.
MAE: 80°C/30 min/100W;
TT: 121°C/20 min/15 psi;
O_3: 30 sec of ozonation

This tendency suggests a high reactivity of ozone for compounds containing double bonds and functional groups with high electron density. Thus, lignin oxidation is promoted as it has a high content of C=C bonds, that releases water-soluble and low molecular weight compounds, generally as organic acids [125]. Figure 15 shows a possible cleavage mechanism of lignin fraction of softwood, where the alkaline catalysts specifically act on the β-O-4 (aryl ether), β-5 (phenylcumaran), 5-5 (biphenyl), β-1 (diarylpropane) and β-β (resinol) bonds present at different sites in lignin structure. Cleavage of these bonds can release low molecular weight compounds, such as p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol involved in lignin formation [126].

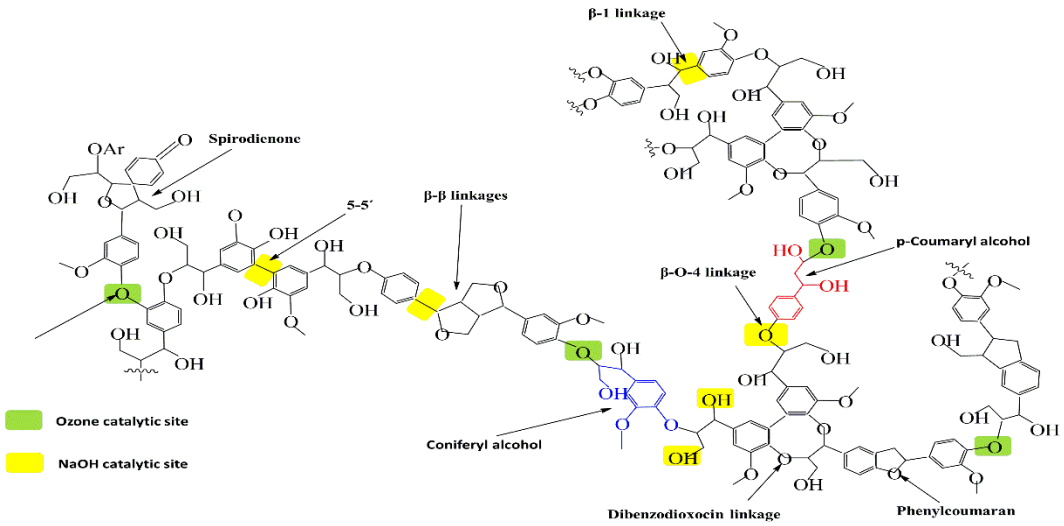

***Figure 15.Catalytic sites of sodium hydroxide and ozone to obtain monolignols compounds from softwood sawdust (Adapted from** [127], [128]).*

The cleavage mechanism relies on the hydrolytic effect of OH- groups in β-aryl ether bonds when they contain a free hydroxyl ion, and this is bound to a phenolic group or alcohol group. Different studies have corroborated the dominant cleavage reaction in aromatic chain bonds when an alkaline delignification is used [129]. Figure 16 shows a possible cleavage mechanism of the lignin fraction of hardwood, where ozone as a complementary treatment promotes oxidation of oxygen in aromatic groups specifically, 4-O-5 (diaryl ether) resulting in an increase in lignin decomposition in their three main monomers. However, this reaction depends on the lignin source and type. For instance, when softwood is used as a feedstock, the reaction can be more complex. In some cases, it can become reversible, as ozone has a deficiency in oxygen electrons, and can directly affect lignin structure, which is rich in electrons [120]. In addition to three monolignols compounds (coniferyl, sinapyl, and p-coumaryl alcohols) other products can be released or formed from the specific cleavage of lignin bonds. There is a wide variety of them, and its formation is highly dependent on treatment conditions used. For instance, from β-O-4 linkage under ozonolysis treatment compounds such as guaiacol derivatives (guaiacyl formiate, 4-vinyl guaiacol, and styrene-β-guaiacyl), quinones (hydroquinone and benzoquinone), ester, homovanillin, among others can be produced [130].

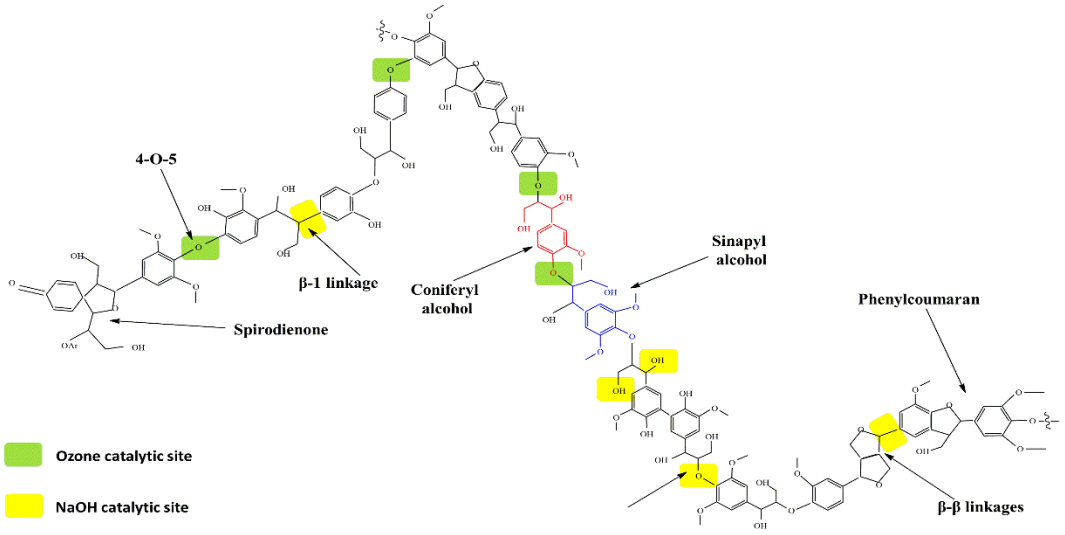

Figure 16.Catalytic sites of sodium hydroxide and ozone to obtain monolignols compounds from hardwood sawdust (Adapted from** [127], [128]**).

Thermal treatment and thermal treatment + ozone effect in monolignols release

Figure 17A shows monolignol concentration for softwood samples after thermal treatment and ozonolysis. As seen in the same Figure (17A), thermal treatment resulted in a 2.4-fold increase in coniferyl alcohol (328.50 mg/kg) concentration in comparison with the concentration obtained after MAE treatment. Likewise, when softwood samples were treated with ozone after thermal treatment, 453.70 mg/kg of sinapyl alcohol was released. On the contrary, a considerable decrease in coniferyl alcohol was observed (47%) in MAE samples. Furthermore, in samples where thermal treatment+ozone was used, an increase of 42% in total monolignol concentration respect to MAE+ozone treatment was observed. Figure 17B shows the highest concentration of sinapyl and coniferyl alcohol that was observed in hardwood samples after thermal treatment at 433 and 418.20 mg/kg, respectively. Likewise, the highest concentration of 16.32 mg/kg for p-coumaryl alcohol was observed in the same sample after ozone treatment. Variance analysis for different monolignol samples released after ozone-treated fractionation showed a significant difference in sinapyl and coniferyl alcohol in softwood sawdust after thermal treatment and between ozone concentrations with p-value=0.001, and 0.002, respectively. Likewise, for sinapyl and coniferyl alcohol monolignols, a significative difference between softwood and hardwood feedstocks with a p-value=0.003 and 0.002, respectively was observed. This tendency is according to general lignin

composition, where coniferyl and sinapyl alcohols are the dominant precursors during lignin biosynthesis in softwood and hardwood. Moreover, both alcohols are linked in lignin structure by ether and carbon-carbon bonds, where ozone treatment has an oxidation effect, improving its release [126], [128].

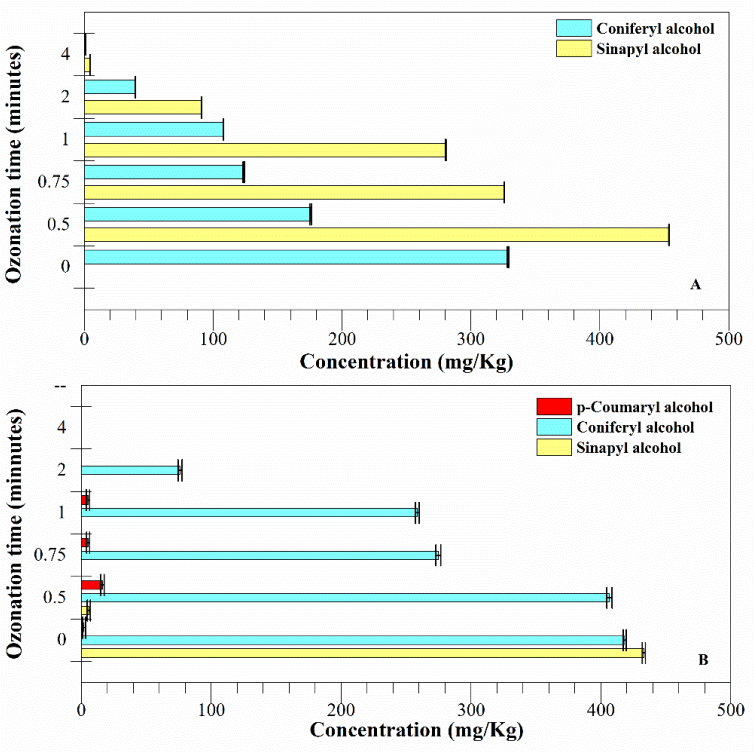

Figure 17.Different compounds obtained using a thermal treatment (TT) + ozonolysis in liquid samples. A) Softwood and B) Hardwood.

Figure 18 shows monolignol fractionation from a solid fraction of the samples. The highest abundance of monolignols for softwood and hardwood was for coniferyl alcohol (25.50 and 6.84 mg/kg), sinapyl alcohol (20.66 and 0.63 mg/kg) and p- coumaryl alcohol (1.00 and 0.09 mg/kg), respectively. The concentration pattern in solid samples is consistent with those obtained in liquid samples, wherein coniferyl and sinapyl alcohol were the two main compounds release in softwood sawdust and coniferyl alcohol in hardwood sawdust. However, the amount of ozone that was used to obtain these concentrations was totally different from liquid samples where the highest concentrations were obtained using the minimum ozone contact time (0.5 min). The results obtained suggested that ozonolysis use as a supplementary treatment in hydrolysates from forestry residues is an efficient treatment to obtain sinapyl, coniferyl and p-coumaryl alcohols. However,

ozone treatment decreased the concentration of coniferyl alcohol in both hardwood and softwood samples and sinapyl alcohol in hardwood samples. One of the reasons for this tendency is the ozone effect on carbon-carbon bonds (5-5', and β-1 linkage). Because carbon-carbon bonds are responsible for conferring the branched lignin structure of this type of biomass, catalytic effect in condensation reactions promoted by alkaline catalyst can generate hydroxyl or methyl substituents in arene groups, resembling two of the main dimers present in lignin (coniferyl and p-coumaryl alcohol) [131].

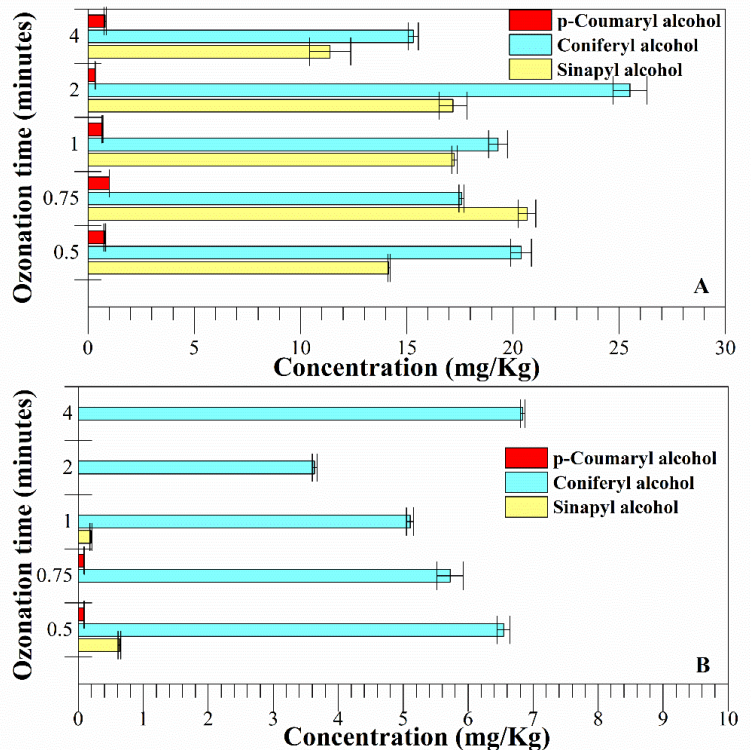

Figure 18.Different compounds obtained using a thermal treatment (TT) + ozonolysis in solid samples. A) Softwood and B) Hardwood.

Likewise, the source and type of lignin are particularly important to choose, design and optimize delignification and ozonolysis treatments. For example, softwood lignin is guaiacyl type or Type-G, with a higher level of coniferyl alcohol polymerization. On the other hand, lignin from hardwood source is a guaiacyl-syringyl type or Type G-S, with a high presence higher level of sinapyl and coniferyl alcohol polymerization [132]. On the other hand, the use of ozone in solid samples showed positive effects on fractionation of coniferyl and p-coumaryl alcohol. Variance

analysis in hardwood and softwood solid samples did not show a significant difference in coniferyl alcohol, between 0.5, 0.75, 1, 2, and 4 mg/min of ozone concentration (p-value=0.953, and 0.487, respectively), but a significative difference (p-value=0.001) between softwood and hardwood was observed. Furthermore, the higher yields were obtained using softwood sawdust.

The results obtained in this study are consistent with other investigations reported in the literature, where they have used pretreatments for relatively short periods of time (≤30 min), low pressures (≤5MPa) and low temperatures (≤200 °C) in the presence of an alkaline catalyst [133]. The delignification can promote the release of monomers and dimers present in the lignin structure through cleavage of ether and carbon-carbon bonds [129]. Additionally, ozone as a complementary treatment with thermal treatment has the potential to increase the production of lignin monomers through oxidation. The above results and characteristics reveal that an increase in exposure to ozone has a negative effect during monolignols release from forestry residues. Likewise, it is necessary to consider that treatments performed under conditions of high temperature, high pressures, or acid catalyst can lead to oxidation, hydrolysis, and dehydration reactions, resulting in the formation of different by-products, such as phenolic compounds, aldehydes, and hydroxybelzadehydes [6]. So far, very few methods about tailored monolignol production from lignocellulosic biomass have been developed as most of the research has been focused on its derivatives such as furans (furfural and 5-hydroxymethyl furfural), organic acids (levulinic and fumaric acids) or phenolic compounds (vanillin, and vanillic acid). So far, a very few works have been developed regarding monolignols obtention, where the research has been performed primordially using biocatalytic routes trough designed microorganisms or enzymatic complex using mainly synthetic media as a substrate. Furthermore, the research has been focused mainly on coniferyl and p-coumaryl alcohols. For instance, Overhage et al. [134] and Chen et al. [135] produced de novo coniferyl alcohol through a designed *Amycolatopsis* sp. HR167 and *Escherichia coli* (respectively), using synthetic media as a substrate. Moreover, Tramontina et al. [136], produced p-coumaryl alcohol from wheat arabinoxylans through enzymatic and whole-cell biocatalytic route. In this sense, MAE+ozonolysis or thermal+ozonolysis treatments have not been tested yet with the purpose to obtain single monolignols. The results obtained in this work are in agreement with previous works regarding coniferyl and p-coumaryl alcohol. However, we observed an important release of sinapyl alcohol in both substrates.

One of the main bottlenecks for any production process is the cost linked with energy consumption to obtain the desired product. In this sense, thermal treatment (autoclave) used in this study uses a 6000 W heater to reach and maintain a temperature of 121°C during the pretreatment time (50 minutes). With the above conditions, the energy consumption of the pretreatment is 5kWh, equivalent to 50 cents per cycle (1 kWh = 10 cents). On the other hand, pre-treatment carried out using microwave extraction presents an energy consumption of 0.54 kWh operated at 1000W by 30 minutes, equivalent to 5 cents per cycle. This energy consumption is equivalent to 5 cents per cycle. Finally, ozone as complementary treatment resulted in energy consumption of 0.6 kWh, equivalent to 0.10 cents per ozone production/min. The difference in the cost of two pretreatments used + ozone regarding the fractionation of different monolignol compounds from lignin is a key factor to establish a viable process for monolignol fractionation. Currently, different industrial systems of thermal treatment and ozone production in the market are affordable. Hence, thermal treatments + ozone could provide a potential alternative method to improve the extraction of coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol from lignin. However, this research is a preliminary screening to improve the extraction of this type of value-added compounds. Thus, future research on optimization of different parameters and scale-up studies could prove the potential of this method in commercialization.

Fourier-transform infrared spectroscopy (FTIR) analysis

It was observed that FTIR spectra for hardwood and softwood have similar signal patterns between 4,000 and 500 cm-1. However, the band intensity varied for each treatment. In the case of softwood, the presence of bands at 3,400 to 3,600 cm^{-1}, corresponding to phenolic and aliphatic OH- groups, as well as between 2,797 to 2,820 cm^{-1} corresponding to covalent bond stretching in methyl and methylene groups indicated the presence of side chains. The presence of carbonyl groups at 1,100 cm^{-1} and 1,800 cm^{-1} suggested that C=O bands could be conjugated or unconjugated with the aromatic rings. The appearance of bands at 1,600 and 1,400 cm^{-1} is characterized by aromatic rings of the phenylpropane skeleton, which is an intermediate to incorporate the three main monolignols into lignin in form of guaiacyl, syringyl and p-hydroxyphenyl phenylpropanoids [129], [137], [138]. The bands at 1,388 cm^{-1} are characterized by vibrations of covalent bonds linked with aromatic rings and aliphatic stretch in methyl groups or hydroxide from phenyl groups, respectively. Finally, for bands between 500 to 1,000 cm^{-1},

general groups such as methyl, ether, carboxylic acid, aldehyde deformation, stretching guaiacyl rings, stretching carbonyl group, and aldehydes are present in the whole mass (cellulose, hemicellulose, and lignin).

Conclusion

Forestry residues treated with a physicochemical process is a suitable option to obtain value-added compounds from lignin fraction. Ozonolysis treatment shows a positive effect on total monolignols release in liquid and solid samples from softwood sawdust after microwave-assisted or thermal treatments, where sinapyl and coniferyl alcohols were the most abundant monolignols. On the other hand, in samples from hardwood sawdust, the highest monolignol concentration obtained was coniferyl alcohol. This work can serve as a baseline to design and improve the release of monolignol compounds, specifically, sinapyl alcohol and coniferyl alcohol in softwood and p-coumaryl alcohol in hardwood feedstocks.

CRediT author statement

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Supplementary data for this work can be found in ANNEX 1.

CHAPTER FIVE: *WOOD HYDROLYSATE AS A SUBSTRATE FOR LIPID PRODUCTION USING THE OLEAGINOUS YEAST RHODOSPORIDIUM TORULOIDES*

5.1 Lipid production in *Rhodosporidium toruloides* using C-6 and C-5 wood hydrolysate: A comparative study

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Abstract

Rhodosporidium toruloides is an oleaginous yeast that can accumulate up to 70% of its dry biomass as lipids and can use C5 and C6 sugars as a carbon source, that are present in hydrolysates from different lignocellulosic biomass. *R. toruloides* can be an alternative for the valorization of forestry residues and the obtention of higher value-added products. In this study, five strains of *R. toruloides* were evaluated to find the most efficient yeast strain for C5 and C6 sugars utilization, using lignocellulosic hydrolysates as a culture media. Sugar consumption was similar between all strains. Nevertheless, it was different among C6 and C5 hydrolysates, with maximum sugar utilization of 98%, and 60% for C6 and C5 hydrolysate, respectively. Among the studied strains, the highest lipid production was observed in *R. toruloides*-1588 with 23.33 g of lipids/L in C6 hydrolysate after 112 h. The highest lipid production in C5 hydrolysate was observed in *R. toruloides*-7191 with 14.67 g of lipids/L after 120 h. Predominantly, fatty acids for *R. toruloides*-1588 was oleic, palmitic, and stearic acids and pentadecanoic, palmitic, and heptadecanoic acids for *R. toruloides*-7191. Moreover, both strains grew and produced lipids in the presence of inhibitor compounds, such as levulinic acid (13.88 mgL^{-1}), 5-HMF (80.86 mgL^{-1}), furfural (153.54 mgL^{-1}), vanillin (17.17 mgL^{-1}), vanillic acid (85.25 mgL^{-1}), syringaldehyde (41.83 mgL^{-1}), ferulic acid (1.66 mgL^{-1}), and 4-hydroxybenzoic acid (0.85 mgL^{-1}). The study revealed that *R. toruloides*-1588 and *R. toruloides*-7191 are promising strains for C5 and C6 sugar utilization from lignocellulosic hydrolysates for lipid production.

Keywords: Fatty acids; lipids; *Rhodosporidium toruloides*; sugar utilization; lignocellulosic biomass

Introduction

Oleaginous yeast can consume fermentable sugars and convert them into single cell oil (SCO). These lipids are mostly triacylglycerols (TAGs) or free fatty acids (FAs) [52]. These microbial lipids containing essential fatty acids, such as gamma-linoleic acid, eicosapentaenoic acid, arachidonic acid, and docosahexaenoic acid are especially valuable in food, chemical, and pharmaceutical industries [93], [139]. Likewise, lipids with fatty acids in a range from C10 to C18 are an attractive feedstock for the production of renewable fuels such as biodiesel. Above all, due to their high carbon ratios and lower sulfur content, carbon monoxide and hydrocarbon emissions are reduced as compared to fossil fuels [64]. Nevertheless, one of the most important challenges

to make the process economically viable is the use of substrates that do not compromise food security, renewable, and low cost. An alternative substrate that has increased interest among the academic and industrial community during the last decades is the use of hydrolysates obtained from lignocellulosic biomass [84]. Sitepu et al. [140] evaluated the capacity of 45 species of oleaginous yeasts, such as *Y. lipolytica*, *C. curvatus*, *L. starkeyi*, *R. glutinis*, and *R. toruloides* to use carbohydrates and tolerate toxic compounds present in lignocellulosic hydrolysates. However, one of the challenges that these strains presented in their natural state was the use of specific substrates to obtain high yields. This limitation largely restricts its use on substrates where a mixture of different carbon sources is present. These substrates are derived from hydrolysates obtained from different lignocellulosic biomass, such as sugarcane bagasse, switchgrass, forestry residues, straw, and corn stover, among others. For this reason, it is necessary to use more robust strains that have the ability to grow in a wide variety of raw materials. Recent research has shown that short and long-term microorganism adaptation, random mutagenesis, and genetic modification are important tools for increasing lipid production, tolerance to inhibitors and improve the use of different sugars [46], [83], [141]. An example is a work done by Bonturi and coworkers [52] where the expression of ribulose 5-phosphate epimerase (RPE1) gene in an *R. toruloides* strain was increased 5.8-fold, to evaluate xylose utilization and lipid production using sugarcane bagasse hydrolysate as a substrate.

Rhodosporidium toruloides is a red yeast capable of accumulating up to 70% of the lipids during the log and stationary phase and can produce up to 100 gL^{-1} of biomass. The fatty acids present in the lipids accumulated by this strain are mainly palmitic, stearic, oleic, and linoleic acids [44]. Along with lipid production, this yeast produces carotenoids, such as β-carotene, torulene, torularhodin, and astaxanthin, which are of high value in chemical, pharmaceutical and food industries [66]. During the last decade, the genus *Rhodosporidium* has generated an increasing interest due to the amount of lipid that it accumulates, which are precursors for the elaboration of different products such as adhesives, waxes, surfactants, creams, and biofuels [1], [46], [142], [143]. However, nowadays research on this strain has focused mainly on obtaining all the possible knowledge about its genome [37]. This has allowed to carry out some metabolic, proteomic, and genetic modification studies; as well as the development of different culture strategies [44], [52], [86], [88], [141], [144]–[146]. Several research has focused mainly on improving lipid accumulation and carotenoid production [46], [66], [93]. Nevertheless, the research has begun to

expand to other fields, such as the use of different carbon sources and the development of tolerant strains to inhibitory compounds that are present in some of the culture media currently used [47], [51], [54], [58], [69], [75], [147]. The aim of this study was to evaluate the use of C5 and C6 sugars present in a hydrolysate of woody biomass on the growth and accumulation of lipids in five different *R. toruloides* strains. Likewise, the effect of some inhibitor compounds present in the hydrolysates was studied since it is essential to know the tolerance or degradation of these inhibitors during fermentation.

Material and Methods

Wood feedstock

For lipids production, two different wood hydrolysates were used as a culture media, which was kindly donated by Greenfield Global Inc., Varennes, Quebec, Canada.

Microorganism, culture maintenance, inoculum preparation, and cell growth

The different strains used in this work are listed in Table 7 All the strains were procured from the Agricultural Research Service (NRRL) Culture Collection (USA). Laboratory stocks of the culture were grown in YM agar (yeast extract 3 g/L; malt extract 3 g/L; peptone 5 g/L; glucose 10 g/L; agar 15 g/L). Figure 19 shows the process required to prepare the seed culture. Each yeast was pre-cultured up to three generations in a Multitron® shaker (Infors Canada).

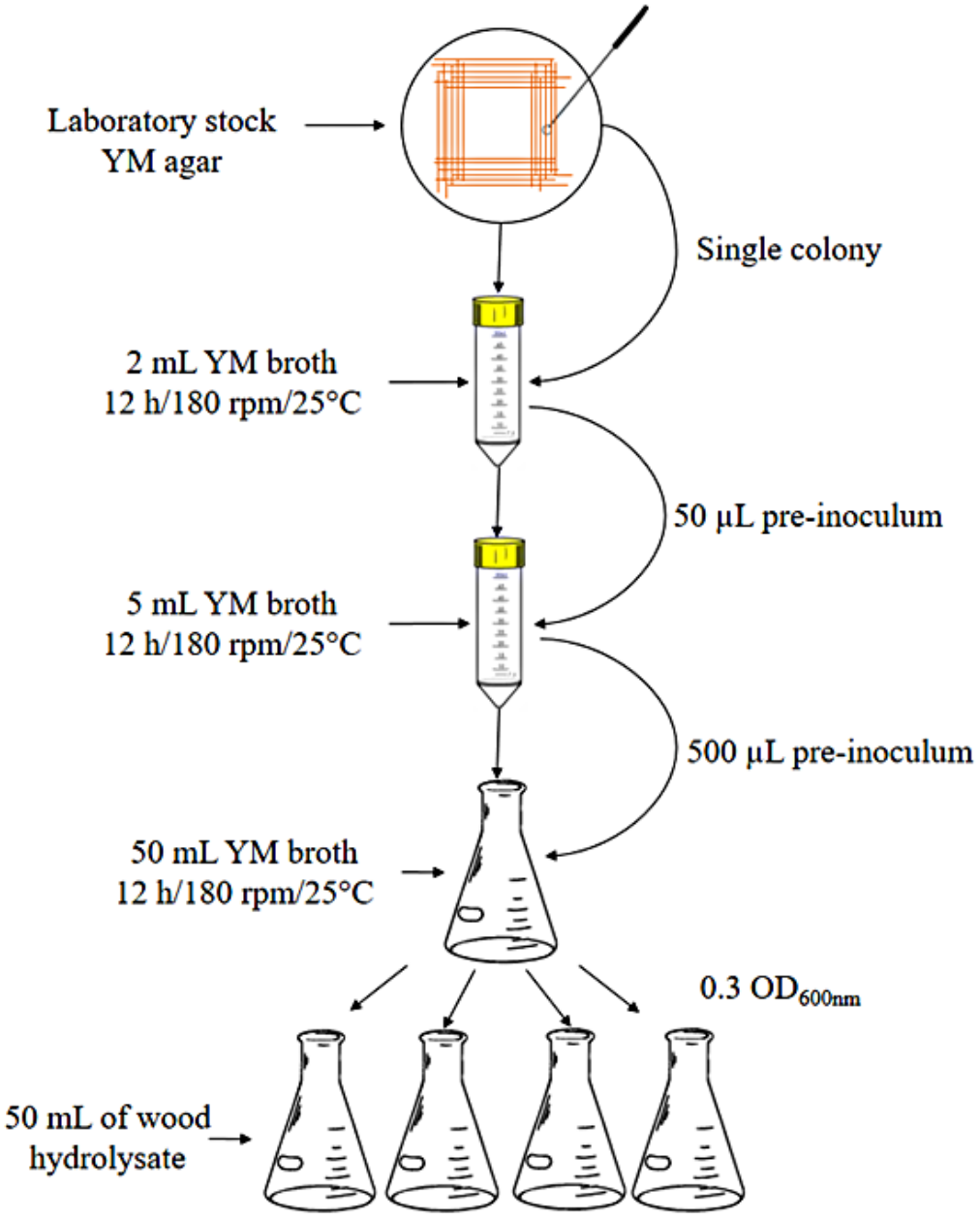

Figure 19.The process to prepare seed culture.

Table 7.Strains used to evaluate lipid production and sugar utilization in R. toruloides using C-6 and C-5 wood hydrolysate as a substrate.

Strain	Features	Purpose
R. toruloides NRRL-1091	Isolated from wood pulp, however, it has been reported that this yeast could not grow in undetoxified lignocellulosic hydrolysates	Sugar utilization and lipid production
R. toruloides NRRRL-1588	Isolated from the soil, with the capacity to produce up to 100 g/L of biomass and accumulate up to 60% of its weight in lipids	
R. toruloides NRRL-6984	Isolated from wood pulp and has been reported that this yeast could not grow in undetoxified lignocellulosic hydrolysates	
R. toruloides NRRL-6987	Diploid isolated from a hyphal conjugate of NRRL Y-1091 and NRRL Y-1588, capable of producing 0.24 g biomass/g glucose and up to 79 mg oil/g glucose in media with nitrogen restriction	
R. toruloides NRRL-7191	Isolation source of this strain is not available, and there are no reports of its use in the production of lipids	
Yarrowia lipolytica NRRL-63746	Unconventional yeast able to accumulate up to 50% of its dry weight in lipids.	A control for lipid production
Scheffersomyces stipitis NRRL-17104 *Scheffersomyces stipitis* NRRL-11545	These yeasts in the native form had the highest capacity to use C5 sugars (xylose and arabinose) than any other known microorganism	A control for C5 sugar utilization
Saccharomyces cerevisiae NRRL-12632	These yeast present high tolerance to aldehyde inhibitors such as furfural and 5-HMF	A control for C6 sugar utilization and negative control for lipid production

Lipid production

For microbial lipid production, *R. toruloides* cultures were grown in separate 250 mL-flask containing 50 mL of C6 hydrolysate (50 g/L glucose) and C5 hydrolysate (50 g/L xylose). As a source of nitrogen 1 g/L ammonium sulfate was added, and pH was adjusted to 6. Both substrates

were inoculated with seed culture to an initial OD_{600nm}=0.3, and cultivated at 25°C, 180 rpm in a shaker incubator). During fermentation, the samples were drawn every 18 h during exponential phase (first 72 h), and afterward, until 224 h, the sampling was every 8 h. All experiments were done in duplicates.

Lipid extraction

The cells were harvested by centrifugation (15 000 *xg*/2 min), followed by drying at 60°C to obtain a constant weight. The cell lysis was carried out according to Thakur et al. [148], where the dry biomass was subjected to acid hydrolysis (boiling with 1 M/L HCl for 1 h). Lipids were extracted by the method of Singh et al. [66]. Briefly, after cell lysis, 3.75 mL of chloroform/methanol (2:1 v/v) was added, the mixture was vortexed for 15 min. Later, 1.25 mL of chloroform was added, vortexed again for 1 min, and lastly, 1.25 mL of NaCl (1 M) was added. The resulting mixture was centrifuged at 7000 *xg* for 15 min to separate the two phases (aqueous and organic phase). The evaluation of lipid production was carried out according to Ma et al. [149], through lipid content (Eq. (1)), lipid concentration (Eq. (2)), and lipids yield (Eq.(3)):

$$Lipid\ content = \frac{lipid\ weigth\ (g)}{cell\ dry\ weight\ (g)} \quad \text{Eq. (1)}$$

$$Lipid\ concentration = \frac{lipid\ weight\ (g)}{culture\ medium\ volume\ (L)} \quad \text{Eq. (2)}$$

$$Lipid\ yield = \frac{lipid\ concentration\ (g/L)}{total\ sugar\ consumed\ (g/L)} \quad \text{Eq. (3)}$$

Analytical methods

Cell growth and carbohydrate analysis

Cell growth was measured by dry constant weight (DCW). Briefly, the cells were harvested by centrifugation (15000 x g/2 min), washed with phosphate-buffered saline (PBS), and dried at 60 °C, cell biomass was expressed as g/L. Total reducing sugars (TRS) concentration during fermentation was determined by DNS method using glucose as the standard. Briefly, 500 µL of the sample was mixed with 1.5 mL of DNS reagent and boiled for 10 min. After the reaction, the samples were then cooled in a cold-water bath to stop the reaction. Later, 4 mL of water was added

and vortexed [150]. The amount of TRS was determined by UV–visible spectrophotometer (Cary-50, Varian) at 540 nm.

C-5 and C-6 sugars and inhibitors analysis by LC-MS method

During fermentation, sugars and microbial growth inhibitors were quantified using a Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer (LC-MS). To determine sugars, a 5 μm, 150mm ID, 4.6mm df column; acetonitrile: water (89:11 v/v) was used as a mobile phase and D6 glucose was used as an internal standard. In case of inhibitor analysis, a BetaBasic-18 100 mm×2.1 mm; 3 μm column was used at 30 °C, with water: methanol (80:20 v/v) as mobile phase and phenyl ethanol-D5 as an internal standard. All data presented are average values from duplicate samples.

Fatty acid composition

To determine fatty acid compositions, the method reported by Seo et al. [151] was used. Briefly, crude lipid was subjected to rapid methyl esterification using methanol and sulfuric acid to 100 °C/20 min. Later, the samples were cooled for 10 min to room temperature and FAME was extracted with hexane. Fatty acids were determined and quantified by comparing peak areas and the retention times with the Supelco 37 Component FAME Mix (Sigma-Aldrich, USA), using an Agilent 7890B gas chromatograph, with a flame ionization detector (FID), equipped with an Agilent 122–2362 DB-23 column with 60m×250 μm and 0.25 μm film thickness.

Results and Discussion

Sugar utilization from C5 and C6 sugars in the wood hydrolysate

For the culture media, an initial concentration of 50 g/L of C6 or C5 carbon source was used separately. The consumption of the substrate was evaluated in the fermentation broth at regular intervals for each of the strains. The profile of microbial growth was observed to be similar for both hydrolysates during the lag phase of growth (first 6 h). Later, in the case of C6 hydrolysate, the cell growth continued to be in exponential phase until 72 h followed by the beginning of the stationary phase (until 224 h). On the other hand, when C5 hydrolysate was used as culture media, microbial growth tendency was observed to be different, because of faster growth (attained stationary phase within 54 h for all strains studied). This was followed by continuous growth in

stationary phase until 108 h. Then a second slight exponential phase of 18 h (shorter duration), which was followed by the beginning of a second stationary phase. This tendency suggests a longer adaptation process to the culture media that can lead to slower consumption of nitrogen source. This indicates that yeast requires a higher amount of ATP during its lag and exponential phase in substrates with xylose as a sole source of carbon [152]. These results are promising from point of view of scale-up. As in biological processes, a rapid microbial growth theoretically leads to an early obtention of the expected products. Consequently, this decreases the tot al time of production and the concomitant reduction in costs associated with energy consumption and other utilities.

In general, the use of glucose was very rapid and more than 95–98% was consumed in the first 72 h of fermentation (exponential phase) in the control strains (*S. stipites*-17104, *S. stipites*-11545, and *S. cerevisiae*-12632, respectively). The similar tendency was observed in *R. toruloides* strains (1091, 6984, and 7191, respectively). The strains, *R. toruloides*-6987, and *Y. lypolytica*-63746 consumed the substrate at 96 h of fermentation and finally, *R. toruloides*-1588 presented the slowest substrate consumption until 128 h (Figure 20A). The residual glucose in the culture media was less than 2% after the 128 h of fermentation. Conversely, the consumption of xylose when C5 hydrolysate was used as culture media, was in the range of 40–50% during the first 18 h of fermentation for all strains and it remained in the same range until the end of fermentation (Figure 20B). Sugar utilization profile in *R. toruloides* sp. strains showed a rapid use of C6 and C5 sugars, using wood hydrolysate as culture media. This demonstrated the yeast growth capacity in culture media without nutrient supplementation, such as vitamins or amino acids. These results are promising because only less than 20% of known yeasts have this ability. Thus, the strains used in this study are suitable for their cultivation in hydrolysates obtained from lignocellulosic biomass, and specifically, in hydrolysates obtained from forest industry residues.

In this context, the study showed that the time of sugar consumption was fast and biomass production was higher than reported by Braunwald et al. [153] for a strain of *Rhodotorula glutinis* cultivated in a similar proportion of C/N in the medium (57 g/L glucose and 1 g/L ammonium sulfate). In addition, several studies have shown that the use of the high proportion of carbon source (≥65 g/L of carbon present in the culture media) with respect to nitrogen content, allows the oleaginous yeast to reach its maximum yield and the maximum storage capacity of lipids. This is due to the osmotic shock that the carbon source causes when it is in high proportion in the culture

medium since the activity of the Krebs cycle decreases due to the intracellular increase of citric acid [64], [67]. In addition to glucose and xylose, the hydrolysates contained other sugars, such as fructose, galactose, sucrose, and lactose. However, these sugars did not show considerable changes in their concentration throughout fermentation (ANNEX 2). This observation is in concordance with the study reported by Wiebe et al. [88], where strains of the genus *R. toruloides* consumed only glucose (100%), xylose (50%) and arabinose (18%).

The kinetics of cell growth and the substrate consumption of *R. toruloides* in C6 hydrolysate was similar for strains *R. toruloides*-1091 (0.21 gL^{-1} h^{-1}), *R. toruloides*-6984, *R. toruloides*-6987 (0.20 gL^{-1} h^{-1}), and *R. toruloides*-7191 (0.17 gL^{-1} h^{-1}), except strain *R. toruloides*-1588, which showed a maximum production (120 g/L) and a slower substrate consumption (0.11 gL^{-1} h^{-1}). This behavior is the same when a synthetic medium was used as a culture medium, where strain *R. toruloides*-1588 was the one with the highest biomass production with 52.5 g/L (data not shown). On the other hand, the substrate consumption in the *S. cerevisiae*-12632, *S. stipites*-17104, and *S. stipites*-11545 was similar (0.21 gL^{-1} h^{-1}) and the maximum biomass values obtained was lower (59 g/L, 81 g/L, and 80 g/L, respectively) than that obtained in the five strains of *R. toruloides*. Likewise, when the strains were cultivated in C5 hydrolysate, an increase in biomass production was observed at 36 h of fermentation. This profile suggests that the xylose consumed (50%) by different yeasts, showed the same tendency when glucose was used as a carbon source. However, after this time xylose consumption remained constant. The maximum production of biomass was obtained with *R. toruloides*-7191 (92 g/L), followed by *R. toruloides*-1588, *R. toruloides*-1091, and *R. toruloides*-6987 with 80, 77 and 75 g/L, respectively. Finally, the lower value was obtained by *R. toruloides*-6987 and *S. cerevisiae*-12632 with 70 g/L and 61 g/L. In general, the production of biomass using C5 hydrolysate was lower as compared to the value obtained when C6 hydrolysate was used as culture media. Microbial growth and biomass production obtained in this study demonstrated that strains of *R. toruloides* were robust. This is related to the rapid production, ability to use different carbon sources, ability to grow in culture media with minimal or null nutrients, and tolerance to stress factors as inhibitory compounds present in the culture media.

These results demonstrated the efficient use of glucose as a carbon source by the strains evaluated from the lignocellulosic hydrolysate. This is reflected in the faster time of consumption and the high production of biomass. This is due to the use of the nitrogen source at the beginning of the

fermentation, which allows a complete utilization of the sugars in the hydrolysate. On the other hand, xylose consumption was only 50% during the first 18 h of fermentation and remained so until the end of the process. This demonstrates the ability to use C5 sugars by strains of this genus in their native state. These results agree with those reported by different studies, where lignocellulosic hydrolysates with C5 and C6 sugars were used as a substrate for lipid production using *R. toruloides* strains. For instance, Wiebe et al. [88]; evaluated two different mixtures of sugars (g/L): i) 25 g/L glucose, 33 g/L xylose and 11 g/L arabinose and ii) 6 g/L glucose, 56 g/L xylose, and 9 gL^{-1} arabinoses for lipid production using *R. toruloides*. In this research, glucose was consumed during the first 40 h (100%), followed by xylose in 78 h (98%), and finally arabinose until 96 h (15%). By another hand, Bommareddy et al. [86]; reported a profile glucose consumption used in combination with raw glycerol, they found that 100% of glucose was consumed during the first 24 h of fermentation. Likewise, Fei et al. [144]; found a total consumption of sugars (100 g/L glucose and 10 g/L xylose) at 78 and 96 h of fermentation, respectively. Evely, Bonturi et al. [52]; also reported total consumption of sugars until 144 h of fermentation (glucose, xylose, and a mixture) for native and adapted *Rhodosporidium* strains, using a hydrolyzate from bagasse sugar cane as substrate. The results obtained in this research reinforce the statement on the difference in individual sugar utilization. However, in some microorganisms, such as *R. toruloides* (native strains), the use of C5-sugars such as xylose has certain limitations, mainly at metabolic levels. Nevertheless, to obtain an increase in rate-use of xylose by *R. toruloides*, different methodologies and processes have been proposed and developed.

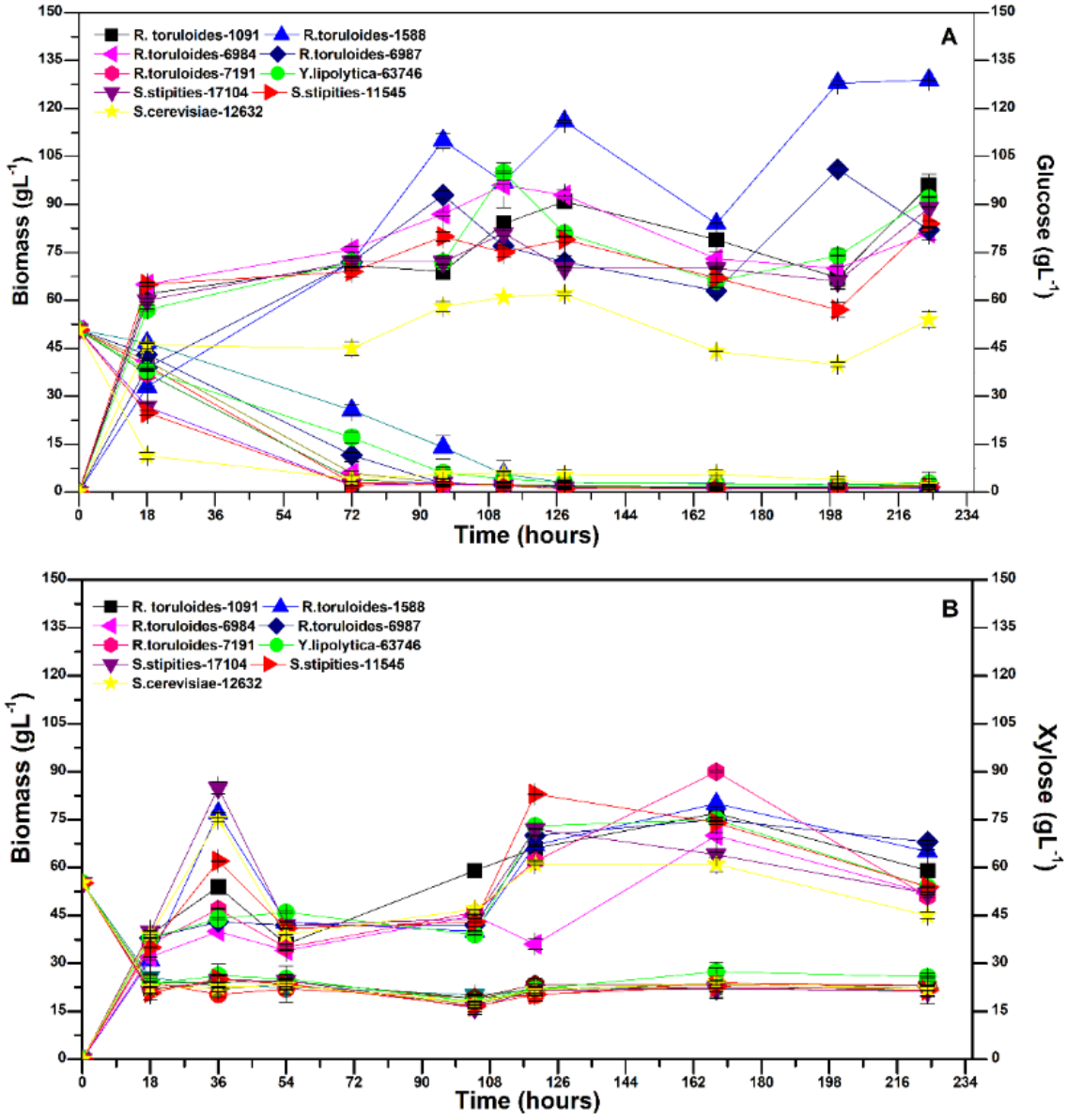

Figure 20.Sugar utilization and biomass production in C6 (A) and C5 (B) wood hydrolysate.

Different alternatives (conventional methods and genetic modification) to increase the use of C5 sugars by this strain exist. A conventional and attractive method due to its simplicity and effectiveness is the short- or long-term adaptation of microorganisms to different compounds or conditions that they particularly want to improve [52]. This method is based on phenotypic heterogeneity. In the case of a short-term adaptation, a transcriptional state of the most tolerant phenotype is inherited and prevails through the generations (transcriptional memory), which

generates an epigenetic inheritance. On the other hand, long-term adaptation has an evolutionary engineering approach since it generates changes in microorganism genotype. Another method that can be used to overcome this limitation or increase lipid accumulation, is mutagenesis. By definition, random mutagenesis is the process used for genetic information of microorganisms that can be modified. These processes can be naturally generated by changes in the environment in which they coexist or can be induced by physical methods through UV and X-ray irradiation or atmospheric room temperature plasma (ARTP). Similarly, another option is through the use of chemical agents such as bisulfite, aminopurine, and nitroguanidine. In addition, another option is directed mutagenesis or site-directed mutagenesis, which uses nucleotides or specific oligonucleotides to induce the mutation in the DNA sequence of a gene or any of its products. However, mutagenesis methods are limited by mutation type because they are not as specific as genetic methods used nowadays [46], [145], [154]. Finally, there is genetic manipulation, directed to expression or silencing of genes or key enzymes such as Ribose-5-phosphate isomerase (RKI1) or Ribulose-phosphate3-epimerase (RPE1), through the use of genetic engineering. Likewise, the conventional route of C6 sugars utilization can be modified through the deletion of key enzymes that promote the degradation of lipids such as long-chain acyl-CoA synthetase (FAA1, FAA2, FAA3, FAA6), and directed towards a better conversion of a carbon source into lipids.

Nowadays, advances in genomics and synthetic biology provide a better and more comprehensive understanding of metabolic pathways to develop engineered microbial strains, specifically with enhanced microbial performance in terms of carbon consumption and lipid production. Precursors, such as NADH and NADPH have been identified and exploited in several strains to increase lipid biosynthesis [37]. In general, NADPH involves capturing electrons during substrate catabolism and then later acting as a reducing agent for lipid biosynthesis during de-novo lipogenesis. Hence, high activity of NADPH will result in increased lipid accumulation even though whether the electrons come from pentoses or hexoses. For instance, Bandhu et al. [155] recently reported the increase in lipid by 1.18-fold using xylose as a carbon source, when NADP-malic enzyme was expressed in *Rhodotorula mucilaginosa*-IIPL32.

So far, engineering strategies in several *Rhodosporidium* strains has been focused on increasing lipid production through overexpression of various other enzymes, such as acetyl CoA carboxylase (ACC1), glycerol 3-phosphate dehydrogenase (GPD), diglyceride acyltransferase (DGA),

triglyceride lipase (TGL), and ATP citrate lyase (ACL). Nevertheless, literature and research focused on genes and enzymes involved in the pentose-phosphate pathway in *Rhodosporidium* genre is limited or null. This opens up a large number of possibilities to study genes and enzymes involved to improve sugar utilization and increase lipid production.

Lipid production and fatty acid composition

Summary of lipid production in wood hydrolysate to each strain evaluated is presented in Figure 21. When using C-6 hydrolysate as culture media, the strain *R. toruloides*-1588 with the highest biomass yield corresponds to the highest lipid yields (0.35 g lipids/g biomass). On the other hand, when C-5 hydrolysate was used as culture media, a similar trend was observed, since the highest amount of lipids was obtained with the strain *R. toruloides*-7191 (0.32 g lipids/g biomass). However, time is a key factor in the process of lipid accumulation since the highest concentration of lipids was obtained at 112 h of fermentation when hydrolyzed C6 was used and 120 h of fermentation when C5 hydrolysate was used. On the other hand, the difference in lipid production with control strain *Y. lipolytica*-63746 was lower (0.15 g lipids/ g biomass, and 0.21 g lipids/g biomass) compared to that obtained with *R. toruloides*-1588 and *R.toruloides*-7191, respectively. In general, the low lipid yield (≈30%) suggested that the two strains had the highest accumulation, and the nitrogen source was not consumed in its entirety. This tends to generate a deficiency in the lipid accumulation process since the yeasts use the carbon source for the generation of biomass and not for lipid synthesis. Additionally, the lipid accumulation could be affected by the acidifying effect that the nitrogen source produces during its absorption by the cells, affecting the lipogenesis pathway, causing a decrease in lipids accumulation.

The lipid composition showed that the fatty acid distribution was similar among the yeasts when C6 hydrolysate was used as the substrate. The fatty acids mainly consisted of chains from 12 to 24 carbon atoms. The composition of the fatty acids presented in the lipids that were obtained using C6 wood hydrolysate is shown in Figure 22. The main distribution of fatty acids, in order of abundance, was C18:1 (oleic acid), C16:0 (palmitic acid), and C18:0 (stearic acid), for the five strains of *R. toruloides*, without showing substantial changes in the abundance range. The fatty acid distribution in this work is consistent with that reported by several authors in strains of the same genus cultivated in different lignocellulosic hydrolysate as a substrate. Wu et al. [71]; reported 30% palmitic acid, 14% stearic acid, and 49% oleic acid, Zhao et al. [69]; found in fed-batch fermentation about 35% palmitic acid, 6% stearic acid, and 49% oleic acid. Likewise, Fei et

al. [144] reported 25% of palmitic acid, 10% stearic acid, and 45.9% oleic acid. Thus, it can be inferred that: i) lipids produced by *R. toruloides* using C6 and C5 wood hydrolysate as culture media are promising feedstock for different applications of industrial interest, ii) lipid composition from *R. toruloides* is similar between the species, and iii) fatty acids distribution in lipids produced from *R. toruloides* is similar when lignocellulosic biomass hydrolysates are used as a culture media. Nevertheless, diligent handling of culture media formulation and fermentation processes are key factors in single-cell oil production and composition.

On the other hand, in the control strain used as a positive control in lipids production (*Y. lypolytica*-63746), the main distribution was the same although in lower abundance than the strains of *R. toruloides*. This trend is due to the less utilization rate of C5 (≈40%) and C6 (≈80%) sugars by control strain. Additionally, *Y. lypolytica*-63746 in their native state lacks some of the key enzymes involved in the oxidoreductase pathway, such as XYL1 (xylose reductase), and XYL2 (xylitol dehydrogenase). Both enzymes are necessary to increase the metabolic flux and convert xylose into xylulose to improve the amount of xylulose-5-phosphate, a crucial intermediate in the pentose phosphate pathway [156]. Oleic acid was the most abundant, representing approximately 50% of the total and was followed by palmitic acid and stearic acid. This composition is comparable with some vegetable oils, such as palm, soy, and sunflower, which indicated that the microbial lipids produced from the lignocellulosic hydrolysate have great potential as a biodiesel raw material [1].

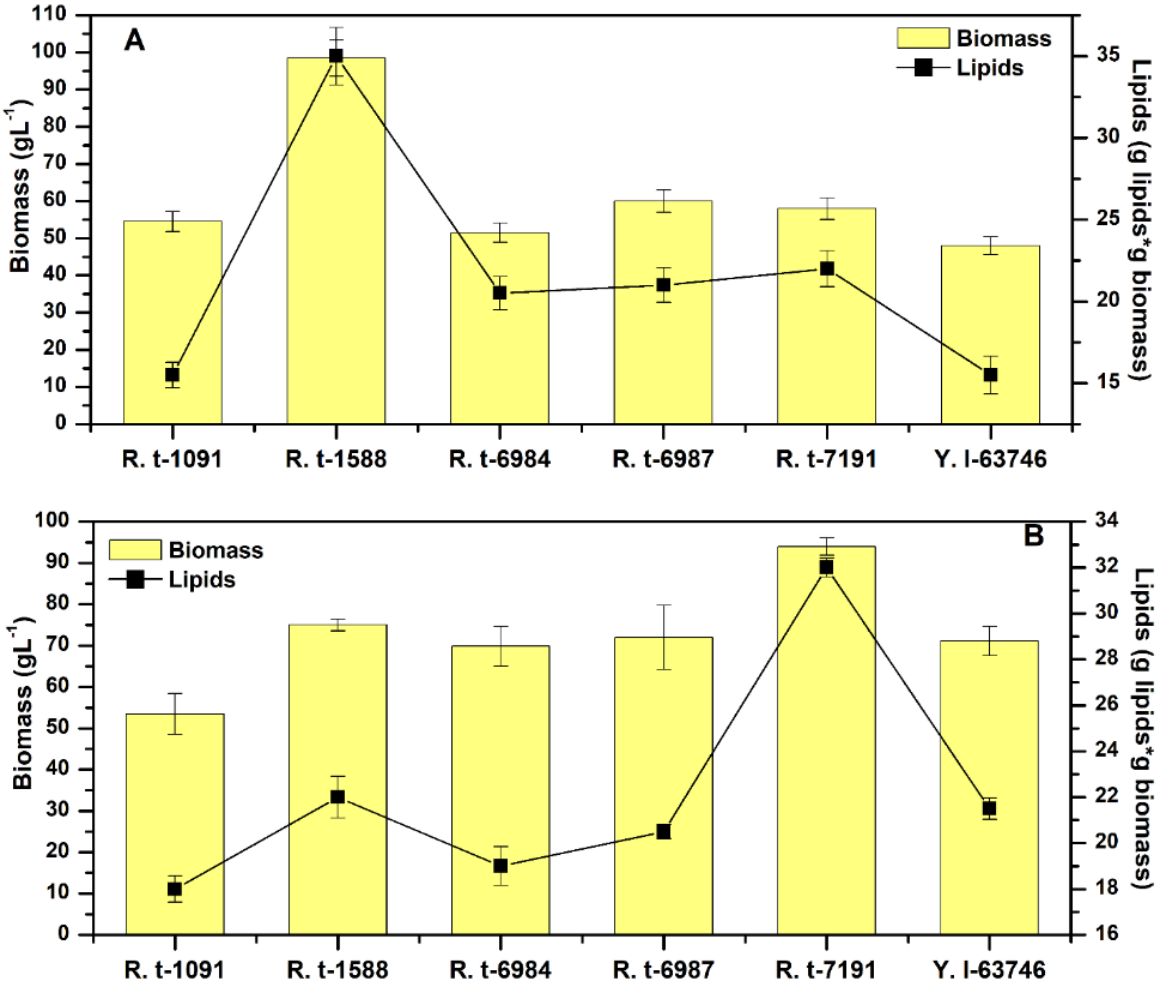

Figure 21.Lipid accumulation from different R. toruloides strains using C-6 (A) and C-5 (B) wood hydrolysate as substrate.

Additionally, the presence of other longer chain carbon fatty acids (polyunsaturated fatty acids) was produced in smaller quantities than the previous ones (Table 8). The presence of some of these fatty acids has been reported in other investigations where they used strains of *R. toruloides* for lipid production in lignocellulosic hydrolysates [139]. Nevertheless, their concentration and presence of them are dependent on the source of the substrate (lignocellulosic biomass) and the composition of culture media [157]. A parameter that affects the composition of the lipids obtained is the source of nitrogen. An example is the synthesis of linoleic acid (C18:3), it increases when the nitrogen is limited in the culture medium and the fermentation times are prolonged. On the

other hand, fatty acid profiles changed with the C/N ratio in the media. It has been reported that a low ratio of C/N (≤20), saturated fatty acids such as palmitic (C16:0) and stearic (C18:0) was decreased. However, for unsaturated fatty acids, such as oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), the trend was opposite showing a higher presence [153]. Likewise, when detoxified and undetoxified hydrolysate are used as substrates, the lipid composition is different. It is due to the fact that the proportion of unsaturated fatty acids is slightly higher in the undetoxified hydrolysates. This suggested that the toxic compounds present in the hydrolysates obtained from lignocellulosic biomass do not change the lipid composition, but rather promote the synthesis of the unsaturated fatty acids [158]. On the other hand, although the concentration of these polyunsaturated fatty acids is lower than the monounsaturated, they have a high commercial value due to their use in the food, chemical or pharmaceutical industry [93], [154].

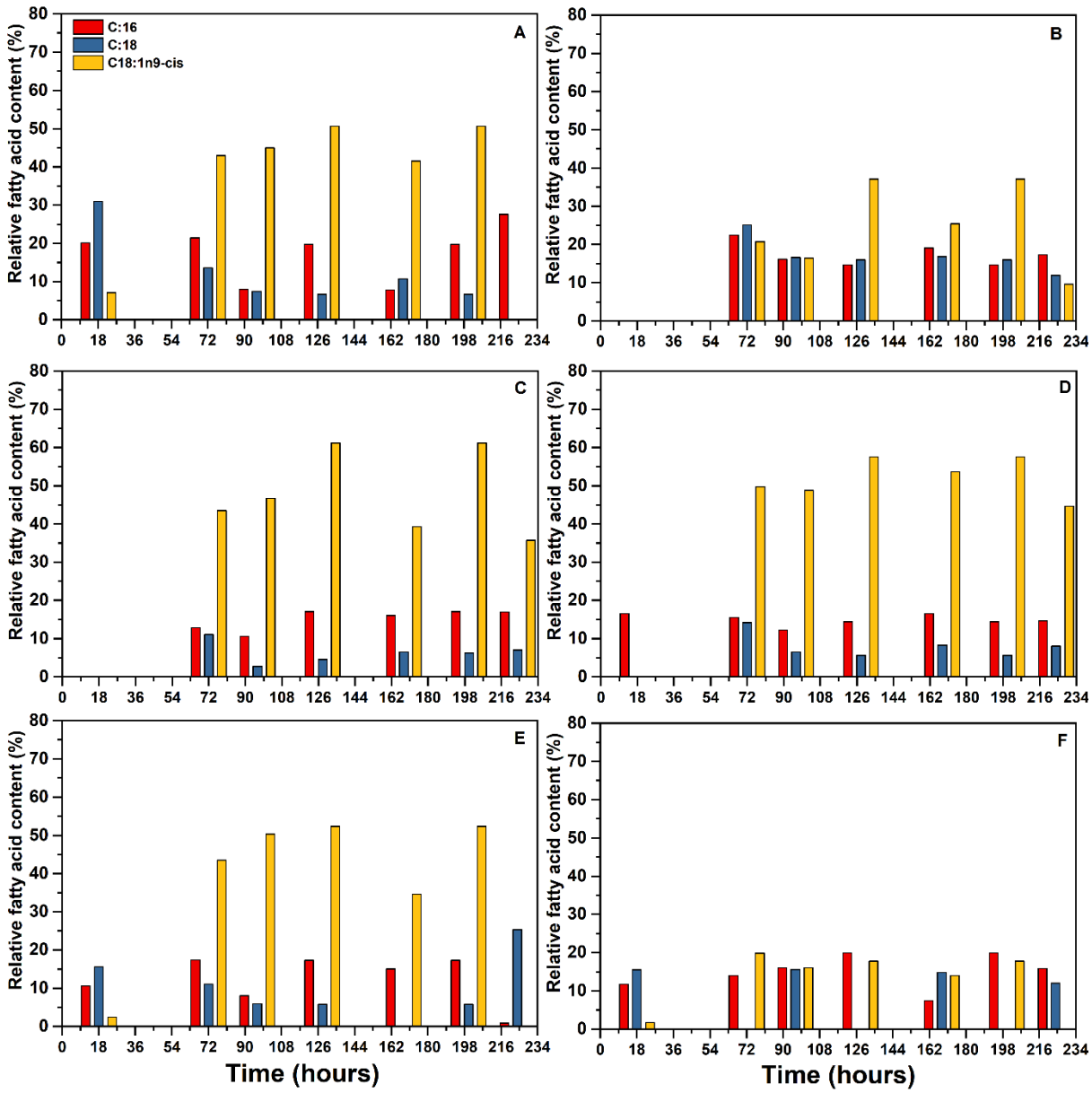

Figure 22.Fatty acid composition from C6 wood hydrolysate. A) R. toruloides-1091, B) R. toruloides-1588, C) R. toruloides-6984, D) R. toruloides-6987, E) R. toruloides-7191, and F) Y. lipolytica-63746.

Some studies reported that yeasts of the genus, *Rhodosporidium* grown in C5 sugars had a lower diversity of fatty acids compared to those grown in C6 hydrolysate as the only source of carbon [142]. This fact is in concordance with the results obtained in this work when C5 hydrolysate was used as a substrate unlike the distribution obtained with C6 hydrolysate. Figure 23 shows that the

order of abundance was completely different for the three main fatty acids obtained when C6 was used as culture media. The specific distribution was during the first 36 h of fermentation pointed toward the most abundant fatty acid being tridecylic acid (C:13), linolelaidic acid (C18:2n6t), and palmitoleate acid (C16:1n7). From 54 h to 104 h, palmitic acid (C:16), stearic (C:18), and oleic acid (C18:1n9-cis) were the most abundant. Finally, from 120 h to 224 h of fermentation, abundant fractions comprised pentadecanoic acid (C15:1), and Cis-10-heptadecanoic acid (C17:1), with substantial changes in the abundance range. On the other hand, in the *Y. lipolityca*-63746 used as a positive control in lipids production, the main distribution was similar in abundance to the strains of *R. toruloides*.

Table 8.Relative polyunsaturated fatty acids concentration produced using C6 and C5 hydrolysates.

Polyunsaturated fatty acids	**C6 wood hydrolysate**						**C5 wood hydrolysate**					
(%)	***R. toruloides***					**Control**	***R. toruloides***					**Control**
Omega-3	1091	1588	6984	6987	7191	63746	1091	1588	6984	6987	7191	63746
α-Alpha linolenic acid (C18:3n3)	0.29	0.19	0.18	0.18	0.37	nd	nd	nd	nd	nd	1.92	nd
Stearidonic acid (C18:4n3)	nd	nd	0.25	0.08	0.24	0.17	0.54	nd	nd	nd	2.92	nd
Eicosatrienoic acid (C20:3n3)	0.18	0.45	0.15	0.12	nd	0.40	1.22	1.03	0.55	nd	nd	nd
Eicosapentanoic acid (C20:5n3)	0.26	0.38	0.06	0.21	0.58	0.45	nd	nd	nd	nd	nd	nd
Docosapentaenoic acid (C22:5n3)	0.36	0.69	0.31	0.37	0.55	0.22	nd	nd	nd	nd	nd	nd
Omega-6												
Linoleic acid (C18:2n6)	5.87	5.64	6.63	5.95	4.73	0.90	9.81	2.68	3.96	2.55	2.94	1.69
Trans-linoleic acid (C18:2n6)	2.01	nd	1.33	0.33	0.54	0.64	10.62	2.76	4.16	2.9	7.50	1.51
Gamma linoleic acid (C18:3n6)	0.04	0.03	nd	nd	0.03	nd	0.97	0.89	0.73	nd	nd	nd
Eicosadienoic acid (C20:2n6)	0.21	nd	0.44	0.82	0.51	nd	nd	nd	nd	nd	nd	nd
Dihomogamma linolenic (C20:3n6)	0.01	nd	nd	nd	0.02	0.10	1.41	nd	6.49	1.77	2.72	1.94
Arachidonic acid (C20:4n6)	0.05	0.33	0.23	0.24	0.39	0.30	nd	nd	nd	nd	nd	nd
Omega-9												
Elaidic acid (C18:1n9)	5.85	nd	4.25	6.07	6.42	10.36	6.23	nd	12.05	18.48	14.33	12.22
Erucic acid (C20:1n9)	0.83	1.66	0.53	0.95	1.56	2.71	nd	nd	nd	nd	nd	nd
(Z)-Docos-13-enoic acid (C22:1n9)	nd	0.70	0.42	0.35	0.34	nd	nd	nd	nd	nd	nd	nd

nd: not detected

Meanwhile, long-chain fatty acids values (> 20 carbons) obtained in this hydrolysate for each one of the strains is shown in Table 8. Hence, the composition of the lipids obtained using *R. toruloides* strains can be directed towards the production of specific fatty acids, such as neutral lipids, glycolipids, sphingolipids, or phospholipids, modifying the media culture composition. The most common way to do this is by adding different types of mineral salts as NH_4Cl, KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$. This effect is observed directly in the metabolic pathway of microorganisms during lipogenesis and storage of lipids [91]. In this work, the fatty acids obtained are those that microorganism produces natively due to the hydrolysate used as media culture which was not supplemented. In general, the lipid fractions and fatty acid compositional profiles indicated that microbial lipids produced from lignocellulosic biomass hydrolysate as a substrate may be explored as a sustainable feedstock for use as food additives, chemical precursors, conventional biofuel, and advanced drop-in biofuels such as biodiesel, alkanes, lubricants among others [143]. Nevertheless, another key factor for the efficient use of sugars and lipid production is the inhibitor compounds present in the hydrolyzate at the time of fermentation. These compounds mainly cause a partial or total inhibition of microbial growth. Consequently, this inhibition causes a decrease in the consumption of the carbon source, thus directly influencing the accumulation of lipids in oleaginous microorganisms [75].

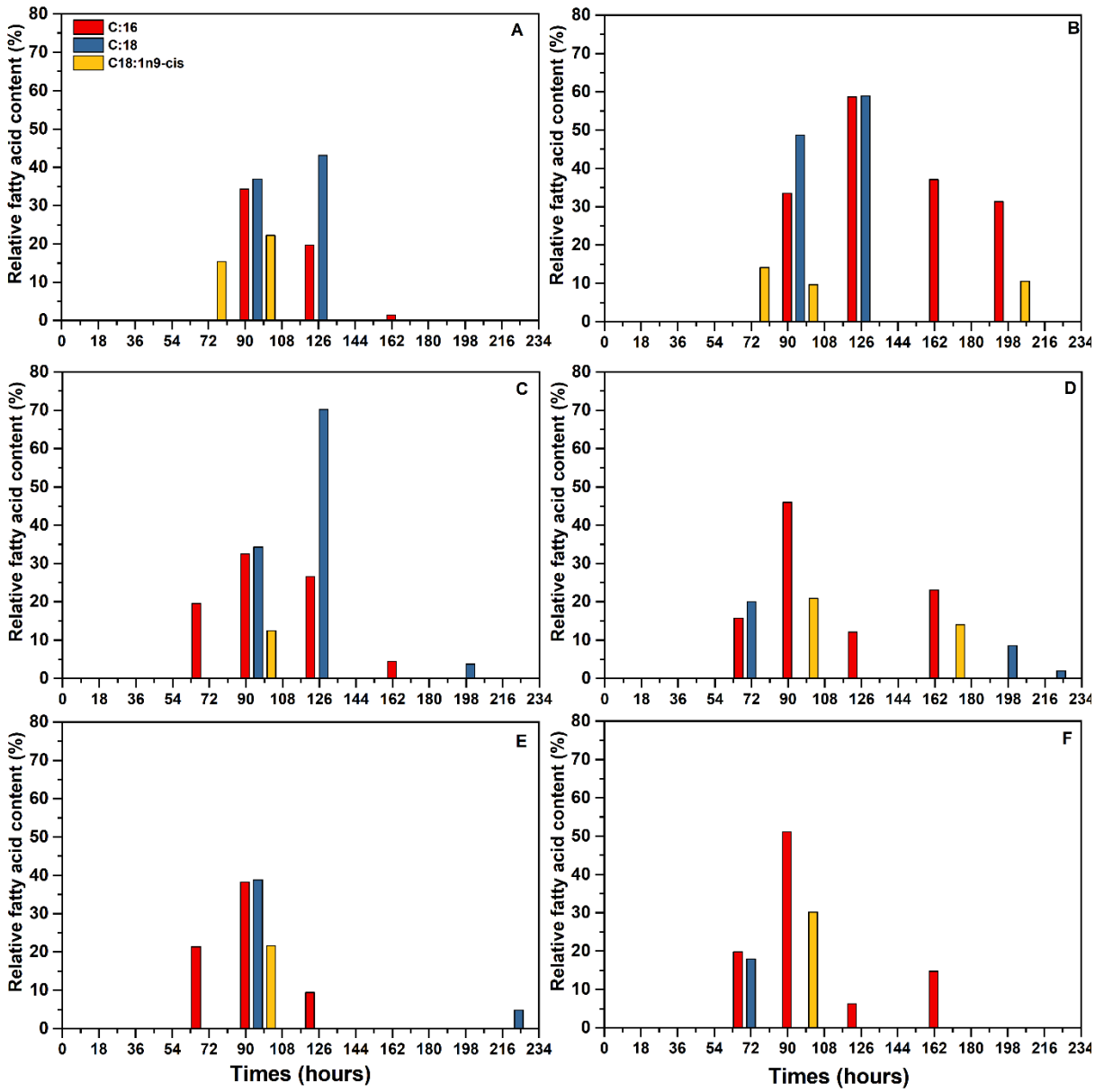

Figure 23.Fatty acid composition from C5 wood hydrolysate. A) R. toruloides-1091, B) R. toruloides-1588, C) R. toruloides-6984, D) R. toruloides-6987, E) R. toruloides-7191, and F) Y. lipolytica-63746.

Inhibitor effect on growth and lipid production

Tolerance and degradation rates of 5-HMF (80.86 mg/L), furfural (67.70 mg/L), levulinic acid (13.88 mg/L), vanillin (1.20 mg/L), vanillic acid (85.25 mg/L), 4-aminobenzoic acid (0.85 mg/L), and ferulic acid (1.66 mg/L) were quantified in C6 and C5 wood hydrolysate on the five *R. toruloides* strains during fermentation. According to results obtained in C6 hydrolysate, the

amount of these compounds was partially degraded or metabolized by all *R. toruloides* strains (Figure 24). However, degradation time and the amount were different for each of these compounds. Two major compounds of sugars dehydration (5-HMF and furfural) and total degradation was observed during the first 18 h. For phenolic compounds, 33% of vanillin degradation was observed during 72 h in four strains, except for *R. toruloides*-6987 where the concentration was constant (0.77 mg/L) in the later period. In the case of vanillic acid, *R. toruloides*-1091, *R. toruloides*-7191, and *R. toruloides*-6984 carried out total degradation during the first 18 h and 72 h, respectively. Nevertheless, the total degradation in *R. toruloides*-1588 and *R. toruloides*-6987 was until 96 h. On the other hand, only 23% of 4-aminobenzoic acid degradation was obtained during the first 18 h in *R. toruloides*-1091, *R. toruloides*-1588, and *R. toruloides*-6987, for *R. toruloides*-6984, and *R. toruloides*-7191 concentration was constant (≈0.66 mg/L). Finally, for organic acids, total degradation was in the first 18 h except *R. toruloides*-1588 where total degradation was until 72 h in the case of ferulic acid, and finally, for levulinic acid, 90–95% of degradation in all strains was seen during 72 h. Subsequently, the concentration was constant (≈1–1.6 mg/L).

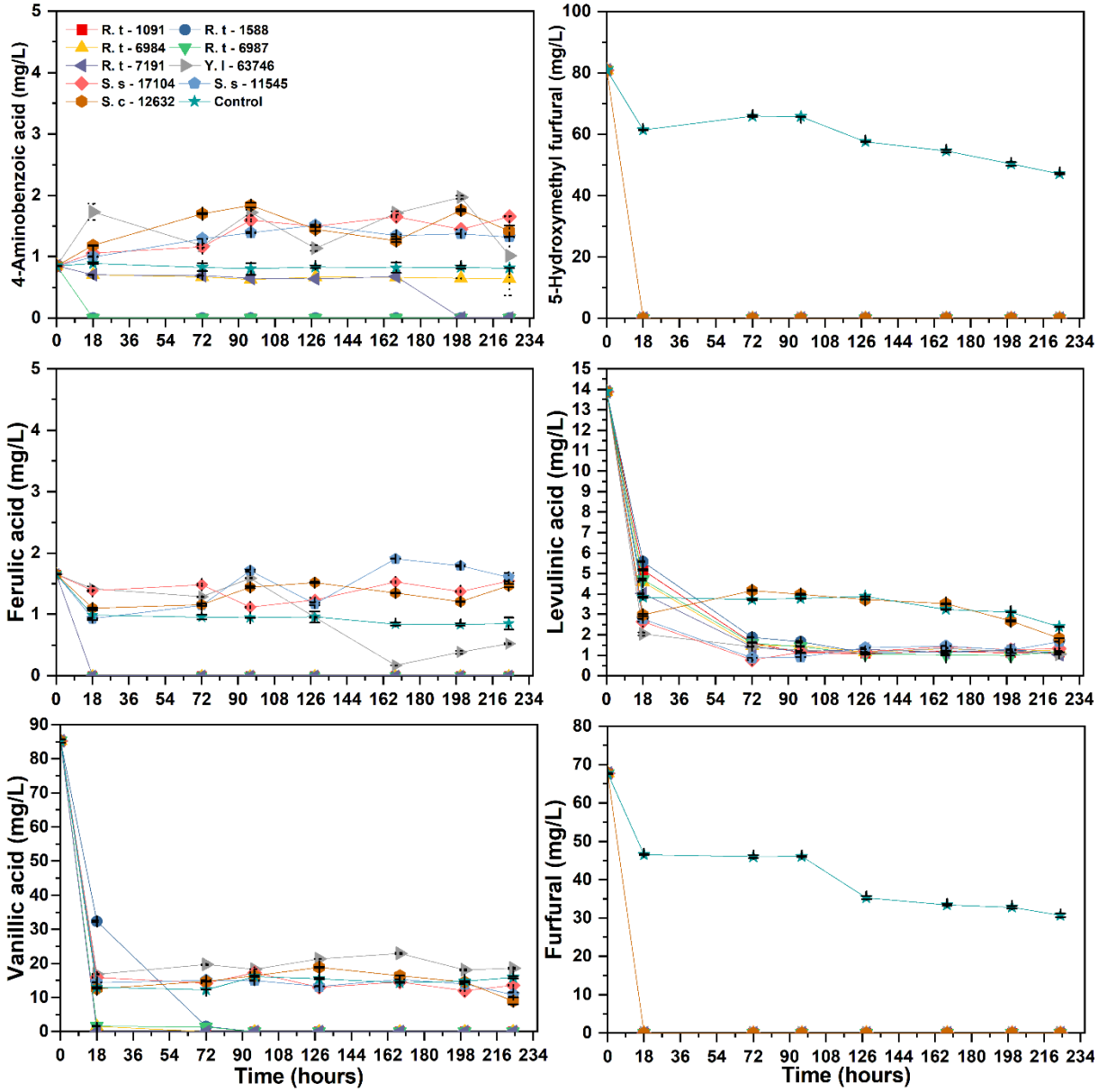

Figure 24.Inhibitor concentration and degradation in the C6 wood hydrolysate.

On the other hand, the degradation profile of inhibitor compounds in C5 hydrolysate was completely different since all compounds were present until the end of fermentation. Figure 25 shows a decrease in different compounds in each strain evaluated. Nevertheless, only 38%, 50%, 59%, 75%, and 35% were metabolized (with respect to the control sample) by *R. toruloides*-1091, *R. toruloides*-1588, *R. toruloides*-6984, *R. toruloides*-6987, and *R. toruloides*-7191 at 54 h of fermentation, respectively. On another hand, degradation of levulinic acid, furfural, vanillin,

vanillic acid, and syringaldehyde, was observed through fermentation at the same or lesser amounts compared with the control sample. This degradation can be associated with fermentation conditions, such as pH, salt content, or temperature. In addition, ferulic acid and 4-aminobenzoic acid were not present in the hydrolysate. These results suggested that strains evaluated in this work showed tolerance to the presence of these compounds. Since the growth and lipid production was not affected by these compounds. Some studies have reported inhibition in the growth of *Rhodosporidium* sp. in hydrolysate obtained from lignocellulosic biomass. This phenomenon mainly occurs during the pretreatment used for the breakdown of lignocellulose. Some of the methods can release the inhibitor compounds, that partially or inhibit the growth of this yeast [52] and affect the metabolism of microorganisms during fermentation having a negative impact on overall process performance [18].

The main problems caused by the inhibitors in the cells vary depending on the compound. Compounds obtained from the dehydration of sugars, such as furfural and 5-HFM cause problems during the assimilation of amino acids, inhibit enzymes for the production of precursor compounds necessary for the synthesis of lipids [159]. On the other hand, the main effect that organic acids cause on cells is on the structure of the plasma membrane during its diffusion within the cell. This causes a high demand for adenosine triphosphate (ATP), which causes inequity in the respiratory chain and oxidative phosphorylation of adenosine diphosphate (ADP) [86]. Likewise, the phenolic compounds derived from the hydrolysis of lignin (vanillin, vanillic acid, syringaldehyde, and aminobenzoic acid), like organic acids affect the plasma membrane, allowing the entry of other compounds into the cell. Additionally, they cause changes in the electrochemical gradient of the mitochondrial membrane [90].

Several mechanisms can thus explain the inhibitory effects that these compounds cause to microorganisms during fermentation, and they have been well-studied [47], [75]. Nevertheless, it is advisable to study in more detail the process of degradation of these compounds by yeast, to improve the fermentation. This can also help us to develop new detoxification strategies since the concentration of these compounds in the culture medium varies according to the raw material and the pre-treatment that is used to obtain the final fermentable sugars, a feedstock for the drop-in fuels.

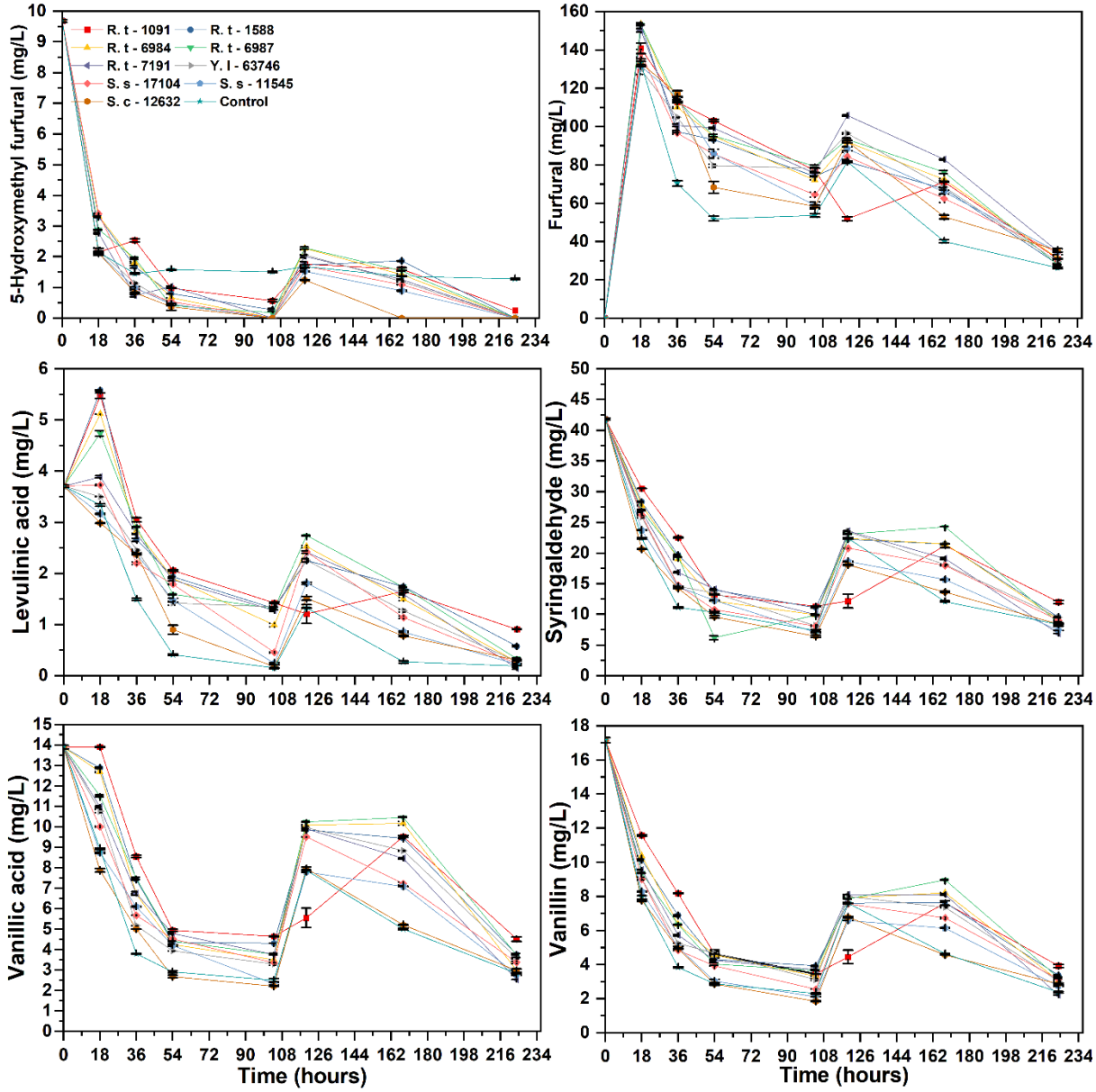

Figure 25.Inhibitor concentration and degradation in the C5 wood hydrolysate.

Conclusion

R. toruloides is an alternative due to its growth characteristics and ability to utilize various sugars present in lignocellulosic hydrolysates. The strains evaluated showed an increase of 30% in sugar utilization when grown in C6 hydrolysate. Additionally, the evaluated strains showed tolerance to the presence of toxic compounds, such as organic acids, furans, and phenolic compounds. This was due to the fact that sugar utilization and lipid synthesis in both hydrolysates was unaffected with total lipid content of 0.33 g of lipids/g of dry biomass, and 0.32 g of lipids/g of dry biomass,

respectively. This study determined that hydrolysate from lignocellulosic biomass could be used as a suitable substrate for microbial lipid produced by *R. toruloides*-1588 and *R. toruloides*-7191 without the addition of external nutrients.

CRediT author statement

Carlos S. Osorio-González.: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft. **Krishnamoorthy Hegde**.: Supervision, Writing-Review & Editing. **Pedro Ferreira**.: Investigation. **Satinder Kaur Brar**.: Funding acquisition, Supervision, Writing-Review & Editing. **Azadeh Kermanshahipour**.: Writing-Review & Editing. **Carlos Ricardo Soccol**.: Writing-Review & Editing. **Antonio Avalos-Ramírez**.: Funding acquisition, Supervision.

Supplementary data for this work can be found in ANNEX 2.

5.2 Carbon/nitrogen ratio as a tool to enhance the lipid production in *Rhodosporidium toruloides*-1588 using C5 and C6 wood hydrolysates

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Abstract

The interest in microbial lipids has recently increased because of their wide use to produce several value-added compounds in the biofuel, pharmaceutical and food industries. Oleaginous yeast such as *Rhodosporidium toruloides* could be an efficient option because of its ability to consume five-carbon sugars, high lipid accumulation, and tolerance to toxic compounds such as furans, phenolic compounds, and organic acids. The present study aims to investigate the effect of different initial sugar ratios, in a combination of different carbon/nitrogen ratios, and the use of dibasic sodium phosphate (Na_2HPO_4) as an inducer on cell biomass production, sugar consumption, and lipid accumulation by *Rhodosporidium toruloides*-1588. The investigation showed a maximum lipid accumulation of 5.35 g/L (0.28 g of lipids/g of sugar) under the culture conditions of initial glucose: xylose ratio of 1:1, C/N ratio of 70, and Na_2HPO_4 concentration of 1.05 g/L. The predominant lipids composition was palmitic, stearic, oleic, and linoleic acids which could be used as a suitable feedstock for biofuel production. Additionally, under the optimal conditions (initial glucose: xylose ratio of 1:1, 1.19 g/L of Na_2HPO_4 and C/N ratio of 70.50) an increase in 10.5% and 7.5% of lipids was observed, compared with glucose and xylose control treatments. In addition, the study shows the ability of *R. toruloides*-1588 to tolerate inhibitors, a feature that could be a promising alternative to increase the feasibility of microbial lipid production process using undetoxified wood hydrolysate as a sustainable culture media.

Keywords Fatty acids; inhibitor effect; lipid production; lipid inducer; *Rhodosporidium toruloides*; wood hydrolysate

Introduction

In the past decade, microorganisms capable of accumulate lipids, in the form of triacylglycerols (TAGs), have been received great attention from the global scientific community. These lipids can be used for different purposes, such as a precursor for surfactants, pharmaceutical compounds, food additives, creams, adhesives, waxes, as well as feedstock for biofuel production. Among their key characteristics, microbial lipids have a similar fatty acid composition in comparison with the vegetable oils, such as palm, soy, and sunflower [39], [160]. An alternative is to produce microbial lipids using the oleaginous yeast *Rhodosporidium toruloides*, which stands out because of its three distinct features: i) capacity to accumulate high lipid content (up to 70%), ii) capacity to use simultaneously and efficiently glucose and xylose as a carbon source, allowing the use of

hydrolysates obtained from lignocellulosic biomass; and iii) high tolerance to inhibitory compounds such as furfurals, 5-HMF, syringaldehyde, vanillin and vanillic acid [47], [48], [51], [69], [147], [161]. In addition, recent research has reported different methods and tools to increase the lipid yield, assimilate different carbon sources, and tolerate inhibitory compounds present in lignocellulosic hydrolysates by *R. toruloides* [43], [86], [88], [141], [162], [163].

One of the premises to increase the process feasibility is using renewable and economical substrates as well as an increase on lipid accumulation in the yeast cells. Few studies have reported the effect of carbon source, pH, nitrogen content, lipid inducer, and inhibitors on the lipid accumulation in several strains of *R. toruloides* [47], [75], [88], [164]. One of the main parameters that contribute to an increase in lipid biosynthesis is the type of carbon source and its proportion regarding the nitrogen content in the culture media, which affects the biomass and lipid production, as well as the fatty acid distribution of the produced lipids. In this sense, the carbon-nitrogen ratio (C/N) is a parameter that promotes microbial growth and activates lipid biosynthesis. Additionally, a recent approach in lipid production is the use of different salts because they affect the microbial growth and fatty acid distribution. So far, one of the most promising compounds that have been tested as lipid inducers with *R. toruloides* strains is dibasic sodium phosphate (Na_2HPO_4), because it can induce the uptake of carbon source for essential activities as well as lipid biosynthesis [70], [165]–[167]. To improve the lipid yield using *R. toruloides*, it is necessary to optimize the cultivation parameters through optimization process [61], [153]. However, most of these optimization studies used synthetic media, industrial residues, and detoxified hydrolysates from different sources [56], [153], [168]–[170]. A very few studies have been performed using undetoxified wood hydrolysates as a substrate with the aim to produce microbial lipids and none of them have optimized the cultivation parameters[164]. Hence, this work aims at increase on lipid production by *R. toruloides*-1588 through the optimization of C/N and glucose: xylose ratio, as well as three different concentrations of Na_2HPO_4 as a lipid inducer using two types of undetoxified wood hydrolysates as a culture media.

Materials and Methods

Wood hydrolysate

Two wood hydrolysates obtained from forestry residues of poplar (*Populus* sp.) transformation were used as a culture media. One with a total xylose content of 120 g/L and 10.1 g/L of glucose (C5) and the other with a total glucose content of 117 g/L and 11.8 g/l of xylose (C6). Both hydrolysates were donated by Greenfield Global Inc. (Varennes, Canada).

Microorganism, culture maintenance, inoculum preparation

Rhodosporidium toruloides NRRL-1588 was used for lipid production. The strain was purchased from the Agricultural Research Service (NRRL) Culture Collection (USA). The yeast was preserved in YM agar (yeast extract 3g/L; malt extract 3 g/L; peptone 5 g/L; glucose 10 g/L; agar 15 g/L). The inoculum seed was pre-cultured three times in YM broth at 25°C, 180 rpm for 36 hours.

Flask fermentation

R. toruloides-1588 was cultured in 250 mL-flasks containing 50 mL of wood hydrolysate, with a total initial sugar content of 50 g/L distributed in three initial glucose: xylose ratios (1:1, 1:2, 2:1), three C/N ratios (20, 70, and 120), and three initial concentrations of Na_2HPO_4 (0.1, 1.05, and 2.0 g/L). Glucose and xylose treatments without phosphate addition were used as a control. Each flask was inoculated adding a seed culture with an initial OD_{600nm} of 0.1 and incubated for 176 h at 25°C in a Infors Multitron Incubator Shaker (USA). Ammonium sulfate $(NH_4)_2SO_4$ was used as a nitrogen source and the C/N ratio of each treatment were calculated based on the total carbon content (glucose + xylose).

Lipid extraction

Lipid extraction and quantification were performed according to Osorio-González et al. [171]. Briefly, *R. toruloides*-1588 cells were harvested by centrifugation (15 000 *x g*/2 min), dried at 60ºC to constant weight and the lipids were extracted with a chloroform/methanol solution (2:1 v/v). Total lipids were quantified gravimetrically according to Equation 1.

$$Lipid\ content = \frac{lipid\ weigth\ (g)}{dry\ cell\ weight\ (g)} \quad \text{Eq.1}$$

Analytical methods

Cell growth, carbohydrate, and inhibitor analysis

Cells were harvested by centrifugation (15000 *x g*/2 min) and biomass and supernatant fractions were kept separately for further analysis. Cell biomass production was determined by dry constant weight (DCW) and expressed as g/L and cell yield was quantified according to Equation 2.

$$Cell\ yield = \frac{dry\ cell\ biomass\ \ (g)}{sugar\ consumed\ (g)} \quad \text{Eq.2}$$

Sugars were analyzed using Liquid Chromatography coupled to Mass Spectrophotometry (Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer), equipped with a 5 µm x150mmx, 4.6mm HILIC column. The mobile phase was an aqueous solution of NH_4OH (0.2%) and acetonitrile: NH_4OH (0.2%), in a ratio of 11:89 v/v with a flow rate of 300 µL/min. Finally, 20 µL as total injection volume, and 20 µL/mL of an internal standard of D6 glucose was used [171]. The specific sugar consumption was calculated according to Equation 3 [172].

$$Specific\ sugar\ consumption = \frac{total\ sugar\ consumed(\frac{g}{L})}{biomass\left(\frac{g}{L}\right)/time\ (h)} \quad \text{Eq.3}$$

To analyze microbial inhibitory compounds, the protocol reported by Osorio-González et al. [124] was used. Briefly, a BetaBasic-18 column (100 mm length x 2.1 mm of internal diameter, and 3µm pore size) was used at 30°C. The mobile phase was an aqueous solution of acetic acid (0.1%) and methanol: acetic acid (0.05%) in a ratio of 82.5:17.5 v/v with a flow rate of 300 µL/min. As an internal standard, 20 µL/mL of phenyl ethanol-D5 was used. The mass detector was operated in a mode of SRM negative detection for sugar analysis and positive detection for microbial inhibitory compounds. All compounds were determined and quantified according to the peak area and retention time of its standards (Sigma-Aldrich, USA).

Fatty acid methyl esters composition

Fatty-acid methyl esters (FAMEs) were obtained by transesterification of extracted lipids. Methanol was used as a reactant and sulfuric acid as catalyst at 100±1°C for 20 minutes. Later, the samples were cooled to room temperature and centrifuged at 9800 *x g* during10 minutes. Fatty acid

methyl esters were analyzed by gas chromatography (GC) using an Agilent 7890B gas chromatograph, equipped with a flame ionization detector (FID). Each FAME was identified and quantified according to the retention time and peak area of a standard mixture of FAME's (Supelco 37 Component FAME Mix, Sigma-Aldrich, USA).

Statistical analysis

The effect of C/N ratio, lipid inducer addition and different initial sugar proportions in cell biomass, sugar utilization, and lipid production by *R. toruloides*-1588 was analyzed using a Box-Behnken experimental design with three central points. The statistical analysis of the data was performed using Minitab® 17.1.0 (LEAD Technologies, USA). Parameter interaction effects were analyzed by One-Way ANOVA (Fisher test) at 95% confidence. All the experiments were carried out in duplicates.

Results and discussion

Effect of initial sugar content on sugar consumption and biomass production

Figure 26 shows the sugar consumption and cell biomass production by *R. toruloides*-1588 under the tested conditions. In all treatments tested under a C/N ratio equal to 70, 90-98% of glucose was consumed during the first 96 h of fermentation. On the contrary, xylose consume was different for each initial sugar ratio. A high xylose consumption was observed during the first 18 h of cultivation with 49%, 36%, and 17% for 1:2, 1:1, and 2:1 initial glucose: xylose ratio, respectively. Then, during the next 78 hours of cultivation, xylose consumption increases by 7.7%, 8.8%, and 1%, for each glucose:xylose ratios (1:2, 1:1, and 2:1, respectively). In summary, when glucose: xylose ratio of 1:2 was used, a total sugar consumption of 51% was observed, whereas in treatments with a glucose: xylose ratio of 2:1, 80% of sugars were consumed. Finally, 63% of the sugar was consumed when the initial glucose: xylose ratio of 1:1 was used. In the case of treatments tested using carbon-nitrogen ratios of 20 and 120, the glucose consumption was higher than 95% at 96 hours. The sugar consumption observed in this work agrees with other studies, where glucose was consumed faster compared with xylose [38], [60], [86]. In the case of xylose consumption, this study shows that xylose consumption was faster (50% in 150 h) than other studies, for example, Yamada et al. [145] who reported consumption of only 5% in a period of 168 h. Additionally, the sugar consumption pattern observed in the present study was similar to Wiebe et al. [88], where

nearly complete consumption of glucose and 50% of the total xylose content was observed. This result is promising from a process point of view as wood hydrolysates contain both C5 and C6 sugars. Furthermore, these results confirm that this strain has a high tolerance to catabolic repression caused on the pentose phosphate pathway due to the high concentration of xylose in the culture media. For example, in native strains, it has been reported that xylose catabolic repression is observed by the inhibition of xylose transporters and the repression of enzymes involved in the catalytic activity of the pentose phosphate pathway, and this generally can occur after 20% of xylose consumption [86], [173], [174].

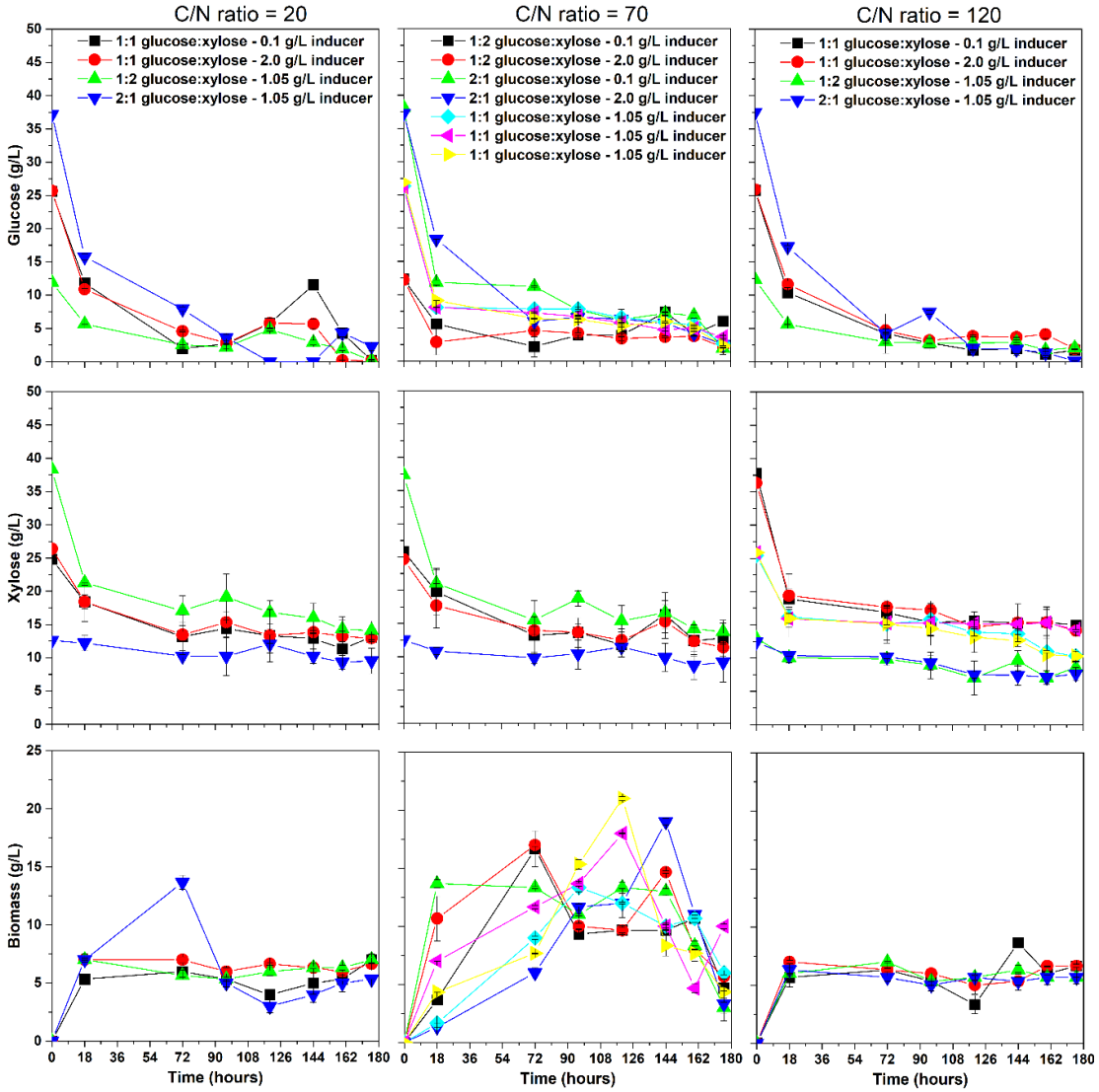

Figure 26.Sugar utilization and biomass production by R. toruloides-1588 during cultivation in a mix of two undetoxified wood hydrolysates.

The microbial growth was similar for the C/N ratio of 20 and 120. In both conditions, the cell growth profile presented a short lag and exponential phases of approximately 6 h and 18 h, respectively. For both C/N ratios, when the stationary phase was reached no more changes in biomass accumulation were observed. For glucose: xylose ratio of 1:2 and 0.1 g/L of Na_2HPO_4 a short lag phase during the first 18 h and an exponential phase of 54 h was observed. At the same initial sugar ratio but using 2.0 g/L of Na_2HPO_4 an imperceptible lag phase (2 h) and long and strong exponential phase (up to 72 h) were observed. A slight decrease in biomass production was observed during the following 48 h, to observe a short second exponential phase of 24 h. Conversely, with the two previous conditions, when initial glucose: xylose ratio of 2:1 and 0.1 g/L of Na_2POH_4 a short exponential phase of 18 h was observed without major changes until the end of the fermentation. Nevertheless, using the same initial sugar ratio with the use of an initial concentration of 2.0 g/L of Na_2HPO_4, a lag phase of 18 h and a long exponential phase (78 h) were observed. After the exponential phase, a similar growth tendency was observed using glucose: xylose of 1:2. This trend suggests a diauxic growth, where one substrate was faster and preferentially catabolized over the other during the growth phase [38], [52], [145], [175]. In this study, the growth tendency suggests that glucose and xylose can be used simultaneously as a carbon source, except in treatments where an initial concentration of 2.0 g/L of Na_2HPO_4 was used, where a diauxic growth was observed. The above trend can be attributed to slower catabolism of xylose provoked by the high amount of glucose in treatments where a high concentration of Na_2HPO_4 was used. Finally, an increase in cell biomass production was observed when a C/N ratio of 70 and initial glucose: xylose ratio of 1:1 and 1.05 g/L were used. Under the above-tested conditions, the microbial growth (19 g/L) and sugar utilization observed in this study were similar to the results reported by Sargeant et al. [143] with a *Rhodotorula minuta* strain, and a total xylose consumption of 50%.

Cell biomass production and sugar consumption within this study showed that the use of mixed sugars in different initial proportions promotes a fast consumption of total sugar content without the addition of external nutrients into the culture media. Moreover, these results showed the efficient use of glucose and xylose present in hydrolysates obtained from forestry residues as the main carbon source to produce lipids. Finally, from an industrial point of view, this feature makes this oleaginous yeast a promising microorganism to use undetoxified wood hydrolysates as a

promising and renewable substrate for lipid production that can be used as a suitable feedstock for biofuel production as well as to produce high added-value fatty acids that can be used in several industries.

Carbon/Nitrogen effect

A Box-Behnken design was used to analyze the effect of C/N ratio, lipid inducer and initial sugar concentration on lipid production. Fifteen conditions were performed to determine the optimal ranges of operating parameters in terms of lipid production. A full quadratic model was developed for lipid production and biomass production (Eq. 4 and Eq. 5, respectively). Figure 27 shows the surface plots constructed with the values obtained for each dependent variable. An ANOVA analysis of the surface regression of both cell biomass production and lipid accumulation with C/N ratio, initial glucose, and initial lipid inducer concentrations was performed. According to the analysis of surface regression, the three parameters and their square interactions affected the biomass and lipid production, being the C/N ratio the parameter with strong effect (p-value=0.001) (ANNEX 3). On another hand, Na_2HPO_4 and initial sugar concentration did not affect biomass production (p-value =0.919 and 0.806, respectively), which was supported by the observed biomass yield.

$$Lipid\ accumulation = -5.67\ 1.017 + 0.1431 C/N + 1.964\ \mathrm{Na_2}HPO_4 + 0.3717\ Glucose - 0.001\ C/N^2 - 1.017\ \mathrm{Na_2}HPO_4{}^2 - 0.00781\ Glucose^2 + 0.00023\ C/N * \mathrm{Na_2}HPO_4 - 0.000062\ C/N * Glucose + 0.0181\ \mathrm{Na_2}HPO_4 * Glucose + e_{ijk} \quad \text{Eq. 4}$$

$$Cell\ biomass\ production = -8.82 + 0.3671\ C/N + 3.19\ \mathrm{Na_2}HPO_4 + 0.578\ Glucose - 0.002622\ C/N^2 - 1.354\ \mathrm{Na_2}HPO_4{}^2 - 0.01102\ Glucose^2 + e_{ijk} \quad \text{Eq.5}$$

For instance, with a C/N ratio of 70, an average yield of 0.44 g of biomass/g of sugar was obtained, which was 2.5-fold higher than treatments where C/N ratios of 20 and 120 (~0.17 g of biomass/g of sugar) were used. These results were different from those observed by Lopes et al. [164] using *R. toruloides* CCT 0783, where microbial growth decreased with the increase of carbon/nitrogen ratio in the culture media. Also, they observed that at lower and higher C/N ratio conditions (20

and 120, respectively), a decrease in the cell biomass yield was observed (5.41 g/L and 5.55 g/L, respectively) resulting in a decrease in lipid accumulation. In the present study, the maximum value for biomass concentration was 13.67 g/L, higher than the cell biomass reported by Braunwald et al. [153] (11.40 g/L) with a *Rhodotorula glutinis* strain using synthetic media (glucose as the main carbon source) supplemented with a mineral solution and cultivated under five C/N ratios up to 216 h. Likewise Lopes et al. [164] observed a decrease in cell biomass production at high C/N ratios. This tendency can be explained by the increase of sugar concentration in the culture media (especially glucose), due can provoke a catabolic repression effect in the cells. As an effect of the catabolic repression, a decrease in the Krebs cycle activity, by increasing the citric acid content at intracellular levels can collapse the cells internally causing their death, causing a decrease in the cell biomass production, which directly impacts the lipid production [64], [67].

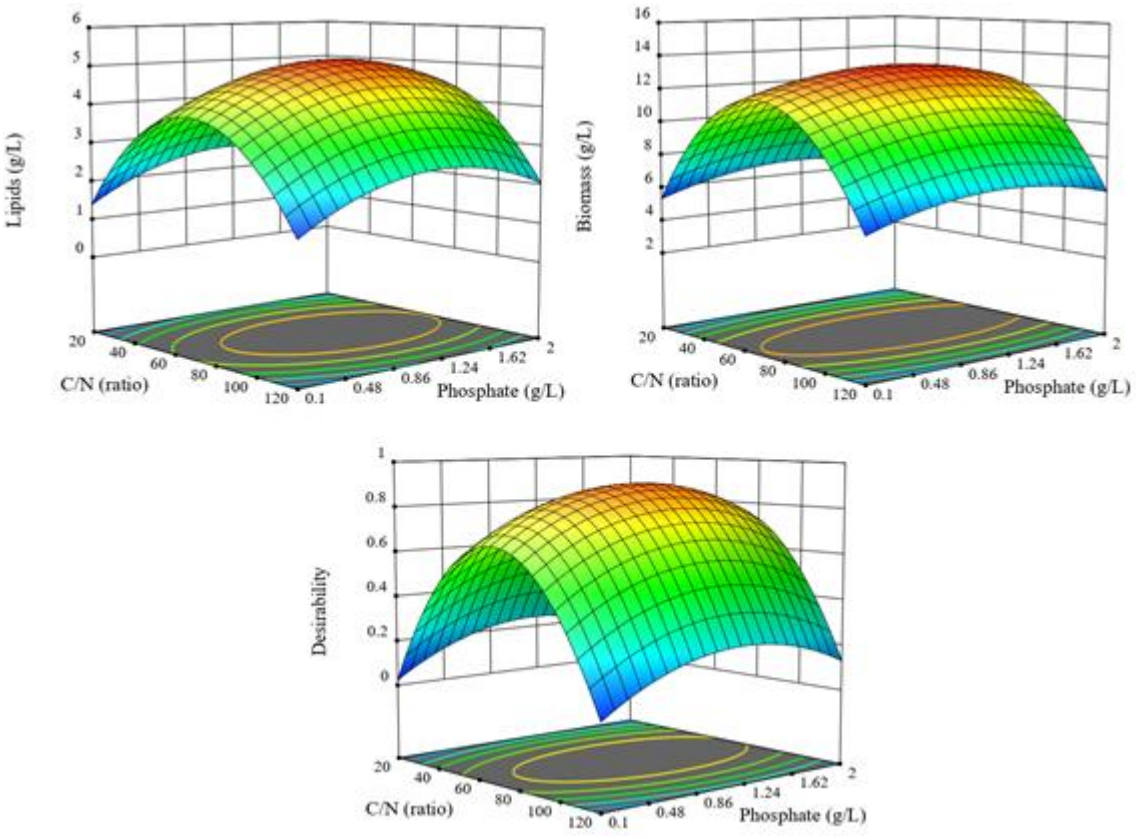

Figure 27.Optimization of biomass and lipid production of R. toruloides-1588 using a mix of two wood hydrolysates as a culture media.

Lipid inducer effect

The use of Na_2HPO_4 as a lipid inducer in *R. toruloides*-1588 grown in wood hydrolysate as a culture media shows that an initial concentration of 1.05 g/L has an effect in cell biomass production and lipid yield with 0.69 g of biomass/g of sugar and 0.28 g of lipids/g of sugar (p-value=0.012). These yields were obtained in combination with an initial C/N ratio of 70 and 1:1 glucose: xylose ratio. However, at the same initial concentration of Na_2HPO_4 in combination with C/N ratio of 20 and 120, and initial glucose: xylose ratio of 1:2 and 2:1, the cell biomass yield was lower, in the range from 0.15 to 0.19 g of biomass/g of sugar and produced lipids in the range of 0.04 to 0.05 g of lipids/g of sugar. Likewise, when a lower and higher initial concentration of Na_2HPO_4 was used (0.1 and 2.0 g/L, respectively) under C/N ratios of 20 and 120 and initial glucose: xylose ratio of 1:1, the biomass and lipid yields were in the same ranges (0.04 and 0.05 g of lipids/g of sugar, respectively). Furthermore, when the same initial Na_2HPO_4 concentrations (0.1 and 2.0 g/L, respectively) were tested in combination with an initial C/N of 70 and initial glucose: xylose ratio of 1:2 and 2:1 a slight increase in biomass yield (0.34, 0.27, 0.37, and 0.28 g of biomass/g of sugars, respectively) and lipid yield (0.096, 0.13, 0.050, and 0.090, respectively) were observed. The above results show that the use of low and high initial concentrations of Na_2HPO_4 decreases the biomass and lipid yield in *R. toruloides*-1588.

It has been reported that oleaginous yeast grown under phosphorus limitation conditions promotes a rapid use of carbon sources for metabolic activities in the cells during the earlier growth steps, increasing lipid accumulation. This phenomenon is stronger, especially in combination with treatments under nitrogen-limiting conditions. Nevertheless, in this work, we observed the opposite behaviour. When *R. toruloides*-1588 was grown using low C/N in combination with low Na_2HPO_4 concentrations resulted in low biomass and lipid production, a tendency that also has been reported on other species of oleaginous yeast. A similar trend was observed in treatment with a high C/N ratio and high Na_2HPO_4 content. For instance, in this work biomass and lipid production by *R. toruloides*-1588 were lower under C/N of 120 and high initial concentrations of Na_2HPO_4 (2.0 g/L). Similar results were observed at high concentrations of Na_2HPO_4 (2.0 g/L) in combination with low C/N ratios such as 20. This result agrees with other works where an excess of phosphorus can cause ribonucleic acid (RNA) degradation as well as a decrease in deoxyribonucleic acid (DNA) synthesis. This can then cause a decrease in cell replication rate,

lowering biomass production, and consequently a decrease in lipid production [70], [166], [167]. In recent years, the use of non-metallic ions such as phosphorus has been utilized to increase the lipid accumulation in *R. toruloides* strains [69], [78], [162]. For instance, in this study, an increase of 7.3-fold and 3.3-fold in biomass and lipid yield were observed than reported by Wang et al. [165] using the yeast *R. toruloides* AS 2.1389 under phosphate-limited and phosphate-repleted conditions using as substrate pure glucose culture media supplemented with trace elements solution.

So far, no studies are available on how phosphate metabolism works in oleaginous yeast using complex media such as undetoxified wood hydrolysates. Likewise, no work has been performed to optimize the culture conditions using phosphate as a lipid inducer in combination with the culture media used in this work. Nevertheless, it is well known that phosphate plays a key role during lipid biosynthesis. Phosphorus contributes to the dephosphorylation of adenosine monophosphate (AMP), which activates the isocitrate dehydrogenase enzyme (IDH); a key enzyme to convert citrate into isocitrate in the tricarboxylic acid cycle (TCA). This helps the acetyl-CoA transportation into the lipid biosynthesis pathway. Furthermore, in this work because of Na_2HPO_4 addition in combination with the culture condition tested, the fatty acid profile showed considerable differences in its distributions regarding control treatments where no addition of Na_2HPO_4 was used. Hence, this work can be used to study and develop detailed research on the use of phosphate as a lipid inducer to increase lipid yield as well as with the purpose to produce a specific fatty acid with high added value in the market.

Lipid accumulation

Table 9 shows the effect of initial glucose:xylose concentration, C/N ratio and lipid inducer on total lipid accumulation by *R. toruloides*-1588. Similarly, as in previous results, the C/N ratio of 70 produced the highest lipid yield with 0.27 g of lipids/g of sugar under initial glucose:xylose ratio of 1:1, and Na_2HPO_4 of 1.05 g/L. On another hand, the lowest yield has been obtained when C/N of 120, initial glucose: xylose ratio of 2:1 and Na_2HPO_4 of 1.05 g/L (0.041 g of lipids/g of sugar). These results confirm that the combination of initial C/N, glucose: xylose ratio and Na_2HPO_4 as lipid inducer, has a strong effect on lipid production by *R. toruloides*-1588 when intermediate concentrations of each parameter were used (70, 1:1, and 1.05 g/L, respectively).

Likewise, the cell biomass production with respect to lipid accumulation shows a positive correlation effect (p-value=0.001, a Pearson's coefficient=0.96, and R^2(adj)=0.92) showing that an increase in cell biomass production increases the lipid accumulation under the optimal condition tested (C/N=70.18, glucose: xylose ratio of 1:1, and 1.19 g/l of Na_2HPO_4) (ANNEX 3). An increase of 7.48% in lipid production was observed when C/N of 70, initial glucose: xylose ratio of 1:1, and 1.05 g/L of Na_2HPO_4. Nevertheless, after comparing the obtained biomass and accumulated lipids obtained under the optimal conditions (13.33 and 5.3 g/L, respectively), with the biomass and lipids obtained in the control treatments using100% glucose, and 100% xylose (separately), with a C/N of 70 and no addition of Na_2HPO_4, an increase in biomass and lipid was observed (27.33 and 8.9 g/L, respectively). Nonetheless, considering the relationship between biomass and total lipid content in glucose and xylose control treatments, the total lipid accumulation was lesser in 7.48% and 10.45%, respectively with the accumulation obtained under optimal cultivation conditions of C/N of 70, initial glucose: xylose ratio of 1:1, and 1.05 g/L of Na_2HPO_4 (treatment with highest lipid accumulation). Furthermore, in this work under the conditions where the highest lipid accumulation was obtained, a decrease of ~30 h of total fermentation time was achieved in comparison with our previous work [171]. The obtained results under the tested conditions enrich the understanding related to the cultivation of this yeast. In addition, the above results can be useful for culture media design to improve the lipid yield using complex culture media such as undetoxified wood hydrolysate as a culture media.

Table 9.Biomass and lipid production by R. toruloides-1588 using a mix of two wood hydrolysate as a culture media.

Tx	C/N	Inductor	Glucose: xylose	Biomass		Lipids		Total sugars	Furfural content	
				Content (g/L)	Yield (g biomass/g sugar)	Content (g/L)	Yield (g lipids/g sugar)	Specific consumption ($g^{-1}g^{-1}h$)	Initial (g/L)	Final (g/L)
T1	20	0.10	1:1	5.33±0.26	0.15±0.007	1.74±0.08	0.050±0.002	0.069±0.003	0.61±0.030	0.04±0.002
T3		2.00	1:1	6.08±0.30	0.18±0.009	1.53±0.07	0.045±0.002	0.073±0.003	0.41±0.020	0.03±0.001
T5		1.05	1:2	5.33±0.26	0.19±0.009	1.41±0.07	0.050±0.002	0.055±0.002	0.47±0.023	0.04±0.002
T7		1.05	2:1	5.06±0.25	0.16±0.008	1.42±0.07	0.044±0.002	0.061±0.003	0.30±0.015	0.02±0.001
T-9	70	0.10	1:2	9.30±0.46	0.34±0.017	2.64±0.12	0.096±0.004	0.038±0.001	0.43±0.021	0.02±0.001
T-10		2.00	1:2	10.03±0.50	0.37±0.018	3.52±0.17	0.130±0.006	0.020±0.001	0.63±0.031	0.04±0.002
T-11		0.10	2:1	11.05±0.55	0.27±0.013	2.07±0.10	0.050±0.002	0.032±0.001	0.34±0.017	0.03±0.01
T-12		2.00	2:1	11.67±0.58	0.28±0.014	3.82±0.19	0.090±0.004	0.020±0.001	0.30±0.015	0.03±0.001
T-13		1.05	1:1	13.33±0.66	0.69±0.034	5.18±0.25	0.280±0.014	0.025±0.001	0.51±0.025	0.04±0.002
T-14		1.05	1:1	13.67±0.26	0.62±0.031	4.92±0.24	0.222±0.011	0.025±0.001	0.41±0.020	0.04±0.002
T-15		1.05	1:1	13.33±0.30	0.53±0.026	5.18±0.25	0.205±0.010	0.021±0.001	0.39±0.019	0.05±0.002
T2	120	0.10	1:1	5.33±0.26	0.16±0.008	1.88±0.09	0.055±0.002	0.066±0.003	0.69±0.034	0.05±0.002
T4		2.00	1:1	6.07±0.30	0.18±0.009	1.72±0.08	0.051±0.002	0.044±0.002	0.47±0.023	0.04±0.002
T6		1.05	1:2	5.33±0.26	0.18±0.009	1.49±0.07	0.051±0.002	0.053±0.002	0.46±0.023	0.04±0.002
T8		1.05	2:1	5.00±0.25	0.15±0.007	1.34±0.06	0.040±0.002	0.086±0.004	0.29±0.014	0.03±0.001
T16	70	0	Control glucose	27.33±0.54	0.54±0.027	8.92±0.44	0.180±0.009	0.019±0.000	0.60±0.030	0.05±0.002
T17		0	Control xylose	13.67±0.52	0.52±0.026	4.05±0.20	0.150±0.007	0.020±0.001	0.55±0.027	0.03±0.001

Fatty acid composition

Figure 28 shows the four main saturated fatty acids (palmitic, stearic, oleic, and linoleic fatty acids) present in lipids produced by *R. toruloides*-1588 grown in the undetoxified wood hydrolysate. The distribution and abundance of fatty acids varied with cultivation conditions. For instance, Figure 28A shows the fatty acid distribution in treatment with lower lipid accumulation (glucose: xylose ratio of 2:1, C/N ratio of 120, and Na_2HPO_4 of 1.05 g/L) where the maximum content of palmitic and stearic acids was observed at 96 h with 23.5%, and 20%, respectively, and the maximum oleic acid content was observed at 168 h (35%). When *R. toruloides*-1588 was cultivated under equal initial glucose: xylose ratio (1:1), C/N of 70, and 1.05 g/L of Na_2HPO_4, the maximum abundance of palmitic, stearic, and oleic fatty acids (13.3%, 11%, and 21%, respectively) were observed during the first 18 h of cultivation. Nevertheless, under these conditions, a higher abundance of linoleic fatty acid (37.5%) was observed at 96 h of fermentation followed by a considerable decrease of all fatty acids at the end of the fermentation (Figure 28B). The fatty acid abundance in control treatments (glucose-Figure 28C and xylose-Figure 28D) was similar to reported in our previous work Osorio-González et al. [171], where the high abundance of palmitic and stearic fatty acids was at 96 h, and oleic and linoleic fatty acids was at 168 h.

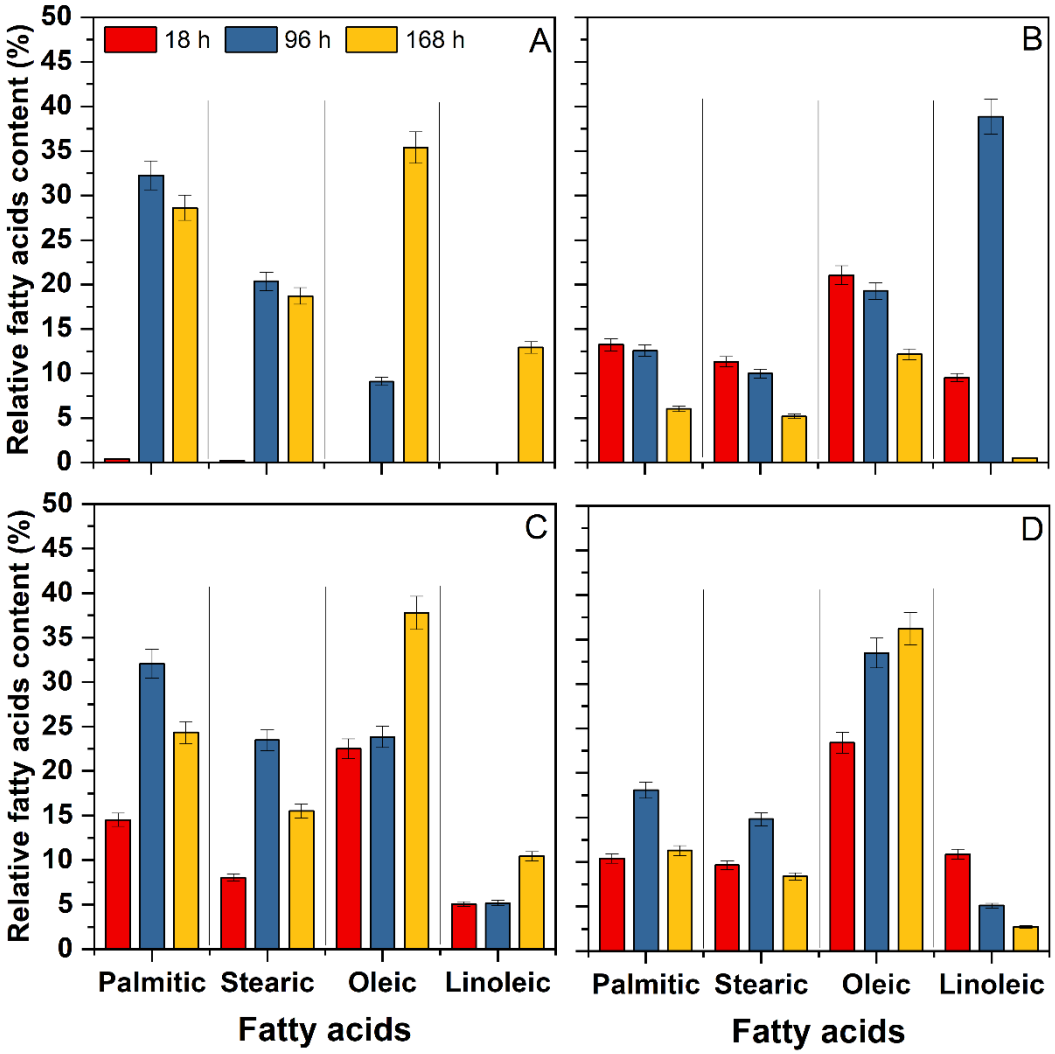

Figure 28.Fatty acid distribution of lipids obtained from R. toruloides-1588 cultivated on wood hydrolysate. A) C/N= 120 – glucose: xylose (2:1), inducer=1.05; B) C/N= 70 – glucose: xylose (1:1), inducer=1.05; C) C/N= 70 – glucose, inducer=0; D) C/N=70 – xylose, inducer=0

Figure 29 shows the distribution of several polyunsaturated fatty acids produced by *R. toruloides*-1588 growth using wood hydrolysate as a culture media. The total abundance of omega-3 fatty acids (alpha-linolenic acid, eicosapentaenoic, and docosapentaenoic acids) and omega-6 fatty acids (linolenic, eicosadienoic, and dihomo-gamma-linolenic) was similar in conditions of C/N of 120, initial glucose: xylose ratio of 2:1, and an initial concentration of 1.05 g/L of Na_2HPO_4, and C/N of 70 (Figure 29A), and glucose control treatment (glucose alone, and no addition of Na_2HPO_4). For omega-9 fatty acid (Eicosenoic), a slightly higher abundance was observed in the

glucose control treatment (Figure 29C). Likewise, when *R. toruloides*-1588 was cultivated under the conditions of C/N of 70, initial glucose: xylose ratio of 1:1, and 1.05 g/L of Na_2HPO_4 (Figure 29B) showed an increase in Omega-3 and Omega-6 fatty acids except for dihomo-gamma-linolenic and eicosenoic fatty acids, that were not present in the produced lipids. Finally, when *R. toruloides*-1588 was grown using xylose alone, and no addition of Na_2HPO_4 (xylose control treatment), the abundance of polyunsaturated fatty acids was higher than observed in the rest of the above-cultivated conditions (Figure 29D). For instance, alpha-linoleic, and eicosapentaenoic fatty acids (Omega-3), was 0.68%, 1.21%, 1.11%, and 0.59%, 1.53%, and 1.37% higher in comparison with the abundance observed in tested conditions with the highest lipid accumulation. Additionally, a lower lipid accumulation was observed in glucose and xylose control treatments. For eicosadienoic fatty acid (Omega-6), an increase of 0.75%, 0.86%, and 0.70% was observed, respectively. Lastly, the highest abundance of Omega-9 (1.71%) was observed under the same cultivated conditions.

The fatty acid profile of the produced lipids in this work meets the required standards by regulatory agencies such as the European Standard EN 14214 for its use as a feedstock for biofuels production such as biodiesel and more recently for drop-in biofuels. The above fact is due to the high presence of saturated fatty acids such as palmitic, stearic, and oleic that are suitable raw materials to obtain short-chain alkanes such as octanes. Furthermore, it has been reported that these types of fatty acids increase the lubricity of the produced biofuels, which from the application point of view is a great advantage due to it has been reported that lipid-based biofuels promote the decrease of engine wear. Additionally, the near balance in saturated fatty acids and the presence of lower polyunsaturated fatty acids help to decrease biodiesel oxidation [176]. Likewise, the produced lipids are a suitable raw material for a wide variety of products different from biofuels because can be used as an intermediate to produce other chemical compounds as well as like a substitute for compounds in the pharmaceutical and food industries.

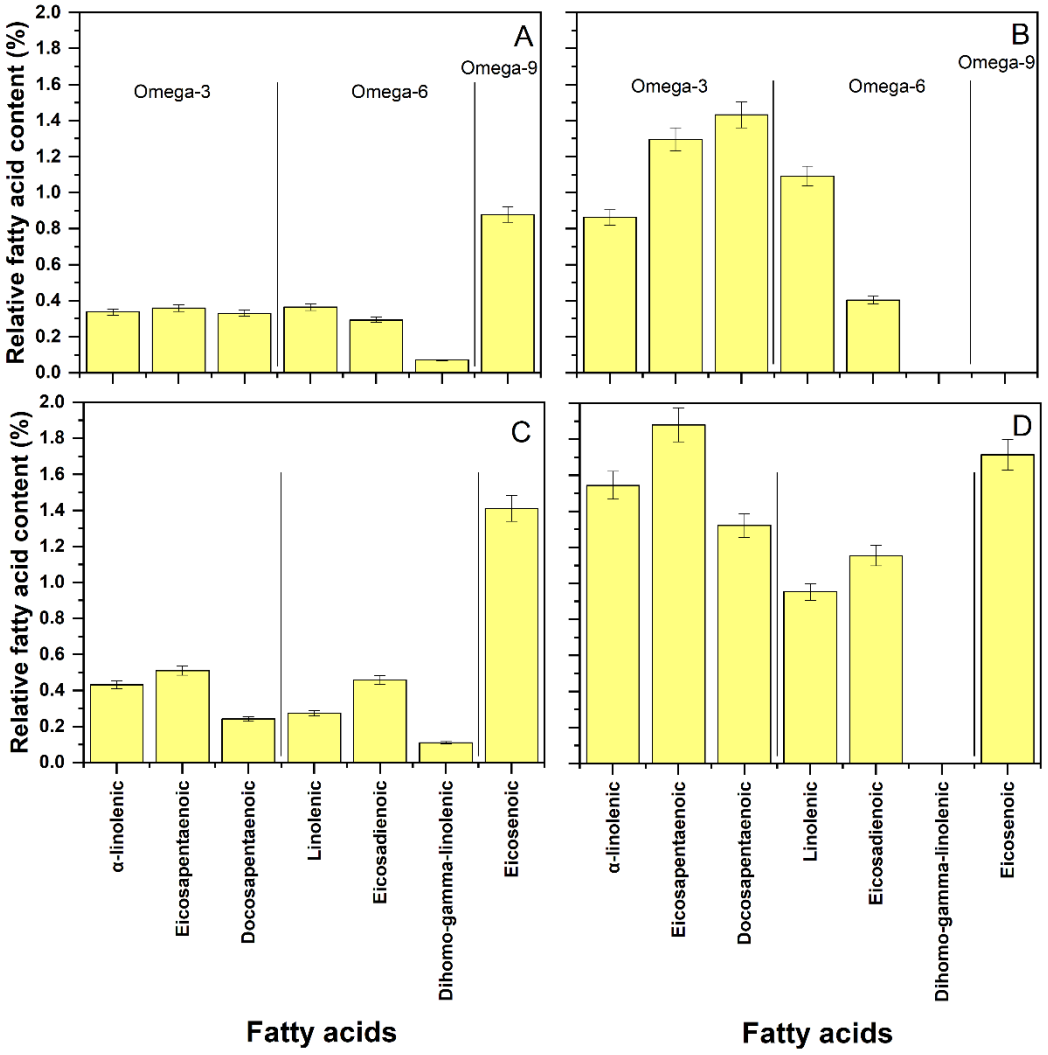

Figure 29.Fatty acid distribution of lipids obtained from R. toruloides-1588 cultivated on wood hydrolysate. A) C/N= 120 – glucose: xylose (2:1), inducer=1.05; B) C/N= 70 – glucose: xylose (1:1), inducer=1.05; C) C/N= 70 – glucose, inducer=0; D) C/N=70 – xylose,

Inhibitor effect

In addition to the release of sugars from lignocellulosic biomass, the pretreatment releases several cell growth inhibitory compounds such as furans, organic acids, and lignin derivatives leading to a considerable decrease in the yeast metabolic activity. It has been reported that among these compounds furfural is the most toxic and abundant in hydrolysates obtained from lignocellulosic biomass that considerably affects the adenosine triphosphate (ATP) production leading to a decrease in the cell biomass yield [78]. Figure 30 shows the amount of furfural that was degraded

in this study. Furthermore, Pearson correlation analysis, shows that furfural has a slight inhibition effect on microbial growth, as well as on lipid production (p-value=-0.173, and p-value=-0.007, respectively). The negative effect could be caused by a decrease or total inhibition of the glyceraldehyde-3-phosphate dehydrogenase enzyme (GPD), and aldehyde dehydrogenase (ALD1) involved in the carbon source uptake into the metabolic process of the yeast cells [6], [75].

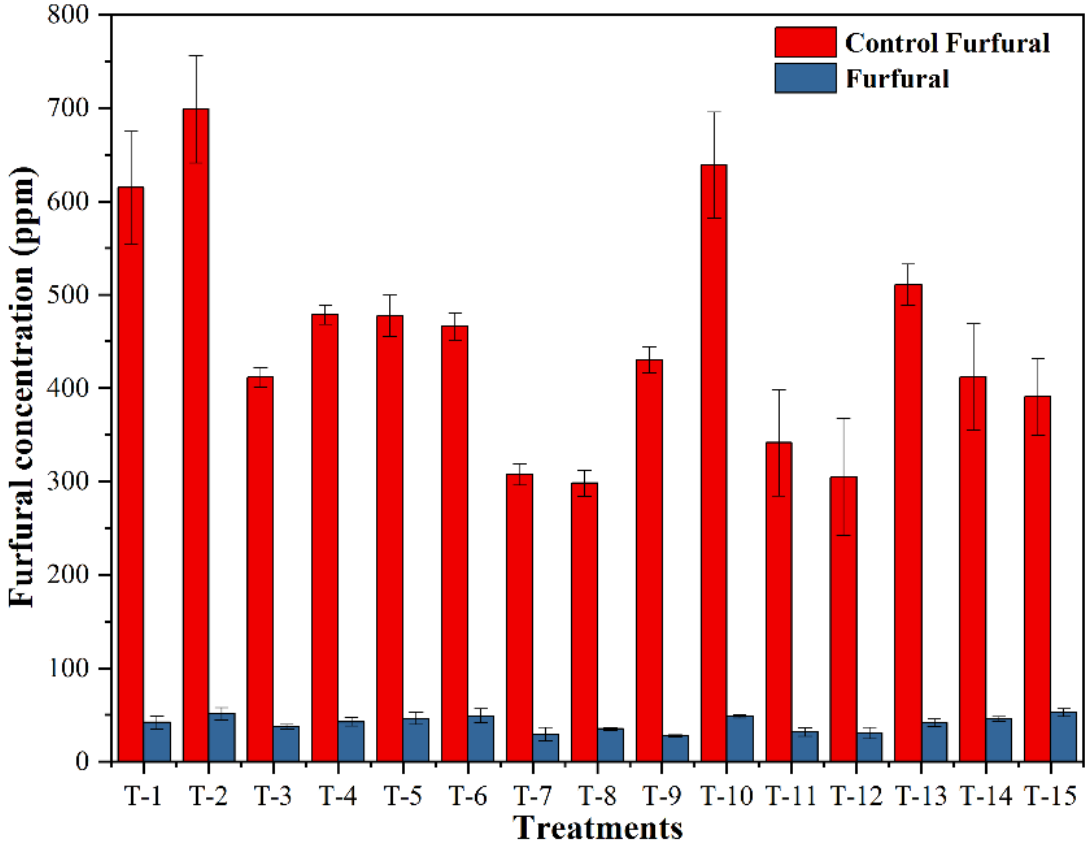

Figure 30.Furfural transformed/degraded by R. toruloides-1588 using a mix of two undetoxified wood hydrolysate as a culture media.

In this work, a remarkable decrease in furfural content of 90.7% at 96 h of fermentation (maximum lipid accumulation) was observed in all tested conditions. Besides, furfural degradation considering the C/N ratios tested (20, 70, and 120) show a difference minor to 1% between them with 91.23%, 90.60%, and 90.36%, respectively. Likewise, the furfural decrease considering the amount of initial Na_2HPO_4 concentrations (0.10, 1.05, and 2.0 g/L) was similar (92.56%, 89.42%, and 91.10%) to the obtained results considering C/N ratios as a factor. Nevertheless, when the effect of the three parameters evaluated was independently analyzed, for instance, a slight

inhibitory effect in microbial growth was observed in treatments where a C/N of 70, 1.05 g/L of Na_2HPO_4, and 1:1 initial glucose: xylose ratio (Pearson coefficient=-0.21, -0.42, and -0.57, respectively) was used. For lipid production, a negative effect was observed in treatments where 1.05 g/L of Na_2HPO_4, and 1:1 and 2:1 initial glucose: xylose ratio (Pearson coefficient=-0.39, -0.47, and -0.003, respectively).

So far, no specific work has been done on genes or enzymes involved in furfural degradation within the genus *R. toruloides*. Nevertheless, in an omic analysis performed by Zhu et al. [177] in an *R. toruloides* Y4 strain, they reported three families of short-chain dehydrogenase/reductase (SDR) proteins in three different genes (TPI1, FAS2, and MDH1, respectively). This family of enzymes can be activated due to the stress caused by the high concentrations of furfural in the culture media. Also, due to the hydrophobic nature of the enzyme, it works as a link channel for the hydrophobic site that can bind to the furfural. However, furfural is not the only compound that could cause a strong toxicity effect on *R. toruloides*-1588 yeast. Compounds derived from the lignin fraction and six-carbon sugar dehydration such as 5-HMF, vanillin, vanillic acid, syringaldehyde, levulinic acid, ferulic acid, and aminobenzoic acid are present in the culture media and can contribute to decreasing the yeast performance during the fermentation process [6]. In the present study, other observed inhibitory compounds (5-HMF, levulinic acid, ferulic acid, vanillic acid, vanillin, syringaldehyde, and aminobenzoic acid) did not show a considerable decrease with respect to the decrease observed in furfural. This may be due to the lower amount of these compounds in the hydrolysate (<100 ppm). The above results confirm that *R. toruloides*-1588 is a strain capable of growing and accumulating lipids in the presence of microbial inhibitory compounds such as furfural, organic acids, and some lignin derivatives, as observed in our previous studies [161], [171].

Conclusion

The effect of three initial C/N and glucose: xylose ratios, as well as three initial concentrations of Na_2HPO_4 on the lipid production, was studied. A C/N ratio of 70, an initial rate of glucose: xylose of 1:1, and 1.05 g/L of Na_2HPO_4 showed a 40% lipid accumulation with a maximum biomass yield of 0.69 g of biomass/g of sugar. The use of Na_2HPO_4 as a lipid inducer showed a 7.48% increase in lipid accumulation. The fatty acid profiles showed that microbial lipids obtained by *R. toruloides*-1588 using wood hydrolysate as a substrate are a potential and suitable feedstock to

produce conventional biofuels and advanced drop-in biofuels, chemical precursors, or food additives. *R. toruloides*-1588 was capable of growth in presence of 0.7 g/L of furfural and other inhibitory compounds. The obtained results in this study confirm that *R. toruloides*-1588 is a promising strain from a process point of view, due to can growth in media without growth supplements, has vigorous growth, has a short period of growth, among others. In summary, the present investigation showed that *R. toruloides*-1588 uses glucose and xylose efficiently in a simultaneous way as a carbon source. Secondly, Na_2HPO_4 used as a lipid inducer can increase lipid accumulation, and thirdly, the culture conditions tested decreased total fermentation time up to 96 hours.

CRediT author statement

Carlos S. Osorio-González.: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft. **Rahul Saini**.: Formal analysis, Investigation. **Krishnamoorthy Hegde**.: Supervision, Writing-Review & Editing. **Satinder Kaur Brar**.: Funding acquisition, Supervision, Writing-Review & Editing. **Antonio Avalos-Ramírez**.: Funding acquisition, Writing-Review & Editing, Supervision. **Alain Lefebvre**.: Resources.

Supplementary data for this work can be found in ANNEX 3.

5.3 Crabtree effect on *Rhodosporidium toruloides*-1588 using C5 and C6 wood hydrolysate as a culture media

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C. S. Osorio-González *et al.*, "Crabtree effect on *Rhodosporidium toruloides*-1588 using C5 and C6 wood hydrolysates as a culture media,". ***Sustainable Energy Technologies and Assessments. To be submitted***

Abstract

R. toruloides-1588 was tested to grow and accumulate lipids using C5 and C6 wood hydrolysates highly rich in sugars as well as the effect of ammonium sulfate as an only supplement in the culture media. Batch fermentation using C5 and C6 wood hydrolysate as a culture media showed a slight substrate and growth inhibition up to 100 g/L with a maximum lipid accumulation of 8.2 g/L with palmitic, stearic, oleic, linoleic and lignoceric acids as predominant fatty acids, which are comparable to vegetable oils. Additionally, this study is the first to report the effect of the presence of high concentrations of xylose in the culture media on *R. toruloides*-1588.

Keywords Substrate inhibition, growth inhibition, wood hydrolysate, lipid production, *Rhodosporidium toruloides*

Introduction

Due to the concern about fossil oil depletion caused by the overuse during the last decades to produce energy, several governments as well as private institutions around the world have put efforts in developing and promoting policies to improve the transition of energy production using biotechnological processes using renewable sources as feedstock [178], [179]. Currently, liquid biofuels such as biobutanol, bioethanol, or biodiesel production using renewable feedstocks such agro-industrial, industrial, crop residues, and lignocellulosic biomass, are a potential alternative to the conventional fossil fuels as well as to the direct development of a sustainability culture and green energy independence [180], [181].

Recent research has been focused on producing biodiesel using lipids from several sources such as raw and refined vegetable oil, cooking oils and animal grease and tallow. Another alternative that has been raised during the last three decades is the production and use of single-cell oil from oleaginous yeast [182]. Oleaginous yeasts are microorganisms that can accumulate between 20% to 70% of lipids within their cellular bodies. Some examples of this microorganism are *Lypomyces starkeyi, Trichosporon fermentants, Cryptococcus curvatus, Yarrowia lipolytica, Rhodotorula glutinis and Rhodosporidium toruloides* [36], [157]. Among these microorganisms, *Rhodosporidium toruloides* stands out as a promising microorganism capable to accumulate up to 70% of its dry weight into lipids, use a wide variety of substrates such as glycerol, sodium chloride, lignocellulosic hydrolysates, and degrade several compounds inhibitory to the microbial growth [44], [171]. Numerous studies have reported the performance of *Rhodosporidium toruloides* in

terms of growth, sugar utilization, and lipid production. Nevertheless, the differences in substrates and culture conditions make it difficult to compare those results. One of these factors is the presence is high sugar concentrations in the culture media, which can cause a catabolic repression effect during the aerobic fermentation process ultimately hindering microbial growth and by consequence lipid accumulation. It has been reported that the excess of sugar in the culture media can cause problems during the adenosine triphosphate (ATP) production, a vital compound in energy metabolism [183]. Some studies have reported that the increase of glucose in oleaginous yeast increased the microbial biomass production and lipids accumulation using high-sugar culture media such as hydrolysates obtained from lignocellulosic biomass [184].

As per the results obtained in our previous work, *R. toruloides*-1588 demonstrated an outstanding capacity to use liquid hydrolysates obtained from forestry residues as a suitable substrate for microbial growth and lipid production [171], [185], [186]. From a scaling-up process point of view, the selection of an optimal sugar concentration is important to achieve the maximum lipid accumulation and prevent loss of the carbon source during the fermentation. In this sense, this study aims to evaluate the capacity of *Rhodosporidium toruloides*-1588 to use high sugar concentrations, growth and accumulate lipids using two wood hydrolysate highly-rich in sugars as a sustainable substrate. Additionally, the study reports the effect of ammonium sulfate as the only culture media supplement.

Materials and Methods

Substrate, microorganism, and inoculum preparation

C5 and C6 wood hydrolysates used as a culture media were produced from Greenfield Global Inc., Varennes, Quebec, Canada from the poplar (*Populus alba*) residues with a glucose content of 10 g/L and 122 g/L and a xylose content of 123 g/L and 11 g/L, respectively. *Rhodosporidium toruloides*-1588 was obtained from the Agricultural Research Service (NRRL) Culture Collection (USA). *R. toruloides*-1588 was preserved and the inoculum seed was prepared using YM media according to Saini et al. [185].

Sugar concentration effect and its impact on microbial growth, sugar utilization and lipid accumulation

To evaluate the effect of sugar concentrations and ammonium sulfate in the culture media on microbial growth, sugar utilization, and lipid production by *R. toruloides*-1588, four initial sugar concentrations (50 g/L, 75 g/L, 100 g/L, and 120 g/L, respectively) and 1 g/L of ammonium sulfate $(NH_4)_2SO_4$ were used. Batch flask fermentations were performed with an initial pH of 6, at 25±1°C, 200 rpm, during 144 h in two sets: i) wood hydrolysate supplemented with ammonium sulfate, and ii) wood hydrolysate without ammonium sulfate supplementation.

Cell harvest and lipid extraction

Cells harvest and lipid extraction was performed according to Osorio-Gonzalez et al. [171]. Briefly, the cells were harvested by centrifugation and dried at 60±1°C for 18 hours. After that, were treated with a solution of hydrochloric acid for 1 hour. After that, lipids were extracted using a chloroform: methanol solution (2:1 v/v).

Analytical methods

Microbial growth

Microbial growth was quantified by microbial biomass dry constant weight (DCW) and reported as g/L of microbial biomass produced (*B*). Furthermore, microbial biomass yield ($Y_{B/S}$) and maximum growth rate (μ_{max}) were calculated according to Equation 1 and 2, respectively.

$$Y_{B/S} = \frac{Total\ biomass\ (g)}{Total\ sugar\ consumed\ (g)} \quad \text{(Eq. 1)}$$

$$\mu_{max} = \frac{Total\ biomass\ (g)}{Total\ time\ (h)} \quad \text{(Eq. 2)}$$

Sugar utilization

To quantify the sugar utilization by *R. toruloides*-1588, samples were drawn across the fermentation, and the analysis was performed according to Saini et al. [185] using a Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer (LC-MS) equipped with a HILIC column (5 µm x 150mm and 4.6mm) and a solution of acetonitrile: water (89:11 v/v) as mobile phase. Total sugar consumed (*S*) was reported as g/L and maximum sugar consumption rate (X_{Smax}) was calculated using Equation 3.

$$X_{Smax} = \frac{Total\ sugar\ consumed\ (g)}{Total\ time\ (h)} \quad \text{(Eq. 3)}$$

Lipid quantification and fatty acid composition

Total lipid content was reported as g/L and lipid yields ($Y_{L/B}$ and $Y_{L/S}$) were calculated according to Equation 4 and 5, respectively. Fatty-acid methyl esters (FAMEs) were obtained by transesterification from extracted lipids according to Osorio-Gonzalez et al. [171]. Briefly, methanol was used as a reactant and sulfuric acid as a catalyst at 100±1°C for 20 min. FAMEs were extracted with hexane and analyzed using an Agilent 7890B Gas Chromatography-Flame Ionization Detector (GC-FDI) using an Agilent 122-2362 DB-23 column (60 m x 250 µm and 0.25 µm film thickness). All fatty acid was identified and quantified using a Supelco 37 Component FAME Mix as a standard (Sigma-Aldrich, USA).

$$Y_{L/B} = \frac{Total\ lipids\ (g)}{Total\ biomass\ (g)} \quad \text{(Eq. 4)}$$

$$Y_{L/S} = \frac{Total\ lipids\ (g)}{Total\ sugar\ consumed\ (g)} \quad \text{(Eq. 5)}$$

Total nitrogen content

Total nitrogen content (N dissolved + N suspended particles which do not settle) in liquid samples was analyzed using a Shimadzu Total Organic Carbon Analyzer (TOC-VCPH) with a detection limit of 0.02 mg/L.

Statistical analysis

The statistical analysis of the data was conducted using Origin Lab-Pro® 20.0. Sugar concentration effect on biomass production, sugar utilization and lipid accumulation were evaluated by One-Way ANOVA with a Fisher test at 95% level of confidence. All experiments were performed in duplicate.

Results and discussion

Substrate inhibition analysis

Figure 31 shows glucose and xylose consumption by *R. toruloides*-1588 using C5 and C6 wood hydrolysates as a substrate. When C6 wood hydrolysate + $(NH_4)_2SO_4$ was used as a substrate, slow glucose utilization was observed during the first 18 h at initial glucose concentrations of 50 g/L (5.77%), 75 g/L (6.66%), and 100 g/L (20%), respectively. However, an increase of two times on glucose utilization (43.75%) was observed at an initial glucose concentration of 120 g/L. After 18 hours, pronounced glucose consumption during the next 54 hours was observed in all treatments as 64.9%, 61.95, 42.5%, and 28.75%, respectively. The maximum glucose utilization was observed at different times between the four initial glucose concentrations. For instance, when the initial concentration of 50 g/L maximum glucose consumption was observed at 128 h (99.7%). For initial concentrations of 75 and 100 g/L, the times were similar (144 h), but the maximum consumption was different (97.0% and 86.5%, respectively). Nevertheless, when an initial glucose concentration of 120 g/L was used, the maximum glucose consumed was at 96 h (80%). As compared to previous results, when C6 wood hydrolysate was used alone (without $(NH_4)_2SO_4$ addition), an increase of 16.29%, 53.0%, 20.0%, and 4.16% in glucose consumption was observed during the first 18 hours. The times at which the maximum glucose consumption was observed comprised treatments employing 75, 100 and 120 g/L of initial glucose concentration using C6 wood hydrolysate + $(NH_4)_2SO_4$ as a substrate. Maximum glucose consumption was at 144 h with 97%,81%, 80%, respectively. Conversely, treatments with an initial glucose concentration of 50 g/L, showed a decrease of 32 h with respect to the rest of the treatments to achieve the maximum glucose consumption (>98%).

The above stated results showed a catabolic repression effect on *R. toruloides*-1588 in both C6 wood hydrolysate when initial sugar concentrations of 100 g/L and 120 g/L were used. In the case of treatments where C6 wood hydrolysate was supplemented with $(NH_4)_2SO_4$, a decrease of 13.4% and 25% of glucose utilization, respectively. On the other hand, in C6 wood hydrolysate without $(NH_4)_2SO_4$ supplementation, a decrease of 18.29% and 24% in glucose utilization was observed, respectively. In both cases, the catabolic repression effect was observed after 96 hours of fermentation. Nevertheless, the obtained results in this research are different in comparison with the observed by Li et al. [187] using *R. toruloides* Y4. They observed that the microbial growth was highly repressed in treatments with a glucose concentration between 150 g/L to 300 g/L. It should be stated that this work used an undetoxified wood hydrolysate as a culture media with $(NH_4)_2SO_4$ as an only supplement, in comparison with the minimal media (Glucose=10 to 400 g/L,

$(NH_4)_2SO_4$=12 g/L, KH_2PO_4=1 g/L, $MgSO_4 \cdot 7H_2O$=1.5 g/L, and yeast extract=0.5 g/L) used by Li et al.[187]. Thus, the increase in tolerance to high glucose concentration could be linked to the availability of the nitrogen source in the culture media that promoted the yeast metabolism increasing the glucose catabolism.

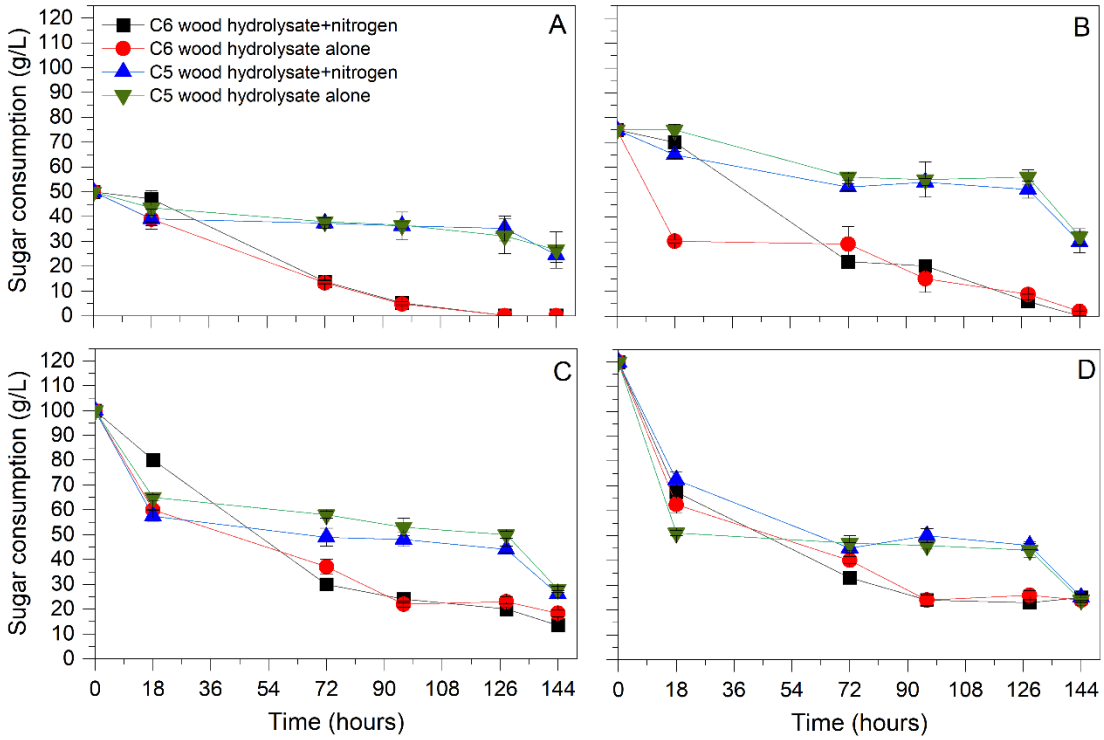

Figure 31.Sugar consumption profile by R. toruloides-1588 using wood hydrolysate as a substrate. A) 50 g/L; B) 75 g/L; C) 100 g/L; D) 120 g/L.

The xylose consumption was similar between C5 wood hydrolysate + $(NH_4)_2SO_4$, and C5 wood hydrolysate without nitrogen addition, due to the total xylose consumption at 144 h fermentation was similar between treatment with the same initial xylose concentration. For instance, in treatment where an initial xylose concentration of 75, 100, and 120 g/L using C5 wood hydrolysate + $(NH_4)_2SO_4$ as a substrate, a maximum xylose consumption of 60%, 73% and 79%, was observed. A similar consumption was observed using C5 wood hydrolysate without $(NH_4)_2SO_4$ addition (57%, 72%, and 80%, respectively). Nevertheless, the maximum difference in xylose consumption (10%) between the substrates was observed when an initial xylose concentration of 50 g/L was used (48% and 38%, respectively). Although the trend of xylose consumption was similar between

the two hydrolysates and the four initial concentrations, a faster consumption during the first 18 hours of fermentation in treatments with higher initial xylose concentration (100 and 120 g/L) was observed. Likewise, an increase of 30% and 45% in xylose consumption was observed in comparison with the xylose consumed in treatments with 50 g/L of initial xylose, and 32.1% and 41.9% in treatments with 75 g/L of initial xylose concentration. After 18 h of fermentation, the trend of xylose consumption was similar, except during the last 18 h of fermentation where an increase in xylose consumption was observed. These results showed that *R. toruloides*-1588 encountered a slight inhibition in xylose utilization. These results are however promising as very few studies have reported the use of xylose as a single carbon source and in a concentration higher than 25 g/L [168], [188]. Additionally, no literature, to the best of our knowledge, has been reported with studies about substrate inhibition using high xylose concentrations.

Biomass production analysis

Figure 32 shows the microbial biomass production by *R. toruloides*-1588 using C5 and C6 wood hydrolysate as substrate. When C6 wood hydrolysate was used as a substrate the exponential phase was observed during the first 18 h of cultivation. Nevertheless, the stationary phase was different for each initial sugar concentration, for instance in treatments with an initial concentration of 50 g/L an increase of 5 g/L in biomass concentration was observed in C6 wood hydrolysate + $(NH_4)_2SO_4$ in comparison with the obtained biomass using C6 wood hydrolysate without supplementation, with a well-shaded stationary phase and "exponential-stationary" constant phase, respectively. Now, when 75 and 100 g/L of initial sugar concentration were used, a similar tendency was observed in C6 wood hydrolysate + $(NH_4)_2SO_4$ with a defined stationary phase of 78 h.

Hence, *R. toruloides*-1588 can use glucose effectively during the biomass production process. For instance, in this research, the maximum biomass production using C6 wood hydrolysate + $(NH_4)_2SO_4$ was 21.5 g/L with 100 g/L of initial glucose concentration. Conversely, Li et al. [187] reported 18.6 g/L using an initial glucose concentration of 90 g/L, showing a dropping decrease after the above concentration. Similarly, they observed a small decrease in the specific growth rate when the initial sugar concentration increased from 40 g/L to 150 g/L and a drastic decrease when glucose concentrations were higher than 200 g/L. Opposite trends were reported by Li et al. [187], and Fei et al. [144] with a total biomass production of 36.2 g/L using a clarified lignocellulosic hydrolysate with an initial glucose concentration of 110 g/L.

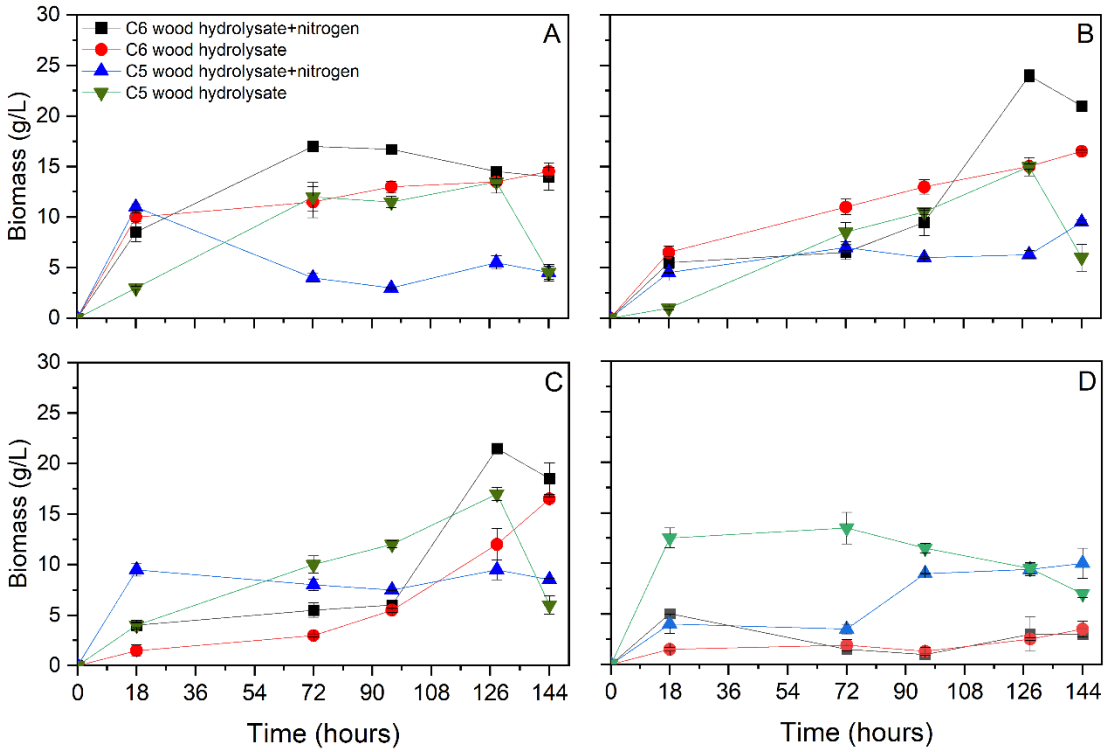

Figure 32.Biomass production by R. toruloides-1588 using wood hydrolysate as a substrate. A) 50 g/L; B) 75 g/L; C) 100 g/L; D) 120 g/L.

In treatments using C5 wood hydrolysate as a culture media, the maximum biomass concentration was obtained at 128 hours in treatments where an initial sugar concentration of 50 g/L, 75 g/L, 100 g/L and 72 hours when an initial sugar concentration of 120 g/l was used using C5 hydrolysate without $(NH_4)_2SO_4$ addition. The growth profile was different between the initial sugar concentration and with reference to the addition of $(NH_4)_2SO_4$ to supplement the hydrolysates. For instance, when an initial sugar concentration of 50 and 100 g/L was used, the presence of lag phase was very short (less than 6 hours, data not shown). In the case of treatments with an initial sugar concentration of 50 g/L an exponential phase of 72 hours followed for 56 hours of stationary phase were observed. Conversely, in treatments with initial sugar of 100 g/L a constant exponential phase was observed until 128 hours. The growth tendency in treatments where an initial sugar concentration of 75 g/L was used, a short lag phase of 18 hours was observed, followed by a pronounced exponential phase of 110 hours. In the particular case of treatments where an initial sugar concentration of 120 g/L a short lag phase (less than 6 hours, data not shown), followed for 18 hours of exponential phase and 24 hours of stationary phase. The above observations along with the calculated values of the maximum growth rate showed a catabolic repression effect in the

microbial growth of ~50% between the two C5 wood hydrolysates used as a culture media in relation to the increase of the initial sugar concentration. Nonetheless, akin to the xylose utilization, the biomass production by *R. toruloides*-1588 using C5 wood hydrolysate is promising. This is the first study analyzing the effect of wood hydrolysate containing high xylose concentration as a single carbon source.

Nitrogen effect

It is well known that lipid accumulation in oleaginous microorganisms is triggered by the limitation of one primary nutrient, commonly the nitrogen source. Overall, total nitrogen consumption was more than 60% but not higher than 90% in both hydrolysates. Nevertheless, a different consumption pattern was observed between the initial sugar concentrations. For instance, when the yeast was growing in C6 wood hydrolysate with an initial sugar concentration of 50 g/L and 75 g/L, the total nitrogen consumption was higher (83.3% and 69.3%, respectively) on treatments where the hydrolysate was supplemented with $(NH_4)_2SO_4$. Conversely, when initial sugar concentrations of 100 g/L and 120 g/L were used, the highest nitrogen consumption (79.8% and 89.7%, respectively) was observed in treatments where C6 wood hydrolysate was used alone. In treatments where C5 wood hydrolysate was used as a substrate, the addition of $(NH_4)_2SO_4$ increases the nitrogen utilization at lower and higher initial sugar concentrations (50 g/L and 120 g/L) with 75.2% and 91.7%, respectively. On the contrary, at 75 g/L and 100 g/L of initial sugar concentration, maximum nitrogen consumption (89.7% and 86.2%, respectively) was observed using C5 wood hydrolysate alone.

Table 10 shows the effect of $(NH_4)_2SO_4$ addition on the performance of *R. toruloides*-1588 using C5 and C6 wood hydrolysate as a culture media. The addition of $(NH_4)_2SO_4$ doesn't affect total sugar consumed (p-value=0.215). In terms of microbial biomass production using C6 wood hydrolysate as a substrate, the addition of $(NH_4)_2SO_4$ promotes biomass production no matter the initial sugar concentration. On the contrary, in treatments where C5 wood hydrolysate was used as a substrate, $(NH_4)_2SO_4$ did not show an effect (p-value=0.256) on biomass production as highest values were observed when C5 wood hydrolysate was used without $(NH_4)_2SO_4$ supplementation.

Table 10. R. toruloides-1588 performance using C5 and C6 wood hydrolysates as a culture media.

Substrate	Initial sugar (g)	S (g/L)	B (g/L)	$Y_{B/S}$ (g/g)	μ_{max} (h^{-1})	X_{Smax} (h^{-1})	$Y_{L/S}$ (g/g)
C6 hydrolysate + $(NH_4)_2SO_4$	50	49.90±0.01	17.00±0.42	0.34	0.47	0.50	0.16
C6 hydrolysate		49.90±0.01	14.50±0.45	0.29	0.56	0.61	0.14
C5 hydrolysate + $(NH_4)_2SO_4$		25.43±2.82	11.00±0.32	0.43	0.61	0.60	0.12
C5 hydrolysate		23.46±7.31	13.50±1.13	0.58	0.17	0.88	0.06
C6 hydrolysate + $(NH_4)_2SO_4$	75	74.82±0.07	24.00±0.63	0.32	0.31	0.74	0.11
C6 hydrolysate		73.09±0.13	16.50±0.14	0.23	0.36	2.49	0.11
C5 hydrolysate + $(NH_4)_2SO_4$		45.00±4.24	9.50±0.21	0.21	0.25	0.56	0.06
C5 hydrolysate		43.00±3.25	15.00±0.91	0.35	0.12	1.34	0.03
C6 hydrolysate + $(NH_4)_2SO_4$	100	86.60±1.54	21.50±0.14	0.25	0.22	1.11	0.05
C6 hydrolysate		81.71±1.36	16.50±0.21	0.20	0.11	2.22	0.05
C5 hydrolysate + $(NH_4)_2SO_4$		73.96±1.44	9.50±0.98	0.13	0.53	2.36	0.02
C5 hydrolysate		72.00±1.24	17.00±0.63	0.24	0.22	2.57	0.02
C6 hydrolysate + $(NH_4)_2SO_4$	120	97.00±1.69	5.00±0.14	0.05	0.28	2.92	0.05
C6 hydrolysate		96.00±2.34	3.50±0.77	0.04	0.08	3.19	0.02
C5 hydrolysate + $(NH_4)_2SO_4$		95.00±1.07	10.00±1.48	0.11	0.22	2.64	0.03
C5 hydrolysate		96.00±1.21	13.50±1.54	0.14	0.69	4.00	0.01

S (g/L): total sugar consumed; B (g/L): total biomass; $Y_{B/S}$: biomass yield/substrate consumed; μ_{max}: maximum growth rate; X_{Smax}: maximum sugar consumption rate; $Y_{L/S}$: lipid yield/ substrate consumed.

Performance of *R. toruloides*-1588 in lipid accumulation

Lipid accumulation by *R. toruloides*-1588 using C5 and C6 wood hydrolysate is shown in Figure 33. Overall, the highest lipid accumulation was observed in treatments where C6 wood hydrolysate was used as a substrate. Maximum lipid accumulation (8.2 g/L) were obtained using an initial sugar concentration of 75 g/L in C6 wood hydrolysate without $(NH_4)_2SO_4$ addition, followed by treatments with an initial sugar concentration of 50 g/L and 75 g/L using C6 wood hydrolysate supplemented with $(NH_4)_2SO_4$ where the lipid accumulation was 8 g/L. Minimum lipid accumulation (1.5 g/L) was obtained in treatments with an initial sugar concentration of 120 g/L using C6 wood hydrolysate without $(NH_4)_2SO_4$ addition. Furthermore, a decrease of ~50% in lipid accumulation was observed in treatments where high initial sugar concentrations were used (100 g/L and 120 g/L). The above results are in accordance with Li et al. [187] and Fei et al. [144], where a decrease in lipid accumulation was observed along with the use of initial sugar concentration higher than 90 g/L. On another hand, the lipid production using C5 wood hydrolyzate was significantly lower (p-value=0.05) with a maximum value of 3 g/L in treatments with an initial sugar concentration of 50 g/L and 120 g/L using C5 wood hydrolysate supplemented

with $(NH_4)_2SO_4$. Similarly, the minimum lipid values (1.3 g/L and 1 g/L) were observed at the same initial sugar concentrations but using C5 wood hydrolysate without $(NH_4)_2SO_4$ addition. This result showed that the presence of high concentrations of xylose in media rich in nitrogen decreases lipid accumulation and improves xylose consumption. *R. toruloides*-1588 can use xylose to produce sugar alcohols such as D-arabitol, ribitol or erythritol instead of lipids [189]. In addition, a recent transcriptome and metabolomic analysis performed by Jagtap et al. [190], confirms a high expression of xylitol dehydrogenase (XDH) and xylose reductase (XR), and a downregulation of xylulokinase (XKS1), which is the responsible enzyme to convert D-xylulose into D-xylulose-5-phosphate, a key intermediate in the non-oxidative pentose phosphate pathway. Likewise, the authors observed a 42-fold increase in the arabitol dehydrogenase (ARD1) enzyme, which in the presence of nicotinamide adenine dinucleotide (NADH) as a cofactor, promotes the conversion of xylulose into arabitol.

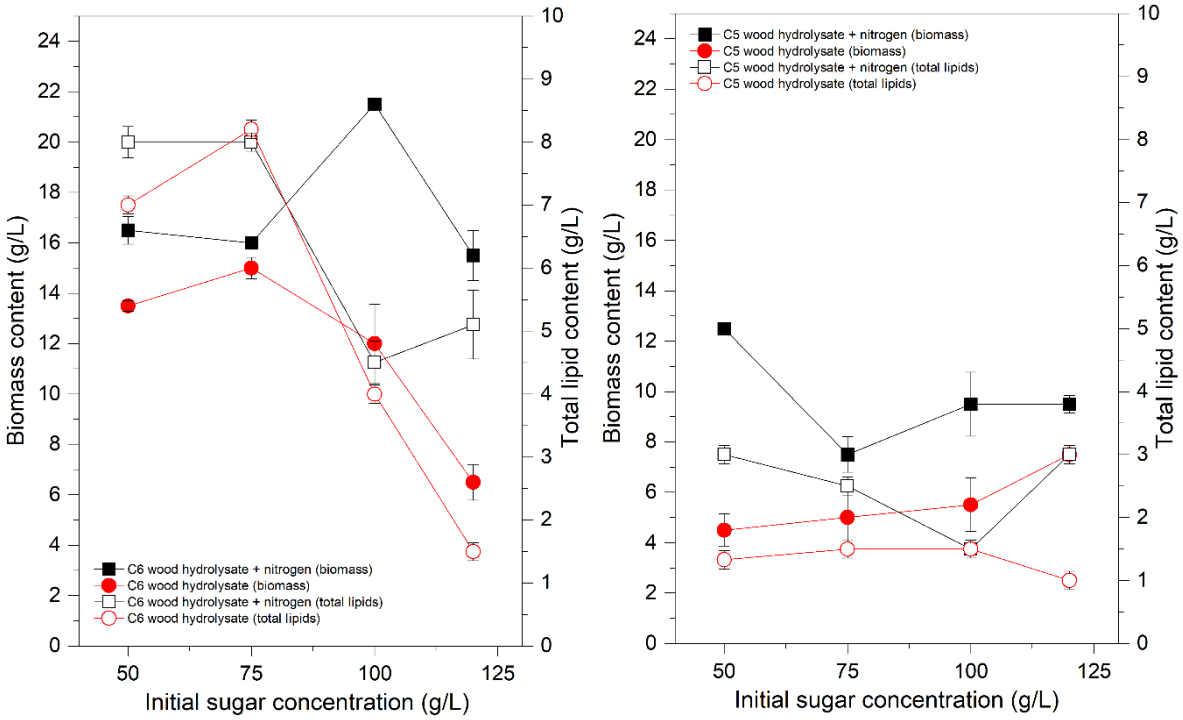

Figure 33.Lipid accumulation profile by R. toruloides-1588 using wood hydrolysate as a culture media. A) C6 wood hydrolysate; B) C5 wood hydrolysate.

Fatty acid distribution on *R. toruloides*-1588.

The fatty acid profile from lipids produced by *R. toruloides*-1588 using C5 and C6 wood hydrolysates as a substrate is shown in Table 11. The saturated fatty acid content was ~1% myristic, palmitic (>25%) and stearic (>15%). In addition, the presence of monounsaturated fatty acid was oleic acid (>40%) and linoleic (>10%) was observed in both hydrolysates (C5 and C6, respectively). Finally, the two most abundant polyunsaturated fatty acids were linolenate (≥5%) and lignoceric acid (>3%). In general, the fatty acid composition observed in the accumulated lipids by *R.toruloides*-1588 agrees with the fatty acid profile of *Rhodosporidium spp*. and several vegetable oils [144], [168], [171], [186], [187], [191]. The most significative difference was observed on the produced lipids using C6 wood hydrolysate + $(NH_4)_2SO_4$ shown a 2-fold increase in palmitic and linolenic fatty acids and a slight decrease in stearic and linoleic fatty acids in treatments with an initial glucose concentration of 50 g/L, after that no significant changes in fatty acid distribution and content were observed. On another hand, the produced lipids using C5 wood hydrolysate with and without $(NH_4)_2SO_4$ addition, do not show major differences in the fatty acid content.

Table 11.Fatty acid distribution in lipids produced by R. toruloides-1588 using wood hydrolysate as a substrate.

Relative fatty acid content (%)	C6 wood hydrolysate + nitrogen				C6 wood hydrolysate			
	Initial glucose content (g/L)				Initial xylose content (g/L)			
	50	75	100	120	50	75	100	120
Palmitic	23.39±1.16	23.37±1.16	20.15±1.00	21.20±1.06	11.45±0.57	23.06±1.15	22.69±1.13	19.99±0.99
Stearic	14.92±0.74	15.73±0.78	13.09±0.65	18.83±0.94	17.91±0.89	15.53±0.77	11.97±0.59	17.69±0.88
Oleic	39.05±1.95	44.60±2.23	47.48±2.37	34.74±1.73	37.7±1.88	35.33±1.76	41.86±2.09	35.76±1.78
Linoleic	11.30±0.56	6.28±0.31	7.415±0.37	8.31±0.41	15.38±0.76	12.19±0.60	12.33±0.61	8.96±0.44
Linolenate	3.26±0.16	1.92±0.09	3.09±0.15	5.10±0.25	8.40±0.42	5.91±0.29	3.38±0.16	5.41±0.27
Lignoceric	2.19±0.10	2.36±0.11	2.38±0.11	3.88±0.19	1.54±0.07	1.47±0.07	1.79±0.08	3.74±0.18
	C5 wood hydrolysate + nitrogen				C5 wood hydrolysate			
Palmitic	17.50±0.87	17.62±0.88	17.4±0.87	16.49±0.82	16.72±0.83	16.89±0.84	17.47±0.87	17.81±0.89
Stearic	16.73±0.83	18.25±0.91	17.64±0.88	18.17±0.90	17.01±0.85	17.08±0.85	17.81±0.89	19.40±0.97
Oleic	41.49±2.07	41.28±2.06	40.35±2.01	40.18±2.00	41.60±2.08	41.63±2.08	38.8±1.94	38.67±1.93
Linoleic	8.45±0.42	7.345±0.36	8.04±0.40	7.87±0.39	8.28±0.41	8.16±0.40	7.77±0.38	6.96±0.34
Linolenate	5.05±0.25	4.565±0.22	5.15±0.25	4.73±0.23	4.94±0.24	4.99±0.24	4.73±0.23	3.90±0.19
Lignoceric	3.47±0.17	3.345±0.16	3.51±0.17	3.45±0.17	3.77±0.18	3.56±0.17	3.23±0.16	3.70±0.18

Conclusion

High sugar concentrations using C5 and C6 wood hydrolysate as a culture media clearly demonstrated the catabolic repression effect on *R. toruloides*-1588. Small substrate inhibition effect was seen on the capacity of the strain to grow (18.6 g/L) and accumulate lipids (8.2 g/L) up to a sugar concentration of 120 g/L. These results confirm that wood hydrolysates from forestry residues are a suitable culture media for microbial lipid production. Thus, R. toruloides-1588 possesses the potential to be a convenient oleaginous strain for industrial-scale operations.

CRediT author statement

Carlos S. Osorio-González.: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft. **Rahul Saini**.: Formal analysis, Investigation. **Krishnamoorthy Hegde**.: Supervision, Writing-Review & Editing. **Satinder Kaur Brar**.: Funding acquisition, Supervision, Writing-Review & Editing. **Alain Lefebvre**.: Resources. **Antonio Avalos-Ramírez**.: Funding acquisition, Writing-Review & Editing, Supervision.

Supplementary data for this work can be found in ANNEX 4.

5.4 Microbial lipid production in undetoxified C5 and C6 wood hydrolysate by *Rhodosporidium toruloides*-1588

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C. S. Osorio-González *et al.*, "Microbial lipid production in undetoxified C5 and C6 wood hydrolysate by *Rhodosporidium toruloides*-1588,". ***Process Safety and Environmental Protection. To be submitted***

Abstract

The cultivation of Rhodosporidium toruloides-1588 was performed at a bench-scale to accumulate lipids using two undetoxified wood hydrolysates as a culture media. *R. toruloides*-1588 is an oleaginous yeast that can accumulate 30.79%, 57.14%, and 43.75% of lipids using undetoxified wood hydrolysates as a culture media (C5, C6 and C5/C6, respectively). The total sugar consumption was 83.6%, 95.12%, and 93.32% with a biomass production of 0.18, 0.38, and 0.27 g of biomass/g of sugar consumed, respectively. The study revealed that *R. toruloides*-1588 is a strong potential strain for microbial lipid production at an industrial scale using undetoxified wood hydrolysate as a culture media.

Keywords *Rhodosporidium toruloides*-1588, wood hydrolysates, microbial lipids, inhibitors, biorefinery

Introduction

The growing demand for energy causes the depletion of limited reserves of fossil resources. To prevent the damage caused to the environment by fossil fuel production, the interest to develop new technologies for producing biofuels from renewable feedstocks has aroused [192], [193]. Biofuels can be defined as a fuel obtained from biomass or its derivatives by biotechnological or thermochemical processes to generate thermal energy by combustion. The above fact has deepened in the use of alternative feedstock sources with specific characteristics, such as all-year availability, cheap, renewable, and easy to process [194], [195]. In this sense, a promising option is a lignocellulosic biomass, such as crops, agri-food, and forestry residues are renewable feedstocks available worldwide, with low cost and the most important characteristic is this raw material do not have any conflict with the food production system. Lignocellulosic biomass can be treated to obtain liquid hydrolysates highly rich in fermentable sugars that can be used as suitable substrates to produce biofuels or feedstock to produce it [196], [197].

A potential feedstock to produce biodiesel that has been explored during the last three decades are microbial lipids using oleaginous microorganism. Oleaginous yeast, *Rhodosporidium toruloides* sp. has high capacity to accumulate lipids (up to 70%) and produce carotenoids, and its ability to use a wide variety of substrates, and its tolerance to inhibitory compounds [198], [199]. While it is true, that intense research has been performed about this strain during the last decade, however, most of these studies have been focused on genomics or genetic modification with the purpose to

improve the yield of microbial lipid production. Likewise, the research has been performed using synthetic media and detoxified hydrolysates obtained from agri-food, food, or industrial residues [52], [144], [200], [201].

So far, apart from the research performed in our previous works [171], [186], [202], a very few studies have been performed to produce microbial lipids using *Rhodosporidium toruloides* and undetoxified wood hydrolysate as a culture media [53], [203], [204]. Because of all the above facts, this study aimed to evaluate the performance of *Rhodosporidium toruloides*-1588 to accumulate lipids using two undetoxified wood hydrolysates as a culture media at a bench-scale as a promising bioprocess to produce biofuel.

Materials and methods

Microorganism and maintenance medium

Rhodosporidium toruloides Y-1588, from Agricultural Research Service (NRRL) Culture Collection (USA) was used in this study for lipid production. Laboratory yeast maintenance and inoculum preparation was done according to Osorio-Gonzalez et al. [171]. Briefly, yeast was preserved in YM agar (g/L): glucose 10; peptone 5; yeast extract 3; agar 15; malt extract 3. To prepare *R. toruloides*-1588 inoculum seed, yeast cells were pre-cultured by three generations in YM broth for synthetic media batch, and C6-C5 wood hydrolysate (1:1 ratio and 20 g/L of carbon source) for wood hydrolysates batch fermentations. The growth temperature was 30°C at 200 rpm during 36 hours in a Multitron® shaker (Infors Canada, Montreal, Canada).

C5 and C6 wood hydrolysate as a substrate

C5 and C6 wood hydrolysates were obtained from *Populus* sp. (Greenfield Global Inc., Varennes, Quebec, Canada) and used as a culture media. Table 5.4.1 shows the main components of both hydrolysates.

Table 12.Composition of C5 and C6 wood hydrolysate obtained from forestry residues.

Compound	Wood hydrolysate		
Sugars (g/L)	C5	C6	C5/C6
Xylose	25.41	0.87	12.52
Fructose	ND	ND	ND
Glucose	0.54	25.35	12.82
Sucrose	ND	ND	ND
Lactose	ND	ND	ND
Trehalose	ND	ND	ND
Lignocellulosic inhibitors (mg/L)			
Levulinic acid	ND	ND	ND
5-Hydroxymethyl furfural	120.20	352.47	284.10
Furfural	350.45	78.36	130.39
4-amino benzoic acid	ND	ND	ND
Vanillic acid	ND	ND	ND
Vanillin	65.54	50	75.63
Syringaldehyde	856.26	85.23	135.52
Ferulic acid	ND	ND	ND
Sinapyl alcohol	24.71	27.80	<10
Coniferyl alcohol	10.04	<10	<10
p-Coumaric acid	ND	ND	ND
m-coumaric acid	ND	ND	ND

ND: Not determine

Bioreactor culture conditions

All cultures were performed in 3 liters (2.5 liters working volume) stirred tank reactor (Labfors 4, Infors USA), equipped with a Rushton turbine (radial flow) and operated at 30°C. Experiments were performed at 400 rpm and airflow ratio of 1 vvm to maintain 35% of dissolved oxygen. The experiments were performed using synthetic media, C5 and C6 wood hydrolysate inoculated with 0.1 OD_{600nm} of seed culture and Antifoam 204 at 0.05% v/v were used. The initial pH was adjusted

to 6.0±0.5 before inoculation and kept constant during the inoculation by the addition of a sterile solution of sodium hydroxide (1N) or hydrochloric acid (1N). The pH and dissolved oxygen were monitored using Hamilton EasyFerm plus and Mettler Toledo probes, respectively. Samples were taken each 8 hours for a total fermentation time of 96 h to determine the microbial growth, cell biomass production, xylose consumption, and lipid accumulation.

Determination of culture yields and kinetics parameters

Lipid content ($Y_{L/X}$), and lipid yield ($Y_{L/S}$) were calculated according to Equation 1 and 2, respectively. In equation 1 X_i and L_i are the dry cell weight and lipid concentration on day t_i, respectively and X_0 and L_0 are the dry cell weight and lipid concentration on the first day (t_0), respectively. In equation 2, S_i and S_0 are the sugar concentration on day t_i and the first day (t_0), respectively. L_i and L_0 are the lipid concentration on day t_i and the first day (t_0), respectively.

$$Y_{L/X} = \frac{L_i + L_0}{X_i - X_0} \quad \text{Eq. (1)}$$

$$Y_{L/S} = \frac{L_i - L_0}{S_0 - S_i} \quad \text{Eq. (2)}$$

Biomass productivity ($Biomass_{prod}$) and lipid productivity ($Lipid_{prod}$) were calculated according to Equation 3 and 4, respectively; were in Equation 3 where, X_0 and X_i is biomass concentration on day t_0 and t_i, respectively. On other hand, in lipid productivity, Lip_{max} is the maximum lipid concentration on day t_i.

$$Biomass_{prod} = \frac{X_i + X_0}{t_i} \quad \text{Eq. (3)}$$

$$Lipid_{prod} = \frac{Lip_{max}}{t_i} \quad \text{Eq. (4)}$$

Sugar consumption rate ($Sugar_{rate}$) and specific sugar consumption ($Sugar_{specific}$) were quantified using Equation 5 and 6, respectively; where S_i and S_0 are the sugar concentration, X_i is the total biomass on day t_i and the first day (t_0), respectively.

$$Sugar_{rate} = \frac{S_i - S_0}{t_i - t_0} \quad \text{Eq. (5)}$$

$$Sugar_{specific} = \frac{S_i/X_i}{t_0}$$ Eq. (6)

Analytical methods

Lipid extraction

Lipid extraction was performed according to [185]. Briefly, *R. toruloides*-1588 cells were harvested by centrifugation (15000 *x* g/2 min) and dry at 60ºC until constant weight. After that, cell wall disruption was performed using acid hydrolysis (HCl 1M). Lipids were extracted using liquid-liquid extraction with a chloroform/methanol solution (2:1 v/v). Finally, the aqueous phase was removed, and the remaining organic phase was evaporated in an oven at 50°C for 18 hours.

Liquid Cromatography-Mass Spectrometry (LC-MS) analysis

During the fermentation samples for each fermentation were drawn and analysis for xylose, glucose, and lignin inhibitory compounds were performed using a Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer (LC-MS) according to our previous work [124], [171], [205]. Briefly, sugars were analyzed using a HILIC Pak VG-50 2D column (5 µm x 150 mm x 4.6 mm) using a solution of acetonitrile: water (89:11 v/v), D6-glucose as an internal standard and mass detector in a positive mode. In the case of lignin inhibitory compounds, a BetaBasic-18 column (100mm x 2.1mm; 3µm) was used with a solution of water: methanol (80:20 v/v) as mobile phase and phenyl ethanol-D5 as an internal standard with the mass detector in negative mode detection, except 2-furoic acid and 2-oxoglutaric acid where the mass detector was used in positive mode detection. Finally, lignin monomers were analyzed using a HILICpak VG-50 2D column (5 µm x 150 mm x 4.6 mm) with a solution of acetic acid in water (0.1% v/v) and a solution of acetic acid in methanol (0.05% v/v), with a ratio of 82.5:17.5 (respectively) as a mobile phase. Glycerol was used as an internal standard and the mass detector was operated in a positive mode detection. All compounds were identified and quantified by comparing peak areas and retention times with its standard compounds (Sigma-Aldrich, USA).

Gas Chromatography analysis

Fatty acids, furfuryl alcohol and acetic acid were analyzed using an Agilent 7890B Gas Chromatography-Flame Ionization Detector (GC-FDI) according to [171], [185]. Fatty acid profile from produced lipids was performed through lipid transesterification into fatty acid methyl esters

(FAMEs). Briefly, a solution of methanol-sulfuric acid was used as a catalyst at 100°C for 20 min. After that, FAMEs were extracted with hexane and analyzed using an Agilent 122-2362 DB-23 column (60 m x 250 μm and 0.25 μm film thickness). Fatty acids were identified and quantified by comparing the retention times and peak areas with a Supelco 37 Component FAME Mix (Sigma-Aldrich, USA). For furfuryl alcohol, a NUKOL capillary column (15 m x 0.25 mm x 0.25 μm) was used. Finally, acetic acid was quantified using an HP-INNOWax column. Furfuryl alcohol and acetic acid were determined and quantified according to the peak area and retention time of its corresponding standard compounds (Sigma-Aldrich, USA).

Results

Performance using C5 wood hydrolysate as a culture media

Batch cultivation fermentation was performed using the above-mentioned C5 wood hydrolysate with an initial xylose concentration of 25 g/L. Figure 34 shows the performance of *R. toruloides*-1588 using C5 wood hydrolysate. The obtained results showed 83.6 % of the total xylose during the first 16 hours, after that xylose content remained without major changes. A sugar consumption rate of 0.22 h^{-1} and a specific sugar consumption of 0.07 g h^{-1} was observed. The maximum cell biomass production was 3.8 g/L with a lipid yield of 0.18 g of biomass per g of sugar consumed and a maximum growth ratio of 0.04 h^{-1}. In terms of lipid accumulation, the maximum content was 1.17 g/L with a lipid yield of 0.31 g of lipids per g of biomass, and lipid productivity of 0.01 g L h^{-1}. The fatty acid profile of the produced lipids was constant being palmitic and stearic acid the only two fatty acids present in the lipids. Finally, the initial microbial inhibitors present in the hydrolysate were transformed at different rates. For instance, furfural was transformed totally with the concomitant production of furfuryl alcohol with a maximum concentration of 0.56 g/L. In the case of 5-HMF, vanillin and syringaldehyde, a degradation efficiency of 22.12%, 62.61%, and 72.18%, respectively were observed.

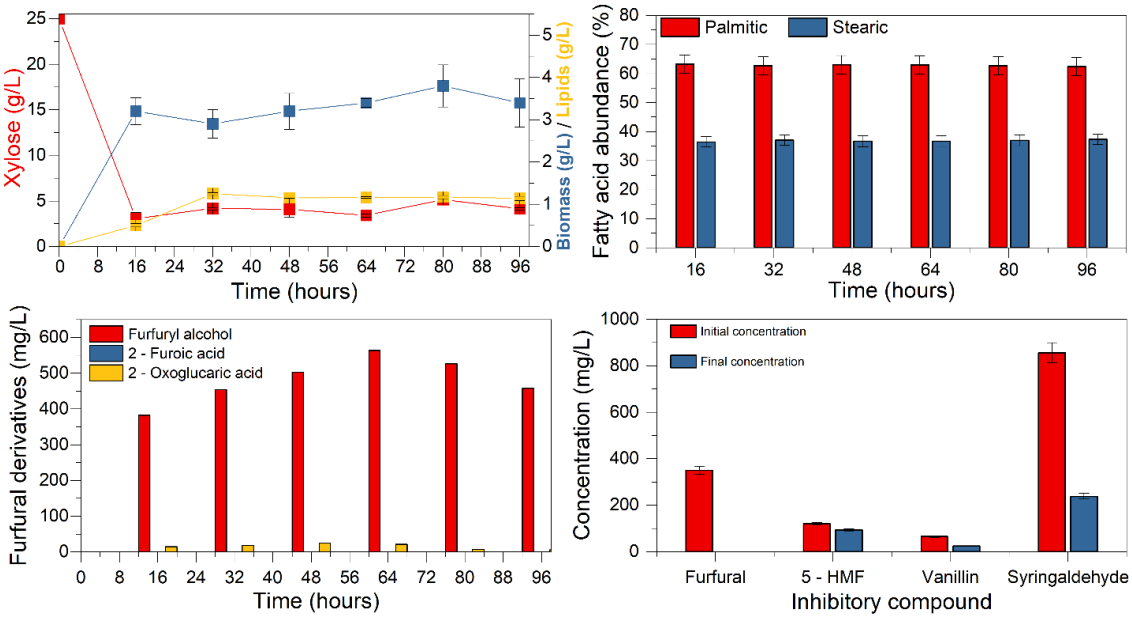

Figure 34.R.toruloides-1588 performance using C5 wood hydrolysate as a culture media.

Performance using C6 wood hydrolysate as a culture media

Rhodosporidium toruloides-1588 cultivation using C6 wood hydrolysate is shown in Figure 35. A maximum cell biomass production of 9.1 g/L with a lipid yield of 0.57 g of biomass per g of sugar consumed and a maximum growth ratio of 0.09 h^{-1} was observed. Total glucose consumption of 95.1% was observed with a sugar consumption rate of 0.25 h^{-1} and specific sugar consumption of 0.03 g h^{-1}. The maximum lipid accumulation was 5.20 g/L with a lipid yield of 0.57 g of lipids per g of biomass, and lipid productivity of 0.05 g L h^{-1}. The main fatty acids present in the produced lipids were palmitic, stearic oleic and linoleic acids. In the case of four main microbial inhibitory compounds transformation, furfural, vanillin and syringaldehyde were transformed and 94.36% of 5-HMF was transformed. Along with furfural transformation, 0.36 g/L furfuryl alcohol and 0.08 g/L of 2-furoic acid were produced. Likewise, from 2-furoic acid, 0.21 g/L of 2-oxoglutaric acid was produced.

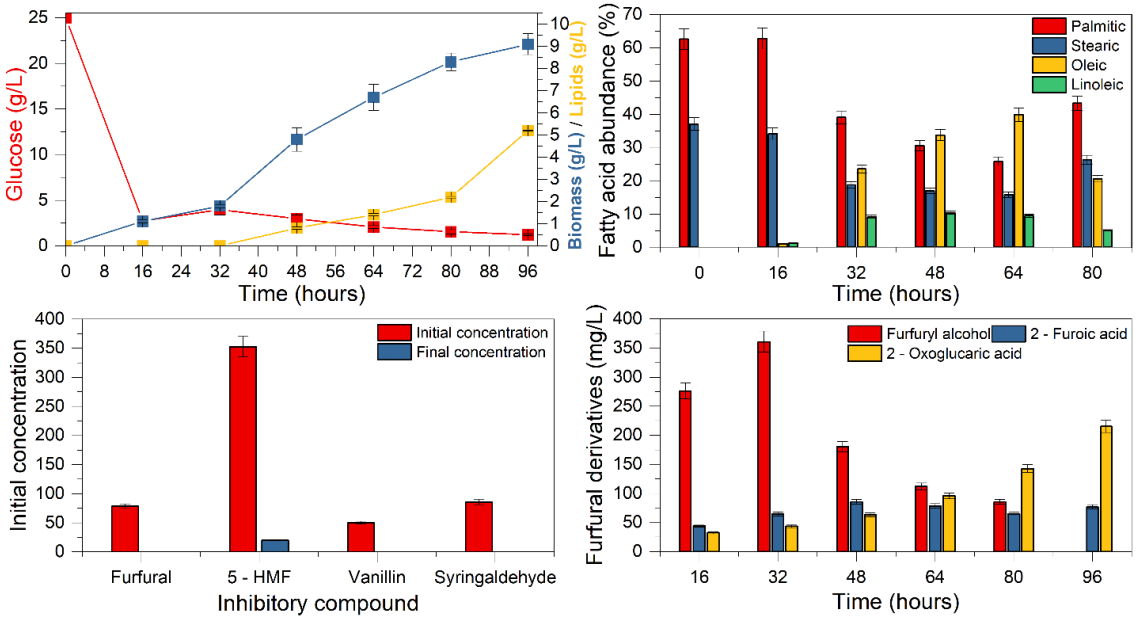

Figure 35.R.toruloides-1588 performance using C6 wood hydrolysate as a culture media.

Performance using C5/C6 wood hydrolysate as a culture media

Figure 36 shows the performance of *R. toruloides*-1588 cultivation using C5 and C6 wood hydrolysate. The maximum lipid accumulation was 2.8 g/L with a lipid yield of 0.44 g of lipids per g of biomass, and lipid productivity of 0.03 g L h^{-1}. The main fatty acid present in the produced lipids were palmitic, stearic oleic, linoleic acids, eicosenoate, and eicosadienoic acids. Total sugar consumption of 93.3% was observed with a specific sugar consumption of 0.04 g h^{-1} and a sugar consumption rate of 0.24 h^{-1}. The maximum cell biomass production was 6.4 g/L with a biomass yield of 0.27 g of biomass per g of sugar consumed and a maximum growth ratio of 0.07 h^{-1} was observed. Finally, furfural was transformed and 5-HMF, vanillin and syringaldehyde microbial inhibitory compounds were 56.59%, 77.19%, and 69.56% transformed, respectively. From furfural transformation, 0.22 g/L furfuryl alcohol and 0.03 g/L of 2-furoic acid were produced. In addition, 0.05 g/L of 2-oxoglutaric acid was produced.

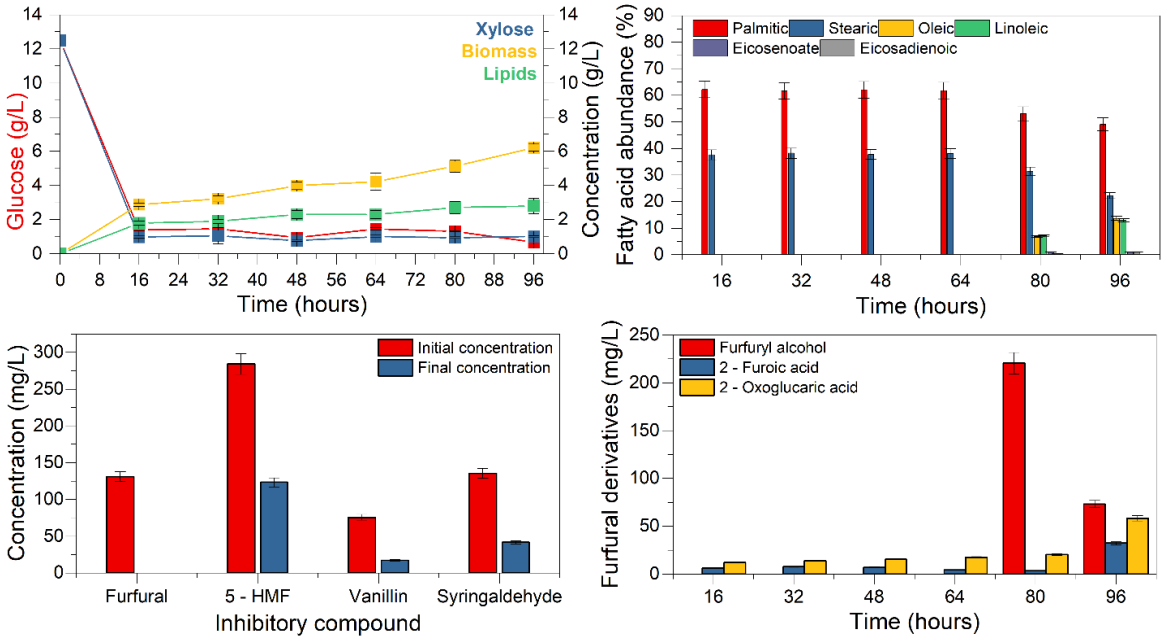

Figure 36.R.toruloides-1588 performance using C5 and C6 wood hydrolysate as a culture media.

Discussion

Sugar utilization

The substrate consumption in this study was very similar between the tested conditions and occurred during the first 16 hours of cultivation. The results in this study using C5/C6 wood hydrolysate in terms of glucose consumption agree with the reported by Bonturi et al. [52] using sugar cane bagasse hydrolysate supplemented with glycerol, where more than 90% of the glucose was consumed during the first 24 h. Nevertheless, an opposite consumption pattern was observed in xylose with ~50% versus the 90% observed in this study. A similar consumption tendency was reported by Chaiyaso et al. [206] using *R. paludigenum* KM281510 and corncob hydrolysate as a culture media containing glucose, xylose, and arabinose with an 83% of total sugar consumption. Nevertheless, a high proportion of the total percentage reported is because of glucose consumption (>90%), due to xylose and arabinose utilization being ~50% in both cases. In comparison with the results observed in this study, where *R. toruloides*-1588 shows an increase of 7% in the total sugar consumption (>90%) using C5/C6 wood hydrolysate as culture media. In addition, in this study, the maximum sugar utilization was observed during the first 16 hours of fermentation, in

comparison with the ~6 days observed by Chaiyaso et al. [206]. The above facts, support the efficiency of *R. toruloides*-1588 to utilize as single carbon source glucose or xylose or both simultaneously, in short periods. This information is highly useful for the next step such as fed-batch or continuous cultivation, which will help to develop a more feasible microbial lipid production process using wood hydrolysate as a culture media.

Microbial growth

Rhodosporidium toruloides-1588 was characterized using undetoxified C5 and C6 wood hydrolysates under aerobic batch conditions. A similar short lag phase (less than 8 hours) in C5 and C5/C6 wood hydrolysates was observed versus the observed in C6 wood hydrolysate (~32 hours). Respect to the exponential phase, a constant growth until the end of the fermentation in C6 and C5/C6 wood hydrolysates was observed after 16 h and 32 h, respectively. Conversely, in C5 wood hydrolysate, an exponential phase of ~12 h was observed followed by a stationary phase until the end of the fermentation. On another hand, the obtained results in this study regarding biomass yield on a substrate using wood hydrolysate show a slight decrease in C5 wood hydrolysate (0.18 g/g), and a similar yield in C6 and C5/C6 wood hydrolysates (0.38 g/g and 0.27 g/g, respectively) in comparison with the reported by Bonturi et al. [52] using an *R. toruloides* CCT 0783 adapted in a sugarcane bagasse hemicellulosic hydrolysate and grown using as a culture media with glucose, xylose and xylose-glucose (6:1 ratio) as carbon source (separately) with 0.35 g/g, 0.26 g/g, and 0.27 g/g, respectively. Likewise, the biomass yield obtained in this study using C6 wood hydrolysate agrees with the obtained by Soccol et al. [201] (0.36 g/g) using sugarcane juice as a culture media and *R. toruloides* DEBB 5533. Is worth to noted that the culture media used by Bonturi et al. [52] was supplemented with a trace mineral solution, improving the performance of the yeast. Nevertheless, according to the report by Fei et al. [144], in a strain *Rhodotorula toruloides* GEM grown in a mineral media using xylose as an only carbon source, a specific growth rate of 0.06 h^{-1} was observed, slightly higher than the specific growth observed in this work (0.04 h^{-1}). The above results are positive in terms of efficient conversion of the substrate into microbial biomass, specifically using C6 and C5/C6 wood hydrolysate as a culture media. On another hand, the low specific growth rate using C5 wood hydrolysate in this work can be associated with the complexity of the culture media and the presence of inhibitory compounds derived during the wood hydrolysate production.

Lipid accumulation

R. toruloides-1588 possesses the ability to grow and accumulate lipids using undetoxified wood hydrolysates as culture media, making it a suitable candidate to be used in fermentation using renewable substrates obtained from different lignocellulosic biomasses. The lipid yield observed in this study was slightly higher than the obtained by Galafassi et al. [200] with a strain of *R. graminis* DBVPG 7021 using glucose, glucose:xylose (1:1) and corn stover hydrolysate with 0.17 g/g, 0.17 g/g, and 0.26 g/g. Conversely, the lipid productivity values in this study were lower by 0.046 g L h^{-1}, 0.081 g L h^{-1} and 0.096 g L h^{-1}, respectively. The difference in the productivity values obtained by the above authors can be due to the cultivation strategy using a culture media-rich in nitrogen at the beginning of the fermentation that helps to increase the biomass production until its depletion, followed by the increase of the carbon source, provoking the ideal stress conditions to trigger a fast lipid accumulation. Similarly, to the above results and tendency, Soccol et al. [201] using *R. toruloides* DEBB 5533 obtain a similar lipid yield (0.34 g/g) with the obtained in this work using C5 wood hydrolysate (0.31 g/g) but lower compared when C6 and C5/C6 wood hydrolysate was used (0.57 g/g and 0.44 g/g, respectively). Nevertheless, the production values in this study were lower than the obtained from the above authors with 0.28 g L h^{-1}. Same as Galafassi et al. [200], the strategy implemented by Soccol et al. [201] (fed-batch) in addition to the type of substrate (sugarcane juice) decreases the time during the lipid accumulation but without major changes in the lipid yield. The above results show the similar capacity of *R. toruloides*-1588 to accumulate lipids in comparison with other strains cultivated in a different type of culture media. The above fact elucidates two main points that: i) undetoxified wood hydrolysates are a suitable culture media for microbial lipid production using *R. toruloides*-1588 and ii) the development of a proper cultivation strategy according to the culture media and strain.

Inhibitory compounds transformation

Lignocellulosic biomass has been identified as a renewable and sustainable feedstock to produce liquid hydrolysates. Nevertheless, one of the main challenges that this feedstock is facing up is the presence of microbial inhibitory compounds such as furans, phenols, and organic acids. Overall, *R. toruloides*-1588 is an appropriate bio factory to produce microbial lipids using undetoxified wood hydrolysates as a culture media. The obtained results confirm the tolerance and capacity of the yeast to transform microbial inhibitory compounds such as furfural into furfuryl alcohol and 2-furoic acid as well as the partial or total degradation of 5-HMF, vanillin and syringaldehyde.

Furthermore, the fact that in this work no additional supplementation was used (besides ammonium sulfate as a nitrogen source), the transformation capacity and tolerance exhibited by *R. toruloides*-1588 is superior or similar to some strains of its main genre but different family, like *R. glutinis* 2.107, *R. glutinis* 2.704, and *R. toruloides* 2.1389, *R. toruloides* Y4, *R. toruloides* BIS3 and *R. toruloides*-1091 [78], [84], [200], [204], [207], [208]. Likewise, similar to our previous work [171], [185], [186], in this study the authors do not observe an inhibition on the microbial growth and lipid accumulation. Nevertheless, the complete transformation and degradation pathway in this type of microorganisms has not been elucidated and because of the complexity of the microorganism specific studies using proteomic or transcriptomic studies are needed to understand the defense mechanism of *R. toruloides*-1588 against the toxic effects caused by these compounds.

Conclusion

This study confirms that R. toruloides-1588 is a suitable microorganism to be used in bioprocess using undetoxified wood hydrolysates from forestry residues as a culture media without the addition of external nutrients. R. toruloides-1588 shows the ability to utilize five and six-carbon sugars separately and simultaneously as a carbon source as well as the growth capacity, accumulate lipids, and transform and degrade microbial inhibitory compounds present in the hydrolysate. In conclusion, this study provided important data to develop a fed-batch or continuous cultivation strategies to increase the lipid yield and lipid productivity.

CRediT author statement

Carlos S. Osorio-González.: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft. **Rahul Saini**.: Formal analysis, Investigation. **Krishnamoorthy Hegde**.: Supervision, Writing-Review & Editing. **Satinder Kaur Brar**.: Funding acquisition, Supervision, Writing-Review & Editing. **Antonio Avalos-Ramírez**.: Funding acquisition, Writing-Review & Editing, Supervision. **Alain Lefebvre**.: Resources.

Supplementary data for this work can be found in ANNEX 5.

CHAPTER SIX: *MICROBIAL INHIBITOR DEGRADATION BY THE OLEAGINOUS YEAST RHODOSPORIDIUM TORULOIDES-1588*

6.1 Inhibitor degradation by *Rhodosporidium toruloides* NRRL 1588 using undetoxified wood hydrolysate as a culture media

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Abstract

Lignocellulosic biomass has been identified as a renewable and sustainable feedstock to produce liquid hydrolysates as a suitable substrate to produce a variety of compounds through biochemical processes. Nevertheless, the main challenge to using this substrate is the hydrolysis needed to release fermentable sugars. This pretreatment leads to the production of microbial toxic compounds such as furans, phenols, and organic acids. In this work, an oleaginous yeast, *R. toruloides* NRRL 1588, was used to study its ability to degrade inhibitors in C5 and C6 wood hydrolysates obtained from forestry residues. The study showed that *R. toruloides* NRRL 1588 can grow, accumulate lipids, and degrade up to 8.01 mgL^{-1} h^{-1} of furfural, 5.63 mgL^{-1} h^{-1} of 5-hydroxy methyl furfural, 1.70 mgL^{-1} h^{-1} of levulinic acid, 1.15 mgL^{-1} h^{-1} of syringaldehyde, 0.67 mgL^{-1} h^{-1} of vanillin, and 1.03 mgL^{-1} h^{-1} of vanillic acid. This work confirms the robustness of *R. toruloides* NRRL 1588 when grows in wood hydrolysates containing inhibitory compounds.

Keywords: *Rhodosporidium toruloides*-1588; Wood hydrolysate; Inhibitory compounds; Furans; Organic acids; Phenolic compounds

Introduction

Recently, biofuel production using microbial lipids as a sustainable feedstock has attracted the interest of academic, government, and private industries. This interest is mainly due to the advantages of biofuels, such as low or negligible production during combustion of sulfur oxides, carbon monoxide, and hydrocarbons, with respect to fossil fuels [67], [201], [209]. From the point of view of raw material availability, the production of microbial lipids offers several advantages in comparison with petroleum extraction, or vegetable oil production. For example, the production of microbial lipids needs short life cycles and it is easy to scale up, in addition, it does not depend on climate conditions [6], [210]. So far, several species of oleaginous microorganisms from bacteria, fungi, algae, and yeast have been used for lipid accumulations using a wide variety of substrates. However, among all these substrates, the use of lignocellulosic biomass as hydrolysates rich in sugars has been increasing in importance.

Nevertheless, during the lignocellulosic biomass hydrolysis sugar monomers are released, as well as compounds such as acetic acid, furfural, hydroxymethylfurfural, levulinic acid, ferulic acid, vanillin, vanillic acid, or syringaldehyde. The presence, abundance, and distribution of these compounds are highly dependent on the raw material as well as the pretreatment conditions [6]. It

has been reported that these compounds are toxic for most microorganisms and can cause partial or total inhibition of microbial growth. Furthermore, it affects the cell metabolism resulting in a decrease in the product concentration, making difficult the use of undetoxified hydrolysates and turning its exploitation into a great challenge [2], [140], [211], [212]. To use this resource as a feedstock to produce microbial metabolites like lipids, the hydrolysates rich in fermentable sugars need to be detoxified before fermentation. Another alternative is the use of strains that exhibit tolerance to inhibitory compounds such as furans, organic acids, and phenolic compounds. Several studies have investigated the effect of several compounds with an inhibitory effect on microbial growth and lipid production on some of the most popular oleaginous yeast species, such as *Cryptococcus curvatus, Trichosporon cutaneus, Lipomyces starkeyi, Trichosporon fermentants,* and *Rhodosporidium toruloides* [75], [78], [84], [213]. The effect of four factors involved in degradation tests of microbial growth inhibitory compounds has been studied: i) the type of culture media (basic, minimal, or synthetic), ii) the addition of trace mineral solution to improve the microbial growth, iii) the use of single carbon source (usually glucose), and iv) the presence of specific inhibitory compounds as well as its concentration. Nevertheless, the type and concentration of inhibitory compounds are highly dependent on the hydrolysis method. Therefore, the use of synthetic culture media to mimic the composition of lignocellulosic hydrolysates may not be a "real-effective" way to determine the inhibitor tolerance by the microorganisms. The use of raw lignocellulosic hydrolyzate lead to a better understanding of microbial response that can help to design a detailed scale-up process. In this sense, *Rhodosporidium toruloides* is a promising microorganism that has shown high tolerance to these compounds and it has been used to produce lipids that can be destined as feedstock for biofuels [6], [75], [78], [164], [185], [186]. The main aim of this research is to study the degradation of inhibitory compounds by *Rhodosporidium toruloides*-1588 using C5 and C6 wood hydrolysates as a substrate.

Materials and methods

Culture media and yeast preparation

Forestry residues of *Populus alba* were used by Greenfield Global Inc. (Varennes, Quebec, Canada) to produce C6 and C5 hydrolysates. The initial composition (before sterilization process) of C6 wood hydrolysate contains (g/L): glucose 117, xylose 11.8, formic acid 0.3, acetic acid 3.2, hydroxymethylfurfural 0.2, furfural 0.2, and C5 wood hydrolysate (g/L): xylose 120, glucose 10.1,

formic acid 5.6, acetic acid 33.7, hydroxymethylfurfural 0.1. Both hydrolysates were diluted using MilliQ water to obtain the specific sugar concentration (described below). Two hydrolysates were used separately as culture media to analyze the degradation of inhibitory compounds by *R. toruloides*-1588, obtained from the Agricultural Research Service (NRRL) Culture Collection (USA). *R. toruloides*-1588 was preserved using YM plates according with [171].

Inhibitor degradation study

Degradation studies were performed in a 250 mL flask using 50 mL of wood hydrolysate (separately) with initial pH of 6, 200 rpm for 128 hours at 25°C. The effect of four initial sugar concentrations (50 g/L, 75 g/L, 100 g/L, and 120 g/L) and the addition of four initial concentration of ammonium sulfate (0.87 g/L, 1.30 g/L, 1.74 g/L, and 2.08 g/L, respectively) as nitrogen source (only supplement in the culture media) on microbial inhibitory degradation compounds were tested. Total inhibitor degradation, inhibitor degradation rate (K_D), and specific inhibitor degradation rate with respect to biomass (K_{DB}) and lipid production (K_{DL}) were calculated using Equations 1, 2, 3, and 4, respectively.

$$Total\ inhibitor\ degradation = Initial\ concentration\ (mgL^{-1}) - Final\ concentration\ (mgL^{-1})$$

(Eq. 1)

$$K_D = \frac{Total\ inhibitor\ degraded\ (mgL^{-1})}{Total\ cultivation\ time\ (h)} \quad \text{(Eq. 2)}$$

$$K_{DB} = \frac{Total\ inhibitor\ degraded\ (mgL^{-1}) / Total\ biomass\ (gL^{-1})}{Total\ cultivation\ time\ (h)} \quad \text{(Eq. 3)}$$

$$K_{DL} = \frac{Total\ inhibitor\ degraded\ (mgL^{-1}) / Total\ lipids\ (gL^{-1})}{Total\ cultivation\ time\ (h)} \quad \text{(Eq. 4)}$$

Analytical methods

Cell harvesting

Harvesting of *R. toruloides*-1588 cells was performed according to Osorio-Gonzalez et al. [171]. Briefly, cells were harvested from the culture media by centrifugation at 12,000 *x* g/2 min. The supernatant was kept in a separate tube for further analysis and cells were washed three times by

centrifugation with 2 mL of phosphate buffer saline (PBS) each time, then cells were dried at 60°C, until constant weight.

Inhibitory compounds analysis

During fermentation, microbial inhibitory compounds were quantified following the protocol according to Osorio-Gonzalez et al. [124], [171]. Briefly, microbial inhibitor compounds were analyzed using a Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer (LC-MS), using a BetaBasic-18 column (100 mm x 2.1mm; 3µm) and water: methanol (80:20 v/v) solution as mobile phase. All microbial inhibitory compounds were identified and quantified by comparing with their respective standard (Sigma-Aldrich, USA).

Lipid quantification and fatty acid composition

Total lipid content was quantified gravimetrically and reported as accumulated lipid (percentage) of total biomass dry cell weight. Lipids were transformed to fatty acid methyl esters (FAMEs) by transesterification according to Saini et al. [185]. Briefly, a methanol:sulfuric acid solution was used at 100°C during 20 min. FAMEs were extracted with hexane and analyzed in an Agilent 7890B Gas Chromatography-Flame Ionization Detector (GC-FDI) and Agilent 122-2362 DB-23 column (60 m x 250 µm and 0.25 µm film thicknes). All fatty acid was identified and quantified by comparing with a Supelco 37 Component FAME Mix (Sigma-Aldrich, USA).

Statistical analysis

The statistical analysis of the data was conducted using OriginPro® 20.0 (OriginLab Corporation, USA). Sugar concentration and addition of ammonium sulfate effect on inhibitory compound degradation were analyzed by One-Way ANOVA with a Fisher test at 95% level of confidence. All data presented are average values from duplicate samples.

Results and discussion

Coactive inhibitory effect on *R. toruloides*-1588 growth and lipid accumulation

After the sterilization process, a significant change in the content of inhibitory compounds was observed in both hydrolysates. Table 13 shows the initial concentration of inhibitory compounds in which *R. toruloides* NRRL1588 was able to grow. The compounds found corroborated with the

ones reported in the literature as the main compound released by the dehydration of C5 and C6-carbon sugars (furfural, 5-HMF, levulinic acid, acetic acid) as well as by the oxidation of lignin fraction (vanillin, vanillic acid, syringaldehyde, aminobenzoic acid, ferulic acid) [6].

Table 13.Wood hydrolysate composition at the beginning of the fermentation.

Compounds (mg/L)	Initial sugar (g/L)	C5 wood hydrolysate				C6 wood hydrolysate				
		50	75	100	120	50	75	100	120	
Furfural		475.77	630.73	906.99	930.38	156.32	252.87	430.27	499.32	
5-Hydroxymethyl furfural		70.22	49.51	58.25	50.05	352.47	477.02	700.15	751.91	Supplemented
Levulinic acid		108.43	167.77	196.34	223.81	50.00	50.17	64.10	118.52	with
Vanillin		50.00	64.72	91.00	95.90	50.21	55.10	60.08	53.10	ammonium
Vanillic acid		50.00	124.25	106.71	187.55	50.13	55.30	60.40	53.03	sulfate
Syringaldehyde		80.50	120.72	137.77	154.83	50.09	50.15	63.41	72.95	
Furfural		574.56	806.19	995.16	1298.53	181.89	369.21	508.08	571.15	
5-Hydroxymethyl furfural		86.73	50.00	52.73	60.79	324.33	510.64	731.52	832.10	
Levulinic acid		120.79	181.05	178.45	257.30	55.00	69.03	55.10	223.91	Without
Vanillin		52.73	69.06	87.66	121.84	50.17	60.10	57.36	52.10	ammonium
Vanillic acid		81.35	115.96	140.04	195.53	54.05	60.26	57.18	52.71	sulfate
Syringaldehyde		99.17	108.60	147.12	207.73	66.00	48.16	68.63	79.05	

R. toruloides-1588 growth was different for each wood hydrolysate, and it was affected by the initial sugar concentration (Figure 37). For instance, in C6 wood hydrolysate, when an initial glucose concentration of 50 g/L was used, a slight lag growth phase was observed, followed by an exponential phase of 54 h. In treatments where an initial 75 g/L of glucose was used, a constant growth until 96 h was observed using C6 wood hydrolysate supplemented with $(NH_4)_2SO_4$. In treatments where initial 100 g/L and 120 g/L of glucose were used, an opposite growth tendency in comparison with the previous patterns was observed. For instance, in treatments with 100 g/L of initial glucose concentration a diauxic growth was observed with a slight growth phase during the first 18 h followed by a stationary phase of 54 h. Then a second exponential phase was observed in comparison with treatments where 120 g/L of initial glucose was used, where the microbial growth was constant until the end of the fermentation. For treatments where C5 wood hydrolysate was used, a similar growth tendency in all initial sugar concentrations was observed, showing an exponential growth from the beginning of fermentation until 96 hours, where a growth decrease was observed. The microbial growth observed in treatments where C5 wood hydrolysate was used as a culture media was used is similar to the reported by Jagtap and Rao [189], where *R. toruloides* shows short lag phase and a fast increase in microbial biomass using substrates rich in xylose and nitrogen. Nevertheless, the author reported a decrease in lipid production under the above condition, due to *R. toruloides* can use xylose to produce different sugar alcohols. Likewise, the lipid accumulation in this research agrees with the previous statement, due to the maximum lipid accumulation in treatments using C5 wood hydrolysate was only 30% of the dry cell weight. Furthermore, total glucose and xylose consumption was different between initial sugar concentration and between hydrolysates. Likewise, no effect was observed due to addition of ammonium sulfate as a supplement (Supplementary Figure 1). In overall, when C6 wood hydrolysate was used, a total glucose consumption was >90% for initial concentration of 50 g/L and 75 g/L, and <80% for initial concentrations of 100 g/L and 120 g/L. In treatment where C5 wood hydrolysate was used, a total xylose consumption was 50%, 60%, 70 and 80% for initial concentration of 50 g/L, 75 g/L, 100 g/L and 120 g/L, respectively. Moreover, the specific sugar consumption was observed during the first 18 hours of fermentation for all the tested treatments (Supplementary Figure 2).

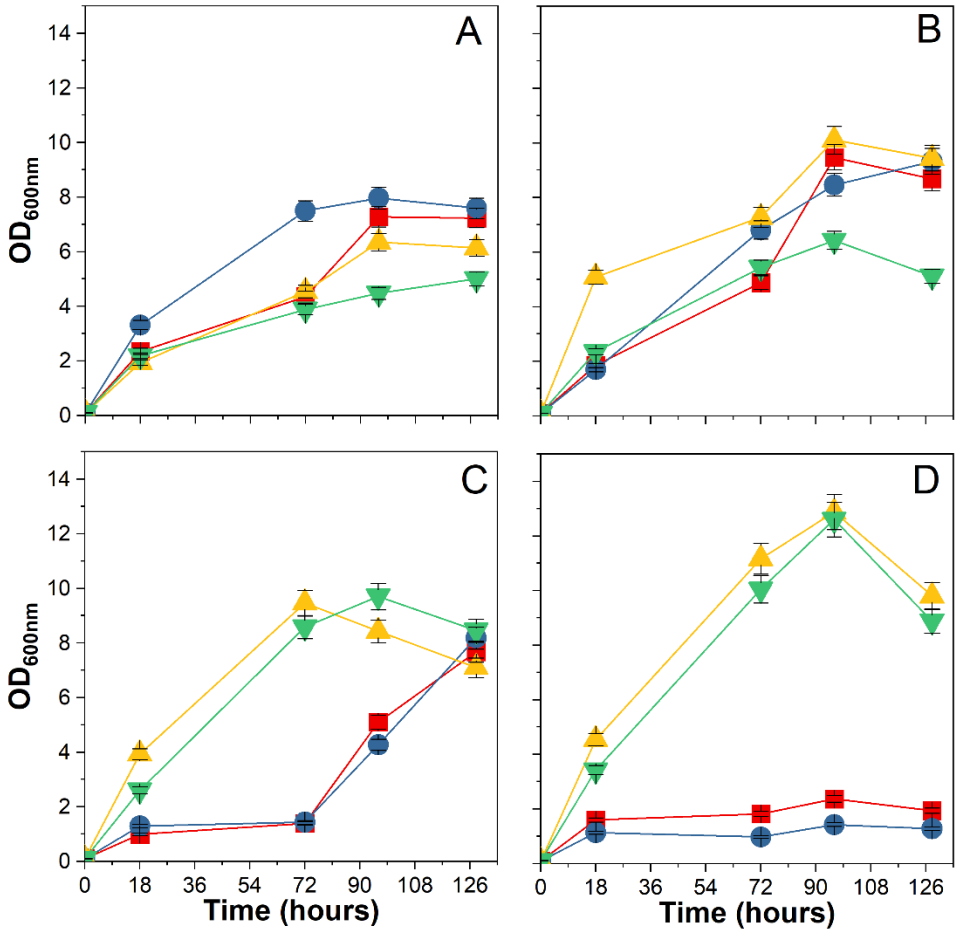

Figure 37.Growth of Rhodosporidium toruloides NRRL-1588 using C6 and C5 undetoxified wood hydrolysates as substrate. A) 50 g/L; B) 75 g/L; C) 100 g/L; D) 120 g/L. Red (C6 wood hydrolysate + ammonium sulfate); Blue (C6 wood hydrolysate); Yellow (C5 wood hydrolysate + ammonium sulfate); Green (C5 wood hydrolysate).

The coactive inhibitory effect of furfural, 5-HMF, levulinic acid, vanillin, vanillic acid and syringaldehyde on *R. toruloides*-1588 growth was observed after 96 h at 75 g/L of initial sugar concentration, with a partial growth inhibition (0.7 to 1.3-folds). Likewise, at the same initial sugar concentration, no inhibition of the growth was observed in treatments where C6 wood hydrolysate without $(NH_4)_2SO_4$ supplementation was used, where the microbial growth remained on exponential phase until the end of the fermentation. On another hand, no inhibition was observed in treatments where C6 wood hydrolysate at 100 g/L of initial sugar concentration was used.

Nevertheless, a slowdown in microbial growth was observed as a prolonged lag phase, this growth tendency suggests partial catabolic repression caused for the high amount of glucose in the culture media as well as for the simultaneous degradation of the inhibitory compounds. On other hand, at the same initial sugar concentration but using C5 wood hydrolysate as a culture media, inhibition of 1.33-folds was observed after 72 h in the hydrolysate supplemented with $(NH_4)_2SO_4$ and after 96 h in the hydrolysate without $(NH_4)_2SO_4$ addition. Last but not the least, to sum up, treatments where 120 g/L of initial sugar concentration was used, after 18 h a total inhibition was observed in C6 wood hydrolysate and after 96 h in C5 wood hydrolysate. Finally, we observe that the presence of the six inhibitory compounds in both hydrolysates caused a noticeable inhibition effect on microbial growth, especially at high initial sugar concentrations. Nevertheless, due to the variable concentration of each inhibitory compound, the possible number of chemical and biochemical reactions and their effects on *R. toruloides*-1588 turn into a very complex interactions, that can need the development of a more specific fermentation process.

Overall, *R. toruloides* NRRL 1588 is a suitable strain to produce microbial lipids using undetoxified wood hydrolysates. Furthermore, the obtained results confirm the tolerance and degradation capability of the yeast to microbial inhibitory compounds derived from lignocellulosic biomasses. Figure 38 shows the performance of *R. toruloides* NRRL 1588 in terms of lipid accumulation, where the highest lipid accumulation (>50%) was observed in treatments using C6 wood hydrolysate as a culture media. The maximum lipid accumulation (55%) was observed in treatments where C6 wood hydrolysate without $(NH_4)_2SO_4$ supplementation with an initial sugar concentration of 75 g/L. On another hand, the lipid production using C5 wood hydrolyzate as a culture media was similar between the four initial sugar concentrations. The maximum lipid accumulation (31%) was observed in treatments supplemented with $(NH_4)_2SO_4$ and 120 g/L of initial sugar concentration. The lipid composition profile under the tested conditions does not show major changes. Palmitic, stearic, and oleic acid were the three predominant fatty acids in all the samples with 10-25%, 5-20% and 30-50%, respectively (ANNEX 7).

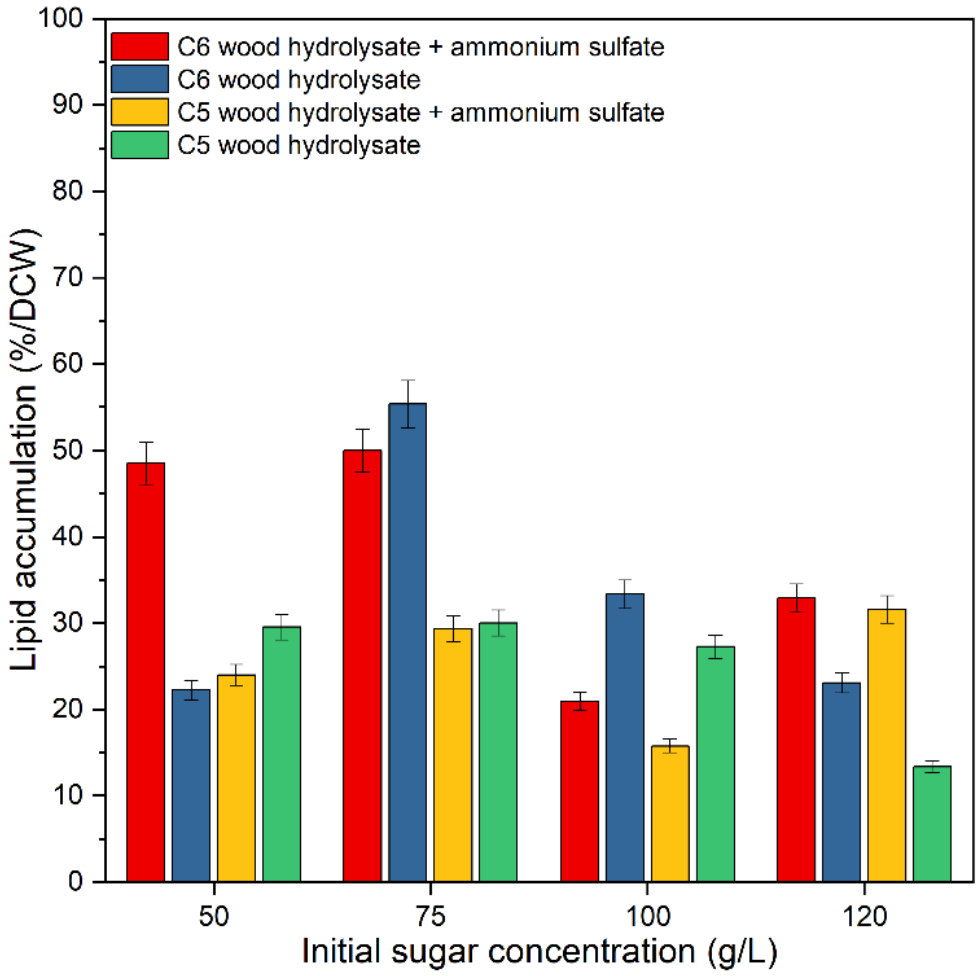

Figure 38.Growth of Rhodosporidium toruloides NRRL-1588 using C6 and C5 undetoxified wood hydrolysates as substrate. A) 50 g/L; B) 75 g/L; C) 100 g/L; D) 120 g/L. Red (C6 wood hydrolysate + ammonium sulfate); Blue (C6 wood hydrolysate); Yellow (C5 wood hydrolysate + ammonium sulfate); Green (C5 wood hydrolysate).

Furfural and 5-hydroxymethyl furfural degradation profile

Two of the most toxic compounds in hydrolysates obtained from lignocellulosic biomass are furfural and 5-HMF. Figure 39 shows furfural and 5-HMF degradation by *R. toruloides*-1588 using C6 and C5 wood hydrolysate. Overall, C6 wood hydrolysate with and without $(NH_4)_2SO_4$ addition does not show a significant effect (p-value=0.575) on furfural degradation, with 96.3% and 96.2% of total furfural degradation, respectively. Furthermore, approximately 50% of total furfural was degraded during the first 18h of fermentation regardless of the initial sugar concentration.

Thereafter, furfural degradation was negligible from 18 to 72 h, where a secondary degradation step was observed until the end of the fermentation when almost all furfural was degraded. In the cases where C5 wood hydrolysate without $(NH_4)_2SO_4$ addition was used, the furfural degradation was 82.5% and 80.7%, respectively. According to the initial sugar concentration, the degradation profile presented a similar tendency to C6 wood hydrolysate. This means that from 40% to 50% of furfural degradation was observed during the first 18 h, followed by a no degradation step up to 54 h, for increasing the degradation after 54 h up to 75%. The obtained results support the statement that *R. toruloides* NRRL-1588 is a robust strain capable to degrade high concentrations (>1 g/L) of furfural in comparison with other strains such as *R. glutinis* 2.107, *R. glutinis* 2.704, and *R. toruloides* 2.1389 (Table 14). Chen et al. [84] found that to cease the growth, 1 g/L of furfural was needed in the case of *R. glutinis* 2.107 and 0.5 g/L for *R. glutinis* 2.704, and *R. toruloides* 2.1389, using synthetic media supplemented with yeast extract and micronutrients as a substrate. Similar results were observed by Zhao et al. [78] in *R. toruloides* 2.1389 with a decrease of 60% on cell biomass accumulation using sugarcane bagasse hydrolysate as substrate. Nevertheless, the toxicity effect of furfural on *R. toruloides* is highly dependent on each strain. Some studies indicate that even low furfural concentrations, as low as 1 mM and 8 mM, can cause total growth inhibition on *R. toruloides* Y4 and *R. toruloides* NRRL Y-1091, respectively [75], [208].

Table 14.Degradation studies of inhibitory compounds using Rhodosporidium toruloides.

	Inhibitor degradation studies							
	Liu et al. [211]	Liu et al. [208]	Bonturi et al. [52]	Poontawee et al. [47]	Zhao et al. [78]	Chen et al. [84]	This study	This study
Oleaginous yeast	*R. toruloides* 1091	*R. toruloides* 1091	*R. toruloides* CCT 0783	*R. fluviale* DMKURK 253	*R. toruloides* AS 2.1389	*R. toruloides* 2.1609	*R. toruloides* 1588	*R. toruloides* 1588
Substrate	Wheat straw hydrolysate	Synthetic media[§]	Sugar cane bagasse hydrolysate	Synthetic media[t]	Detoxified sugarcane bagasse hydrolysate	Corn stover hydrolysate	C6 wood hydrolysate	C5 wood hydrolysate
Furfural (mg/L)	81	768	1700	500	1000	320	600	1300
5-HMF (mg/L)	8	378	200	4000	nr	1000	750	100
Levulinic acid (mg/L)	427	1700	nr	nr	nr	1490	120	260
Ferulic acid (mg/L)	nr	1900	nr	nr	nr	nr	nr	nr
Syringaldehyde (mg/L)	42	728	nr	nr	nr	nr	80	210
Vanillin (mg/L)	22	456	nr	1000	1500	61	60	120
Vanillic acid (mg/L)	19	1600	nr	nr	nr	nr	60	120
p-hydroxybenzaldehyde (mg/L)	37	244	nr	nr	nr	103	nr	nr

nr: no reported

[§]Synthetic media was supplemented with leucine and glucose and inhibitory compounds were tested individually.

[t]Synthetic media was supplemented with a trace element solution.

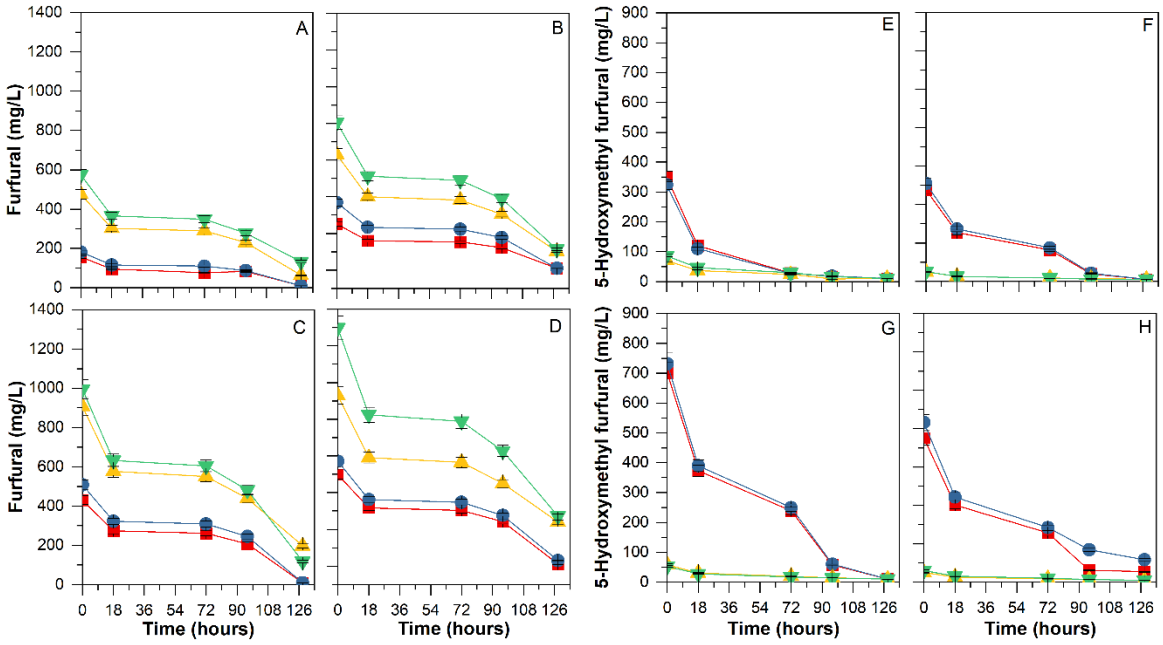

Figure 39.Furfural and 5-HMF degradation by R. toruloides-1588 using wood hydrolysate as a culture media. A,E) 50 g/L; B,F) 75 g/L; C,G) 100 g/L; D,H) 120 g/L. Red (C6 wood hydrolysate + ammonium sulfate); Blue (C6 wood hydrolysate); Yellow (C5 wood hydrolysate + ammonium sulfate); Green (C5 wood hydrolysate).

Table 15 shows the total and specific degradation rates of furfural and 5-HMF by *R. toruloides*-1588 using undetoxified wood hydrolysate as a substrate. In summary, furfural and 5-HMF do not show an inhibitory effect in microbial growth of *R. toruloides*-1588. It is worth noting that the maximum degradation rate (K_D) and specific degradation rate with respect to biomass production (K_{DB}) for both compounds were observed in treatments with high initial sugar concentration (120 g/L) in both hydrolysates. Nevertheless, a different pattern was observed on specific degradation rate with respect to lipid production (K_{DL}), where the maximum K_{DL} for furfural was observed at low initial sugar concentration (50 g/L) in both hydrolysates. Nevertheless, in the case of 5-HMF, the maximum degradation was observed in treatments where hydrolysates supplemented with $(NH_4)_2SO_4$ and 100 g/L of initial sugar concentration. These results confirm that furfural is more difficult to degrade by *R. toruloides*-1588 than 5-HMF, considering the same initial sugar concentration. The obtained results show that the increase of furfural in the culture media is proportional to the increase of initial sugar content and inversely proportional to the microbial

growth. The decrease in microbial growth because of high concentrations of furfural in the culture media can be caused by the inhibition of enzymes with an essential role in metabolic activity (pyruvate dehydrogenase, alcohol dehydrogenase, acetaldehyde dehydrogenase) as has been reported [100]. Some of the consequences caused by inhibition of some of these enzymes is a decrease of nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide hydrogen (NAD(H)), both essential electron donors used as reducing power for redox balance and anabolic reactions as well as for energy production in form of adenosine triphosphate (ATP). Furthermore, these compounds can induce the activation of energy flux change to repair the damages that cause the accumulation of reactive oxygen species to the vacuole and mitochondrial membranes [6], [100], [214].

Table 15.Inhibitory compounds degradation by R. toruloides-1588 using C5 and C6 wood hydrolysate as a culture media.

	Treatments															
	C6 hydrolysate + nitrogen				C6 hydrolysate				C5 hydrolysate + nitrogen				C5 hydrolysate			
	50	75	100	120	50	75	100	120	50	75	100	120	50	75	100	120
Furfural degradation																
K_D	1.143	1.897	3.283	3.823	3.222	4.131	5.543	5.398	1.343	2.806	3.891	4.23	3.453	5.399	6.854	8.017
K_{DB}	0.084	0.082	0.156	1.3	0.676	0.782	0.746	0.773	0.105	0.192	0.331	1.785	0.333	0.42	0.457	1.068
K_{DL}	0.002	0.001	0.001	0.001	0.034	0.033	0.028	0.025	0.002	0.001	0.001	0.002	0.058	0.047	0.018	0.028
5-Hydroxymethyl furfural degradation																
K_D	2.676	3.649	5.392	5.450	0.470	0.309	0.377	0.313	2.456	3.911	5.637	5.590	0.600	0.313	0.334	0.397
K_{DB}	0.185	0.152	0.251	1.817	0.086	0.049	0.040	0.033	0.182	0.261	0.470	2.236	0.044	0.021	0.020	0.042
K_{DL}	0.054	0.053	0.067	0.056	0.032	0.013	0.007	0.004	0.049	0.059	0.073	0.059	0.034	0.016	0.007	0.005
Levulinic acid degradation																
K_D	0.313	0.314	0.423	0.805	0.741	1.146	1.272	1.444	0.352	0.461	0.291	0.458	0.73	1.199	1.177	1.703
K_{DB}	0.027	0.016	0.023	0.309	0.154	0.208	0.161	0.186	0.032	0.036	0.036	0.237	0.07	0.094	0.082	0.212
K_{DL}	0.002	0.001	0.001	0.001	0.007	0.007	0.005	0.004	0.002	0.001	0.002	0.001	0.012	0.011	0.004	0.004
Syringaldehyde degradation																
K_D	0.313	0.313	0.417	0.433	0.327	0.586	0.687	0.800	0.438	0.298	0.458	0.446	0.350	0.426	0.728	1.151
K_{DB}	0.022	0.013	0.019	0.144	0.059	0.093	0.072	0.085	0.032	0.020	0.038	0.179	0.026	0.028	0.043	0.121
K_{DL}	0.006	0.005	0.005	0.004	0.022	0.024	0.012	0.011	0.009	0.005	0.006	0.005	0.020	0.022	0.015	0.015
Vanillin degradation																
K_D	0.313	0.352	0.391	0.336	0.242	0.318	0.503	0.532	0.313	0.391	0.367	0.328	0.174	0.311	0.456	0.671
K_{DB}	0.022	0.015	0.018	0.112	0.044	0.050	0.053	0.057	0.023	0.026	0.031	0.131	0.013	0.021	0.027	0.071
K_{DL}	0.006	0.005	0.005	0.003	0.016	0.013	0.009	0.007	0.006	0.006	0.005	0.003	0.010	0.016	0.009	0.009
Vanillic acid degradation																
K_D	0.313	0.352	0.391	0.336	0.181	0.219	0.518	0.465	0.344	0.391	0.367	0.328	0.296	0.569	0.762	1.032
K_{DB}	0.020	0.017	0.014	0.110	0.033	0.035	0.054	0.049	0.025	0.026	0.031	0.131	0.022	0.038	0.045	0.109
K_{DL}	0.006	0.005	0.005	0.003	0.012	0.009	0.009	0.006	0.007	0.006	0.005	0.003	0.017	0.030	0.015	0.014

50, 75, 100, 120= Initial sugar concentration

K_D= Degradation rate (mgL h-1)

K_{DB}= Specific degradation rate (mgL^{-1} of inhibitor/gL h^{-1} of biomass) K_{DL}= Specific degradation rate (mgL^{-1} of inhibitor/gL h^{-1} of lipids)

Levulinic acid degradation profile

Other inhibitory compounds such as levulinic, formic and acetic acids can be formed due to the hydration of hydroxymethylfurfural and the deacetylation of hemicellulose. Figure 40 shows the levulinic acid degradation by *R. toruloides*-1588 using C6 and C5 wood hydrolysate as substrate. Unlike observed in furfural degradation, the supplementation of C6 wood hydrolysate with $(NH_4)_2SO_4$ increased by 4.7% the levulinic acid degradation (82.85%) in comparison to C6 wood hydrolysate without supplementation (78.10%). The same tendency was observed in C5 wood hydrolysate supplemented with $(NH_4)_2SO_4$ with an increase of 2.3% in the levulinic acid degradation, in comparison to C5 wood hydrolysate without supplementation. In addition, the degradation rate of levulinic acid and 5-HMF by *R. toruloides* NRRL 1588 presented a similar tendency, where the high degradation rates (K_D, and K_{DB}) were observed at high initial sugar concentration (120 g/L). However, levulinic acid degradation showed an opposite pattern with respect to the lipid production (K_{DL}). High degradation rates were observed at low initial sugar concentration (50 g/L) in both hydrolysates (Table 15). This result suggests that the addition of an external nitrogen source such as $(NH_4)_2SO_4$ can act as a buffer towards the acidity in the media caused by the presence of levulinic acid. Weak acids can traverse the membrane and reach the cytosol because of their low pKa value (4.79) which let to their diffusion through the membrane. Once in the cytosol, weak acids decrease the pH. Furthermore, due to the damage caused to the plasma membrane, the cell requires high amounts of ATP to be used in the conversion of adenosine diphosphate phosphorylation (ADP) and in microbial respiration. These compounds can also inhibit the maleic enzyme, which is a key enzyme to produce nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor used in the tricarboxylic acid cycle (TCA cycle) to carry out anabolic reactions [6], [100]. Nevertheless, some studies reported that the individual inhibition effect caused by levulinic acid in *R. toruloides* strains is minor in comparison to the inhibition effect induced by acetic or formic acids. The inhibitory effect is also defined by the metabolism of each strain. For instance, Chen et al. [84] reported that *R. toruloides* 2.1389, *R. glutinis* 2.704 and *R. glutinis* 2.107 were capable of growing and accumulating lipids in culture media containing 10 g/L of levulinic acid. Conversely, Liu et al. [208] observed total growth inhibition in *R. toruloides* NRRL Y-1091 with a concentration of 1.74 g/L of levulinic acid in the media.

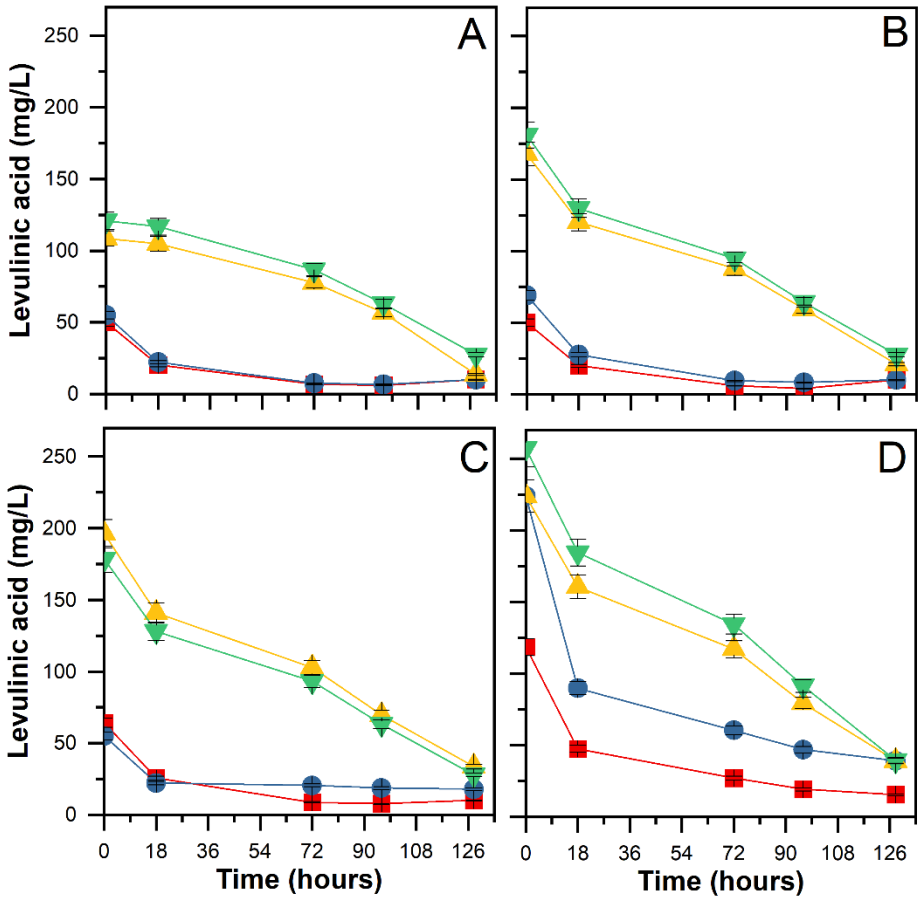

Figure 40.Levulinic acid degradation by R. toruloides-1588 using wood hydrolysate as a culture media. A) g/L; B) 75 g/L; C) 100 g/L; D) 120 g/L. Red (C6 wood hydrolysate + ammonium sulfate); Blue (C6 wood hydrolysate); Yellow (C5 wood hydrolysate + ammonium sulfate); Green (C5 wood hydrolysate).

Phenolic compounds degradation profile

Among the most common toxic compounds released from the lignin fraction, there are aldehydes, acids, and alcohols that are classified as phenolic compounds because all of them share an aromatic ring in their chemical structure. Overall, the phenolic compounds affect the microorganism in two ways: i) affect the cell membrane by changing its hydrophobicity allowing the entrance of external compounds to the cell, and ii) cause DNA mutagenesis by increasing the reactive oxygen species [215], [216]. Figure 41 shows the degradation profile of syringaldehyde, vanillin and vanillic acid. In the case of syringaldehyde, 80% and 60% of total degradation were observed in C6 and C5

wood hydrolysate, respectively. Furthermore, the addition of $(NH_4)_2SO_4$ does not show a significant effect on syringaldehyde degradation (p-value=0.245 and p-value=0.568, respectively). These results are promising in comparison to reported results in which, growth inhibition of *R. toruloides* NRRL Y-1091 and *R. toruloides* Y4 has been observed at 7mM and 12 mM, respectively [75], [208]. On the other hand, *R. toruloides* NRRL-1588 used in this study was capable of growing in wood hydrolysate with a syringaldehyde concentration of 200 mg/L. Similarly, a maximum total degradation of 81% and 66% was observed in the case of vanillin in C6 and C5 wood hydrolysates, respectively. Furthermore, in treatments where C5 wood hydrolysate supplemented with $(NH_4)_2SO_4$ was used as a culture media, an increase of 5.66% in comparison with syringaldehyde degradation was observed. These are positive results because vanillin has been considered a strong hazardous compound for several microorganisms included oleaginous yeast such as *Rhodosporidium toruloides* [154], [217]. The maximum degradation of vanillic acid in C6 and C5 wood hydrolysates was 86% and 61%, respectively. Nonetheless, a decrease of 10% in the total degradation was observed in treatments where C5 wood hydrolysate was supplemented with $(NH_4)_2SO_4$. Although vanillic acid degradation decreases using C5 wood hydrolysate supplemented with $(NH_4)_2SO_4$, the results remain promising to use *R. toruloides* NRRL-1588 for lipid production. The above fact is supported with microbial growth, where a short lag phase (18 h) was observed. Conversely, Liu et al. [211] reported an increase of the microbial lag phase due to the increase of vanillic acid in the culture media.

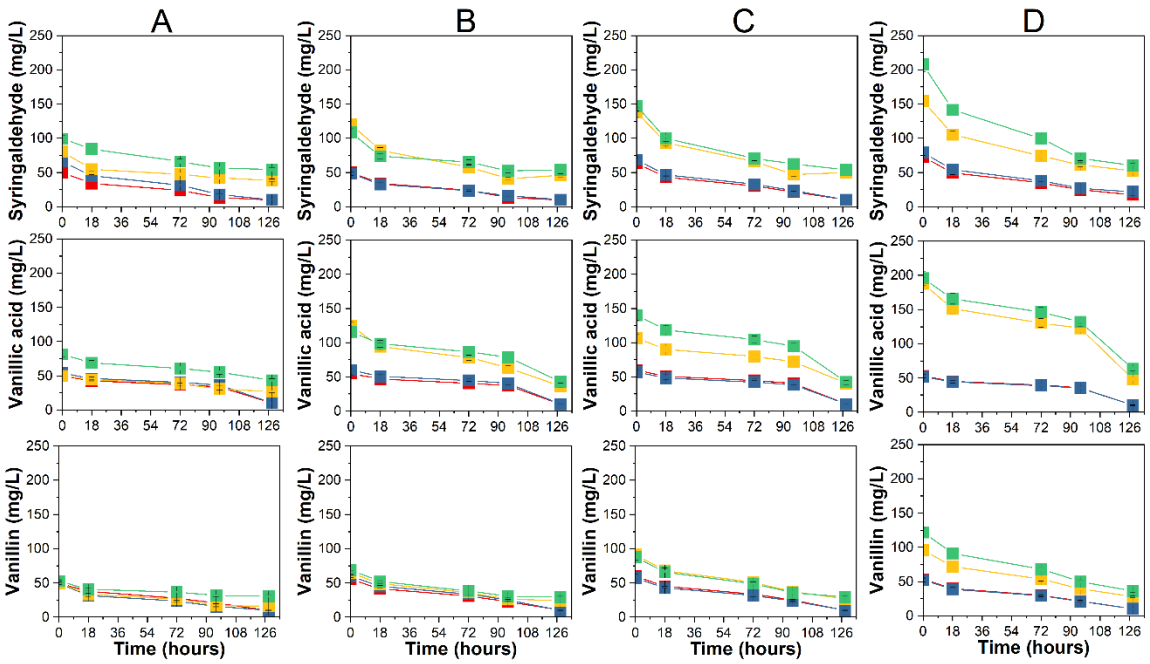

Figure 41.Phenolic compounds degradation by R. toruloides-1588 using C5 and C6 wood hydrolysate as a culture media. A) 50 g/L; B) 75 g/L; C) 100 g/L; D) 120 g/L. Red (C6 wood hydrolysate + ammonium sulfate); Blue (C6 wood hydrolysate); Yellow (C5 wood hydrolysate+ ammonium sulfate); Green (C5 wood hydrolysate).

Conclusion

Rhodosporidium toruloides-1588 was capable to grow in raw and undetoxified C5 and C6 wood hydrolysate obtained from forestry residues. Furthermore, was able to degrade up to 96% of furfural, and 5-HMF, 85% of levulinic acid, 80% of syringaldehyde, 81% of vanillin and 86% of vanillic acid. In addition, 5-hydroxymethyl furfural was the compound with the fastest degradation (5.450 mgL^{-1} h^{-1}). This means that *R. toruloides*-1588 is a promising microorganism to use lignocellulosic biomass hydrolysates to produce microbial lipids. Likewise, this work can be used as a baseline to develop specific studies at a proteomic level to understand in a better way the effects of these compounds on this promising yeast.

CRediT author statement

Carlos S. Osorio-González.: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft. **Rahul Saini**.: Formal analysis, Investigation. **Krishnamoorthy Hegde**.:

Supervision, Writing-Review & Editing. **Satinder Kaur Brar**.: Funding acquisition, Supervision, Writing-Review & Editing. **Alain Lefebvre**.: Resources. **Antonio Avalos-Ramírez**.: Funding acquisition, Writing-Review & Editing, Supervision.

Supplementary data for this work can be found in ANNEX 6.

6.2 Furfural degradation and its effect on *Rhodosporidium toruloides*-1588 during microbial growth and lipid accumulation

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C. S. Osorio-González *et al.*, "Furfural degradation and its effect on Rhodosporidium toruloides-1588 during microbial growth and lipid accumulation,". ***Bioresource Technology. Under revision.***

Abstract

The presence of furfural in the hydrolysates obtained from lignocellulosic biomass sources represents an enormous challenge during the performance of fermentation. *Rhodosporidium toruloides*-1588 has shown the capacity to grow and accumulate lipids in presence of 1 g/L of furfural using wood hydrolysate as substrate. In this study, the capacity of *R. toruloides*-1588 to growth, accumulate lipids has been tested using four initial concentrations of furfural with the absence of glucose in the media. *R. toruloides*-1588 degraded furfural into furfuryl alcohol (1.8 g/L), and 2-furoic acid (0.9 g/L) as well as 2-furoic acid into 2-oxoglutaric acid (0.1 g/L). Furthermore, *R. toruloides*-1588 was able to grow and accumulate lipids with a maximum biomass of 8.4 g/L and a lipid accumulation of 4.0 g/L. *R. toruloides*-1588 also accumulated lipids using furfural as a potential energy source.

Keywords Degradation, *Rhodosporidium toruloides*-1588, Furfural, Furfuryl alcohol, Furoic acid, Oxoglucaric acid

Introduction

Currently, more than 80% of the liquid fuel consumed around the world is from fossil sources, causing irreparable damage to the environment. To decrease the environmental damage, options like bioethanol and biodiesel have been developed during the last two decades [218]. Especially, biodiesel is a renewable biofuel produced using several lipid sources (vegetable oil, cooking waste oil, animal grease and tallow) as a feedstock. However, this production process is not profitable and requires subsidies to allow its commercialization [219], [220]. The lack of profitability is caused mainly by four main factors: i) feedstock availability, ii) feedstock cost, iii) production cost and feasibility, and iv) the ethical dilemma of food vs fuels due to the increase of population with the demand of both goods, the necessity of alternatives is needed.

Currently the research community has been proposed two alternatives to overcome the above-raised concerns. Firstly, the use of lignocellulosic biomass as a renewable raw material to produce suitable substrates that can be used as a carbon source to produce biofuels [188], [218]. Secondly, microbial cell oil is a renewable and sustainable feedstock that does not compete with food security. Thus, lignocellulosic biomass has been considered a sustainable feedstock during the past two decades due to its high abundance (180 billion tones/year), low cost, renewable nature, high sugar content in form of cellulose and hemicellulose (~60%). These characteristics make

lignocellulosic biomass a suitable raw material to produce substrates rich in sugars that can be used as an energy source in biochemical processes to produce biofuels, chemicals, flavours, among others. However, to recover the sugars, lignocellulosic biomass has to be pretreated to overcome the recalcitrate nature of the matrix, where extensive research and technology have been developed on different strategies to obtain sugars from lignocellulosic biomass [221]–[224]. Nevertheless, as a result of the use of these pre-treatments, several by-products are released along with the sugars. The abundance and type of these compounds depend specifically on the method and conditions in which the lignocellulosic biomass has been treated. These compounds are classified mainly into three groups or categories: phenolic compounds (syringaldehyde, vanillin, aminobenzoic acids, catechol), furan derivatives (furfural and 5-hydroxymethyl furfural) and aliphatic acids (levulinic acid, ferulic acid, acetic acid) [6]. Among these compounds, furfural is the predominant (from 0.4 to 2.8 g/L) and the hazardous compound obtained in most of the treatments used [85]. Likewise, it has been reported that this compound cause inhibition of microbial growth, as well as key enzymes such as alcohol, acetaldehyde, and pyruvate dehydrogenases, causing a decrease in bioprocess performance [100], [208].

On the other hand, oleaginous microorganisms such as algae, bacteria, fungi, and yeast are a prominent alternative to produce microbial lipids, due to these microorganisms can accumulate more than 20% of lipids of their total cell mass. Among those microorganisms, the yeast *Rhodosporidium toruloides* has gained attention due to its high capacity to accumulate lipids (up to 70%), produce carotenoids, ability to use a wide variety of substrates as an energy source, capacity to tolerate microbial inhibitory compounds, among others [44], [225]. It has been reported that the presence of furfural in lignocellulosic biomass hydrolysates and its tolerance by oleaginous yeast are critical steps during the lipid production process [140]. So far, the furfural metabolic pathway has not been totally elucidated in prokaryotes and the research about the subject for eukaryotes is very limited or almost null. For instance, it has been observed that several strains of the *Rhodosporidium toruloides* genre have the capacity to degrade furanic aldehydes. Nevertheless, the degraded pathway and identification of the two main by-products like furfuryl alcohol and 2-furoic acid has not been reported. The above fact raises the question of whether *R. toruloides* can degraded or transform these compounds [226].

As per previous works, [171], and [161], [185], *R. toruloides*-1588 has shown tolerance to high furfural concentrations (~1 g/L) present in wood hydrolysates obtained from forestry residues. This ability makes this strain a promising strain to be used at an industrial scale to produce lipids and carotenoids using liquid hydrolysates obtained from lignocellulosic biomass. The main aim of this work is to study the degradation and degradation of furfural by *R. toruloides*-1588 as well as its effect on lipid accumulation and yeast performance. This study also tests the capacity of *R. toruloides*-1588 to use furfural as a potential energy source, due to the degradation has been observed in presence of a carbon source [227].

Materials and methods

Culture media and seed culture preparation

Oleaginous yeast R. toruloides-1588 was purchased from the Agricultural Research Service Culture Collection (NRRL, USA). YM media (yeast extract 3g/L; malt extract 3 g/L; peptone 5 g/L; glucose 10 g/L; and bacteriological agar 15 g/L) was used for the growth and preservation of the yeast. A three-generation culture seed was produced at 25°C, and 200 rpm in a Multitron Shaker Incubator (Infors, USA).

Furfural metabolize-degradation and lipid accumulation studies

R. toruloides-1588, was cultured in a 250 mL-flask with 50 mL of YM broth initial pH of 6, for 72 hours, at 25°C and 200 rpm. To study furfural degradation, and its effect on *R. toruloides*-1588 biomass production, sugar utilization, lipid accumulation, and fatty acid distribution , YM broth containing four initial furfural concentrations (0.5, 1.0, 2.0, and 4.0 g/L, respectively) was used. A separate set of treatments with the same furfural concentration using YM broth without glucose (yeast extract 3 g/L; malt extract 3 g/L; peptone 5 g/L) was used to test the ability of *R. toruloides*-1588 to use furfural as an alternative energy source.

Analytical analysis

Furfural, furfural derivates and glucose analysis

Furfural, 2-furoic acid, 2-oxoglutaric as well as glucose was quantified using an Orbitrap Elite Liquid Chromatography coupled to a Mass Spectrometry detector (Thermo Fisher Scientific, Inc., USA). For furfural analysis, a Beta-C18® column (20 cm x 200 Å, 5 µm, and 150 µm) was used with a mobile phase of formic acid (0.1%), and 0.1% of formic acid in acetonitrile in an isocratic

gradient of 75:25, respectively, with mass spectrometry detector in positive mode. In the case of 2-furoic acid and 2-oxoglutaric acid, column, and mobile phase were the same as described previously for furfural analysis, with the difference that the mass spectroscopy detector was used in negative mode. Glucose detection was performed using a HILIC column (5 μm x 15 cm and 4.6 mm) using a solution of acetonitrile: water (89:11 v/v) as mobile phase and D6 glucose as an internal standard. Finally, furfuryl alcohol was quantified using an Agilent 7890B Gas Chromatography-Flame Ionization Detector (GC-FDI) and a NUKOL® capillary column (15 m × 0.25 mm and 0.25 μm). All compounds were determined and quantified according to the peak area and retention time of their corresponding standard compound (Sigma-Aldrich, USA).

Cell growth and lipid extraction

Microbial growth, lipid extraction and lipid quantification were quantified according to our previous work [161], [171], [185]. Briefly, microbial biomass was harvested at 12000 x *g* for 2 minutes, and biomass concentration was determined by dry constant weight (DCW) and expressed as g/L. Microbial biomass yield was quantified and expressed as g of microbial biomass/g of sugar consumed. Briefly, for lipid extraction, cells were harvested (12000 xg/2 min) and dried at 60ºC for 24 hours. After that, an HCl solution was used for cell wall disruption and a solution of chloroform/methanol (2:1 v/v) for lipid extraction. Total lipids were quantified gravimetrically and expressed as g/L.

Fatty acid methyl ester quantification

A transesterification reaction of extracted lipids using methanol-sulfuric acid as reactant-catalyst at 100±1°C for 20 minutes was used to obtain fatty acid methyl esters. Then, an incubation time of 10 minutes at room temperature was performed. After that, hexane was added in a ratio of 1:1 v/v to carry on the fatty acid methyl esters extraction. Then, samples were centrifuged at 5000 rpm for 10 minutes at room temperature, and fatty acid methyl esters were analyzed using an Agilent 7890B Gas Chromatograph coupled to a Flame Ionization Detector (GC-FID) [171], [185]. All fatty acid methyl ester were identified and quantified according to the retention time and peak area of a Supelco 37 Component FAME Mix standard mixture (Sigma-Aldrich, USA).

Statistical analysis

The statistical analysis of the data was performed using OriginPro® (OriginLab2021, USA). Parameter interaction and effects on all response variables were analyzed by One-Way ANOVA (Fisher test) with an $\alpha=0.05$. Additionally, a Pearson correlation analysis was performed with 95% of confidence. All the experiments were carried out in duplicates.

Results and discussion

Furfural metabolize-degradation studies

Figure 42 shows furfural degradation by *R. toruloides*-1588. A total degradation of furfural was observed in YM media at 8 h and 24 h of fermentation on treatments where initial furfural concentrations of 0.5 g/L and 2 g/L were used. However, when an initial furfural concentration of 4 g/L was used, a total furfural degradation of 71% was observed. When YM media without glucose was used as a culture media, a total degradation was observed only in treatments where an initial furfural concentration of 0.5 g/L was used. In treatments with an initial furfural concentration of 1, 2 and 4 g/L, a total degradation of 97%, 82% and 54% was observed, respectively. These results confirm the statement that *R. toruloides* NRRL-1588 is capable of degraded furfural concentrations higher than 1 g/L in comparison with similar strains such as *R. toruloides* Y4, *R. glutinis* 2.704, *R. toruloides* 2.1389, *R. toruloides* NRRL Y-1091 and *R. glutinis* 2.107, where furfural concentrations of 0.1, 0.5, 0.7 and 1 g/L caused total inhibition of the microbial growth [75], [78], [84], [208]. In addition to the above results, *R. toruloides*-1588 show the capacity to grow using furfural as a possible energy source which from the process point of view is an excellent result due to a wide variety of renewable substrates obtained from lignocellulosic biomass containing high furfural concentration making it difficult to use it as a culture media in the fermentation.

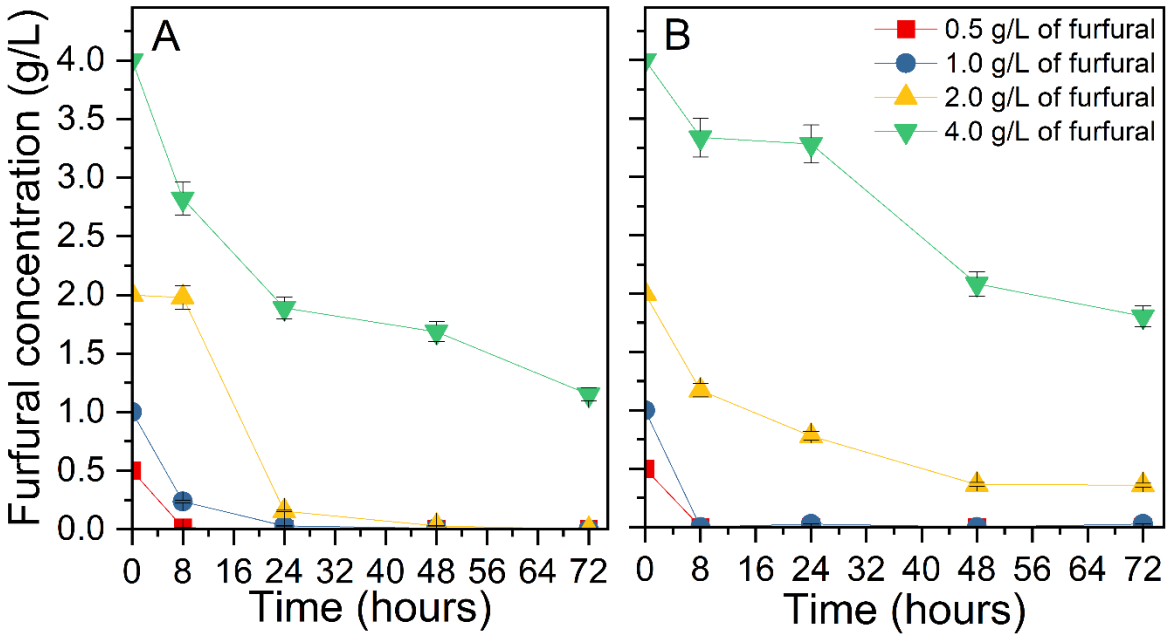

Figure 42.Metabolize-degradation kinetic of furfural by R. toruloides-1588. Furfural concentrations: Square – 0.5 g/L; Circle – 1 g/L; Up-Triangle – 2 g/L; Down-Triangle – 4 g/L.

Furfural metabolize products

It has been reported that some native species of bacteria like *Ureibacillus thermosphaericus, Coniochaeta ligniaria, Enterobacter* sp, and *Issatchenkia occidentalis,* can degraded furfural through the Trudgill pathway, a series of redox reactions catalyzed by furoyl-CoA dehydrogenase, furoyl-CoA synthetase or aldehyde dehydrogenase with furfuryl alcohol or 2-Furoic acid as by-products [33], [35]. Nevertheless, so far, no proteomic studies have been performed on *R. toruloides* to identify and characterize the specific enzymes responsible for furfural degradation. In this sense, we hypothesize the furfural degradation by the use of aldehyde dehydrogenase (RHTO_05838, RHTO_04425, RHTO_04310, RHTO_06724, RHTO_04543), an alcohol dehydrogenase (RHTO_05600) or through an oxidoreductase (RHTO_01466, RHTO_05740) enzymes that have been reported by Zhu et al. [177] in a multi-omic map analysis of *Rhodosporidium toruloides* NP11.

Furfuryl alcohol content

Furfural degradation into furfuryl alcohol can be carried on through an alcohol dehydrogenase or an oxidoreductase enzyme. The exact mechanism to reduce furfural in *Rhodosporidium toruloides* has not been elucidated or studied in any way. Figure 43A shows the proposed pathway using an

alcohol dehydrogenase that consists of a redox reaction using CH-CH groups as electron donors, reducing NAD^+ or NADH. In the case of oxidoreduction, due to the aromatic nature furfural is reduced into alcohol by a hydrogen-mediated reduction in presence of NAD^+ or unpaired electrons.

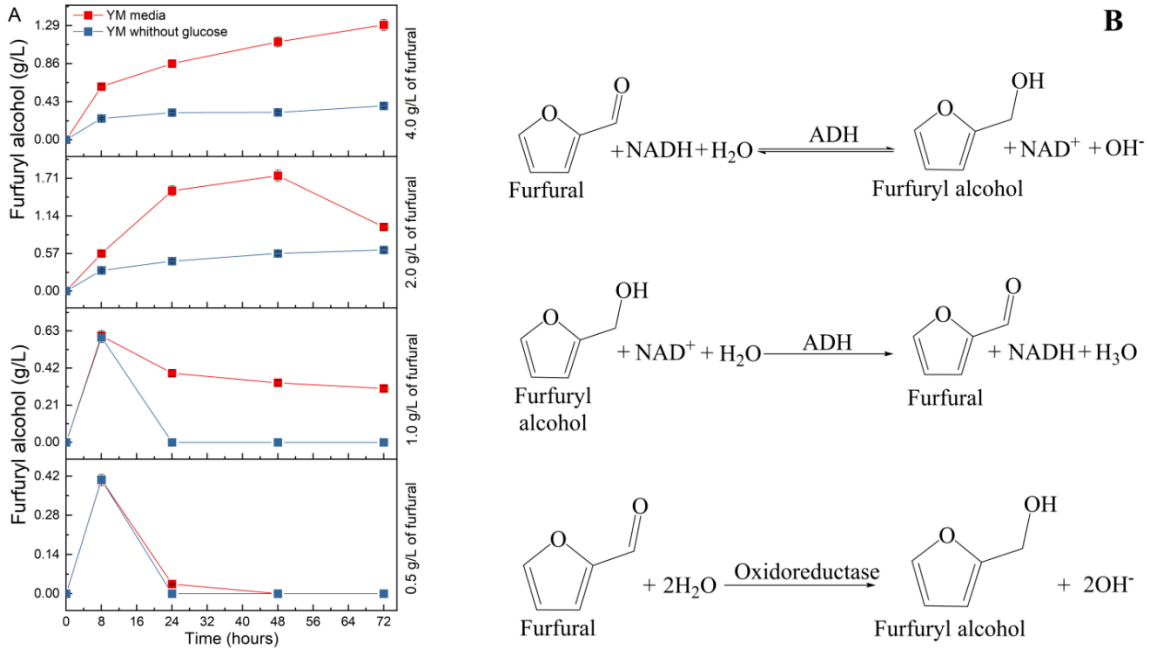

Figure 43.Furfuryl alcohol production by R. toruloides-1588. A) Enzymatic degradation reactions; B) Furfuryl production. ADH (Alcohol dehydrogenase). Furfural concentrations: Square – 0.5 g/L; Circle – 1 g/L; Up-Triangle – 2 g/L; Down-Triangle – 4 g/L.

Figure 43B shows the production of furfuryl alcohol from furfural degradation by *R. toruloides*-1588. The observed results show a similar production of ~0.4 g/L of furfuryl alcohol in both culture media with an initial furfural concentration of 0.5 g/L was used. For treatments where an initial furfural concentration of 1 g/L was used, maximum production of 0.6 g/L was observed in both culture media. However, a fast decrease was observed in treatments where YM media without glucose was observed in the next 8 hours after reaching the maximum production (8 h) in comparison with the observed decrease in treatment using YM media with a decrease of ~50% at the end of the fermentation. In treatments where an initial furfural concentration of 2 g/L, the maximum furfuryl alcohol concentration was observed in YM media (1.7 g/L) at 48 h with a decrease of 55% during the next 24 h. Conversely, in treatments where YM media without glucose was used, a constant production was observed through all the fermentation reaching the maximum

production of 0.6 g/L at 72h. Finally, furfuryl alcohol production in treatments where YM without glucose an initial furfural concentration of 4 g/L shows a maximum production of 0.3 g/L, 50% less production in comparison when 2 g/L of furfural was used. In treatments where YM media was used, maximum production of 1.2 g/L was obtained at the end of the fermentation. Along with the fact that the obtained results are very promising from the point of view to use undetoxified lignocellulosic biomass hydrolysates as a culture media in bioprocess using *R. toruloides*-1588. The degradation of furfural into furfuryl alcohol with *R. toruloides*-1588 can be an attractive alternative to decrease the environmental concern due to the disposal of copper-chromite catalysts used during its chemical synthesis [228]. In addition, the biological production of furfuryl alcohol along with the lipid accumulation using *R. toruloides*-1588 can improve the economic feasibility of microbial lipid production process, due to furfuryl alcohol can be recovered and used for several industries as an intermediate compound to produce lysine, flavourings, fragrances, resins, lubricants among others [229].

2-Furoic acid content

Figure 44A shows the production of 2-furoic acid from furfural degradation by *R. toruloides*-1588. The observed results show the maximum production of 2-furoic acid at the end of the fermentation in both culture media with ~0.1 g/L for treatments with an initial furfural concentration of 0.5 g/L. In treatments with 1 g/L of initial furfural concentration, maximum production of 0.2 g/L was observed in treatments using YM media without glucose. Conversely, in treatments using YM media an increase of ~3.5 folds were observed. For treatments with an initial furfural concentration of 2 g/L, the maximum production of 2-furoic acid was observed in YM media (~1 g/L), a 3-fold high production in comparison (0.3 g/L) with the observed in treatments where YM media without glucose was used. Finally, the maximum production of 2-furoic acid in treatments with an initial furfural concentration of 4 g/L was similar in both culture media with 0.19 and 0.20 g/L, respectively. Figure 44B shows the proposed degraded reactions through oxidoreductase and aldehyde dehydrogenase enzymes. Similarly, to furfuryl alcohol, furfural degradation is reduced by hydrogen-mediated reduction in the presence of unpaired electrons. In the case of aldehyde dehydrogenase, furfural is oxidized in presence of NADH, which accepts two electrons and one proton. However, sometimes NADH is used to account for that second hydrogen that gets removed from the substrate being oxidized.

In comparison to furfuryl alcohol, 2-furoic acid is an important chemical intermediate in different industries such as food, pharmaceutical, and cosmetology. 2-furoic acid is used during perfumes, fragrances, medicines, preservatives, and bactericides [230]. Nevertheless, it has been reported that 2-furoic acid can be degraded into 2-oxoglutaric acid by a series of reactions with the characteristic to be CoA-dependant, to finally enter into the Tricarboxylic Acid Cycle (TCA) to be part of metabolism to produce energy.

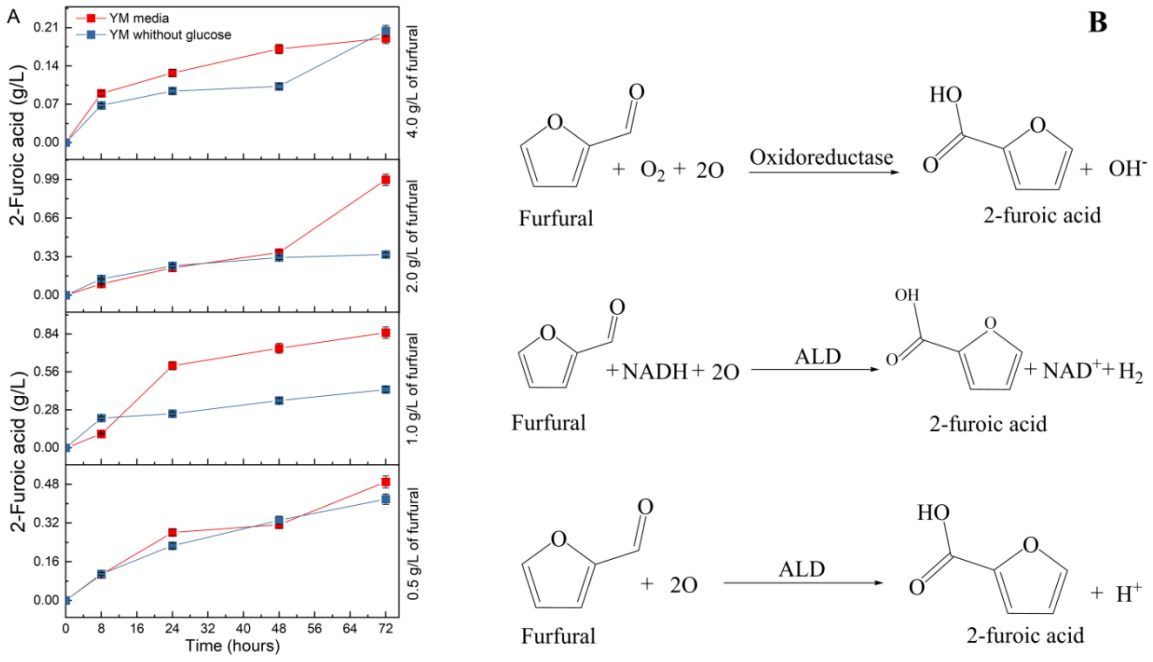

Figure 44.2-Furoic acid production by R. toruloides-1588. A) 2-Furoic acid production; B) Enzymatic degradation reactions. ALD (Aldehyde dehydrogenase). Furfural concentrations: Square – 0.5 g/L; Circle – 1 g/L; Up-Triangle – 2 g/L; Down-Triangle – 4 g/L.

2-Oxoglutaric acid

It has been reported that most of the microorganisms with the ability to degraded furfural have two main products, furfuryl alcohol and 2-furoic acid due to the aldehyde group present in the furan ring is just oxidized or reduced [227]. Figure 45 shows the degradation proposed route of 2-furoic acid into 2-oxoglutaric acid by redox reactions produced by transitional intermediates due to the presence of CoA groups [33], [231]. 2-oxoglutaric acid is a stable compound involved in the

tricarboxylic acid cycle (TCA cycle) or during the synthesis of the amino acid. In the first option, it acts as a carbon source that can be oxidized or reduced by the action of α-ketoglutarate dehydrogenase (KGD) or isocitrate dehydrogenase to obtain succinate or isocitrate, respectively. In the case of amino acid synthesis, 2-oxoglutaric acid participates as a starting compound during the formation of glutamate and glutamine in the central nitrogen metabolism, as well as participate during the formation of proline and arginine amino acids [177]. In this study, production of 2-oxoglutaric acid was observed just in treatments using YM broth as a culture media. Maximum production was observed in treatments with an initial furfural concentration of 1 g/L, 2 g/, and 4 g/L with 112 mg/L, 17 mg/L, and 9 mg/L, respectively. Finally, no production of 2-oxoglutaric acid was observed in treatments where 0.5 g/L of initial furfural concentration was used.

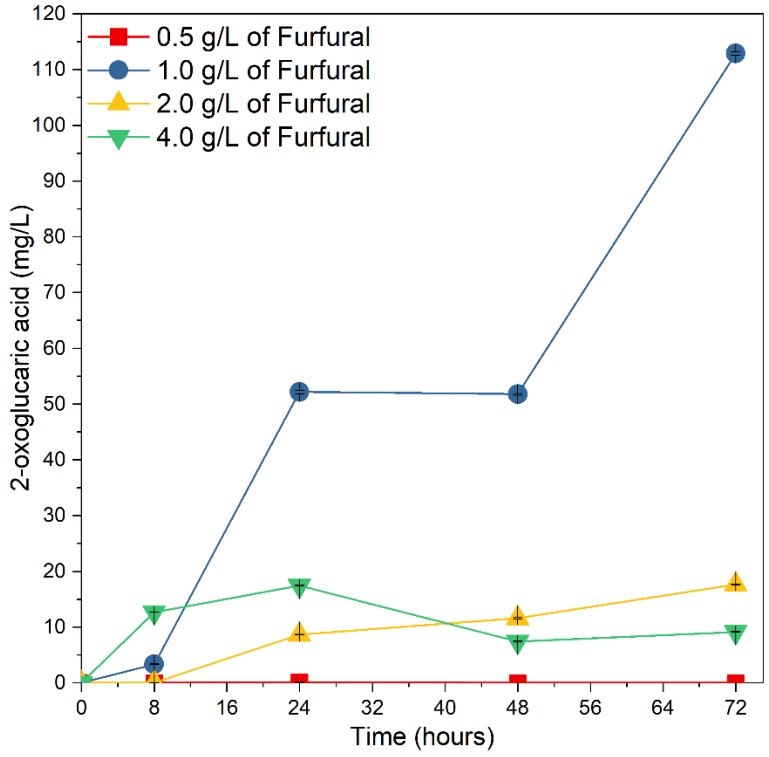

***Figure 45.Proposed degradation of 2-furoic acid into 2-oxoglutaric acid. Modified from** [33].*

***R. toruloides*-1588 performance in presence of high furfural concentration**

Biomass production and sugar utilization

Figure 46 shows the microbial growth of *R. toruloides*-1588 in presence of high furfural concentrations. In treatments where YM media was used as culture media, a similar growth tendency was observed in treatments with an initial furfural concentration of 0.5, 1, and 4 g/L with a short lag phase (less than 8 h), followed by an exponential phase of 40 h, 16 h, and 40 h, with a maximum microbial cell as production of 7.1, 5.9, and 2.85 g/L, respectively. Nevertheless, in treatments where 2 g/L of the initial concentration of furfural was used, a constant growth was observed until the end of the fermentation with a total microbial cell mass production of 6.6 g/L. On another hand, in treatments where YM media without glucose was used a similar growth profile was observed in treatments with initial furfural concentrations of 0.5 and 1 g/L with a maximum microbial cell mass production of 3.9 and 4.2 g/L. A similar trend was observed in treatments with an initial furfural concentration of 2 and 4 g/L where 2.9 g/L was the maximum microbial cell mass achieved. In almost all treatments the maximum microbial cell mass was obtained at 48 h of fermentation with the only exception of control treatment in YM media without glucose where the maximum microbial cell mass was observed at 32 h of fermentation. In terms of sugar consumption (YM media), 99% of glucose was consumed in control treatment with 0.5 g/L, 2 g/L, and 4 g/L, 97% in treatments with 1 g/L of furfural, and 50% in treatments with 4 g/L of initial furfural concentration. The maximum consumption time was during the first 48 h for control and 05. g/L, the rest of the treatments maximum consumption was observed until the end of the fermentation.

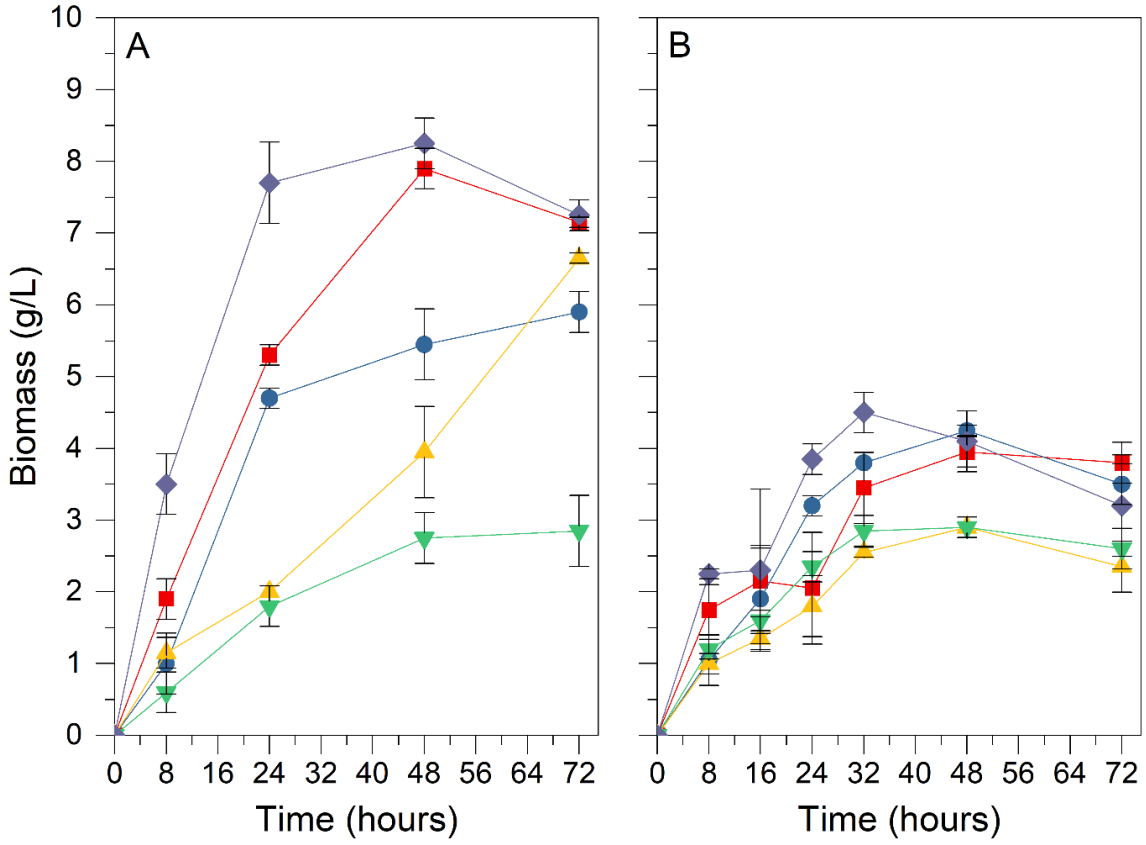

Figure 46.Growth profile of R. toruloides-1588 grew in presence of high furfural concentrations. A) YM media; B) YM without glucose. Furfural concentrations: Red – 0.5 g/L; Blue – 1 g/L; Yellow – 2 g/L; Green – 4 g/L; Purple – Control.

The obtained results are promising and highlight the capacity of *R. toruloides*-1588 to degraded-degrade high furfural concentrations in comparison with other *Rhodosporidium* strains [75], [78], [84], [208] as well as another oleaginous microorganism. For instance, Sitepu et al. [140] found that concentrations of 0.5 and 1 g/L of furfural cause a delay in the growth (increasing the lag phase) as well as partial or total growth inhibition of several oleaginous yeasts such as *Yarrowia lipolytica, Cryptococcus* sp., *Lipomyces* sp., and *Trichosporon* sp. Conversely, with the reported results, this work shows the capacity that *R. toruloides*-1588 can grow in 4 g/L of furfural and can degraded-degrade it. Likewise, *R. toruloides*-1588 can grow in absence of an organic carbon source such as glucose using furfural as an alternative energy source. Furthermore, it is worth highlighting the importance of the short lag phase observed in this study. Industrial applications signify an outstanding advantage in decreasing the energy consumption required to carry on the

fermentation to produce microbial lipids using liquid hydrolysates from lignocellulosic biomass as a renewable substrate [232].

Lipid accumulation and fatty acid profile

Figure 47 shows the performance of *R. toruloides*-1588 in terms of lipid accumulation, where the highest lipid accumulation was higher than 50%. For instance, in treatments where YM media was used, the maximum lipid accumulation (52%) was observed at 32 h in treatments with 1 g/L of initial furfural concentration, followed by treatments with initial furfural concentrations of 0.5 g/L, 2 g/L, and 4 g/L with 47% and 37%, respectively. On another hand, using YM media without glucose as a culture media, lipid accumulation was similar between the four initial sugar concentrations. For instance, maximum lipid accumulation was observed in treatments at 24 h in treatments with an initial furfural concentration of 0.5 g/L, followed by 36%, 35%, and 33% for treatments with an initial furfural concentration of 1, 2 and 4 g/L, respectively. The above results show a proportional decrease in lipid accumulation with the increase of furfural concentration. Nonetheless, these results are promising as the main aim was to test the capacity of *R. toruloides*-1588 to grow, accumulate lipids and use furfural as an alternative energy source as well as the capacity to degraded high furfural concentrations.

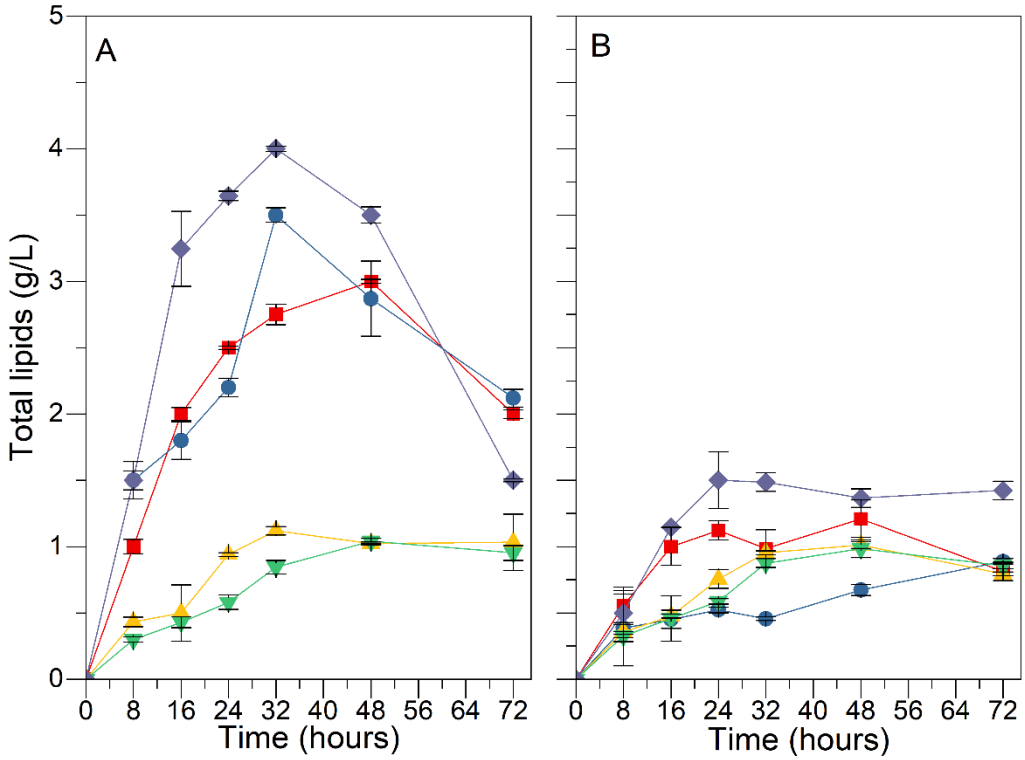

Figure 47.Lipid accumulation by R. toruloides-1588 in presence of high furfural concentration. A) YM media; B) YM without glucose. Furfural concentrations: Red – 0.5 g/L; Blue – 1 g/L; Yellow – 2 g/L; Green – 4 g/L; Purple – Control.

The fatty acid present in the accumulated lipids under the tested conditions does not show major changes in the fatty acid distribution, being palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c), linoleic (C18:2n6c), linolenate (C18:3n3) and lignoceric (C24:0) acids the most predominant in all the treatments. Figure 6 shows the concentration of each fatty acid under the tested conditions. The obtained results show glucose effect in the fatty acid distribution being palmitic (34%), oleic (29%), stearic (67%), and palmitic (31%) the predominant fraction for treatments with initial furfural concentration of 0.5 g/L, 1 g/L, 2 g/L, and 4g/L, respectively (ANNEX 8). As compared to the observed profile in treatments where YM media was used, the fatty acid profile in treatments using YM media without glucose showed a decrease of ~50% of fatty acids concentration as well as a defined pattern for all furfural concentrations (0.5, 1, 2, and 4 g/L). Palmitic acid is the most predominant fatty acid with a relative fatty acid concentration of 49%, 46%, 48%, and 55%, respectively. Likewise, in both culture media, a decrease in fatty acid concentration was observed with the increase of fatty acid unsaturation. This is due to the fact that polyunsaturated fatty acids are more suitable to break down through β-oxidation pathway in mitochondria or in the peroxisomes followed by the glyoxylate cycle to obtain energy after the main carbon source has been depleted.

Conclusion

The present study confirms the capacity of the native strain *Rhodosporidium toruloides*-1588 to degrade up to 4 g/L of furfural into furfuryl alcohol and 2-furoic acid as the main by-products as well as 2-oxoglutaric acid from 2- furoic acid. *R. toruloides*-1588 do not show a retarded lag phase or growth inhibition during the lipid accumulation. A maximum lipid accumulation of 50% was achieved under the tested conditions, being palmitic, stearic, and oleic acid, the main fatty acids present in the produced lipids. This study confirms the capacity of *R. toruloides*-1588 to degrade furfural, growth and accumulate lipids, characteristics that potentially make this strain an excellent candidate for use in large-scale processes.

CRediT author statement

Carlos S. Osorio-González.: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft. **Rahul Saini**.: Formal analysis, Investigation. **Krishnamoorthy Hegde**.: Supervision, Writing-Review & Editing. **Satinder Kaur Brar**.: Funding acquisition, Supervision, Writing-Review & Editing. **Alain Lefebvre**.: Resources. **Antonio Avalos-Ramírez**.: Funding acquisition, Writing-Review & Editing, Supervision.

Supplementary data for this work can be found in ANNEX 6.

CHAPTER SEVEN: A CRITICAL ASSESSMENT OF MICROBIAL LIPID PRODUCTION USING RHODOSPORIDIUM TORULOIDES AS A BIOFACTORY

7.1 An analytical review on *Rhodosporidium* sp.: Critical look and data assessment

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C. S. Osorio-González *et al.*, "An analytical review on *Rhodosporidium toruloides*.: Critical look and data assessment,". To be submitted to ***Biotechnology Advances. Detailed Analysis under progress.***

Abstract
During the last decade, the exploitation of the oleaginous yeast *Rhodosporidium* sp. has been increasing. This fact has generated a large amount of information related to various parameters involved mainly in the yeast performance as well as the different products obtained through its use as a biocatalyst. However, there are questions regarding which substrates, methods, of cultivation strategies are the best suited to improve their performance and increase the final yield of target products. To have a wide perspective about the key parameters involved during lipid accumulation using *Rhodosporidium* sp., 80 research articles focused specifically on the lipid production published within the last decade have been screened from ~300 research articles. Data were collected, classified, and statistically analyzed to study the effect of parameters such as type of substrate (lignocellulosic hydrolysate, industrial residues, food residues, and synthetic media), C/N ratio utilized, sugar concentration, among others on biomass production, lipid accumulation, sugar consumption, lipid yield, and biomass yield were analyzed. In summary, this study will provide a critical discussion focused on the main parameters that influence lipid accumulation. Likewise, the most promising cultivation strategies to produce microbial lipids using *Rhodosporidium* sp.

Keywords: *Rhodosporidium* sp., lipids, carotenoids, analytical assessment, statistical analysis

Introduction

Microbial lipids have been widely accepted as an alternative feedstock for the production of biofuels and oleochemicals. In comparison with plant-based lipids, microbial lipids hold several advantages such as increased accessibility in desired fatty acid production, fast lipid accumulation and easy process optimization [233]. Hence, immense efforts have been made for microbial-based advanced biofuel production [171]. In general, a microorganism capable of accumulating more than 20% lipid of their dry cell weight is known as oleaginous microorganisms such as *Rhodosporidium* sp., *Yarrowia lipolytica*, *Cryptococcus curvatus*, *Trichosporon coremiiforme* and *Rhodotorula glutinis.* Additionally, their genus, media optimization, metabolic pathways and lipid accumulation have been extensively reviewed [225], [234]. Numerous types of substrates have been utilized for microbial-based lipid production such as glucose, xylose, crude glycerol, lignocellulosic biomass, domestic waste, industrial waste and many more [235], [236].

Over the past decade, *Rhodosporidium* sp. has been identified as a potential alternative to produce 50 – 70% of lipids. Moreover, it has been known to thrive on multiple substrates such as glucose,

crude glycerol and hydrolysate derived from lignocellulosic biomass. In addition, *Rhodosporidium* sp. is also known to consume 60 -70% of xylose and tolerate several toxic compounds such as furans, phenols, and organic acids. The fatty acids produced in *Rhodosporidium* sp. include saturated fatty acids and unsaturated fatty acids such as myristic acid, palmitic acid, stearic acid, linoleic acids, oleic acid and docosapentaenoic acid, which could serve as a feedstock for biofuel as well as for food and pharmaceutical industries. In addition, *Rhodosporidium* sp. is also known to accumulate carotenoids such as β-carotene, torulene and lycopene, which have high demand in pharmaceutical industries because of their antioxidant properties [210], [233]. Moreover, extensive reviews on covering information about *Rhodosporidium* sp. such as genomic background, growth and lipid production, metabolomics, metabolic modelling, proteomics, genetic engineering, and advanced drop-in biofuel production [210], [233], [237]. For instance, Yaegashi et al. [67] engineered the *Rhodosporidium* sp. to produced bisabolene (680 mg/L) using corn stover as media. However, most of the articles reported have variations in terms of methodologies, substrates used, fermentation conditions and lipid yields. Besides, a critical review of studies reporting different types of substrates used, biomass and lipid yield, fermentation conditions, use of different inducers on *Rhodosporidium* sp. is currently lacking.

In this sense, the present investigation aims to statistically analyze different fermentation conditions and parameters employed on *Rhodosporidium* sp. The effects of different methodologies are analyzed on lipid accumulation, carotenoid, fatty acids and oleochemicals productions. For this, 80 research articles containing different substrates, carbon to nitrogen (C/N) ratio, inducers used and their effect on lipid accumulation in *Rhodosporidium* sp. are chosen. The analytical paper is divided into four main parts: *i*) assessing the key parameters such as type of substrate, C/N ratio, inducers and toxic compounds and their effect on *Rhodosporidium* sp.; *ii*) statistical analysis of data obtained; *iii*) assessment of results obtained on maximum lipid accumulation; and *iv*) prediction of best suited conditions and parameter to achieve maximum lipid accumulation. For each consideration, the combined and overall reliability score, and the outcome of studies have been discussed. An overview of the lipid yield, lipid compositions, and fatty acids are provided, and their production trends are discussed based on the substrate type, stress condition and fermentation characteristics. Finally, the recommendation has been provided to further improve the lipid accumulation ability in *Rhodosporidium* sp. and key conclusions are summarized.

Methodology

Literature search

The literature research was performed using Google Scholar as a web search engine using the following entries "Rhodosporidium", "Microbial lipids", and "Lipid production". The criteria used to select the articles to be used, four criteria were utilized: i) type of substrate (lignocellulosic biomass, food and industrial residues and synthetic media), ii) cultivation conditions (C/N ratio, sugar concentration, temperature, pH), iii) microbial lipid production as the main aim of the research, and iv) the use of native strains. Furthermore, to have a precise accuracy in the analysis, articles focused or that perform *in-silico* analysis or *in-silico* modelling were not contemplated.

Quantitative data assessment

The statistical assessment was performed through inferential and multivariate statistics using linear regression and correlation analyses as well as cluster and discriminants analysis. All analysis were compared through R^2, Adj-R^2, and p-value with and α=0.05. Furthermore, deterministic, and stochastic models were used for the prediction of culture conditions to achieve the maximum lipid production. All the collected data were analyzed using the software OriginPro©.

This research review will provide an accurate information on the parameters with higher influence during the cultivation of *Rhodosporidium* sp. to produce microbial lipids.

A critical analysis can be obtained on the culture media including its source, economic feasibility, composition, and its effect on the lipid accumulation by *Rhodosporidium* sp.

Finally, this study will serve as a base-model to predict the maximum lipid accumulation using *Rhodosporidium* sp. as a biofactory.

8. CONCLUSIONS

8.1. Forestry residues obtained from hardwood and softwood, in the form of sawdust, is a renewable and sustainable feedstock to produce chemical compounds, such as furfural, 5-hydroxymethyl furfural, vanillin, syringaldehyde, vanillic acid, ferulic acid, and aminobenzoic acids, that can be used as precursors to produce compounds with high value in food, biomedical, pharmaceutical, and chemical industries. The obtained results offer an extra added value to these residues as well as improve the economic feasibility of biochemical processes in which the obtained liquid hydrolysate can be a suitable substrate for microbial growth. In particular, the combination of a thermochemical process followed by an ozonolysis treatment shows a positive effect in the obtention monolignols such as sinapyl, p-coumaryl and coniferyl alcohol from liquid hydrolysates and treated biomass. These monolignols can be used as precursors to produce resins, copolymers, and polymers.

8.2. In a screening study of five *Rhodosporidium toruloides* strains, *R. toruloides*-1588 was identified as the most suitable strain to grow and accumulate 35% of lipids using C5 and C6 undetoxified wood hydrolysates. Likewise, *R. toruloides*-1588 shows tolerance to lignocellulosic inhibitory compounds. In addition, The *predominant fatty acids were oleic, palmitic, and stearic, which are suitable feedstock for biodiesel or advanced biofuel production*. Furthermore, polyunsaturated fatty acids such as omega-3, omega-6, and omega-9 with high added value for food and cosmetic industries were also identified.

8.3. The change in the formulation of C5 and C6 undetoxified wood hydrolysates used as a culture media has an effect on the growth and lipid accumulation by *R. toruloides*-1588. Culture conditions of C/N ratio equal to 70, an initial rate of glucose: xylose of 1:1, and 1.05 g/L of Na_2HPO_4 as a lipid inducer increased the lipid accumulation by 7.48% with a maximum biomass yield of 0.69 g of biomass/g of sugar. The use of Na_2HPO_4 and different initial sugar concentrations also induced the change in the fatty acid profiles. Additionally, due to the wood hydrolysates combination and increase in lignocellulosic inhibitors and under the tested culture conditions, a total fermentation time up to 96 hours was enough to achieve the maximum lipid production.

8.4. The study using high five and six-carbon sugar concentrations highlights the capacity of the *R. toruloides*-1588 to grow (18.6 g/L) and accumulate lipids (8.2 g/L) using up to 120 g/L of sugar.

R. toruloides-1588 shows the ability to utilize C5 and C6 sugars separately and simultaneously as a carbon source as well as transform and degrade microbial inhibitory compounds present in the hydrolysates. These results confirm that *R. toruloides*-1588 is a convenient strain that can be used at industrial-scale operations for microbial lipid production.

8.5. *R. toruloides*-1588 was able to degrade up to 96% of furfural, and 5-HMF, 85% of levulinic acid, 80% of syringaldehyde, 81% of vanillin and 86% of vanillic acid present in wood hydrolysates. Likewise, *R. toruloides*-1588 was able to metabolize and transform furfural into furfuryl alcohol and 2-furoic acid as the main by-product as well as 2-oxoglutaric acid from 2-furoic acid. In addition, 5-hydroxymethyl furfural was the compound with the fastest degradation rate (5.450 mgL h^{-1}), while acetic acid had the lowest degradation rate. The above facts confirm that *R. toruloides*-1588 is a tolerant strain against microbial inhibitory compounds derivate from lignocellulosic biomass. Also, *R. toruloides*-1588 does not show a retarded lag phase or growth inhibition during the lipid accumulation process due to the presence of high inhibitor concentrations. The findings provide useful insight into furfural degradation and require further exploration to improve the use of renewable substrates with a high concentration of this compound.

8.6 Undetoxified C5 and C6 wood hydrolysates, obtained from forestry residues, are an effective culture media to produce microbial lipids using *R. toruloides*-1588. In summary, *R. toruloides*-1588 consumes >95% of glucose and >75% of xylose efficiently, both separately and simultaneously.

RECOMMENDATIONS

The following recommendations are based on the findings of this study:

1. In this dissertation, several culture conditions were investigated to increase lipid accumulation using a native strain, therefore the genetic modification to increase and accelerate the lipid production process is necessary.

2. To explore and optimize the conditions for increased lipid production and accumulation using fed-batch and continuous cultivations.

3. To develop and perform proteomic and transcriptomic analyses and elucidate the detailed metabolization pathway of inhibitor compounds in oleaginous yeast based on the inhibitor's degradation analyses presented in this study.

4. To examine an engineered strain, focused on the overexpression of enzymes involved in the pentose phosphate pathway to channelize xylose into microbial lipids and explain lower lipid yield for xylose than glucose despite its high consumption (>75%).

5. To study the feasibility of the microbial lipid process developed in this dissertation a techno-economic analysis and life cycle assessment are necessary.

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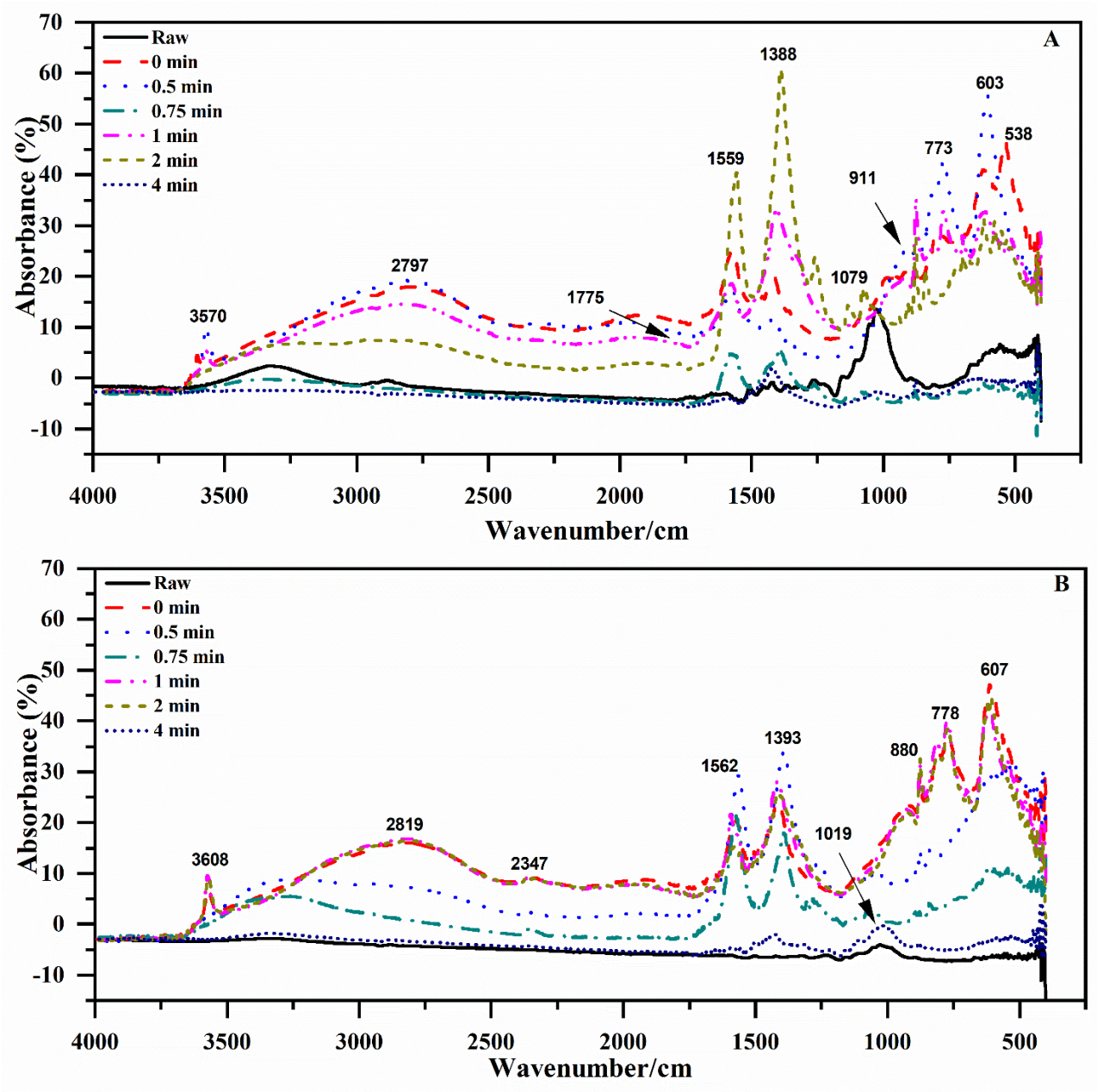
APPENDICES

APPENDIX A. Supplementary information of Section 4.2

Similar studies to produce monolignols.

Compound	Substrate	Treatment	Concentration	Reference
Coniferyl alcohol	Synthetic media	*de novo* biosynthesis using a recombinant *Amycolatopsis* sp. HR167 strain	470 mg L^{-1}	[134]
		de novo biosynthesis using a recombinant *E. coli*	124 mg L^{-1}	[135]
	Wheat arabinoxylan	Enzymatic and whole-cell biocatalytic route	71 mg L^{-1}	[136]
	Softwood hydrolysate	Microwave-assisted extraction + ozonolysis	112 mg/kg	This study
	Hardwood hydrolysate	Thermal treatment + ozonolysis	175 mg/kg	This study
p-Coumaryl alcohol	Synthetic media	*de novo* biosynthesis using a recombinant *E. coli*	501 mg L^{-1}	[135]
	Wheat arabinoxylan	Enzymatic and whole-cell biocatalytic route	58 mg L^{-1}	[136]
	Hardwood hydrolysate	Thermal treatment + ozonolysis	16 mg/kg	This study
Sinapyl alcohol	Softwood hydrolysate	Microwave-assisted extraction + ozonolysis	256 mg/kg	This study
		Thermal treatment + ozonolysis	453 mg/kg	This study

FTIR spectra of liquid samples from thermal treatment (TT) + ozonolysis (min). A) Softwood and B) Hardwood



APPENDIX B. Supplementary information of Section 5.1

Complementary sugars founded in C5 hydrolysate.

Time (hours)	18		36		54		104		120		168		224	
	Fructose (mg/L)													
Yeast	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**
Y-1091	973.14	0.62	1089.76	0.29	1242.21	0.54	1380.59	0.01	1331.03	0.01	1481.71	0.46	1381.71	0.46
Y-1588	788.57	0.21	779.98	0.14	799.94	0.61	772.32	1.10	639.85	0.33	721.13	0.86	621.13	0.86
Y-6984	1368.73	0.12	1118.02	2.55	1061.44	2.38	1431.66	0.14	1283.92	0.61	1362.25	0.19	1213.62	0.91
Y-6987	1339.70	0.54	1475.53	0.42	1483.80	0.33	1599.56	1.30	1448.39	1.00	1392.09	0.67	1383.28	2.67
Y-7191	3643.75	13.92	1505.49	0.38	1407.96	0.74	1553.62	0.11	1421.76	1.30	1282.17	0.78	1211.23	0.83
Y-63746	834.72	0.07	982.51	0.26	806.86	0.60	777.92	0.04	844.44	0.18	717.98	0.06	660.75	0.55
Y-17104	813.53	0.25	1022.32	0.74	788.78	0.09	791.35	0.66	829.83	0.29	866.88	0.90	816.88	0.20
Y-11545	808.73	0.12	1108.46	0.42	746.05	0.07	668.91	0.33	738.81	1.07	571.87	2.27	521.87	1.56
Y-12632	1617.74	1.14	2144.45	1.47	1380.50	0.20	1367.98	0.27	1410.76	0.85	1507.93	0.09	1257.93	0.62
Control	666.33	0.16	946.52	0.31	898.01	0.29	812.06	0.04	717.43	0.14	777.88	0.07	709.38	0.51
	Galactose (mg/L)													
Y-1091	357.85	0.19	444.29	0.05	482.28	0.19	359.81	0.25	302.39	0.08	369.23	0.22	269.23	0.22
Y-1588	404.15	0.01	423.59	0.08	474.79	0.17	356.30	0.07	288.42	0.12	330.82	0.28	230.82	0.28
Y-6984	410.49	0.15	372.00	0.62	382.90	0.70	365.05	0.18	299.86	0.31	311.01	0.05	261.01	0.65
Y-6987	398.01	0.13	444.20	0.04	433.22	0.01	395.45	0.06	336.82	0.21	355.32	0.04	305.32	0.67
Y-7191	473.52	0.12	444.47	0.12	405.71	0.02	401.56	0.44	374.19	0.39	308.82	0.11	282.32	0.27
Y-63746	464.06	0.18	568.08	0.10	390.21	0.31	384.83	0.14	384.38	0.11	321.69	0.36	271.69	0.35
Y-17104	453.10	0.13	559.46	0.40	411.61	0.10	369.98	0.26	384.56	0.08	384.55	0.24	302.98	0.22
Y-11545	441.38	0.15	617.50	0.29	391.63	0.01	324.30	0.17	376.51	0.10	258.86	0.74	288.26	0.36
Y-12632	500.51	0.25	613.76	0.01	370.41	0.16	332.36	0.16	377.08	0.35	369.20	0.40	269.20	0.40
Control	414.82	0.01	564.43	0.10	449.82	0.08	339.57	0.25	348.69	0.23	369.90	0.04	269.90	0.04
	Glucose (mg/L)													
Y-1091	1281.84	0.95	1773.82	0.04	1986.15	1.15	1989.02	1.90	1571.60	0.15	1889.26	0.55	1789.26	0.55
Y-1588	1326.36	0.12	1608.68	0.27	1717.19	0.66	1603.18	1.80	1122.16	0.69	1364.33	1.50	1264.33	1.50

Y-6984	1579.17	0.39	1427.96	3.42	1391.47	3.24	1930.86	0.32	1486.62	0.17	1611.82	0.14	1511.82	0.14
Y-6987	1650.69	0.65	1866.02	0.39	1887.47	0.38	2255.47	2.09	1776.55	0.46	1686.80	0.90	1586.80	0.90
Y-7191	1317.40	6.59	1882.51	0.46	1676.58	0.83	2172.93	0.08	1812.14	1.00	1511.21	1.69	1494.84	1.46
Y-63746	1754.07	0.19	2152.92	0.80	1394.13	0.72	1645.42	1.76	1677.29	0.35	1281.90	0.13	1231.40	0.25
Y-17104	1825.49	0.57	2423.08	2.42	1434.89	0.57	1847.77	0.10	1834.15	0.88	1659.65	0.46	1559.65	0.46
Y-11545	1798.34	0.22	2623.34	1.44	1421.41	0.25	1325.14	0.03	1532.78	1.91	1176.42	4.84	855.92	0.88
Y-12632	2373.08	1.32	3170.12	1.11	1654.18	0.48	1586.74	0.76	1795.25	1.18	2025.09	0.21	1930.10	0.14
Control	1469.98	0.20	2216.22	0.06	1625.84	0.02	1537.06	0.34	1407.22	0.04	1603.11	0.04	1555.88	0.01
	Sucrose (mg/L)													
Y-1091	759.83	0.65	824.49	0.12	759.28	0.50	236.01	0.00	163.31	0.06	95.18	0.04	92.98	0.02
Y-1588	1175.61	0.04	1468.79	0.20	1683.74	1.04	1334.78	2.01	947.77	0.73	1111.34	0.99	961.34	0.28
Y-6984	358.92	0.05	140.01	0.14	87.91	0.04	39.24	0.00	ND	ND	ND	ND	ND	ND
Y-6987	353.45	0.13	142.90	0.05	92.28	0.01	ND	ND	ND	ND	ND	ND	ND	ND
Y-7191	220.37	0.88	113.43	0.00	81.88	0.01	ND	ND	ND	ND	ND	ND	ND	ND
Y-63746	1496.18	0.42	1914.34	1.12	1298.49	0.62	1380.30	0.83	1354.88	0.13	1076.42	0.16	947.95	0.56
Y-17104	1546.92	0.52	2231.99	2.32	1280.97	0.26	1402.41	0.94	1393.38	0.81	1289.72	0.17	1192.69	0.21
Y-11545	1618.82	0.14	2615.34	1.36	1269.95	0.01	1120.45	0.29	1281.35	1.21	987.32	3.81	737.32	1.69
Y-12632	ND	ND	117.36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Control	1444.78	0.12	2270.43	0.11	1472.74	0.35	1259.36	0.22	1158.38	0.25	1331.23	0.14	1222.72	0.33
	Lactose (mg/L)													
Y-1091	1867.70	1.03	1758.67	0.16	1785.70	0.37	1488.79	0.51	1408.75	0.42	1542.19	0.43	1443.97	0.41
Y-1588	1933.95	0.19	1739.61	0.30	1785.15	0.14	1442.94	1.95	1273.76	0.02	1431.85	2.51	1377.43	1.86
Y-6984	1816.01	0.10	1454.48	3.20	1313.28	2.47	1451.41	0.40	1358.99	0.92	1341.08	0.07	1257.93	0.30
Y-6987	1690.13	0.30	1810.16	0.66	1885.57	0.30	1527.23	0.11	1352.21	1.65	1359.84	1.53	1359.84	0.11
Y-7191	1895.31	0.25	1763.63	0.16	1721.52	0.68	1494.62	0.15	1456.55	0.97	1276.93	0.58	1269.31	0.14
Y-63746	1738.07	0.06	1628.25	0.10	1669.31	0.78	1318.36	1.02	1442.39	0.37	1293.35	0.25	1203.35	0.10
Y-17104	1670.73	0.61	1665.94	0.98	1661.65	0.37	1470.61	1.31	1479.49	0.75	1410.66	0.11	1306.60	0.27
Y-11545	1612.29	0.67	1883.24	0.37	1670.08	0.20	1305.12	0.13	1354.48	0.04	1004.83	3.58	854.83	1.45
Y-12632	1939.22	1.27	1749.85	0.51	1489.18	0.41	1408.47	0.06	1469.19	1.45	1405.84	0.52	1316.70	0.37
Control	1533.19	0.13	1961.08	0.26	1608.89	0.04	1528.91	0.05	1450.19	0.13	1312.67	0.11	1307.45	1.23

Complementary sugars founded in C6 hydrolysate.

Time (hours)	18		72		96		128		168		200		224	
	Xylose (mg/L)													
Yeast	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**	**Average**	**Error**
Y-1091	2667.91	0.87	365.86	0.36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-1588	2574.53	0.35	1407.69	0.44	1984.68	0.59	ND	ND	ND	ND	ND	ND	ND	ND
Y-6984	1896.57	0.60	1320.32	5.34	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-6987	1920.47	0.42	1796.28	0.06	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-7191	1787.37	0.04	1275.97	1.73	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-63746	2033.21	3.34	1639.42	0.85	1166.72	0.46	ND	ND	ND	ND	ND	ND	ND	ND
Y-17104	2140.39	0.90	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-11545	2153.67	3.84	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-12632	2275.07	0.19	6874.32	2.00	3118.92	0.81	1721.18	1.95	1723.41	1.26	520.74	0.47	961.96	0.90
Control	1884.67	0.71	2553.07	1.41	2083.65	0.71	1009.77	0.92	1097.84	0.46	938.56	0.89	1041.62	1.18
	Fructose (mg/L)													
Y-1091	2458.37	1.42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-1588	2049.29	0.32	2483.35	1.26	2990.79	1.24	ND	ND	ND	ND	ND	ND	ND	ND
Y-6984	2551.94	1.08	1277.43	8.91	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-6987	2711.85	0.87	2720.64	0.29	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-7191	2517.46	0.29	1304.98	0.80	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-63746	2258.02	2.64	2403.99	0.28	2845.05	0.36	1425.99	1.04	675.20	0.31	391.95	0.42	318.81	1.59
Y-17104	2314.51	0.64	1195.84	0.22	563.47	0.11	ND	ND	ND	ND	ND	ND	ND	ND
Y-11545	2407.19	2.96	926.51	3.55	254.26	1.38	ND	ND	ND	ND	ND	ND	ND	ND
Y-12632	1733.30	1.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Control	2363.33	0.84	2191.97	1.12	2009.75	0.99	1275.99	1.17	1203.06	1.50	ND	ND	1618.19	1.20
	Sucrose (mg/L)													
Y-1091	1253.70	0.12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-1588	1989.27	0.29	555.12	0.33	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-6984	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Y-6987	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-7191	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-63746	1972.29	2.58	2094.13	0.26	2658.01	0.23	2498.88	0.39	3671.47	0.40	2805.62	0.25	2365.03	1.51
Y-17104	1946.13	0.35	2410.38	0.68	1624.62	0.19	ND	ND	ND	ND	ND	ND	ND	ND
Y-11545	1955.61	2.06	1867.06	4.82	941.39	1.94	ND	ND	ND	ND	ND	ND	ND	ND
Y-12632	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Control	1549.89	1.09	2116.94	2.41	1865.95	2.18	1076.39	1.28	1384.09	2.30	ND	ND	ND	ND
	Lactose (mg/L)													
Y-1091	182.23	0.08	126.21	0.14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-1588	191.33	0.09	220.75	0.05	260.60	0.06	ND	ND	ND	ND	ND	ND	ND	ND
Y-6984	181.97	0.01	146.62	0.41	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-6987	137.83	0.09	48.36	0.07	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-7191	166.13	0.05	80.08	0.27	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-63746	250.39	0.48	187.50	0.30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-17104	285.27	0.29	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-11545	316.48	0.47	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Y-12632	256.77	0.02	621.22	1.04	ND	ND	425.49	0.48	317.96	0.98	ND	ND	ND	ND
Control	252.37	0.48	161.58	0.23	ND	ND	166.35	0.23	145.30	0.50	ND	ND	ND	ND

ND: Not determine

APPENDIX C. Supplementary information of Section 5.2

Response optimization analysis

Response	Multiple response prediction			Maximum fit	Composite desirability
	C/N	Lipid inducer	Initial glucose		
Biomass*	70.50	1.17	26.13	13.47	0.9787
Lipids**	70.50	1.19	25.37	5.17	0.9670

* 95% Confidence Interval (12.5, 14.4); 95% Predicted Interval (11.5, 15.3)
** 95% Confidence Interval (4.4, 5.9); 95% Predicted Interval (3.7, 6.6)

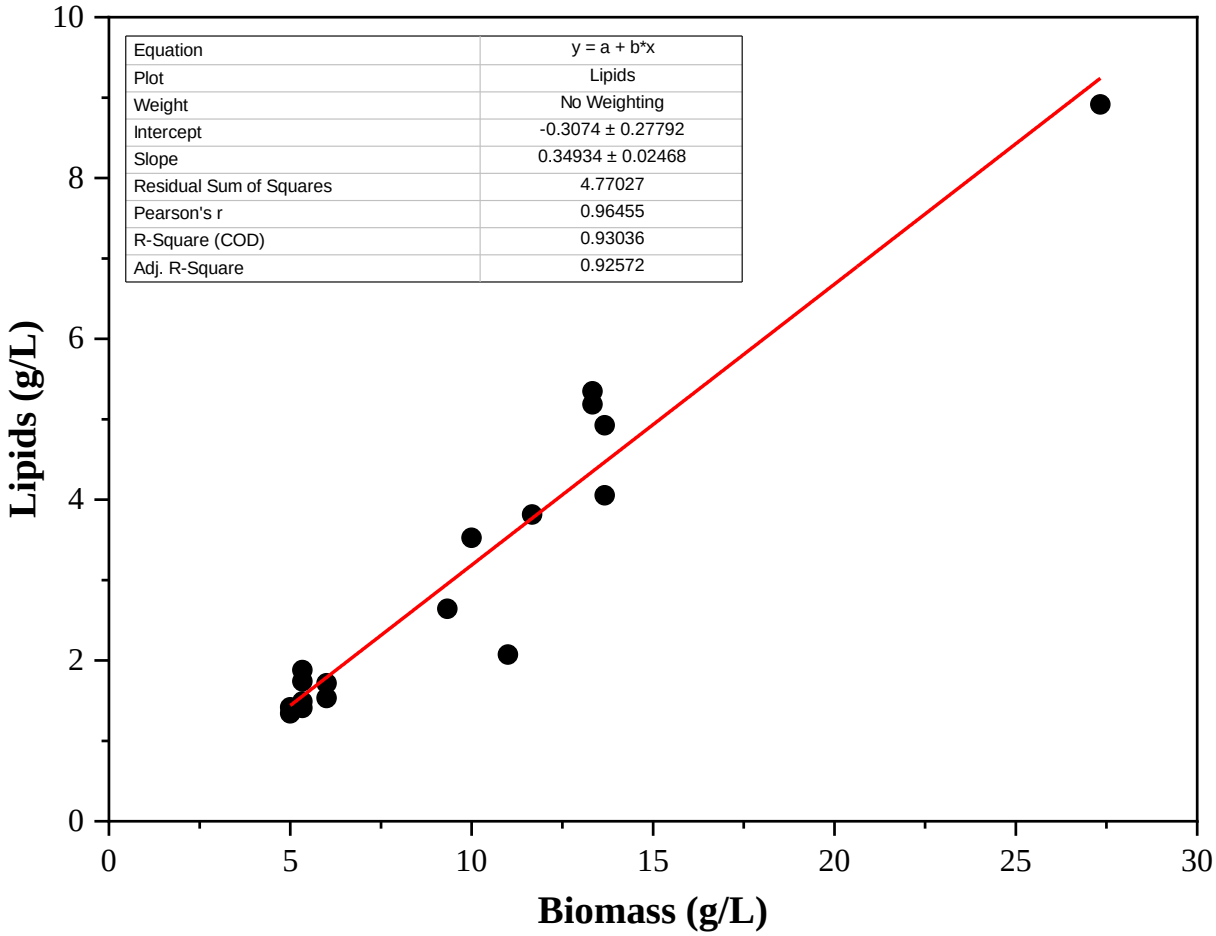
ANOVA analysis of surface regression

Parameter	Constant	p-value	Total degree of freedom	R^2	R^2 (adj)
Biomass production	C/N	1.000	14	98.78	96.57
	Lipid inducer	0.203			
	Glucose	0.203			
	C/N*C/N	0.000			
	Phosphate*Phosphate	0.015			
	Glucose*Glucose	0.004			
	C/N*Phosphate	1.000			
	C/N*Glucose	1.000			
	Phosphate*Glucose	1.000			
Lipid accumulation	C/N	0.824	14	96.04	88.92
	Lipid inducer	0.168			
	Glucose	0.778			
	C/N*C/N	0.000			
	Phosphate*Phosphate	0.016			
	Glucose*Glucose	0.005			
	C/N*Phosphate	0.967			
	C/N*Glucose	0.882			
	Phosphate*Glucose	0.425			

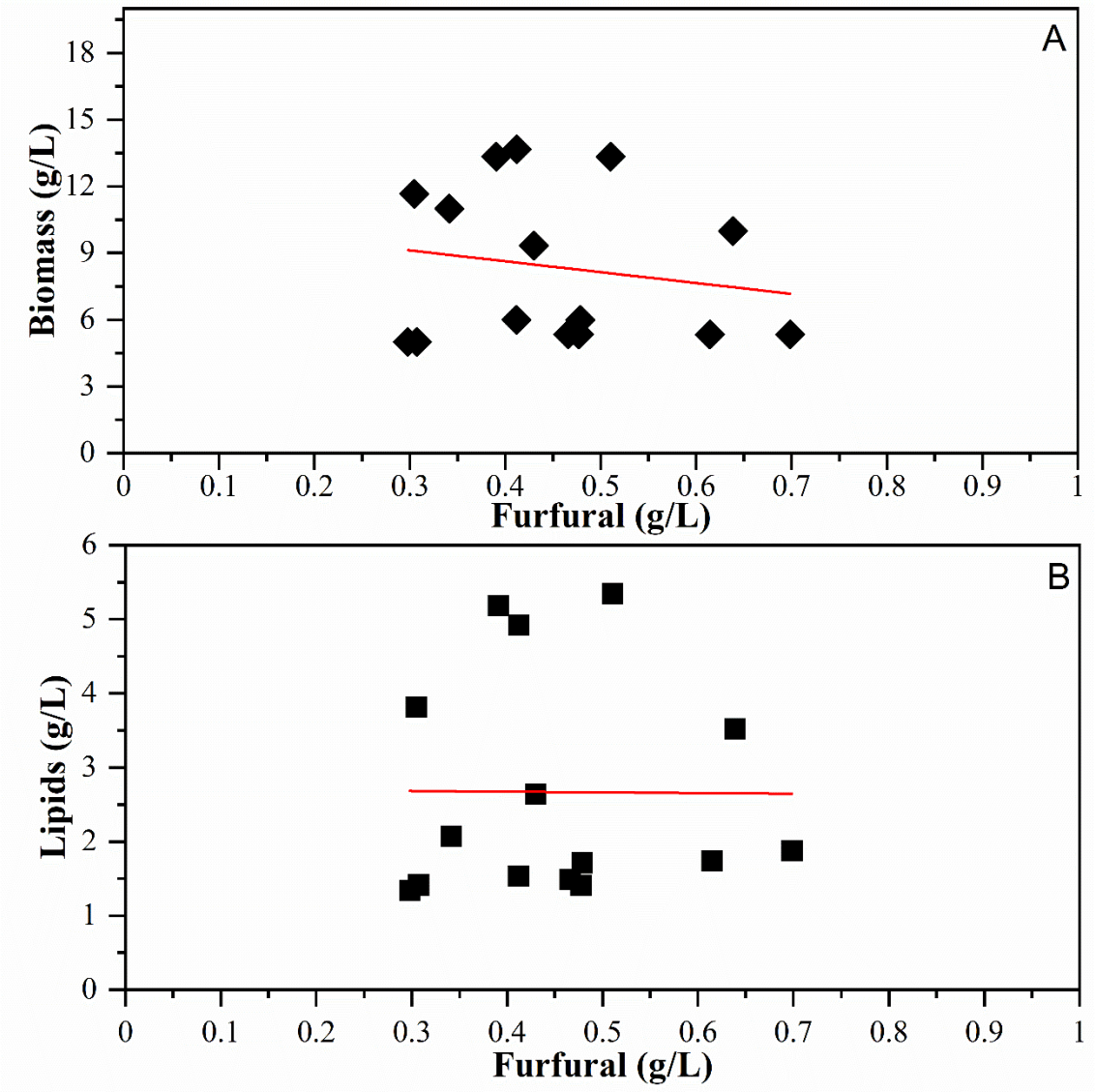
Microbial inhibitor compounds present in the wood hydrolysate

Treatment /Compound	5-Hydroxymethyl furfural	Levulinic acid	Ferulic acid	Vanillic acid	Vanillin	Syringaldehyde	Aminobenzoic acid
T-1	49.14±0.14	18.92±1.44	9.27±0.42	35.64±1.97	31.93±0.37	45.40±4.374	7.91±0.43
T-2	48.40±0.11	18.35±1.12	10.68±0.72	33.68±6.43	31.92±0.93	46.00±0.851	7.50±0.85
T-3	51.79±0.04	20.05±0.42	9.85±0.67	31.31±6.29	31.38±0.35	40.02±0.64	7.43±0.64
T-4	54.43±0.03	18.11±0.35	9.61±0.25	34.23±4.36	33.06±0.04	45.39±1.26	7.14±1.26
T-5	27.72±0.67	23.39±6.72	10.74±0.27	45.17±8.56	36.57±0.26	51.37±3.65	8.11±0.65
T-6	28.08±0.07	22.56±0.67	12.45±1.07	43.83±0.27	36.96±0.34	53.14±0.26	7.84±0.77
T-7	80.84±0.02	23.73±0.25	9.87±0.85	22.98±3.42	24.98±0.43	31.37±0.34	6.99±0.48
T-8	90.52±0.43	15.42±4.27	9.57±0.55	23.50±3.00	24.35±0.32	32.09±4.37	6.99±0.23
T-9	24.85±0.20	25.35±1.12	12.21±1.97	42.84±3.04	36.78±0.77	53.06±3.27	8.14±0.57
T-10	28.28±0.68	22.92±0.42	11.77±0.43	41.14±0.85	36.47±0.80	52.15±1.11	7.66±0.45
T-11	78.07±1.21	15.63±0.35	9.40±0.29	23.86±0.69	24.44±2.43	29.54±0.41	6.81±0.26
T-12	92.76±0.20	18.67±6.72	9.22±0.36	23.23±1.26	23.96±0.85	31.32±0.34	6.85±0.34
T-13	48.48±2.64	21.02±0.67	10.45±0.42	32.79±3.65	30.24±0.37	41.55±6.71	7.06±0.28
T-14	50.55±0.63	20.94±3.65	9.15±0.35	30.98±0.77	31.23±0.93	44.14±0.67	7.30±0.29
T-15	51.64±2.44	20.77±0.77	11.03±0.72	29.90±0.48	32.49±0.35	44.97±0.24	7.37±0.63

Pearson correlation lipids *vs* biomass



Correlation analysis of biomass and lipid production vs furfural content.



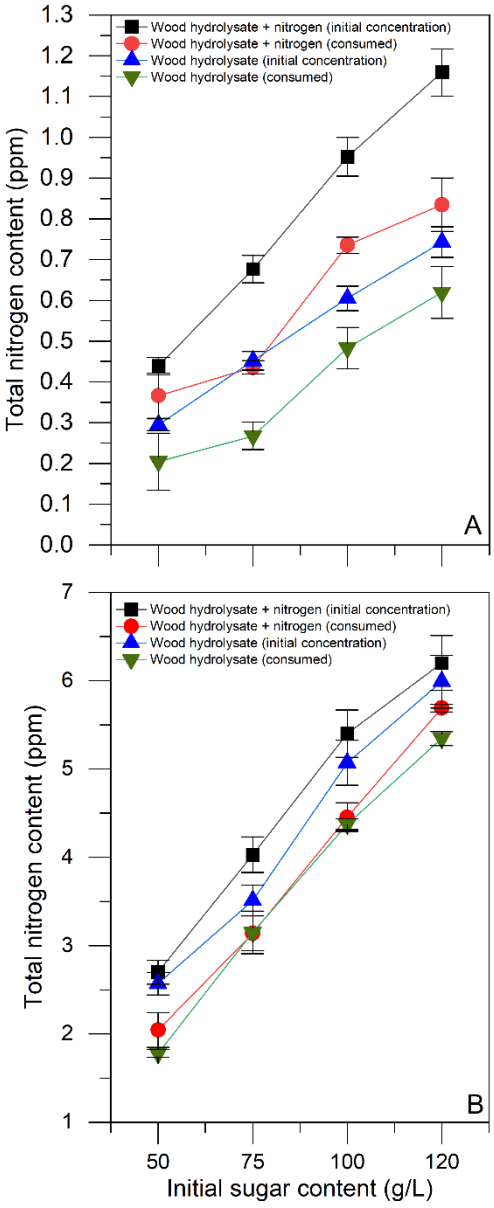
APPENDIX D. Supplementary information of Section 5.3

Set of tested treatments.

Treatment	Wood hydrolysate		Nitrogen
	Type	Initial sugar concentration (g/L)	
T1	C6	50	+
T2		75	+
T3		100	+
T4		120	+
T5	C5	50	+
T6		75	+
T7		100	+
T8		120	+
T9	C6	50	-
T10		75	-
T11		100	-
T12		120	-
T13	C5	50	-
T14		75	-
T15		100	-
T16		120	-

+: treatments with nitrogen source; -: treatments without nitrogen source

Total nitrogen content.

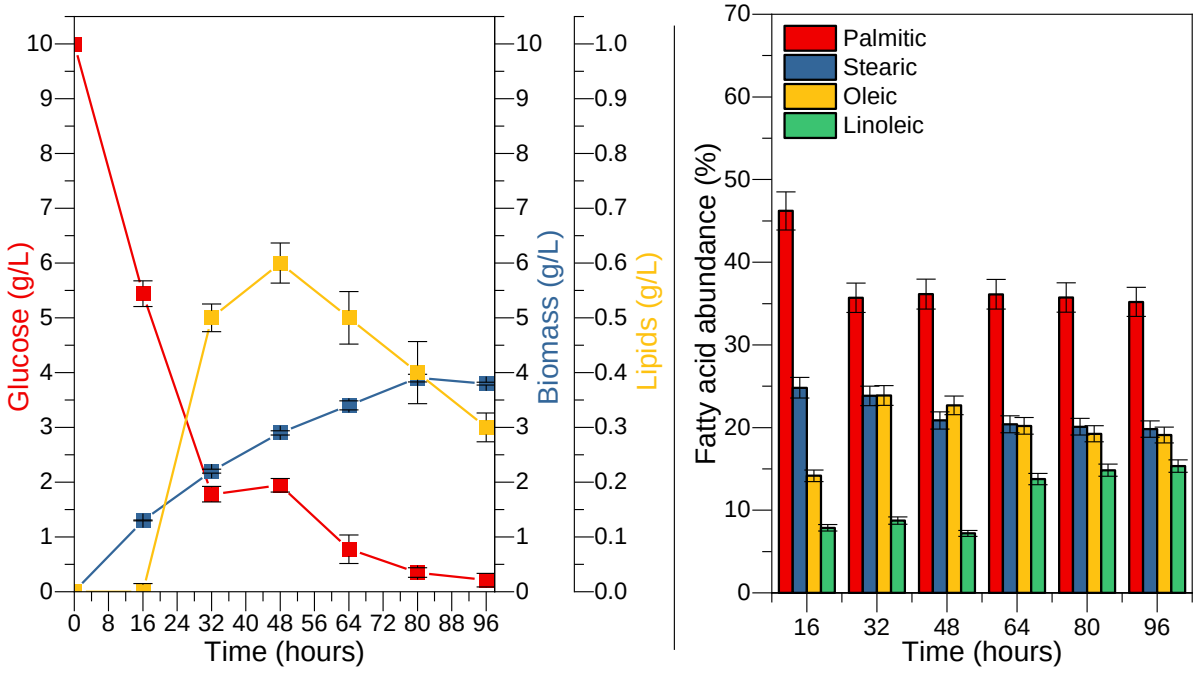


APPENDIX E. Supplementary information of Section 5.4

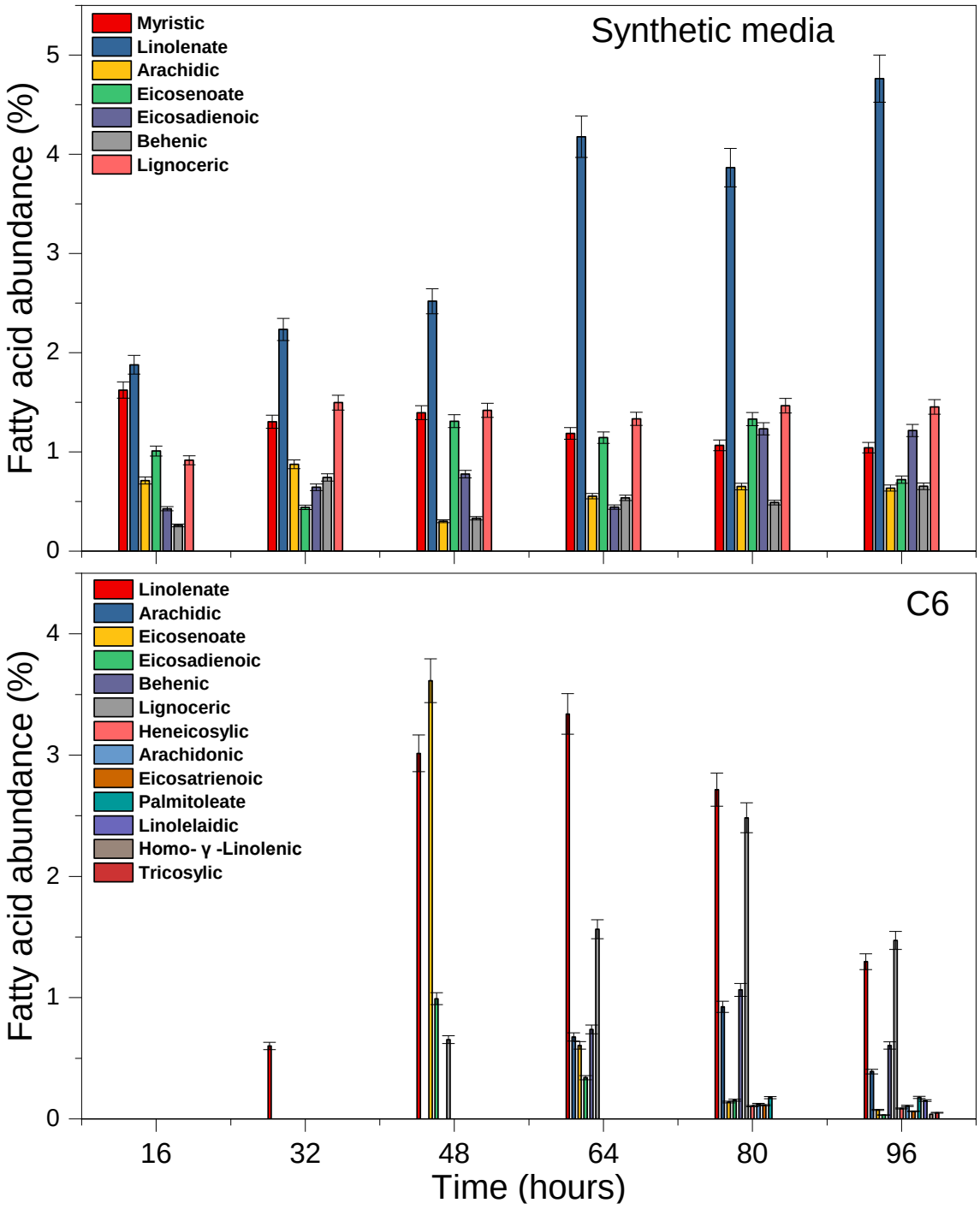
Performance of R. toruloides-1588 using undetoxified wood hydrolysates.

Kinetic parameter	Culture media/Wood hydrolysate			
	Synthetic	C5	C6	C5/C6
$Y_{L/X}$ (%)	15.38	30.79	57.14	43.75
$Y_{L/S}$ (g of lipids/g of biomass)	0.15	0.31	0.57	0.44
$Lipid_{prod}$ (g L^{-1} h^{-1})	0.01	0.01	0.05	0.03
$Biomass_{prod}$ (g of biomass/g of sugar consumed)	0.40	0.18	0.38	0.27
$Sugar_{rate}$ (h^{-1})	0.10	0.22	0.25	0.24
$Sugar_{specific}$ (g g h^{-1})	0.03	0.07	0.03	0.04
μ_{max} (h^{-1})	0.04	0.04	0.09	0.07

***R. toruloides*-1588 cultivation using YM culture media.**

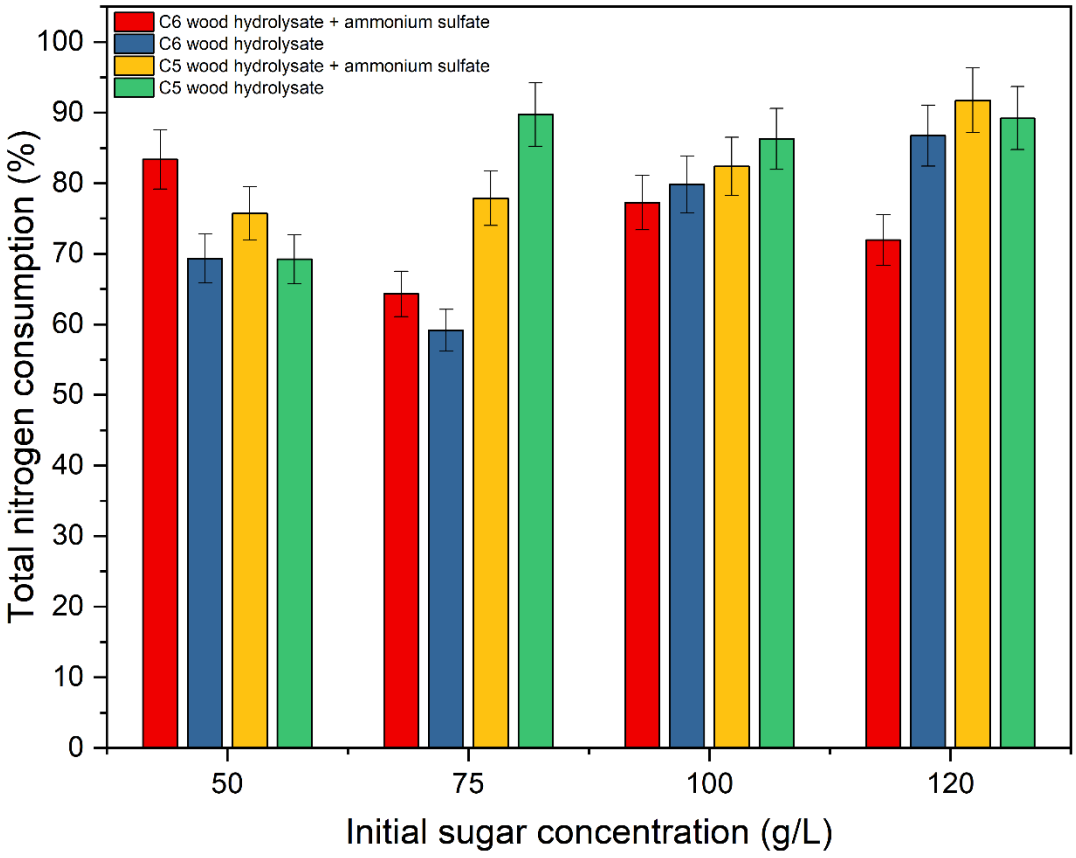


Polyunsaturated fatty acid profile from produced lipids using YM media and C6 wood hydrolysate.

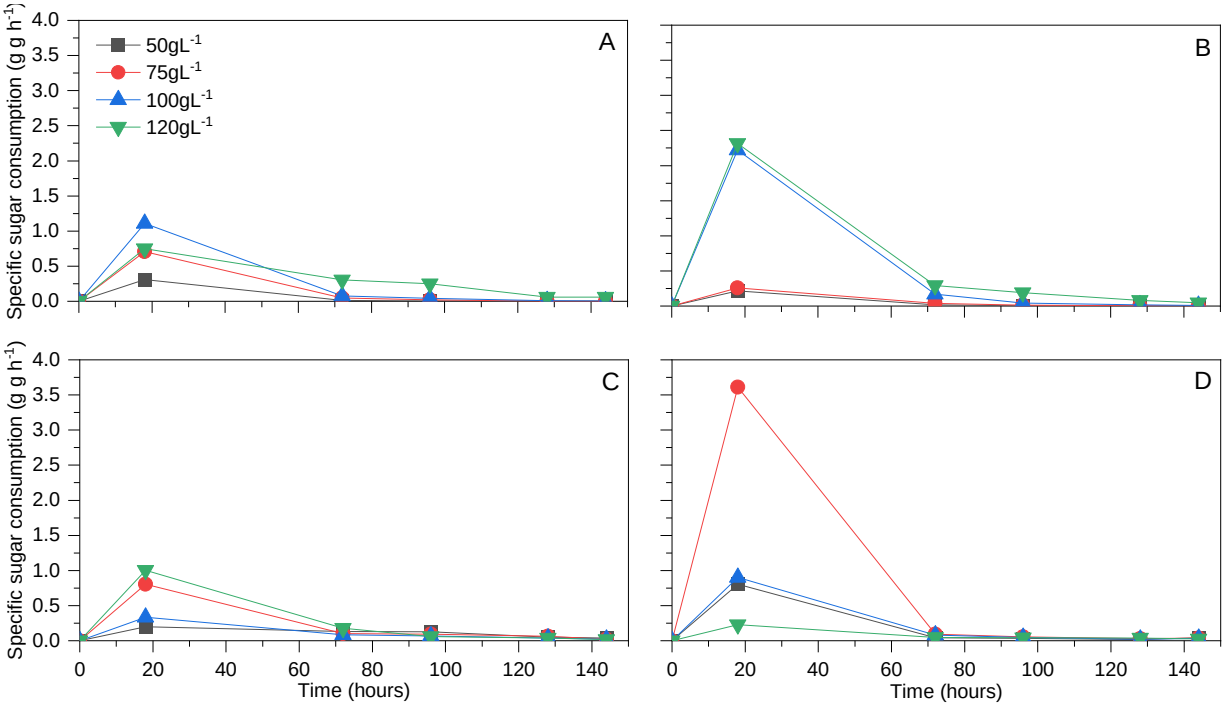


APPENDIX F. Supplementary information of Section 5.5

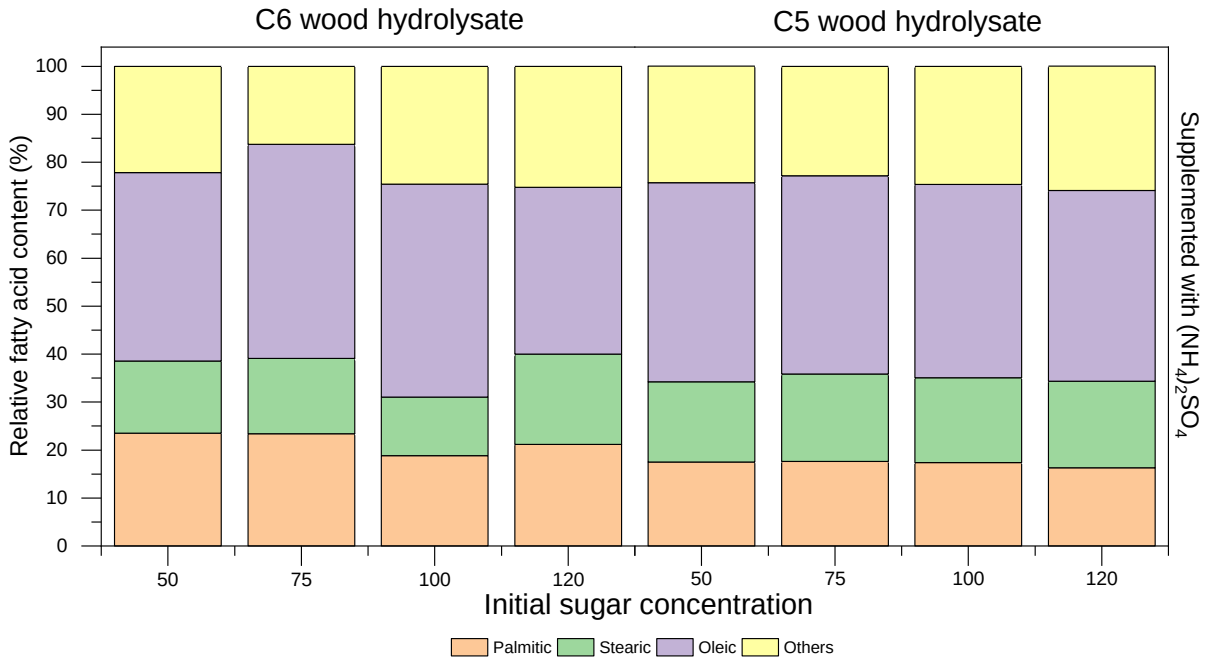
Total nitrogen consumption by *R.* toruloides NRRL 1588.

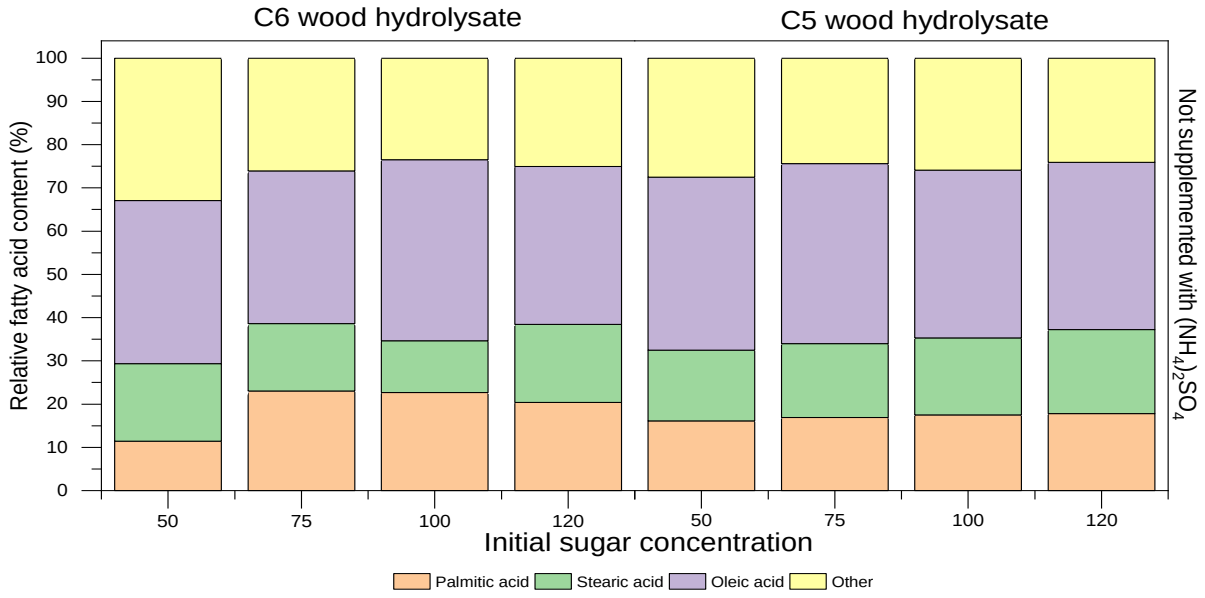


Specific sugar consumption by *R.* toruloides NRRL 1588.



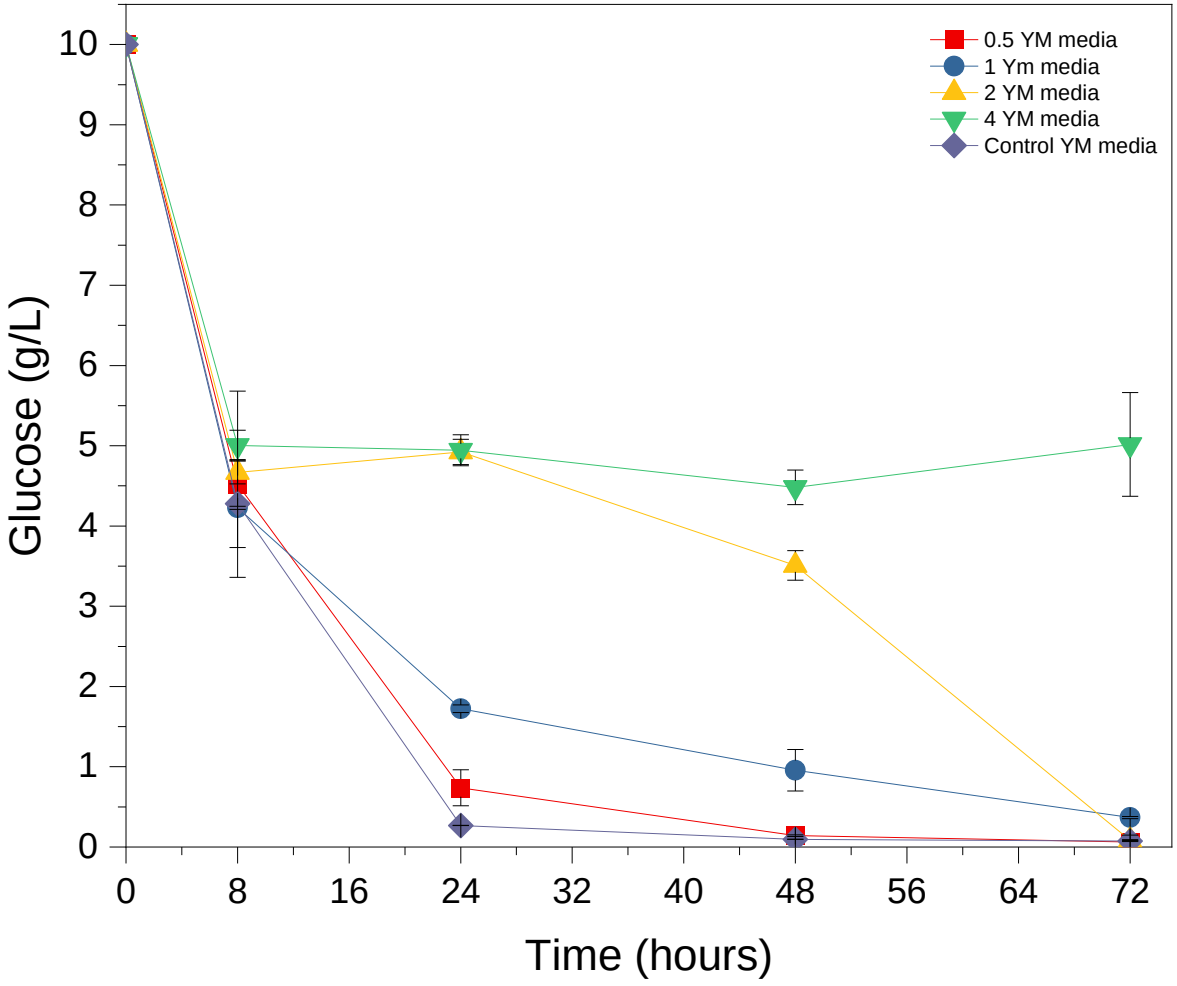
Fatty acid profile of accumulated lipids by *R.* toruloides NRRL 1588.



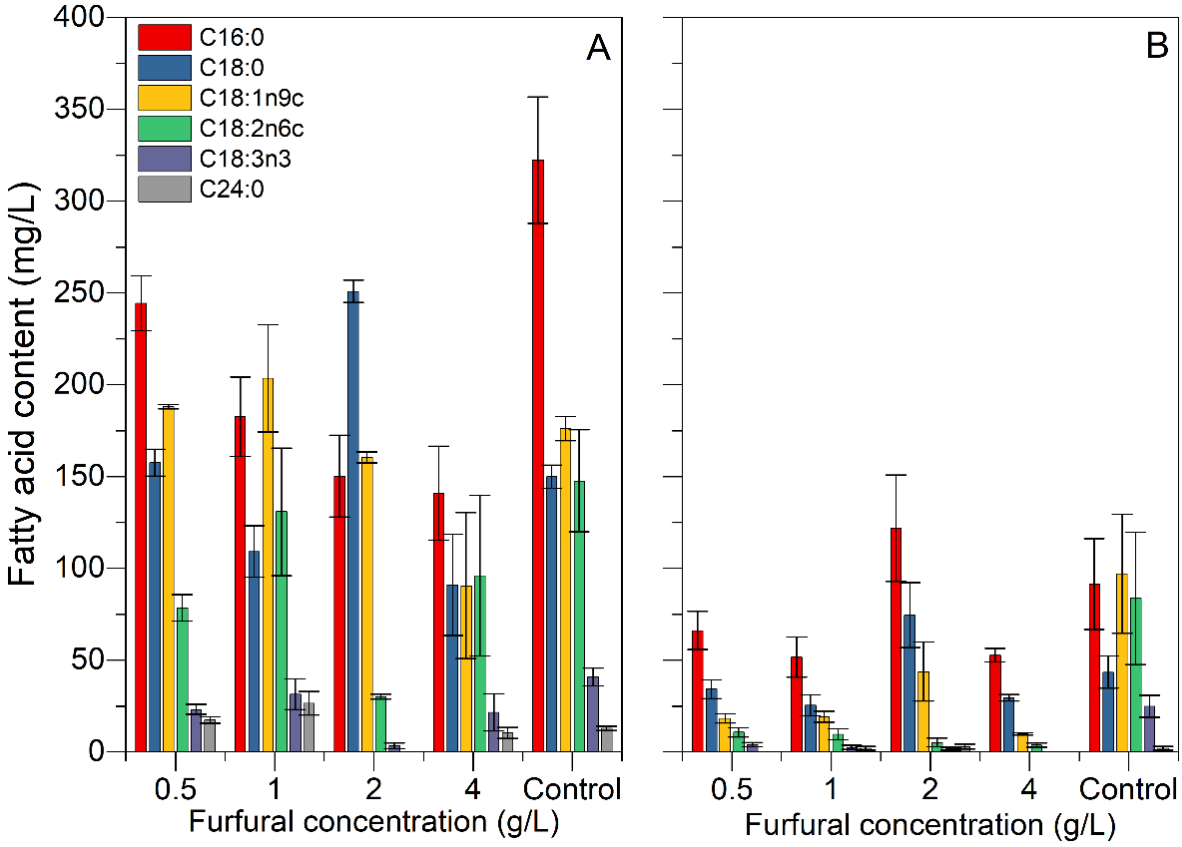


APPENDIX G. Supplementary information of Section 5.6

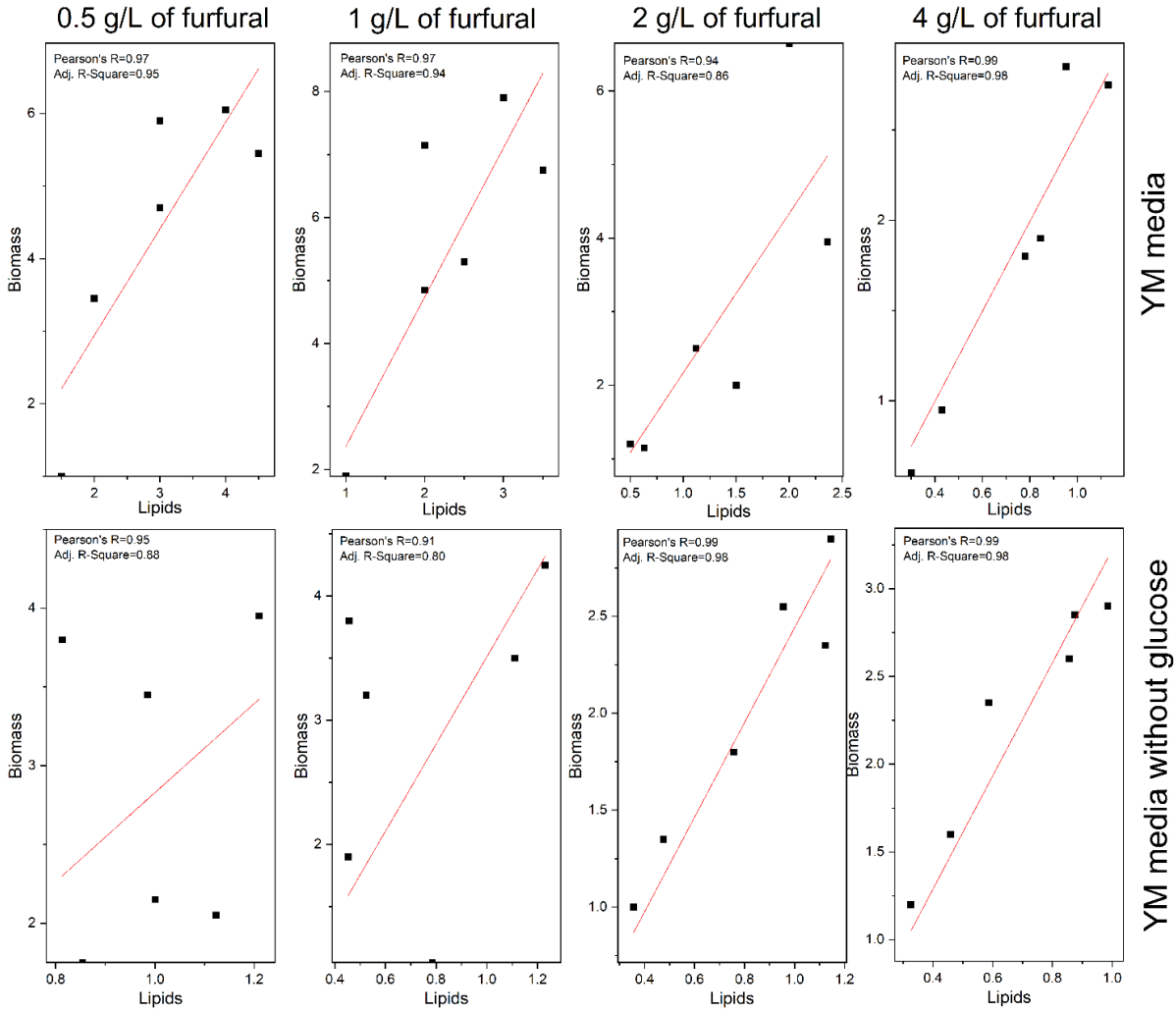
Sugar consumption by *R. toruloides*-1588 under high furfural concentrations.



Fatty acid profile of produced lipids by *R. toruloides*-1588 in presence of high furfural concentration. C16:0 (palmitic acid), C18:0 (stearic acid), C18:1n9c (oleic), C18:2n6c (linoleic), C18:3n3 (linolenate acid), and C24:0 (lignoceric acid).



Correlation analysis of produced biomass vs lipids by *R. toruloides*-1588 in presence of high furfural concentrations.



APPENDIX H. Data assessment *on Rhodosporidium* sp.

Figure 1. Microbial biomass and lipid accumulation by *Rhodosporidium* sp.

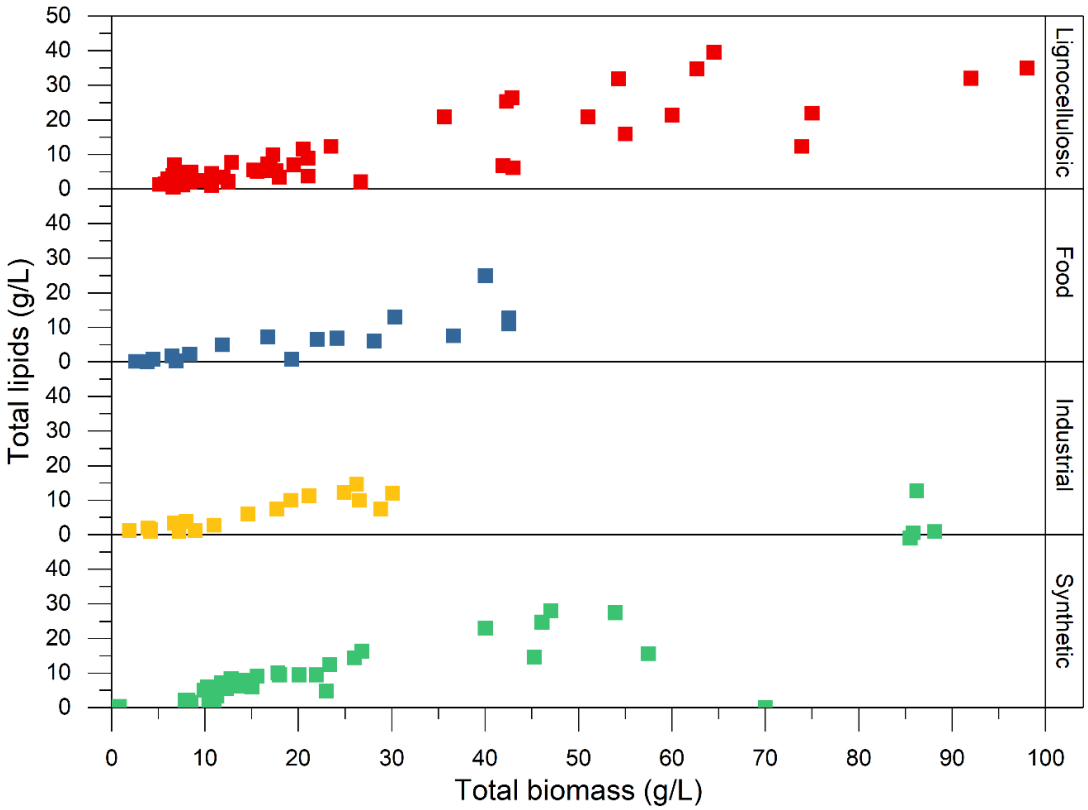


Figure 2. Influence of carbon/nitrogen ratio on microbial biomass and lipid accumulation by *Rhodosporidium* sp.

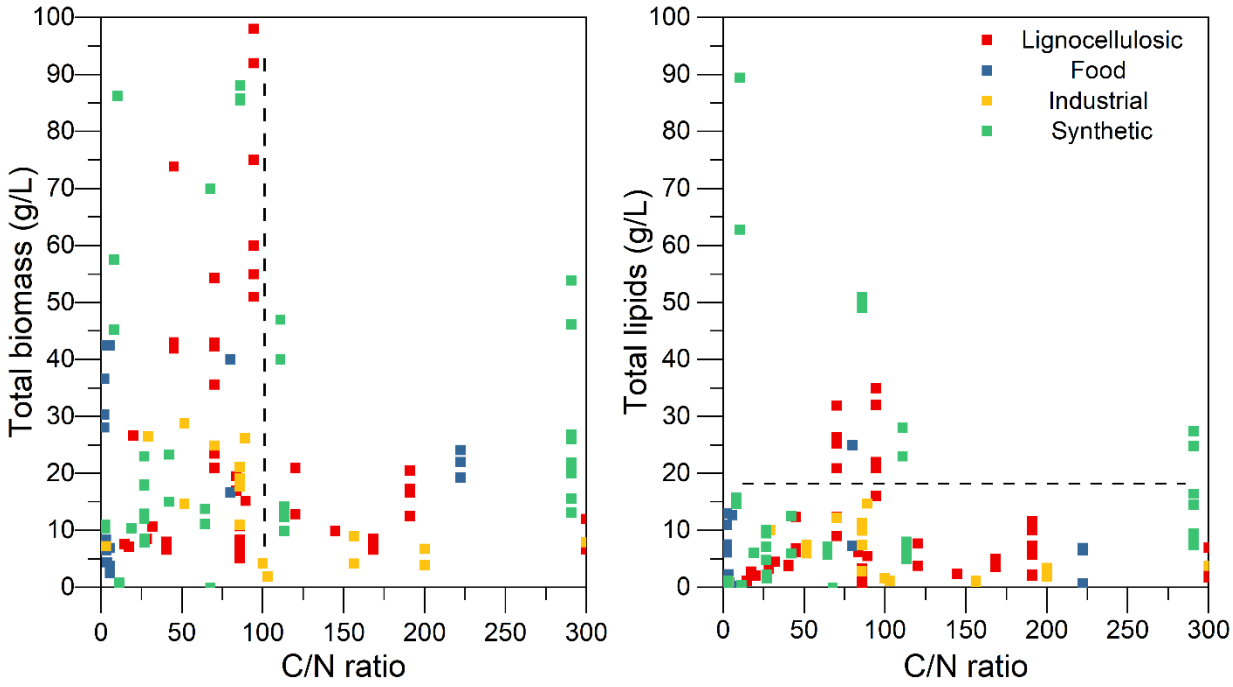


Figure 3. Sugar effect on microbial biomass and lipid accumulation by *Rhodosporidium* sp.

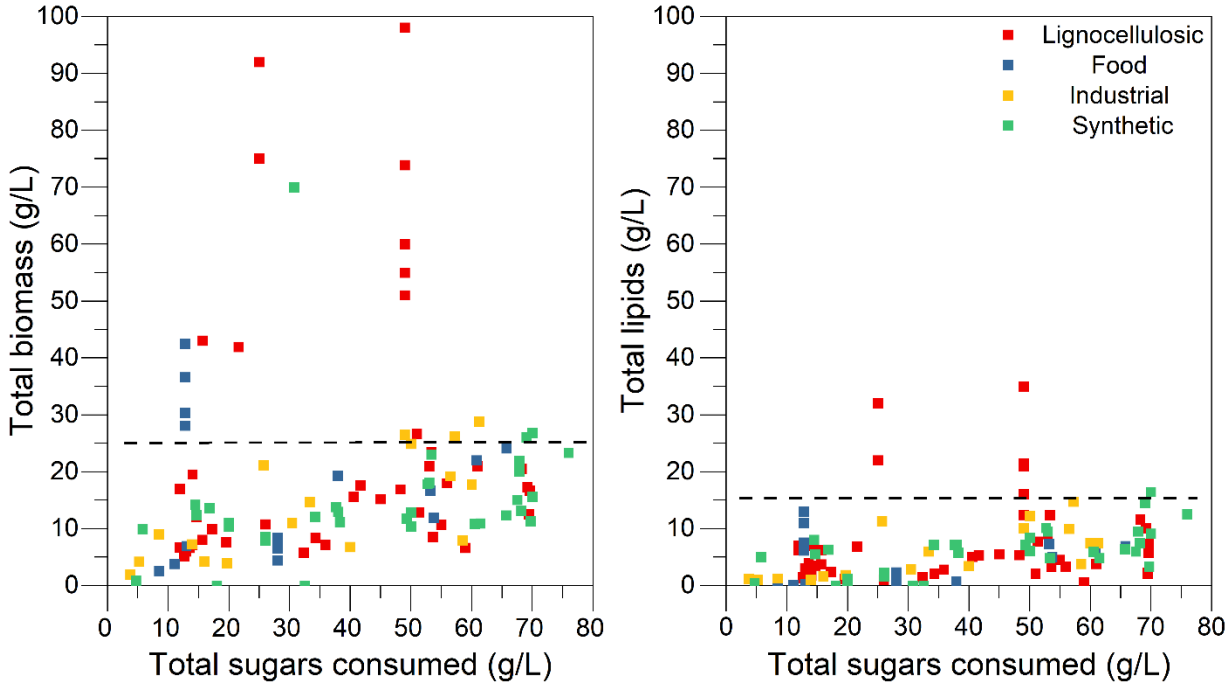
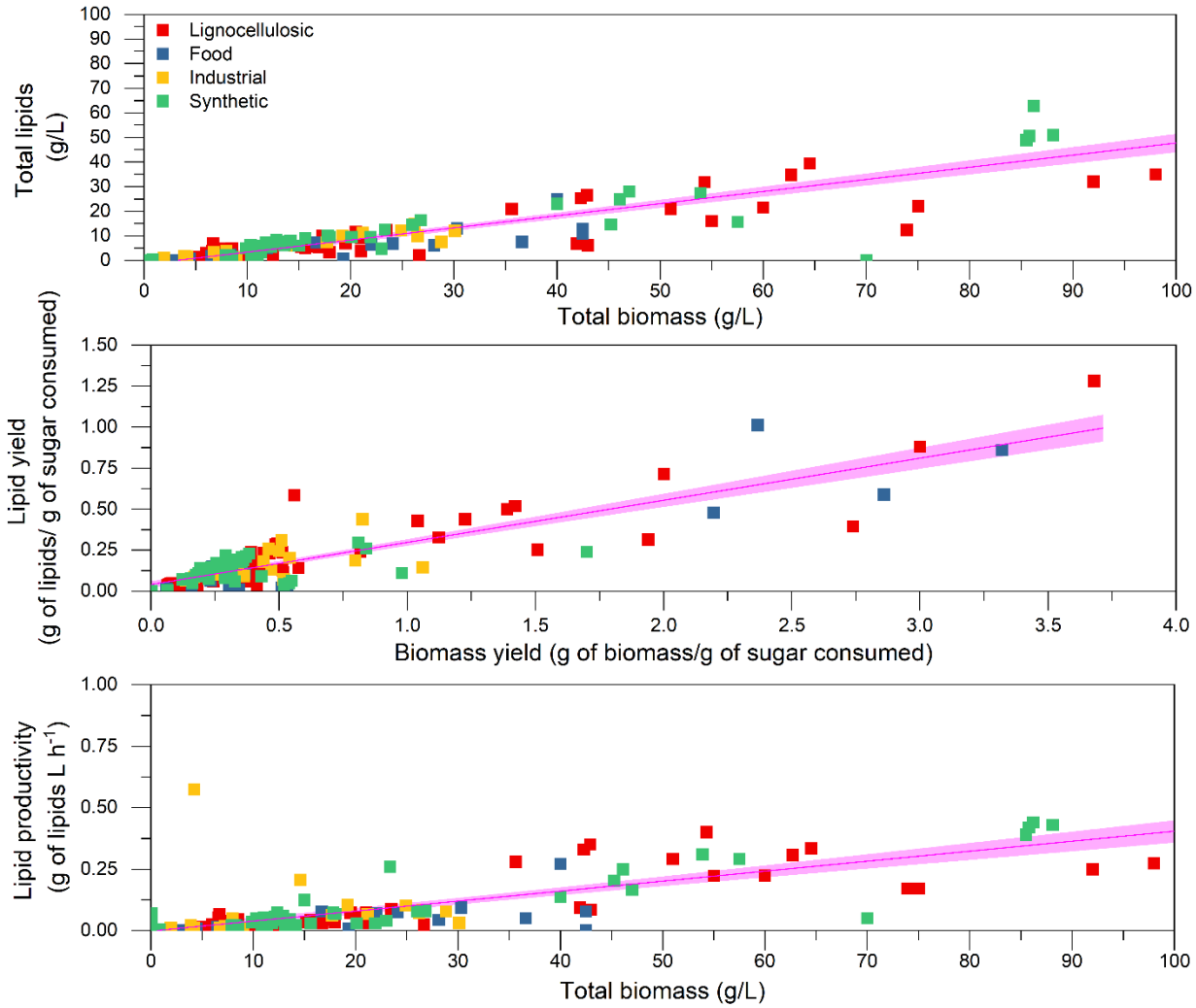


Figure 4. Microbial lipid accumulation by *Rhodosporidium* sp.



APPENDIX I. Materials and methods

Wood feedstock

Softwood sawdust with 35% White Spruce (*Picea glauca* (Moench) Voss), and 65% Fir (*Abies* sp.) and hardwood sawdust from Maple (*Acer* sp), was kindly provided by Quebec Forest Industry Council (QFIC). Both materials were dried at 60°C for 12 hours and ground in a planetary ball mill (PM100; Retsch Corporation) for 15 min at 500 rpm. The resulting milled feedstock was fractioned in a range of 1 mm-300 μm.

Wood hydrolysate feedstock

Two different hydrolysates (C6 and C5) were kindly provided by Greenfield Global Inc., Varennes, Quebec, Canada. Table shows a general composition for each hydrolysate. Before the hydrolysate was used in all the performed experiments, both hydrolysates were centrifuged at 4°C, 9000 rpm for 30 minutes. Liquid and solid fractions were kept separately and stored at -20°C for further experiments. Figure shows the process followed to prepare C5 and C6 hydrolysates before its use during fermentation processes.

Table. General wood hydrolysate composition.

Component (g/L)	C6 hydrolysate	C5 hydrolysate
Glucose	117.0	10.1
Xylose	11.8	120.3
Total nitrogen	0.611	5.3
Formic acid	0.3	5.6
Acetic acid	3.2	33.7
Hydroxy methyl furfural	0.2	0.1
Furfural	0.2	<0.1
pH	5.6	5.2

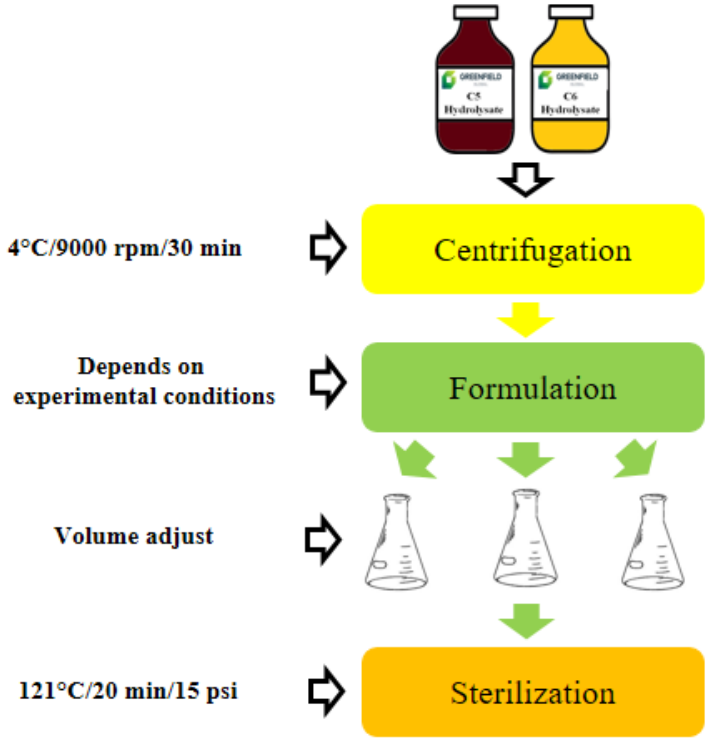


Figure. General process to prepare C5 and C6 wood hydrolysates used as a culture media.

Microorganism, media for culture maintenance and inoculum preparation

The strains used in this work are listed in Table. All the strains were procured from Agricultural Research Service Culture Collection (NRRL, USA). Yeast laboratory stocks and inoculum preparation were grown in YM agar/broth (g/L): yeast extract 3; malt extract 3; peptone 5; glucose 10, bacteriological agar 15. To prepare the inoculum seed, each yeast was pre-cultured up to three generations in a Multitron® shaker (Infors Canada) at 25°C, 200 rpm for 36 hours.

Table. Strains used to evaluate lipid production and sugar utilization in R. toruloides using C-6 and C-5 wood hydrolysate as a substrate.

Strain	Features	Purpose
Rhodosporidium toruloides NRRL-1091	Isolated from wood pulp, however, it has been reported that this yeast could not grow in undetoxified lignocellulosic hydrolysates	Lipid production
Rhodosporidium toruloides NRRL-1588	Isolated from the soil, with the capacity to produce up to 100 g/L of biomass and accumulate up to 60% of its weight in lipids	
Rhodosporidium toruloides NRRL-6984	Isolated from wood pulp and has been reported that this yeast could not grow in undetoxified lignocellulosic hydrolysates	
Rhodosporidium toruloides NRRL-6987	Diploid isolated from a hyphal conjugate of NRRL Y-1091 and NRRL Y-1588, capable of producing 0.24 g biomass/g glucose and up to 69-79 mg oil/g glucose in media with nitrogen restriction	
Rhodosporidium toruloides NRRL-7191	The isolation source of this strain is not available, and there are no reports of its use in the production of lipids	
Yarrowia lipolytica NRRL-63746	Unconventional yeast can accumulate up to 50% of its dry weight in lipids	Control for lipid production
Scherfenomyces Stipities NRRL-17104 *Scherfenomyces stipities* NRRL-11545	These yeasts in the native form had the highest capacity to use C5 sugars (xylose and arabinose) than any other known microorganism	Control for C5 sugar utilization
Saccharomyces cerevisiae NRRL-12632	These yeast present high tolerance to aldehyde inhibitors such as furfural and 5-HMF	Control for C6 sugar utilization and negative control for lipid production

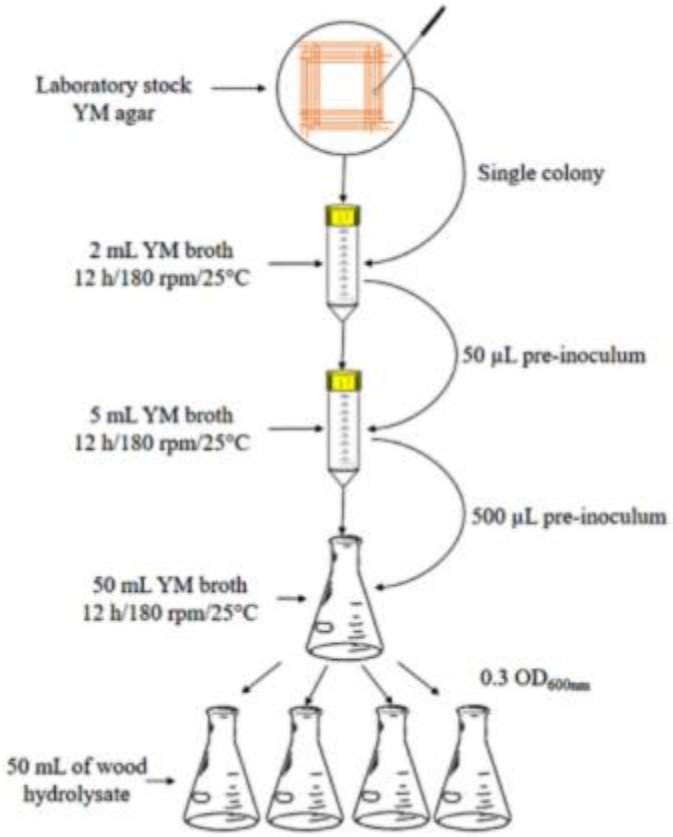


Figure. General process to prepare inoculum seed.

Green Extraction of Valuable Chemicals from Lignocellulosic Biomass using Microwave Method

Hydrophilic compounds derived from hardwood and softwood sawdust were obtained with microwave-assisted extraction method (MAE) using sodium hydroxide (NaOH) as a catalyst. Compounds were extracted separately for each type of wood. Table shows all the tested trials used to study the effect of temperature (65, 80 and 95°C), time (15, 30, and 45 minutes), and NaOH concentration (1, 4, and 7% v/v) on the release of levulinic acid, 5-hydroxymethylfurfural, furfural, 4-aminobenzoic acid, vanillic acid, vanillin, syringaldehyde, and ferulic acid. Briefly, 200 mg of sawdust were treated with MAE at 1000 watts of power using a Perkin-Elmer Anton Paar Multi-Wave Sample Prep System, USA. After MAE, samples were centrifuged at 9000 *x*g for 15 min at room temperature and the supernatant and solids were keep separately in sterile tubes to 4°C for further experiments. The effect of temperature, time, and NaOH in green chemical production was

analyzed using a Response Surface Methodology (RSM) with a Box-Behnken experimental design including three central points. The statistical analysis of the data was performed using Minitab® 17.1.0 (LEAD Technologies, USA). Parameter interaction effects were analyzed by One-Way ANOVA (Fisher test) at 95% of confidence. All the experiments were carried out in duplicates and the average and standard deviation for the data set were calculated.

Table. Treatments tested for green chemical compounds production.

Treatment	Time (min)	Temperature (°C)	Sodium hydroxide (%)
1	15	65	4
2	45	65	4
3	15	95	4
4	45	95	4
5	15	80	1
6	45	80	1
7	15	80	7
8	45	80	7
9	30	65	1
10	30	95	1
11	30	65	7
12	30	95	7
13	30	80	4
14	30	80	4
15	30	80	4

Pulsed-ozonolysis assisted oxidative treatment of forestry biomass for lignin fractionation

To perform a good degree of delignification, parameters for microwave-assisted extraction were selected according to results obtained in our previous work. Briefly, hardwood and softwood samples (200 mg/microwave vessel) were treated separately using MAE at 80°C for 30 min, and 1000 Watts of power using a Perkin-Elmer Anton Paar Multi-Wave Sample Prep System. To carry out the reaction 5 mL of 4% sodium hydroxide solution was used as a delignifying agent using a solid/liquid ratio of 1:25. For thermal treatment, 4 g of hardwood and softwood were treated separately with 4% of sodium hydroxide solution (NaOH) for 20 min at 120°C, and 15 psi of pressure. After MAE and thermal process, samples were centrifuged at 9000 *x*g for 15 min at room temperature and the supernatant and solids were stored separately in sterile tubes at 4°C for further use. Liquid and solid samples obtained previously from MAE and thermal process were treated

with ozone as a complementary treatment to release lignin derivatives. For both fractions (liquid and solid), 30, 45, 60, 120, and 240 sec of direct ozone exposure were used. Ozone was produced in a module Ozonair EMO3-131®, with a flow of 47.195 L/s and a minimum rate of conversion of 0.02 ppm. To select the best treatment, the analysis of the obtained data was conducted using Minitab® 17.1.0 (LEAD Technologies, USA). The statistical significance was analyzed with One-Way ANOVA and compared with the Fisher test at a 95% level of confidence. All the experiments were carried out in duplicates and the average and standard deviation for the data set were calculated.

Lipid Production in *Rhodosporidium toruloides* using C-6 and C-5 Wood Hydrolysate: A Comparative Study

For microbial lipid production, five strains of *R. toruloides* cultures were cultivated separately in C6 hydrolysate and C5 hydrolysate with an initial sugar concentration of 50 g/L, 1 g/L of ammonium sulfate ($(NH_4)_2SO_4$), and initial pH of 6. Both substrates were inoculated with an initial 0.3 OD_{600nm} of inoculum seed and cultivated at 25°C/180 rpm in a Multitron® shaker (Infors Canada). During fermentation, samples were drawn every 18 h until the stationary phase, afterward, sampling was every 8 h. All the experiments were carried out in duplicates and the average and standard deviation for the data set were calculated using Minitab® 17.1.0 (LEAD Technologies, USA).

Carbon/nitrogen ratio as a tool to enhance the lipid production in *Rhodosporidium toruloides*-1588 using C5 and C6 wood hydrolysates

R. toruloides-1588 was cultured in 250 mL-flasks containing 50 mL of wood hydrolysate, with a total initial sugar content of 50 g/L distributed in three initial glucose: xylose ratio concentrations (1:1, 1:2, 2:1), three C/N ratios (20, 70, and 120), and three initial concentrations of Na_2HPO_4 (0.1, 1.05, and 2.0 g/L). Glucose and xylose treatments without phosphate addition were used as a control. Each flask was inoculated adding a seed culture up to an initial OD_{600nm} of 0.1 and incubated for 176 h at 25°C (Infors Multitron Incubator Shaker). Ammonium sulfate $(NH_4)_2SO_4$ was used as a nitrogen source, and three C/N ratios were calculated based on the total carbon content (glucose + xylose). The effect of C/N ratio, lipid inducer addition and different initial sugar proportions in cell biomass and lipid production by *R. toruloides*-1588 was analyzed using a Box-Behnken experimental design with three central points (Table). The statistical analysis of the data

was performed using Minitab® 17.1.0 (LEAD Technologies, USA). Parameter interaction effects on lipid production were analyzed by One-Way ANOVA (Fisher test) at 95% confidence. All the experiments were carried out in duplicates.

Table. Experimental design to optimize lipid production in *R. toruloides*-1588.

Treatment	C/N (ratio)	Phosphate (g/L)	Glucose (g/L)	Xylose (g/L)	Description
T-1	20	0.1	25	25	
T-2	120	0.1	25	25	
T-3	20	2	25	25	
T-4	120	2	25	25	
T-5	20	1.05	12.5	37.5	
T-6	120	1.05	12.5	37.5	
T-7	20	1.05	37.5	12.5	
T-8	120	1.05	37.5	12.5	Response Surface Methodology
T-9	70	0.1	12.5	37.5	
T-10	70	2	12.5	37.5	
T-11	70	0.1	37.5	12.5	
T-12	70	2	37.5	12.5	
T-13	70	1.05	25	25	
T-14	70	1.05	25	25	
T-15	70	1.05	25	25	
T-16	70	0	50	0	Positive glucose control
T-17	70	0	0	50	Positive xylose control
T-18	70	0	0	50	Negative xylose control
T-19	70	0	50	0	Negative glucose control

Lipid production (Catabolic repression)

Lipid production, catabolic repression effect, and nitrogen addition were tested using 50 ml of C6 and C5 wood hydrolysate, separately under the following conditions: 1 g/L of $(NH_4)_2SO_4$ was used as nitrogen source, initial pH of 6, 25°C, and 200 rpm during 198 h. Table shows all trials with the tested conditions. The statistical analysis of the data was conducted using Origin Lab-Pro® 20.0 (USA). The effect of sugar concentration and addition of nitrogen on biomass production, sugar utilization, and lipid accumulation was evaluated by One-Way ANOVA with a Fisher test at a 95% level of confidence. All the experiments were carried out in duplicates.

Table. Treatments to evaluate catabolic repression effect in R. toruloides-1588.

Treatment	Wood hydrolysate		Nitrogen
	Type	Initial sugar concentration (g/L)	
T1	C6	50	+
T2		75	+
T3		100	+
T4		120	+
T5	C5	50	+
T6		75	+
T7		100	+
T8		120	+
T9	C6	50	-
T10		75	-
T11		100	-
T12		120	-
T13	C5	50	-
T14		75	-
T15		100	-
T16		120	-

+: treatments with $(NH_4)_2SO_4$; -: treatments without $(NH_4)_2SO_4$

Lipid production at fermenter scale using native *R. toruloides*-1588 and C5 undetoxified wood hydrolysate as a culture media

All cultures were performed in 3 liters (2.5 liters working volume) stirred tank reactor (Labfors 4, Infors USA), equipped with a Rushton turbine (radial flow) and operated at 30°C. Experiments were performed at 400 rpm and air flow ratio of 1 vvm to maintain 35% of dissolved oxygen. The experiments were performed using synthetic media, C5 and C6 wood hydrolysate inoculated with 0.1 OD_{600nm} of seed culture and Antifoam 204 at 0.05% v/v were used. The initial pH was adjusted to 6.0±0.5 before inoculation and keep constant during the fermentation by addition of sterile solution of sodium hydroxide (1N) or hydrocloridric acid (1N). The pH and dissolved oxygen were monitoring using a Hamilton EasyFerm plus and Mettler Toledo probes, respectively. Samples were taken each 8 hours for a total fermentation time of 96 h to determine the microbial growth, cell biomass production, sugar consumption, and lipid accumulation.

Inhibitor degradation by *R. toruloides*-1588 using undetoxified wood hydrolysate as a culture media

Degradation studies were performed in a 250 mL flask using 50 mL of wood hydrolysate at 25°C, initial pH of 6, 200 rpm for 128 hours. The effect of four initial sugar concentrations (50 g/L, 75 g/L, 100 g/L, and 120 g/L) and the four initial concentrations of ammonium sulfate (0.87 g/L, 1.30 g/L, 1.74 g/L, and 2.08 g/L, respectively) on inhibitor degradation were tested. The statistical analysis of the data was conducted using Origin Lab-Pro® 20.0 (USA) with One-Way ANOVA (Fisher test) at 95% level of confidence. All the experiments were carried out in duplicates.

Furfural degradation by *R. toruloides*-1588 using synthetic media

R. toruloides-1588, was cultured in 250 mL-flask with 50 mL of YM broth initial pH of 6, during 72 hours, at 30°C and 200 rpm. To study furfural, transformation, and its effect in *R. toruloides*-1588 on microbial biomass production, sugar utilization, lipid accumulation, and fatty acid distribution were analyzed. For this purpose, four initial furfural concentrations (0.5, 1.0, 2.0, and 4.0 g/L, respectively) were used. Likewise, a separate set of treatment with the same furfural concentration using YM broth without glucose (yeast extract 3 g/L; malt extract 3 g/L; peptone 5 g/L) was used to test the ability of *R. toruloides*-1588 to use furfural as alternative energy source.

Analytical methods

Cell growth

Cell growth was measured by dry constant weight (DCW). Briefly, the cells were harvested by centrifugation (15000 *x g*/2 min), washed with phosphate-buffered saline (PBS) with pH=7, and dried at 60°C until a constant weight was retained (Figure).

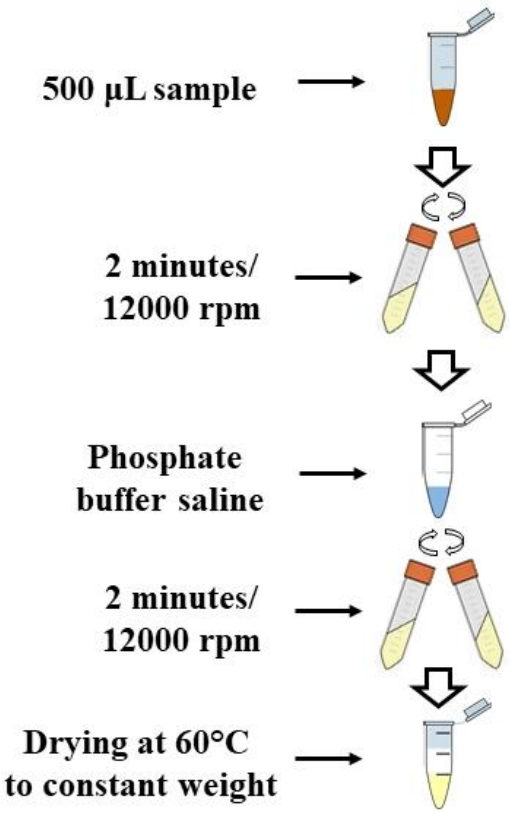


Figure. Process utilized to quantify cellular biomass.

Liquid Cromatography-Mass Spectrometry (LC-MS) analysis

During the fermentation samples for each fermentation were drawn and analysis for xylose, glucose, and lignin inhibitory compounds were performed using a Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer (LC-MS) according to our previous work. Briefly, sugars were analyzed using a HILIC pak VG-50 2D column (5 µm x 150 mm x 4.6 mm) using a solution of acetonitrile: water (89:11 v/v), D6-glucose as an internal standard and mass detector in a positive mode. In the case of lignin inhibitory compounds, a BetaBasic-18 column (100mm x 2.1mm; 3µm) was used with a solution of water: methanol (80:20 v/v) as mobile phase and phenylethanol-D5 as internal standard with mass detector in negative mode detection, with the exception of 2-furoic acid and 2-oxoglutaric acid where the mass detector was used in positive mode detection. Finally, lignin monomers were analyzed using a HILICpak VG-50 2D column (5 µm x 150 mm x 4.6 mm) with a solution of acetic acid in water (0.1% v/v) and a solution of acetic acid in methanol (0.05% v/v), with a ratio of 82.5:17.5 (respectively) as a mobile phase. Glycerol was used as an internal standard and the mass detector was operated in a positive mode detection. All compounds were

identified and quantified by comparing peak areas and retention times with its standard compounds (Sigma-Aldrich, USA).

Gas Chromatography-Flame Ionization Detector (GC-FID) analysis

Fatty acids, furfuryl alcohol and acetic acid were analyzed using an Agilent 7890B Gas Chromatography-Flame Ionization Detector (GC-FDI). Fatty acid profile from produced lipids was performed trough lipid transesterification into fatty acid methyl esters (FAMEs). Briefly, a solution of methanol-sulfuric acid was used as catalyst at 100°C during 20 min. After that, FAMEs were extracted with hexane and analyzed using an Agilent 122-2362 DB-23 column (60 m x 250 µm and 0.25 µm film thicknes). Fatty acids were identified and quantified by comparing the retention times and peak areas with a Supelco 37 Component FAME Mix (Sigma-Aldrich, USA). For furfuryl alcohol, a NUKOL capillary column (15 m x 0.25 mm x 0.25 µm) was used. Finally, acetic acid was quantified using a Thermo Trace 1310 and a Thermo ISQ-MS detector. GC was equipped with a ZB-WAXPlus column (30 m x 0.25mm x 0.25 µm). The acquisition was in SIM mode with the following parameters: Acetic acid: Quantification ion 60 m/z; qualifier ion : 45 m/z, and iso-Butanol as internal standard with a quantification ion 41 m/z; qualifier ion: 56 m/z. Furfuryl alcohol and acetic acid were determined and quantified according to the peak area and retention time of its corresponding standard compounds (Sigma-Aldrich, USA).

Lipid extraction

Figure shows the process followed during the lipid extraction. Briefly, *Rhodosporidium toruloides* cells were harvested by centrifugation (15 000 *x* g/2 min), followed by drying at 60°C to obtain a constant weight. The cell lysis was carried out to acid hydrolysis for 1 hour with 1 M per litre of hydrochloric acid (HCl), obtained from Fisher Scientific (Ottawa, Ontario, Canada). Lipids were extracted with 3.75 mL of chloroform/methanol (2:1 v/v), the mixture was vortexed for 15 min. Later, 1.25 mL of chloroform was added, vortexed again for 1 min, and lastly, 1.25 mL of a 1M solution of sodium chloride (NaCl) obtained from Fisher Scientific (Ottawa, Ontario, Canada) was added. The resulting mixture was centrifuged at 7000 *x g* for 15 min to separate the two phases (aqueous and organic phase), the organic phase was kept and evaporated at 50°C during 18 hours.

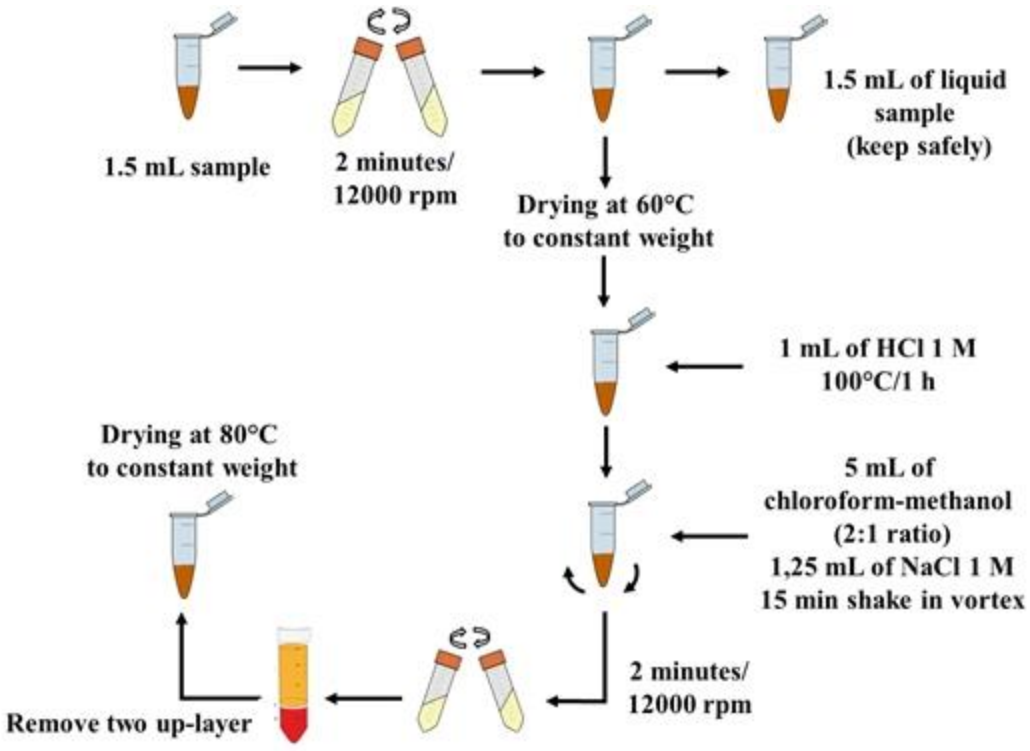


Figure. Process utilized to extract lipids from Rhodosporidium toruloides.

Fourier-Transform Infrared Spectroscopy (FTIR) analysis

To achieve Fourier-Transform Infrared Spectroscopy (FTIR) analysis, the samples were frozen at -20˚C for 24 hours to perform the lyophilization in a freeze-drying system (Dura Freeze Dryer, Kinetics). Then, the lyophilized samples were analyzed by FTIR using Cary 670 FTIR Spectrometer. The spectra were obtained at a resolution of 4 cm-1, in the range of 4000-400 cm^{-1}.