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Conversion of Industrial Waste and Wastewaters into Lipids Suitable for Biodiesel Production

Marta AmirSadeghi

Conversion of industrial waste and wastewaters into lipids suitable for biodiesel production

By

Marta AmirSadeghi

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Conversion of industrial waste and wastewaters into lipids suitable for biodiesel production

By

Marta AmirSadeghi

Approved:

_____
W. Todd French
(Major Professor/Graduate Coordinator)

_____
Rafael Hernandez
(Committee Member)

_____
Santanu Kundu
(Committee Member)

_____
Veera Gnaneswar Gude
(Committee Member)

_____
Abdolhamid Borazjani
(Committee Member)

_____
Jason Keith
Dean
Bagley College of Engineering

Name: Marta AmirSadeghi

Date of Degree: December 9, 2016

Institution: Mississippi State University

Major Field: Engineering

Major Professor: W. Todd French

Title of Study: Conversion of industrial waste and wastewaters into lipids suitable for biodiesel production

Pages in Study 167

Candidate for Degree of Doctor of Philosophy

The potential of oleaginous yeast *Rhodotorula glutinis* for the sustainable production of biodiesel feedstock via fermentation of lignocellulosic biomass in pulp and paper wastewater as a fermentation media was investigated. The overall objective was to increase the levels of lipid feedstock in oleaginous yeast in order to reduce biodiesel production cost. To meet sustainable production of biodiesel, industrial wastewater and waste lignocellulose biomass were used as cultivation media and carbon source, respectively. Pulp and paper wastewater effluent was selected as a source of water and nutrients for the production of microbial lipids due to its environmental pollution as it creates large volume of wastewater discharge with high chemical oxygen demand (COD). Since medium composition and process fermentation condition can significantly affect the fermentative performance of oleaginous microorganisms, to find the optimum cultivation condition, design of experiment combined with RSM optimization technique was performed, which has been shown to be successful to predict the optimum condition for the biomass and lipid production in batch fermentation. In addition, lignocellulosic biomass hydrolysate was used as a substrate to improve the cost associated with

feedstock fermentation. Lignocellulosic hydrolysate, a product of degradation of lignocelllosic biomass, contains degradation by-products such as 5-hydroxymethylfurfural (HMF), furfural and acetic acid that are known as major inhibitors that influences microorganism growth process. Therefore, their impacts on the fermentative performance and lipid productivity of oleaginous yeast were explored. A detailed operating condition and equipment design for the process of biocrude production from pulp and paper wastewater on a commercial scale was developed. A technological assessment of the process was performed to evaluate their technical benefits and limitations.

Results show that pulp and paper wastewater can be used as a cultivation media for the production of microbial lipids using *R. glutinis*. However, its carbon content has to be improved. Analysis of the design and cost of the process showed that acid hydrolysis process using paper mill sludge as lignocellulosic biomass required the smallest process equipment units but at a higher raw material cost compared to fermentation process.

DEDICATION

This work is dedicated to my parents who have scarified a lot that I may have this chance to succeed; and to Saber, my always encouraging, ever faithful husband.

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CHAPTER I

INTRODUCTION

1.1 Biorefineries

1.1.1 The biorefinery concept

Biorefinering refers to the integration of biomass fractionation processes for the production of fuels and chemicals using various technologies (Demirbas, 2009). A biorefinery concept is equivalent to the petroleum refinery, except that biorefinary uses plant resources (renewable material derived from photosynthesizing plants), while a petroleum refinery utilizes non-renewable fossil-derived petroleum. The concept of biorefinery is now worldwide. A wide range of bio-based products including, biofuels (bioethanol and biodiesel), biomaterials, and bio-chemicals through downstream fermentation and refining processes can be provided from technologies based upon the biorefinery concept. Biorefinery term refers to the conversion of biomass feedstock into a variety of valuable chemicals and energy with minimal waste and emissions.

1.1.2 Biorefineries as an alternative to petroleum refineries

There are several advantages in developing a biorefinery, which uses renewable raw materials for the production of bioenergy, biofuels, and biochemicals compared to petroleum refinery of crude fossil oil feedstocks: energy security, environmental benefits, and rural development (Jin et al., 2015).

Plant biomass such as wood, agricultural and forest residues are considered a renewable supply, while crude oil resources are limited, and therefore are being depleted due to its increasing consumption as an energy source. Plant biomass feedstock for production of renewable fuels will consequently increase our energy security. Utilization of renewable fuels as an alternative to petroleum fuels will also reduces the pollution in the environment by reducing waste streams and toxic sulfur-products derived from fossil fuel combustion (Cherubini, 2010). Furthermore, a biorefinery economy will stimulate rural development, generating new businesses that will lead to new jobs, and create wealth for the agro-based countries.

In general, biomass contains too low amount of hydrogen, too high amount of oxygen, and lower carbon fraction compared to petroleum feedstocks, which results in both an advantage and disadvantage. More classes of products from wide variety of raw materials can be produced in biorefineries; however, larger varieties of novel processing technologies, which are being developed, are required. A reasonable combination of various methods and processes (physical, chemical, thermal and biological) is required to prepare biobased products for commercial application. By its nature, a biorefinery is an interdisciplinary science, in which incorporates the different aspects of microbiology, chemistry and biochemistry, and engineering including bioengineering, chemical engineering, and process engineering. Figure 1.1 shows a schematic diagram of the biorefinery concept (Demirbas, 2009).

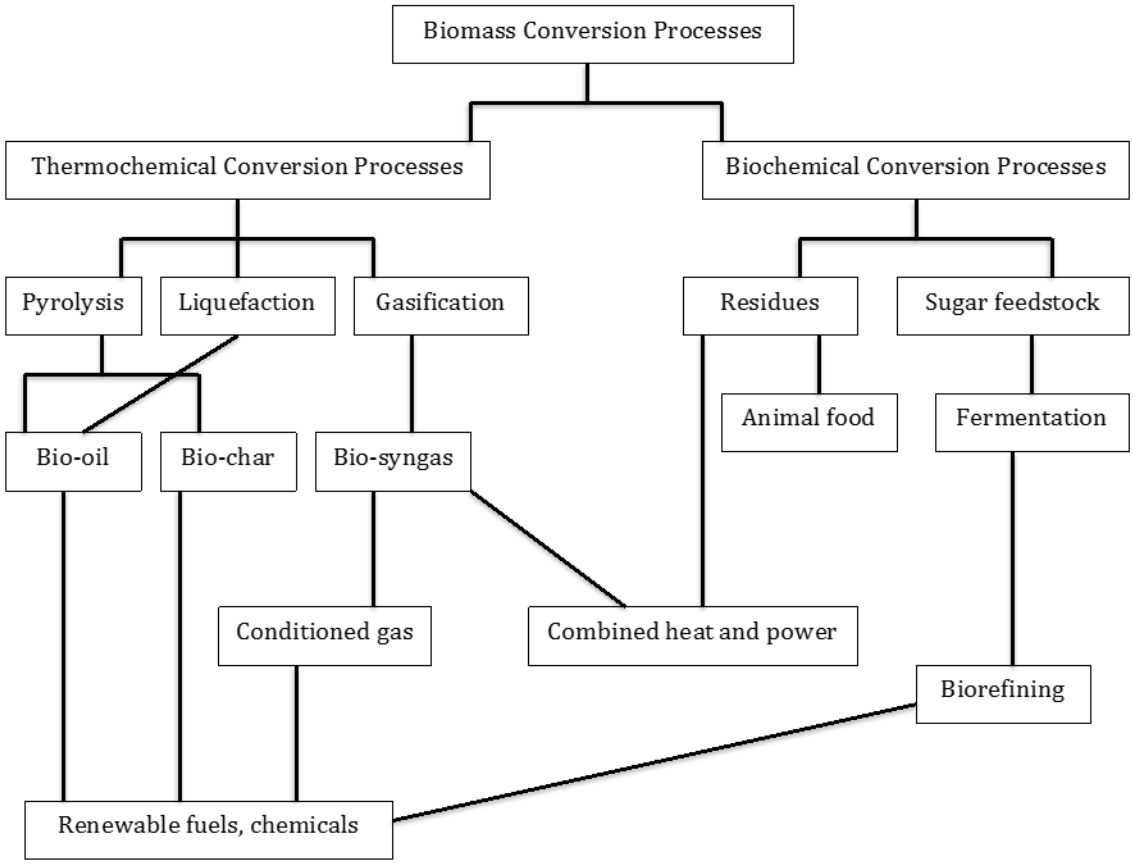


Figure 1.1 Schematic diagram of biorefinery concept

1.2 Production of Biofuels

1.2.1 The necessity of alternative fuels

The unstable price of crude oil, energy security issues, environmental consequences of fossil fuels, and concerns about petroleum supplies have drawn the attention of researchers to develop renewable biofuels. In general, biofuels offer an enormous advantage over fossil fuels. The plant feedstocks capture carbon dioxide from the air through their growth process, therefore, the carbon dioxide that is released from biofuels combustion is not considered a net greenhouse gas addition in the atmosphere (Cherubini 2010; Naik et al., 2010). However, biofuels has lower energy density

compared to petroleum-derived fuels. More than a liter of biodiesel or bioethanol is required to replace a liter of diesel or petrol. Higher combustion efficiency for both biodiesel and bioethanol moderately makes up for the lower energy density (International Energy Agency- IEA, 2004).

Biodiesel is one of the most prominent renewable energy sources that can be considered as a potential substitution for conventional fossil fuels (Nigam and Singh, 2011). Biodiesel is a mixture of fatty acid methyl esters (FAMEs), which can be derived from vegetable oils and animal fats. Biodiesel is more environmentally friendly when compared to other energy sources especially when concerns about global warming are considered. Using biodiesel as a fuel has several advantages over conventional fuels including biodegradability, less toxic, low carbon dioxide emissions, and its production from renewable resources (Nigam and Singh, 2011).

1.2.2 Biofuels

The First generation biofuels are produced from feedstocks that compete with food industries such as ethanol produced from corn, and biodiesel generated from vegetable oil. It is estimated that the biofuel industry is responsible for 20-30% of the global food price spike in 2008 as 125 m tones of cereals were allocated to biofuel production. Currently, the production of first generation biofuels (e.g. ethanol) has been commercialized, with an annual production of almost 50 billion liters (Yousuf, 2010).

During the last two decades, the production of second-generation biofuels has attracted interest to overcome the limitations associated with first generation biofuels. The second-generation biofuels include agricultural and forest residues, woody waste

biomass, municipal solid wastes, which could be used as lignocellulosic feedstock (Yousuf, 2010). The main advantages that second generation biofuels arise are:

- Making use of resources that is not suitable for human consumption
- Not competing with food production
- Using lands that is not suitable for agriculture for human food production
- Environmental impact

Typical second-generation biofuels are:

- Bioethanol (Product of alcoholic fermentation of yeasts)
- Biodiesel from oleaginous microorganisms (triglyceride produced by oleaginous yeasts, bacteria, or microalgae is used as feedstock)

These biofuels are usually produced from microbial fermentation of sugars, which are generated from hydrolysis of lignocellulosic biomass.

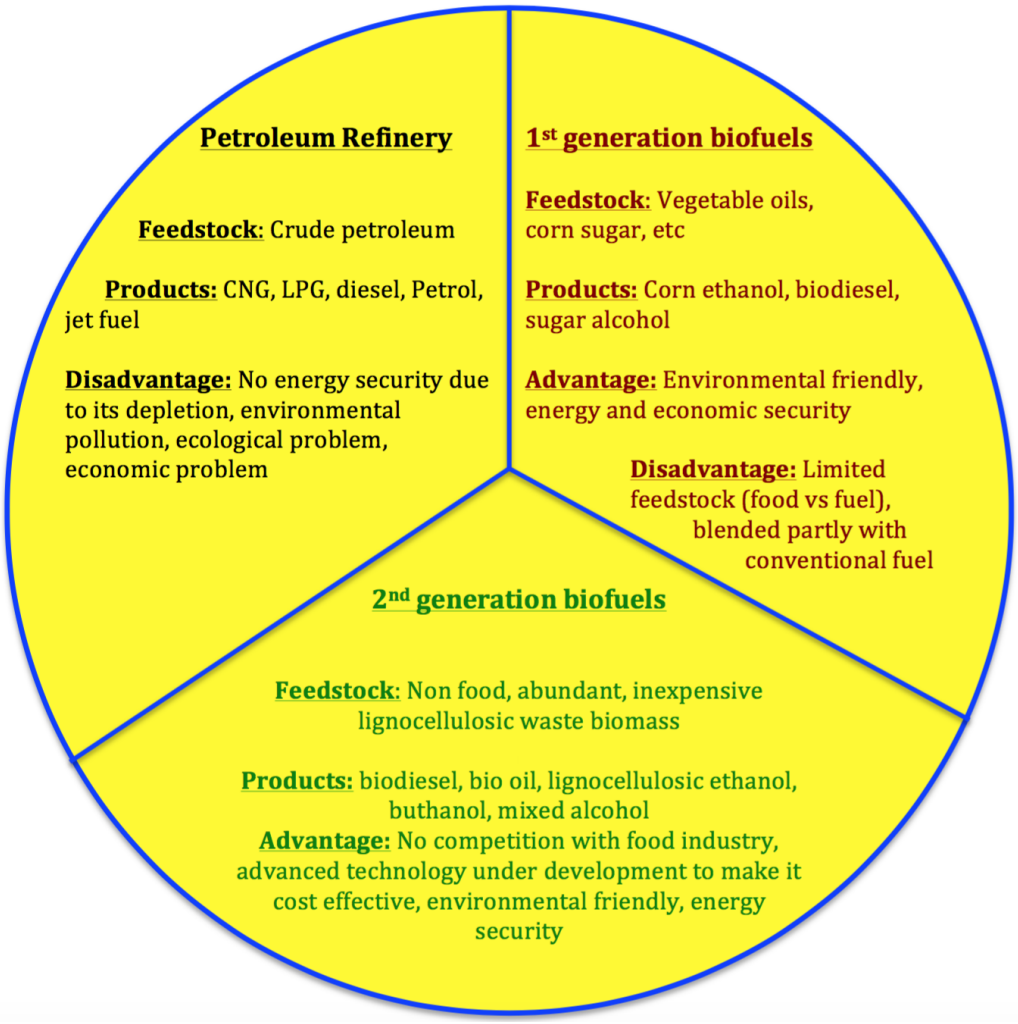


Figure 1.2 Comparison of 1st and 2nd generation biofuel with conventional petroleum fuels

(Yousuf, 2010)

Currently, the 1st generation biofuels: bioethanol produced from sugar and starch, biodiesel from vegetable oil, and green diesel have been commercialized (Bomb et al., 2007). Bioethanol and biodiesel can be blended with gasoline and diesel fuels, respectively, or used in their pure form in slightly modified engines (International Energy Agency -IEA, 2004).

1.2.3 Biodiesel production methods

Vegetable oils (soybean oil in the US, and rapeseed oil in Europe), used cooking oils and animal fats are a typical feedstock for biodiesel production by transesterification of these raw materials. Transesterification is the process where oils and fats mainly consist of triglycerides, reacts with alcohol to produce FAMEs and glycerol in a presence of catalyst. Transesterification of raw materials is carried out in the presence of catalyst, such as alkali, acid, or an enzyme. Biodiesel is commonly produced using alkali-catalyzed transesterification using sodium hydroxide or potassium hydroxide as catalysts. However, this method required strict feedstock specification such as highly refined vegetable oils feedstock and very low water and free fatty acid (FFA) contents in the reaction system (less than 0.1 and 0.5%). High water contents, leads the hydrolysis to become the dominant reaction resulting in lower yield of biodiesel production. High contents of free fatty acid in alkali-catalyzed transesterification leads in the formation of soaps, which makes purification of biodiesel difficult and more expensive (Talebian-Kiakalaieh et al., 2013). Acid-catalyzed transesterification can tolerate high FFA and water contents, which can be used for unrefined oils feedstock. However, to achieve high biodiesel yield, higher reaction temperature (>100 C) and longer reaction time (>48 hours) are required.

Biodiesel can be produced from esterification of free fatty acids. In the esterification reaction, fatty acids react with methanol to generate FAMEs and water (Van Gerpen, 2005). Figure 1.3 and Figure 1.4 show the schematic representation of triacylglyceride transesterification and free fatty acid esterification, respectively.

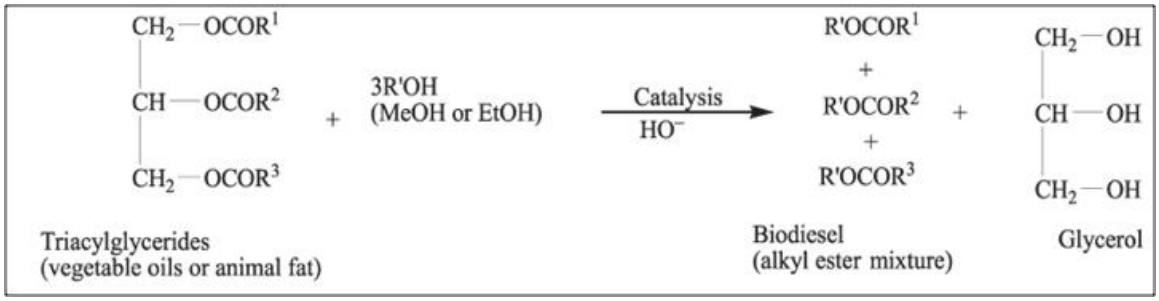


Figure 1.3 Transesterification of triacylglycerides and alcohol for biodiesel production

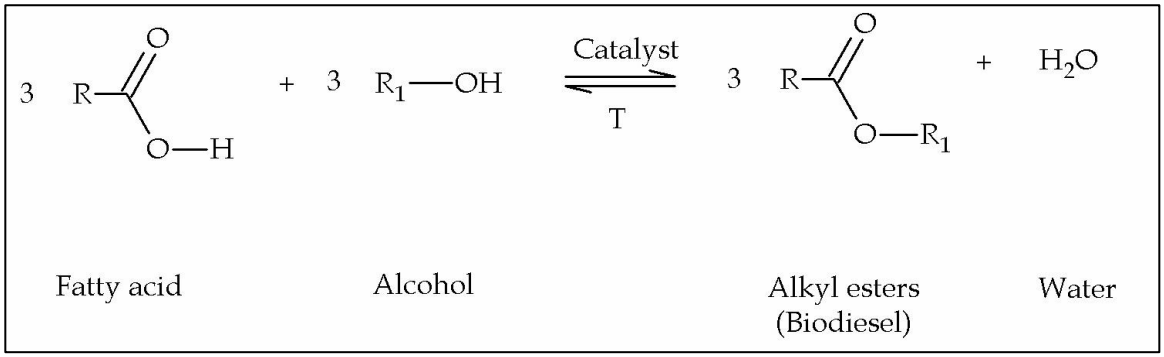


Figure 1.4 Esterification of the free fatty acid for biodiesel production

Many studies have used two-step acid and alkali catalyzed transesterification to eliminate the problems that mentioned in the previous paragraph. In the first step, the esterification of FFA with acid catalysts was performed to decrease FFA levels to lower than 1% and in the second step, the transesterification of feedstock with alkali catalysts carried out.

Contrary to the single-step biodiesel production process using transesterification of oils and two-step acid and alkali-catalyzed transesterification, the use of an enzymatic/acid-catalyzed hybrid process is proposed for the production of biodiesel for the soybean oil (Ting et al., 2008). This hybrid process was selected for the unrefined oil

with high free fatty acid content, since enzymatic hydrolysis can tolerate oils with high FFA contents. In addition, comparing with acid- catalyzed transesterification, the enzymatic/acid-catalyzed hybrid process uses milder reaction conditions.

In this hybrid process, in the first step, commercial grade soybean oil was hydrolyzed using lipase as catalyst, which had been immobilized to chitosan beads using a binary method (Ting et al., 2008). In the second step, the feedstock containing FFA, mono-glycerides, di-glycerides and triglycerides obtained from the hydrolysis was esterified with methanol in the presence of an acid to produce biodiesel.

1.2.4 First-generation biodiesel

Currently, first-generation biodiesel is manufactured using triglycerides from vegetable oils such as soybean oil (Noureddini et al., 2005; Watanabe et al., 2002), rapeseed oil (Yuan et al., 2008), palm oil (Pleanjai and Gheewala, 2009; Salamatinia et al., 2010) and sunflower oil (Orçaire et al., 2006). Among these feedstocks, soybean accounts for approximately 90% of biodiesel production in the US. Producing these oilseed crops requires a complete season and large acreages of land to make sufficient amounts of oil. Also, soybeans are very prominent in the food market; so increased demand for biodiesel production will increase the marketing price of it. Therefore, to satisfy the demand for oils (lipids) for biodiesel production, sources of oils, which do not compete with food supplies, are necessary.

1.2.5 Second generation biodiesel

A variety of raw materials and methods have been reported on production of second- generation biodiesel:

1.2.5.1 Biodiesel from waste cooking oil

People around the world use edible oils for cooking and discard it as waste cooking oil (WCO). It is been shown that waste cooking oils are used as an alternative to vegetable oils for biodiesel production (Zhang et al., 2003). The WCO price is two to three times cheaper than vegetable oils, and it also eliminates the cost of waste product treatment. In addition, utilization of WCO as an alternative to plant oils can significantly decrease the amount of farmland, which is essential for producing corps feedstocks for biodiesel production.

The WCO can be classified based on the FFA content to two groups: (1) yellow grease (<15%) and (2) brown grease (>15%). The prices for these WCO are between ($ 0.04 to $ 0.09) for yellow grease and ($ 0.004 to $ 0.014) for brown grease. However, there are limited amounts of waste cooking oil, which cannot meet the demand for the biodiesel.

1.2.5.2 Biodiesel from animal fats

Animal fats can be used as a raw material because of their triglyceride fraction (Tashtoush et al., 2004). However, additional processing such as using organic solvents to dissolve the fats is required due to its high melting point. A solvent recovery unit needs to be added to the process design, which adds to the final processing cost (Tashtoush et al., 2004).

1.2.5.3 Biodiesel from fermentation of non-lignocellulosic waste

Utilization of agro-industrial residues as nutritional sources for oleaginous microorganisms in a fermentation system has gained the attention of researchers. The

microorganisms are able to use these residual waste sources of nutrients and subsequently accumulate lipids in their cells in a short period of time. Variety of oleaginous microorganisms, such as different types of bacteria, yeast, and microalgae has been implemented along with different waste materials. The agro-industrial wastes such as wastewaters from potato, fruit juice, and lettuce processing (Schneider et al., 2012), kitchen waste, tomato waste, milk industry wastewater, olive oil manufacturing wastewater (Bellou et al., 2014), municipal wastewater (Hall et al., 2011), and sewage sludge (Angerbauer et al., 2008) have been investigated for the production of microbial lipids to be used as a triglyceride source for biodiesel production.

1.2.5.4 Biodiesel from fermentation of lignocellulosic biomass

Lignocellulosic biomass, including forest and agricultural residues and commercial energy crops, are the most abundant natural resource for the production of advanced biofuels. Fermentation of lignocellulosic biomass to biogas or ethanol has been well established. To produce ethanol from lignocellulosic biomass a series of steps needs to be implemented including biomass pretreatment, enzymatic hydrolysis, and yeast (*Saccharomyces cerevisiae*) fermentation. Many studies have investigated the utilization of lignocellulosic biomass as a feedstock for biodiesel production (Ruan et al., 2012; Yu et al., 2011; Huang et al., 2009).

Similar steps to bioethanol production can be used to produce lipids suitable for biodiesel production if a different yeast species rather than *S. cerevisiae* is used for the fermentation step. Various researchers have evaluated the sugar release from different lignocellulosic biomass and investigated the feasibility of cultivation of many oleaginous species in those lignocellulosic biomasses. However, in the field of fermentation of

cellulosic biomass to produce lipids, oleaginous yeast and fungal strains were the main organisms studied (Ruan et al., 2012; Yu et al., 2011).

1.2.5.5 Biodiesel from fermentation of lignocellulosic waste material

Production of microbial oil from lignocellulosic materials is promising since they are the most abundant agricultural residues in nature (Huang et al., 2009). Plant cell walls consist mostly of three organic compounds: cellulose, hemicellulose, and lignin, which are also the main components of lignocellulosic materials. The composition of lignocelluloses is about 30-50% cellulose, 20-35% hemicellulose, and 10-15 % lignin. Cellulose is a linear polysaccharide biopolymer of six-carbon sugar (glucose), which is mostly present in crystalline structure in nature. Hemicellulose is a random, branched amorphous biopolymer of five-carbon sugars (xylose and arabinose), and six-carbon sugars (glucose, galactose, and mannose). Lignin, an amorphous heteropolymer, is a large complex aromatic polymer of phenolpropane and methoxy groups with a 3D cross-linked network that gives rigidity and water permeability to the plant structure. The structure of lignocellulosic material is shown in Figure 1.5.

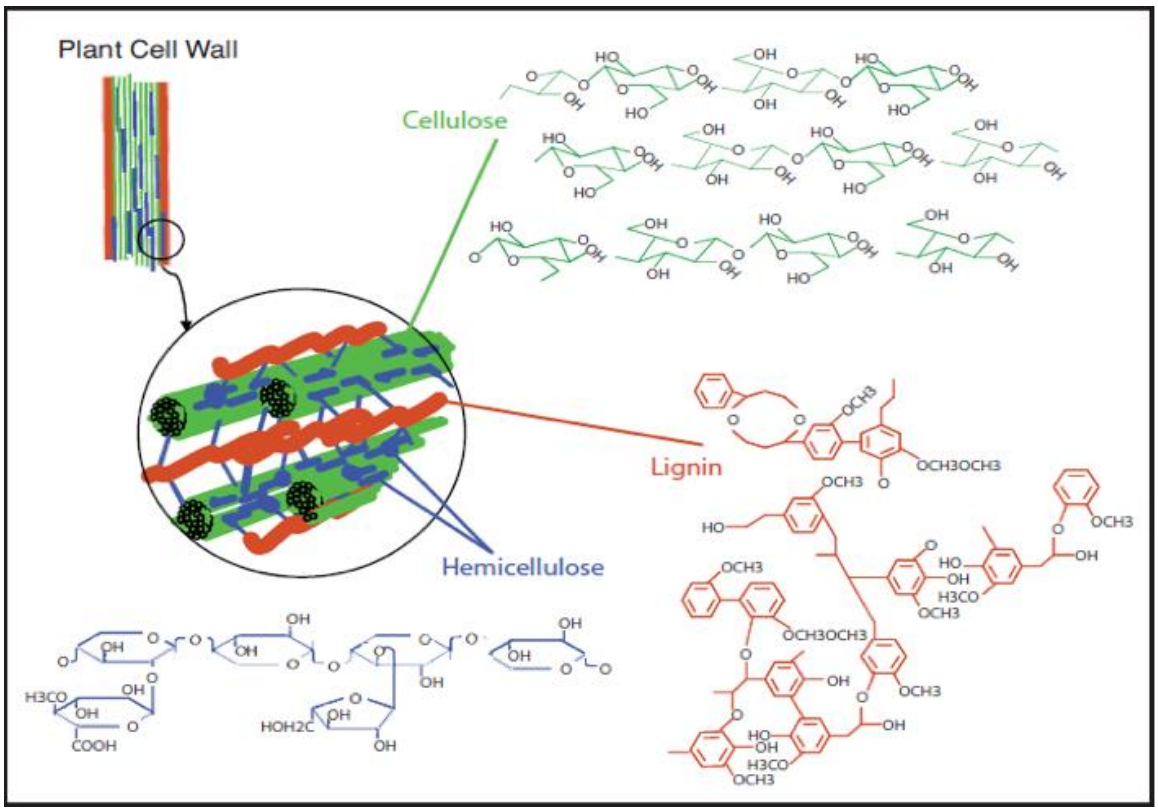


Figure 1.5 Structure of cellulose, hemicellulose, and lignin in lignocellulosic material (Kowsari, 2012)

Production of biodiesel using lignocellulosic waste material such as waste processing wood, waste papers, and wastes paper sludge is promising because these cellulose-based materials are abundant in nature and inexpensive, therefore, can be considered as a sustainable resource for production of renewable biodiesel. However, these lignocellulosic materials must be depolymerized/ degraded to the simpler molecules such as hexose and pentose sugars, then the oleaginous microorganisms can be cultured in a presence of these fermentable sugars for the production of microbial lipids (Huang et al., 2009).

1.3 Oleaginous Microorganisms

Oleaginous microorganisms are defined as microorganism with a microbial lipid content that can accumulate 20-80% of their dry weight in the form of lipids (Subramaniam et al., 2010). Among all oleaginous microorganisms accumulating lipids, yeast, bacteria, fungi, and algae are available for biodiesel production. The lipid content of these microorganisms is generally composed of TAGs (Ttriacylglycerols), which make them chemically equivalent to vegetable oils (Meng et al., 2009).

1.3.1 Microbial oil as a solution for feedstock problem

The feedstock for producing biodiesel is responsible for almost 70-75 % of the total cost of the process (Ma and Hanna, 1999); values as high as 90% have been reported. Biodiesel derived from vegetable oils can only meet a small fraction of fuel demands. In addition, expensive processing cost due to feedstock limitations, forces the need to explore for economical alternatives of feedstocks for biodiesel production. The use of oleaginous microorganisms, which are able to produce and accumulate oils, is promising in order to decrease the cost of oil raw materials for biodiesel production. Table 1.1 shows a list of oleaginous species from various microbial groups that have been recognized to accumulate large amounts of intracellular lipids, which could be potentially used for biodiesel production. The types of lipids produced also vary between prokaryotes and eukaryotes. Eukaryotes like yeasts, fungi, and algae usually produce polyunsaturated fatty acid triacylglycerols (TAG) similar to vegetable oils that can be used for biodiesel or renewable diesel production (Rattray, 1988; Losel, 1988; Wood, 1988). In oleaginous fungi such as *Mucor circinelloides*, the total lipids are only composed of 14% TAG; however, free fatty acids and phospholipids include 32% and

21% of the total storage lipids, respectively (Vicente et al., 2009). It has been also observed that many algal species accumulate substantial amounts of neutral lipids (20-50% w/w), majority of which is TAG (Hu et al., 2008). In contrast, most prokaryotic bacteria tend to make particular lipids such as glycolipids, lipoproteins, and wax esters (Anderson and Dawes, 1990; Schweizer, 1989; Wilkinson, 1988; O'Leary and Wilkinson, 1988). However, TAG accumulation has been revealed in a few bacterial strains to the *actinomycetes* group such as species of *Mycobacterium*, *Streptomyces*, *Rhodococcus*, and *Nocardia* (Alvarez and Steinbuchel, 2002; Brenan, 1988; Shields-Menard et al., 2015). TAG biosynthesis has been discovered only in aerobic heterotrophic bacteria and cyanobacteria, precisely the *actinomycetes* group containing the genera *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia*, or *Gordionia*. To date, *Rhodococcus opacus* is the only bacteria in its native form shown in literature that is able to accumulate more than 20% of its biomass as lipids, producing as high as 87% (w/w) lipids containing TAG (Alvarez and Steinbuchel, 2002).

Table 1.1 Lipid content in various oleaginous microorganisms.

Microbial species	Oil content (% w/w)	Reference
Microalgae		
C. vulgaris	56.6	(Liu et al., 2007)
C. emersonii	63	(Illman et al., 2000)
C. minutissima	57	(Illman et al., 2000)
C. sorokiniana	22	(Illman et al., 2000)
Yeast		
L. starkeyi	52.6	(Kong et al., 2007)
C. curvatus	34.6	(Zhang et al., 2010)
Y. lipolytica	58.5	(Tsigie et al., 2011)
R. glutinis	72	(Meng et al., 2009)
R. toruloides	67.5	(Li et al., 2007)
C. echinula	37.6	(Du et al., 2007)
C. albidus	65	(Meng et al., 2009)
C. potothecoides	46.13	(Han et al., 2006)
Fungi		
M. isabellina	51.7	(Papanikolaou et al., 2008)
C. enchinulata	53.6	(Fakas et al., 2009)
M. vinacea	66	(Subramaniam et al., 2010)
Bacteria		
R. opacus	25	(Subramaniam et al., 2010)
R. rhodochrous	50	(Shields-Menard et al., 2015)
B. alcalophilus	18-24	(Subramaniam et al., 2010)
A. calcoaceticus	27-38	(Meng et al., 2009)

The utilization of microbial oil as feedstocks for biodiesel production has been considered recently (Koutinas et al., 2014; Leiva-Candia et al., 2014; Huang et al., 2014) Oleaginous microorganisms can be considered as an alternative inexpensive oil sources in terms of their independence on locality, season, and climate, and also their low crop acreage. Moreover, as displayed in Table 1.2, the fatty acid compositions of these microbial oils are similar to typical plant-based biodiesel feedstocks and animal fats such as soybean oil and tallow. The fatty acid composition rich in oleic (C18:1) and palmitic (C16:0) acids, could result in enhanced biodiesel characteristics such as cold flow,

oxidative stability, ignition quality (cetane number), and reduced nitrogen oxides (NO_X) emissions (Bringe, 2005).

Table 1.2 The fatty acid composition of oils from various sources.

Species	Reference	Fatty acids						
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Others
Yeasts								
Lipomyces starkeyi	(Li et al., 2008)	33	4.8	4.7	55.1	1.6	-	-
Rhodotorula glutinis	Meng et al., (2009)	37	1	3	47	8	-	-
Rhodosporidium toruloides	(Li et al., 2008)	24.3	1.1	7.7	54.6	2.1	-	-
Yarrowia. lipolytica	(Thevenieau and Nicaud, 2013)	11	6	1	28	51	-	-
Fungi								
Mucor circinelloides	(Wynn et al., 2001)	20	2.3	2	37	14.3	18.5	2
Mortierella isabellina	(Thevenieau and Nicaud, 2013)	29		3	55	3	-	-
Pythium ultimum	(Thevenieau and Nicaud, 2013)	15		2	20	16	1	-
Bacteria								
Rhodococcus opacus	(Zhila et al., 2005)	16.8	5.9	2.8	73.8	-	-	-
Algae								
Botryococcus braunii	(Zhila et al., 2005)	19.1	0.9	4.8	24.4	8.4	19.0	15.4
Chlorella sorokiniana	(Patterson, 1970)	40	4.0	-	5	36	23	32
Chlorella zofingiensis	(Thevenieau and Nicaud, 2013)	23	2	2	36	18	8	-
Tallow	(Bockish, 1993)	26	3.5	19.5	40	4.5	-	3
Soybean oil	(Ferrari et al., 2005)	11.8	-	4	25	55.4	3.8	Trace
Peanut oil	(Thevenieau and Nicaud, 2013)	11		2	48	32	-	-
Palm oil	(Thevenieau and Nicaud, 2013)	44		4	38	10	1	-
Rapeseed oil	(Thevenieau and Nicaud, 2013)	4		2	62	22	10	

(% w/w total fatty acids)

Among all oleaginous microorganisms available for biodiesel production, the use of algae and oleaginous yeast is desirable due to their triacylglyceride-rich lipid

composition. Using oleaginous yeast is promising due to their fast growth rate, and high lipid content compared to algae (Ageitos et al., 2011; Li et al., 2008). Most oleaginous yeasts can storage lipids as much as 70% of their cells under nutrient-limiting conditions (Meng et al., 2009). The process of microbial oil production from algae and oleaginous yeasts must be developed to assess their potential future for biodiesel manufacturing process. In this work we focus on microbial oil production from oleaginous yeast and also different approaches to make this process sustainable.

1.3.2 Mechanism of lipid accumulation in oleaginous microorganisms

Oleaginous microorganisms are able to utilize a wide range of carbon sources such as various sugars. It has been shown that these microorganisms utilized glucose, xylose, glycerol, and food industry waste depending on the type of strain (Subramaniam et al., 2010). These organisms can also utilize multiple sources of carbon such as glucose, xylose, arabinose, mannose, glycerol, and also industrial residues to produce lipids (Amirsadeghi et al., 2015; Easterling et al., 2009; Shields-Menard et al., 2015; Subramaniam et al., 2010). However, oleaginous microorganisms require a large carbon to nitrogen ratio, and the fermentation process requires high amounts of water and nutrients in the media to produce large amounts of storage lipids. The process of lipid accumulation from oleaginous microorganisms requires a medium containing an excess of the carbon source with limited nutrients, typically nitrogen (Papanikolaou and Aggelis, 2011). The oleaginous potential of those microorganisms is affected by growth medium composition and fermentation process variables. The operating temperature, pH, C/N ratio, agitation and aeration rate, fermentation time, and the initial concentration of the seed culture are several factors that can affect the biomass and lipid accumulation of

oleaginous microorganisms (Subramaniam et al., 2010). Among all of these factors, it has been shown that the lipid production by oleaginous microorganisms is critically affected by C/N ratio of the culture medium (Saenge et al., 2011b). At a low C/N ratio, the carbon flux is distributed for cellular proliferation (i.e. increased biomass production). However, in this scenario high lipid production could still be achieved as a result of a higher number of cells present, but with low lipid content as compared to low biomass concentrations containing high lipid content. However, at a high C/N ratio, insignificant microbial growth is detected and when the nitrogen source is depleted, cells cannot multiply; thus, they assimilate the excess carbon substrate for production of lipids. Although high lipid content is achieved, the lipid productivity could be low as a result of low number of cells. Therefore, for enhanced lipid production it is important to determine the optimal composition of the media and the process fermentation condition that optimizes cell concentration as well as lipid concentration.

1.3.3 Lipid content and fatty acid composition of oleaginous yeasts

Lipids are basically molecules that are insoluble in water and soluble in organic solvents with diverse structure: a) isoprene derived molecules such as steroids and carotenoids, b) fatty acids and their long chain linkages such as fatty alcohols, alkanes and alkenes. Plant and animal oils are usually a fatty acid derived type of lipids. Yeasts may contain various types of lipids such as triacylglycerol, diacylglycerols, monoacylgycerols, fatty acids, steryl esters, free sterols, glycerophospholipids, glycolipids, waxes and others, depending on the yeast strain (Sitepu et al., 2014). Oleaginous yeasts accumulate lipids in their cell wall, in a form of triacylglycerols.

Identified oleaginous yeasts are from genera *Yarrowia, Rhodotorula*, Candida, *Cryptococcus*, *Rhodosporidium*, *Lipomyces*, and *Trichosporon* (Ageitos et al., 2011).

Yeast storage lipids are dominantly composed of stearic, palmitic, palmitoleic, oleic, linoleic, and linolenic fatty acids, which their chemical characteristic and energy value are equivalent to those in plant oils, making them suitable for biodiesel and oleochemicals production. The fatty acid composition of the yeast triacylglycerols can vary from species to species and also is a function of environmental growth condition, substrate, and medium composition (Sitepu et al., 2013). Up to 80-90% of the storage lipid in oleaginous yeast is triacylglycerols (Ratledge and Wilkinson, 1988; Sitepu et al., 2014). In the cultivation of most oleaginous yeast, lipid synthesis commonly occurs during nutrients starvation when nutrients (mostly nitrogen) are depleted and the carbon is in excess (Subramaniam et al., 2010). During starvation mode (stationary phase), the stearic and oleic acid synthesis dominates in quantity. However, linoleic acid becomes more prominent in exponential growth phase (Sitepu et al., 2014).

1.3.4 Fatty acid composition and biodiesel properties

Several authors state that the performance of biodiesel is highly dependent on the fatty acid composition of the oils (Knothe, 2005; Pinzi et al., 2009). The standard properties of the biodiesel including cetane number, oxidative stability, kinematic viscosity, and heat of combustion are characterized by chemical structure of its fatty acids, most significantly, chain length and degree of unsaturation and branching (Knothe 2008). Thus, prediction of the quality of the biodiesel can be estimated by analysis of the fatty acid composition of microbial lipids. The study on the effect of fatty acid profile of the biodiesel on its physical and chemical properties has shown that cetane number, heat

of combustion, and oxidative stability increase with chain length and decrease with degree of unsaturation. However, more unsaturated fatty acid and shorter chain improves viscosity and cold flow properties such as cloud point and pour point (Leiva-Candia et al., 2014; Pinzi et al., 2011). Since a production of alternative oil with fatty acid composition being able to provide all fuel characteristics does not exist, the oil with high levels of monounsaturated fatty acids is a target. Oils rich in monounsaturated fatty acids may provide an optimum behavior in terms of fuel stability, low temperature behavior, ignition time, and iodine number. While research on yeast oil-derived biodiesel properties is not sufficient in the literature, statistical models built upon fatty acid composition of the microbial oil is considered as a useful implement to predict the quality of the biodiesel (Leiva-Candia et al., 2014; Pinzi et al., 2011). The fatty acid profile of the most yeast oils is similar to palm oils with high saturated fatty acids (Leiva-Candia et al., 2014), which indicates that they may not be suitable for cold climates unless using it as a blend to diesel fuels (i.e. B20).

1.4 Oleaginous Yeasts in Wastewater

As interest in the field of renewable fuels increases, oleaginous microbes are at the forefront of multi-disciplined research. Advantages of microbial oil over vegetable oils as a biodiesel feedstock include less land use, controlled cultivation environment, and consistent yields. These advantages make microbial oil an interesting alternative to crop or animal-derived biodiesel feedstocks.

1.4.1 Wastewater as a sustainable source of carbon and nutrients

In microbial oil production process, the substrate required for the growth of microorganisms and oil production is the major cost factor of the process. Therefore, using inexpensive waste resources for cultivation of oleaginous microorganisms is desirable. Different sources of wastewaters such as municipals, agricultural, and industrial wastewaters have been studied, and several of those wastewaters have offered the potential for biodiesel production. During the last two decades, many researchers have studied the production of microbial oil or single-cell-oil from industrial wastewaters for renewable biodiesel production. Yeast treatment process is an attractive approach for both wastewater treatment and resource exploitation, which involves removing pollutant from wastewater by oleaginous yeast for the production of yeast biomass and oil. Yeast-derived oils have been investigated for decades, however, substantial improvements such as discovery of new yeast species, understanding of the oleaginicity of the yeast species through their metabolic pathways, optimization of the cultivation conditions and medium components have been implemented in recent years. To achieve higher rate of yeast biomass and oil production, higher pollutant removal, lower need of nutrient supplement, and reduced production cost, highly adaptive oleaginous yeast for the suitable substrate is required. However, the selected oleaginous yeast must be capable of using a variety of carbon sources efficiently, with strong ability to tolerate different inhibitors (organic and inorganic materials present in the wastewater) to produce microbial oils with similar fatty acid composition to vegetable oils.

Base on the information of U.S. Congressional Budget Office, the annual cost venture for wastewater treatments was estimated to be in average between $13.0-$20.9

billion for the years 2000-2019 (Ling et al., 2015). Manipulation of wastewater treatment scenarios to include oleaginous yeast for the production of microbial oil, could lead to a generation of an alternative renewable feedstock for biodiesel manufacturing. To produce renewable biodiesel feedstock from integration of wastewater treatment and oleaginous microorganisms scenarios, two purposes are fulfilled, the production of alternative feedstock for the production of renewable biodiesel and the treatment of industrial wastewater. The oleaginous yeasts available in literature studies that is used for the production of alternative biodiesel feedstock and industrial wastewater treatment purposes are *Lipomyces starkeyi* (Angerbauer et al., 2008; Huang et al., 2011), *Cryptococcus curvatus* (Chi et al., 2011; Gonzalez-garcia et al., 2013), *Rhodotorula glutinis* (Amirsadeghi et al., 2015; Chi et al., 2011; Granger et al., 1993; Xue et al., 2006, 2008), *Trichosporon dermatis* CH007 (Peng et al., 2013), *Trichosporon coremiiforme* (Chen et al., 2012), *Rhodosporidium toruloides* (Ling et al., 2015; Shen et al., 2013). However, the COD removals reported in the literature referenced above is not clear whether or not it would meet permit requirements by these industries. Among these oleaginous yeasts, *Rhodotorula glutinis* is the most commonly used oleaginous yeast for the high potential biodiesel production due to high lipid productivity from industrial wastewater or high pollutant removal efficiencies. A sustainable production of lipid-rich biomass by oleaginous yeast and reutilizing the carbon and nutrients available in the wastewaters can be useful for cost-effective production of biodiesel.

1.4.2 High-Organic-Strength Wastewater

Most of industrial wastewaters are highly acidic with pH of lower than 5, which required a pH adjustment for the treatment process. Yeast treatment process is considered

profitable for high-organic- strength wastewaters, due to their high tolerance to acidity and salinity. Yeasts are able to metabolize various carbon sources, mainly sugars such as glucose, xylose, manose, galactose, sucrose and maltose. Oleaginous yeasts have been reported a good candidate in the production of biodiesel among other microbial sources due to the short doubling time, high lipid content (up to 80% of their dry weights) and production of different classes of lipids from various carbon sources (Ling et al., 2013; Subramaniam et al., 2010). Oleaginous yeasts are effective in degradation of organic substances (COD reduction as high as 68–86%) and have shown a significant growth and lipid productivity in high strength industrial effluents with the initial COD at 15,000–50,000 mg/L in a comparatively short cultivation time compared to microalgae (Ling et al., 2014).

High –organic strength wastewaters available in literature that were used as a substrate for cultivation of oleaginous yeasts include wastewaters from food processing industries such as starch processing wastewater (Liu et al., 2013; Ren et al., 2015; Xue et al., 2010), wastewaters from potato, fruit juice and lettuce processing (Schneider et al., 2012), wastewater from fermentation processes such as butanol fermentation (Chen et al., 2012; Peng et al., 2013), glutamate (Xue et al., 2006, 2008), brewery and distillery wastewaters (Ryu et al., 2013; Schneider et al., 2013), palm oil mill effluent (POME) (Marjakangas et al., 2015; Saenge et al., 2011a), olive oil mill wastewater (Yousuf et al., 2010), pulp and paper wastewater (Amirsadeghi et al., 2015), and etc., with sugar residues as described by chemical oxygen demand (COD). Table 1.3 shows a summary of the various studies involving the growth of lipid-producing oleaginous microorganisms in different types of industrial wastewaters.

Table 1.3 Production of biomass and lipid using oleaginous yeasts from different wastewaters.

Yeast strain	**Wastewater**	**Supplement source**	**Biomass (g/L)**	**Lipid content (%)**	**Initial COD (mg/L)**	**COD Removal (%)**	**Fermentation mode**
Cryptococcus curvatus (Ryu et al., 2010)	Brewery wastewater	Crude glycerol	50.4	37.7	-	-	Flask
Cryptococcus curvatus (Gonzalez-Garcia et al., 2013)	Distillery wastewaters from tequila production	No supplement	5.19	25.2	23,125	78.98	Flask
Rhodotorula glutinis (Xue et al., 2010)	Starch wastewater	Less than 5% waste syrup	40	35	50,000	80	Pilot-scale 300-L fermenter
Rhodotorula glutinis (Xue et al., 2010)	Starch wastewater	No supplement	60	30	50,000	55	5-L fermenter
Rhodotorula glutinis (Xue et al., 2006)	Monosodium glutamate wastewater	No supplement	2.44	9.1	10,000	85	Flask
Rhodotorula glutinis (Xue et al., 2008)	Monosodium glutamate wastewater	Glucose	25	20	40,000	45	5-L fermenter
Rhodotorula glutinis (Schneider et al., 2013)	Brewery wastewater	No supplement	4.5	11	36,000	-	Flask
Rhodotorula glutinis (Saenge et al., 2011)	Palm oil mill effluent	Ammonium sulfate+tween 20	9.15	60.62	25,000	69.6	2-L fermenter
Rhodotorula glutinis (Louhasakul and Cheirsilp, 2013)	Palm oil mill effluent	No supplement	2.06	36.89	37,211	68.7	Flask

Table 1.3 (continued).

Rhodotorula glutinis (Amirsadeghi et al., 2015)	Pulp and paper wastewater	Glycerol+ $(NH_4)_2SO_4$	19	15	-	-	3-L fermenter
Rhodotorula glutinis (Gonzalez-Garcia et al., 2013)	Distillery wastewaters from tequila production	No supplement	6.06	27.02	23,125	84.44	Flask
Y. lipolytica TISTR 5151 (Louhasakul and Cheirsilp, 2013)	Palm oil mill effluent	No supplement	3.5	48	37,211	47.84	Flask
Trichosporon coremiiforme (Chen et al., 2012)	Wastewater after butanol fermentation	No supplement	5.8	19.1	23,560	68	Flask
Rhodosporidium toruloides (Ling et al., 2013)	Distillery wastewater	No supplement	8.12	43.65	52,900	86.11	Flask
Rhodosporidium toruloides Y2 (Zhou et al., 2013)	Bioethanol wastewater	Glucose	3.8	34.9	-	72.3	Flask
Lipomyces starkeyi (Liu et al., 2013)	Potato starch wastewater	Glucose+ $(NH_4)_2SO_4$	2.59	8.88	-	-	Flask
Lipomyces starkeyi (Yousuf et al., 2010)	Olive oil mill wastewaters	No supplement	11	22.4	43,000	-	Flask
Lipomyces starkeyi HL (Huang et al., 2011)	Fishmeal wastewater	Glucose	17.6	-	-	43.4	Flask
Yarrowia lipolytica TISTR 5151 (Cheirsilp and Louhasakul, 2013)	Palm oil mill effluent	4% crude glycerol	3.21	68	86,826	-	Flask

Carbohydrate-rich wastewater from various foods processing manufacturing has investigated for bioconversion of high carbohydrate wastewaters to microbial oils. Starch wastewater is one of the highest organic loaded wastewaters in food industries, which can have a serious effect on the environment. The traditional starch wastewater treatments such as aerobic, and biological treatment processes are only for the purpose of removing organics from the wastewaters without considering a system to recover the

energy contained therein. In addition to carbohydrate, starch wastewater contains nitrogen, phosphorus and minerals such as Fe, Mg, Zn (Ren et al., 2015), which makes is suitable for simultaneous production of energy and wastewater treatment by oleaginous yeasts. The pilot-scale study for the production of microbial oil using oleaginous yeast *Rhodotorula glutinis* by Xue et al. demonstrated the potential industrialization of microbial oil production using starch wastewater. Biomass production of 40 g/L, 35% of lipid content, and 40,000 mg/L of COD removal was achieved, while no nutrient addition, pH adjustment, or sterilization was required (Xue et al., 2010).

The wastewater produced from post butanol fermentation distillation process contains high levels of organic acids, residual sugars with high COD (usually >20,000 mg/L). Chen et al. showed that this wastewater can be used as a substrate with no need of nutrient and trace mineral addition. The cultivation of oleaginous yeast *Trichosporon coremiiforme* in butanol fermentation wastewater resulted in 68% of COD degradation, biomass production and lipid content of 5.8 g/L and 19.1%, respectively.

Wastewaters from beverage industry normally have high organic contents, recognized by a high chemical (COD) and biological (BOD) oxygen demand. The COD of brewery wastewaters ranges between 2000 and 6000 mg/L. Schneider et al. examined a low cost brewery effluents as a growth media to cultivate oleaginous yeast *Rhodotorula glutinis* for lipid and carotenoid production. They observed that brewery wastewater is a suitable fermentation substrate for lipid production since it serves as a sole nitrogen source and contains significant amount of sugar (sucrose, maltose, glucose, and fructose) as carbon source (Schneider et al., 2013). The potential utilization of organic waste from brewery industry was also studied by Ryu et al. using oleaginous yeast *Cryptococcus*

curvatus, which led to 37% lipid content (Ryu et al., 2013). The wastewater contained essential nutrients for the growth of oleaginous yeast, thus, no pretreatment and additional nutrient was needed.

Utilization of raw distillery wastewater without addition of external nutrients, sterilization and pH adjustment was investigated for enhanced lipid production by oleaginous yeast *Rhodosporidium toruloides*. Relatively high lipid production, high COD, total nitrogen and phosphorus removal was achieved in the presence of indigenous microorganisms in distillery wastewater (Ling et al., 2013).

1.4.2.1 Oil-rich wastewater

The food processing industries are responsible for the majority of the wastewater discharges with high quantities of oily wastewaters (e.g., oil and diary mills). Palm oil mill effluent (POME), and olive oil mill wastewater, which contain high amount of fatty acids in their raw vegetable oil is usually discarded as oil-rich industrial wastewater. The POME contains 0.6-0.7% oils and 4–5% solids in water. The large amount of fatty acids, carbohydrates, proteins, and mineral salts in palm oil mill wastewater can stimulate the growth of oleaginous microorganism for the production of oil-rich biomass. It is estimated that the wastewater discharge for the production of each ton of crude palm oil is approximately 2.5 tones annually (Marjakangas et al., 2015). In addition to high organic carbon content, palm oil mill effluent is rich in mineral salts, proteins, and vitamins that could stimulate the growth of oleaginous microorganisms for the production of oil-rich biomass. Cheirsilp and Louhasakul have investigated the potential utilization of palm oil mill industrial wastes for low-cost production of microbial oil through microbial fermentation of several oleaginous yeasts (Louhasakul and Cheirsilp, 2013).

They showed that *Y. lipolytica* TISTR 5151 could effectively grow in POME and produce relatively high lipid production (1.6 g/L) corresponding to a lipid content of (48%) in palm oil mill effluent. The lipid content in *Y. lipolytica* was increased to 68%, when 4% crude glycerol was added to POME (Cheirsilp and Louhasakul, 2013).

A biological treatment process of olive oil mill wastewater is challenging due to antimicrobial activity of its phenolic compounds. Yousuf et al. showed that oleaginous yeast *Lipomyces starkey* was able to survive in untreated olive oil mill wastewater and produce lipids (between 20-30%) without addition of organic supplements while significantly reduced both total organic carbons and phenol content. However, they reported that 50% dilution of OMW results in an increase in the lipid content (22.4% to 28.6%), and a further dilution in wastewater leads to only a minimum improvement (Yousuf et al., 2010).

1.5 Lignocellulosic biomass

Lignocellulosic biomass is the most promising renewable energy sources, which can be considered sustainable carbon source for large-scale energy production. Lignocellulosic biomass mainly composed of carbohydrate polymers (cellulose, hemicellulose), and an aromatic polymer (lignin). It is the fibrous part of plant biomass, which can be categorized into these following sources: 1) agricultural residues (corn stover, sugarcane bagasse, corn stalk); 2) woody crops (willow, switchgrass); 3) forestry residues (wood waste, sawdust); 4) industrial and municipal solid wastes (waste paper, waste newspaper, paper mill sludge) (Wijaya et al., 2014). Table 1.5 illustrates the cellulose, hemicellulose, and lignin contents of some lignocellulosic materials.

Table 1.4 Composition of some lignocellulosic material

Lignocellulosic material	Cellulose %	Hemicellulose %	Lignin %
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Leaves	15-20	80-85	0
Cotton seed hair	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-75	5-15	5-10
Switchgrass	45	31.4	12

1.5.2 Lignocellulosic biomass as a sustainable source of carbon

Currently, the most commonly used biodiesel feedstock is soybean oil, which is responsible for approximately 70-90% of the total cost of biodiesel and uses acres of arable land that is subject to a volatile climate, disease, and market demand for farm land. Research on microbial lipid production from different oleaginous species have shown that pure sugars such as glucose and xylose are the two most favorable carbon sources for lipid production by oleaginous microorganisms. However, the cost of using pure monosaccharide sugars as substrates could prove to be major obstacles for the effective operation of producing oil from oleaginous microorganisms at the commercial scale. As discussed earlier, the growth fermentation media could be industrial or municipal wastewaters, which are nutrient enriched and basically free of cost.

Currently, the production cost of biodiesel using microbial oil feedstock is not comparable to vegetable oil biodiesel. The annual cost of microbial oil production from

glucose is estimated $3.4/kg of microbial oil to produce 10,000 t of microbial oil and zero cost of glucose (Koutinas et al., 2014). However, when the cost for glucose was assumed at $400/t, the cost of microbial oil production and transesterification of oil to biodiesel was reported as $5.5/kg, and $5.9/kg, respectively (Koutinas et al., 2014). Therefore, the process of biodiesel manufacturing from lignocellulosic biomass must be designed to make a full use of all the aqueous and solid products in economically profitable manner. Utilization of pretreated, and non-pretreated industrial waste streams along with their valuable by-products can be a key parameter for developing an economically sustainable manufacture of biodiesel. For example, pretreatment of lignocellulosic biomass will produce lignin, which can be used as valuable fuel additives or merged for power generation (Wijaya et al., 2014).

1.5.3 Pretreatment of lignocellulosic biomass

Conversion of biomass to energy, fuels, and chemicals are still promising to be implemented into an economically feasible processes (Moe et al., 2012). However, for the purpose of bioconversion of lignocellulosic biomass into lipids, the cellulose and hemicellulose must be broken down into soluble sugars (mainly glucose and xylose), which then can be efficiently fermented by oleaginous microorganisms. The relative sugar proportion converted from the hydrolysis, in lignocellulosic material depends on their sources and the operating conversion conditions (Liu et al., 2015). While pretreatment of lignocellulosic material releases sugars, it also produces several other degradation compounds known as microbial growth inhibitors such as acetic acid, furfural, 5-hydroxymethylfurfural, and phenolic lignin compounds.

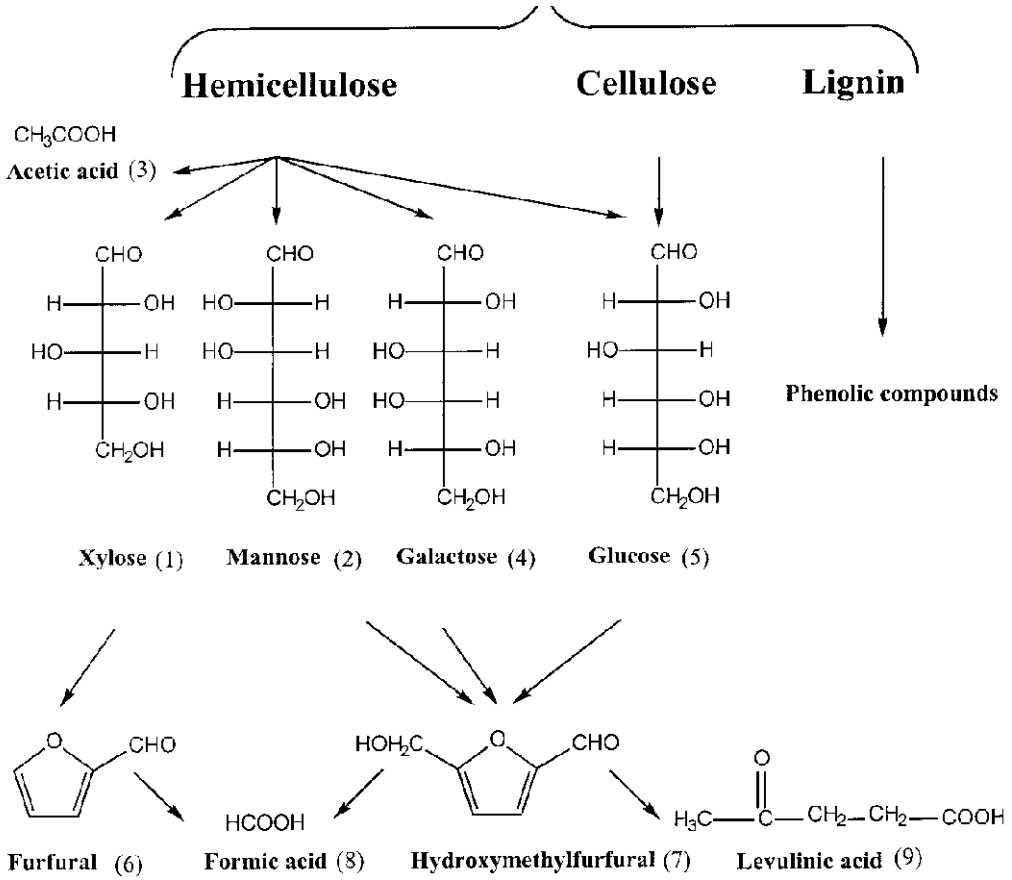


Figure 1.6 Lignocellulosic biomass degradation products

Degradation of hemicellulose releases xylose, mannose, galactose, and glucose along with acetic acid. Glucose is released from break down of cellulose. At high temperature and pressure, xylose is further degraded to furfural. Similarly, 5-hydroxymethylfurfural (HMF) is generated from degradation of hexose sugars (mannose, galactose, and glucose). Formic acid is formed from breakdown of furfural and HMF. Livulinic acid is generated from degradation of HMF, and phenolic compounds are resulted from partial breakdown of lignin.

Not all microbes can tolerate these compounds in their growth environment in fermentation system; however, the level of tolerance of oleaginous microorganisms and their lipid yields depends on the species, and type and concentration of these inhibitory compounds.

In pretreatment of lignocellulosic biomass, the polysaccharides in biomass are hydrolysed to monosaccharides with the aid of acid or enzyme as a catalyst. Acid hydrolysis and enzymatic hydrolysis are the two frequently used methods for pretreatment of lignocellulosic material (Taherzadeh and Karimi, 2007; Wijaya et al., 2014). Enzymatic hydrolysis of biomass commonly generates less inhibitory compounds as a by-product of the process, consequently has less negative effect on the fermentation products. However, the rate of enzymatic hydrolysis is slower than acid hydrolysis and also requires more pretreatment steps compared to acid hydrolysis (Moe et al., 2012; Wijaya et al., 2014). The two methods of acid hydrolysis are dilute acid hydrolysis and concentrated acid hydrolysis. Typically, the acid concentration of less than 10% (w/w) is considered dilute acid, whereas the acid strength in concentrated acid hydrolysis is more than 10% (w/w) (Wijaya et al., 2014).

1.5.4 Lignocellulosic hydrolysate as fermentation substrate

In order to reduce the cost of biofuels arisen from feedstock fermentation, the use of inexpensive and abundant lignocellulosic biomass obtained from agricultural wastes, forestry residues, or municipal solid wastes, is essential to address the food for fuel debate. Utilization of lignocellulosic waste materials as a source of carbon for biofuels production needs to be implemented and this has been presented in several previous studies. Yu et al. studied the microbial oil production from five different oleaginous

yeasts including *Cryptococcus curvatus, Rhodotorula glutinis, Rhodosporidium toruloides, Lipomyces starkeyi*, and *Yarrowia lipolytica* using hydrolysate from dilute sulfuric acid pretreatment. They showed that all the studied yeasts except *Rhodosporidium toruloides* could grow on detoxified and non-detoxified lignocellulosic hydrolysate; However, *Rhodosporidium toruloides* did not grow in non-detoxified hydrolysate (Yu et al., 2011).

Sugarcane bagasse is the main by-product of the sugar cane industry, which contains about 50% cellulose, 25% hemicellulose and 25% lignin (Pandey et al., 2000). Due to its availability, it has been used for ethanol production through fermentation processes (Cardona et al., 2010). However, sugarcane bagasse can serve as an ideal substrate for microbial oil production through yeast fermentation processes for the production of biodiesel. Hydrolysis of sugarcane bagasse to reducing mono sugars has been studied by several researchers. Tsigie et al. showed that hydrolysate that resulted from acid pretreatment using 2.5 % HCl consist of 13.59 g/L xylose, 3.98 g/L glucose, and 2.78 g/L arabinose was suitable for growth of oleaginous yeast *Yarrowia lipolytica*. The maximum lipid content of 58.8% was achieved in detoxified sugarcane bagasse hydrolysate when peptone was added as a nitrogen supplement (Tsigie et al., 2011). Huang et al. showed that oleaginous yeast *Trichosporon fermentans* could utilize sulfuric acid treated sugar cane hydrolysate and produce microbial lipids for biodiesel applications. Their work through optimization of fermentation condition to improve lipid accumulation, lead to a lipid production of 15.8 g/L under optimized condition of C/N ratio 165, inoculum concentration 11%, initial pH 7.6 and fermentation time 9 days

(Huang et al., 2012). Table 1.5 summarizes lipid accumulation using oleaginous yeasts from different pretreated lignocellulosic biomass as a cheap source of substrate.

Table 1.5 Production of lipids from lignocellulosic biomass from oleaginous microorganisms

Feedstock	**Pretreatment**	**Microbial species**	**Fermentation mode**	**Lipid (g/L)**	**Lipid content%**
Corn stover (Ruan et al., 2012)	Dilute acid	*Mortierella isabellina* ATCC 42613	Enzymatic hydrolysate of whole slurry	4.8	34.5%
Corn stover (Galafassi et al., 2012)	Dilute acid	*Rhodotorula graminis*	Enzymatic hydrolysate	14.4	34%
Wheat straw (Yu et al., 2011)	Dilute acid	*Cryptococcus curvatus*	Non-detoxified liquid stream of dilute acid	5.8	33.5%
Wheat straw (Yu et al., 2011)	Dilute acid	*Rhodotorula glutinis*	Non-detoxified liquid stream of dilute acid	3.5	25%
Rice straw (Huang et al., 2009)	Dilute acid	*Trichosporon fermentans* CICC 1368	Detoxified liquid stream of dilute acid	11.5	40.1%
Sugar cane bagasse (Huang et al., 2009)	Dilute acid	*T. fermentans*	Detoxified liquid stream of dilute acid	15.8	N/A
Sugarcane bagasse (Tsigie et al., 2012)	Hydrochloric acid hydrolysis	*Y. lipolytica* Po1g	Detoxified acid hydrolysate	6.7	58.5%
Kraft hardwood pulp (Kurosawa et al., 2013)	None	*Rhodococcus opacus* PD630 Xsp8	Enzymatic hydrolysate	11	45.8%

1.6 Kinetic study

During the last decades, significant developments in cell culturing methods, medium optimization and process control have been implemented; however, the process of culturing cells still suffers from several problems. Although, selection and characterization of the bench-top-bioreactors may not be exactly similar to large-scale industrial bioreactors, similar process and kinetic parameters can be employed. Thus, to predict the behavior of the cells, using mathematical modeling is essential, which results in increasing insight to the performance of the bioreactors and the behavior of the cells outside the studied ranges. In this section, we are aiming to introduce some of the most common kinetic models in the literature that are used for describing microbial cell growth in bioreactors.

1.6.1 Cell Growth in Batch Bioreactors

Cell growth models can be divided into structured, unstructured, segregated, and non-segregated models. Structured models describe the structure and physiology of the cell through intracellular process such as individual reaction or series of reactions in the cells. Structured models are not applicable for most of the cell behaviors, since the effect of growth condition on the behavior of the cell is not implied. Thus, the majority of the cell growth models are considered as unstructured models, which attempts to relate the cell growth to the environmental condition without considering intracellular processes. Unstructured models attempts to describe cells as whole entity interacting with its environment. Based on segregated models, the differences between cells in cell population are considered and each cell in cell population is treated individually. However, non-segregated models describe the whole cell population as one cell, which is

mathematically simple to explain. Unstructured non-segregated models are considered and introduced in this section.

1.6.2 Malthusian Model

Malthusian model is known as the simplest model for cell growth, which is usually known as exponential law. Based on exponential law, when batch fermentation is conducted, the concentration of cells or biomass increases exponentially over time. This phenomenon is described as all the cells in cell population multiply with the same probability. The rate of production of cells is proportional to the concentration of cells. This behavior can be explained by first order reaction, and the rate of biomass production is as follow:

$$r_X = \mu X \tag{1.1}$$

where r_X (g/cm^3. h) is the rate of biomass production or cell growth, X is the concentration of cells (g/cm^3), and μ (1/h) is the constant kinetic parameter, which is referred to as specific growth rate. Equation 1.2, can be described as a first order differential equation:

$$\frac{dX}{dt} = \mu X(t) \tag{1.2}$$

The general solution of this differential equation is

$$X(t) = X_0 e^{\mu t} \tag{1.3}$$

where X_0 is the initial concentration of the cells at $t = 0$. Figure 1.7 shows the solution for exponential or Malthusian model at $X_0 = 0.5 \times 10^5$ (cell/ml) and $\mu = 0.035$ (1/h)

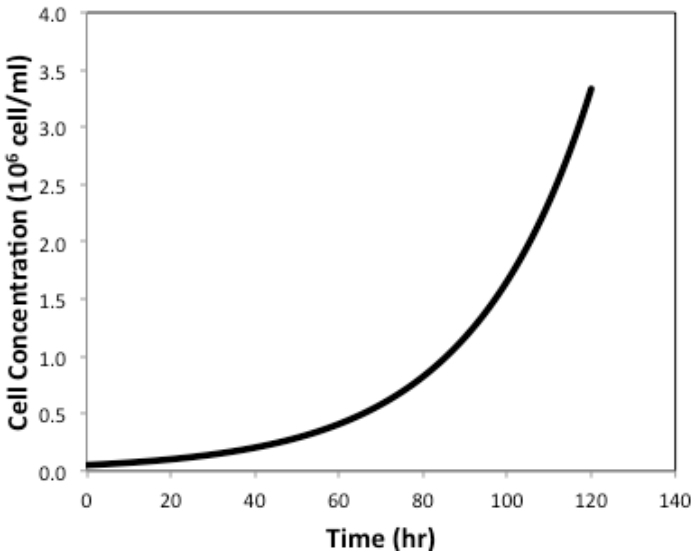


Figure 1.7 The solution for Malthusian model at $X_0 = 0.5 \times 10^5$ (cell/ml) and $\mu = 0.035$ (1/h)

As we can see in Figure 1.6, following Malthusian model, the cell concentration unrealistically increases as time increases without any limits. Malthusian model does not consider that cells needs sufficient environmental conditions for growth such as nutrients, oxygen, space, and etc., and at some point these resources becomes limited.

1.6.3 Logistic Model

In 1838, Pierre-Francois Verhulst proposed a logistic model after he understood the limitation of the Malthusian model. He developed the logistic model as:

$$\frac{dX}{dt} = \mu X\left(1 - \frac{X}{K}\right) \tag{1.4}$$

In addition to parameter μ specific growth rate (1/h), logistic equation has another parameter K carrying capacity (g/cm^3), which is defined as the maximum cell concentration that can be provided by environment. Therefore, in the logistic model, the

concentration of the cell increases with time, then it reaches its maximum value, and finally cells stop growing, as the environment cannot support the cell growth. By integrating Equation 1.4, the solution of logistic equation can be shown as:

$$X(t) = \frac{K}{1 + A\,K e^{-\mu t}} \tag{1.5}$$

where $A = \frac{1}{X_0} - \frac{1}{K}$; the solution for logistic equation using initial condition $X_0 = 0.5 \times 10^5$ (cell/ml), $\mu = 0.07$ (1/h), and $K = 2.5$ (cell/ml) was shown in Figure 1.8.

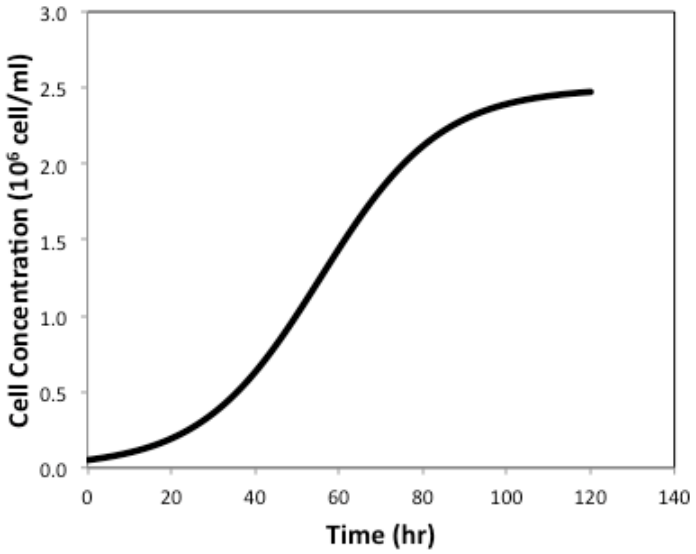


Figure 1.8 The solution for logistic equation using initial condition $X_0 = 0.5 \times 10^5$ (cell/ml), $\mu = 0.07$ (1/h), and $K = 2.5$ (cell/ml)

1.6.4 Monod Model

In 1940s, Monod developed a new model based on environmental conditions, which describes the cell growth as a function of environmental factors. Despite logistic and Malthusian models, Monod model introduces another concept, which is called growth-limiting substrate, indicating that the growth of microorganisms are controlled by

the concentration of substrate. Monod equation is a hyperbolic function, which can be expressed as below:

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{1.6}$$

where, S (g/cm^3) denotes the concentration of limiting substrate in the medium at time t, and constants μ_{max} and K_s stand for maximum specific growth rate and half saturation constant, respectively. Parameter K_s is also called affinity constant, because $\mu = \frac{\mu_{max}}{2}$ when $K_s = S$.

The rate of biomass production based on Monod equation is shown as following equation:

$$\frac{dX}{dt} = \mu_{max} \frac{S}{K_s + S} \; X \tag{1.7}$$

The rate of biomass production is monotonically increases as $S \to \infty$, with a limit of μ_{max}. The most important feature of Monod equation is that, the specific growth rate increases as substrate concentration (S) increases, and it levels out when the substrate concentration becomes limited. The solution of Monod equation at $X_0 = 0.3 \times 10^5$ (g/cm^3), $S_0 = 0.2$ (g/cm^3), $\mu_{max} = 0.1$ (1/h), and $K_s = 0.1$ (g/cm^3) is shown in Figure 1.9.

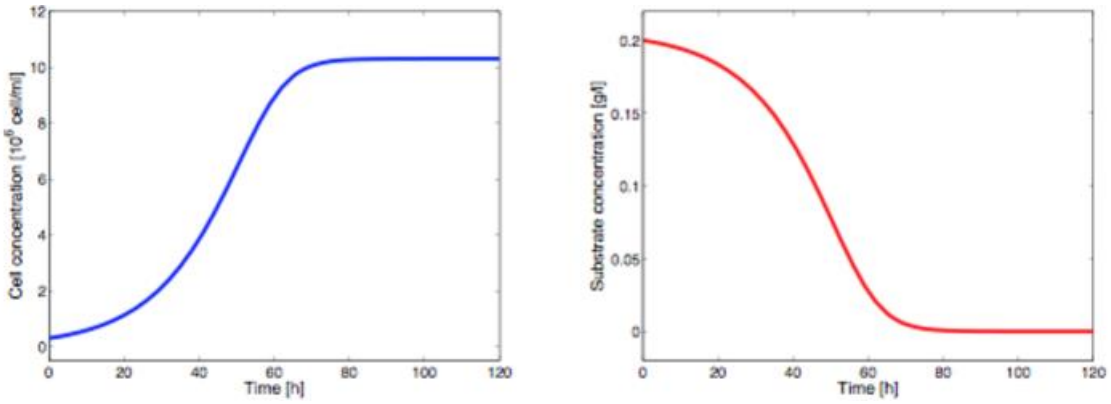


Figure 1.9 Solution of Monod equation at $X_0 = 0.3 \times 10^5$ (g/cm^3), $S_0 = 0.2$ (g/cm^3), $\mu_{max} = 0.1$ (1/h), and $K_s = 0.1$ (g/cm^3)

1.6.4.1 Biological meaning of μ_{max} and K_s

There are two constants for Monod equation μ_{max} and K_s, which both reflects essential physiological characteristics of specific type of microorganisms and used to describe microbial growth. These constants also depend on the environmental condition such as temperature and substrate concentration. The definition of maximum specific growth rate is attributed to the ability of the microorganisms to reproduce in a nutritional environment. Under optimum nourishing condition, when nutrients are not limited (no substrate limited condition), the maximum specific growth rate is expressed as the increase of cell concentration per unit time. However, the half saturation constant is defined as the concentration of substrate, whereas the microorganisms are under substrate-limited condition, which restricts the growth rate to half of its prevalent maximum rate. The empirical constant K_s, mainly depends on the mechanism of substrate transportation through cell membrane; thus, intracellular condition, cell membrane properties, and substrate properties have a significant effect on its value. Therefore, the

value of K_s, can be understood as the desirability of the microorganism for substrate utilization.

The value of both empirical parameters μ_{max} and K_s, predominantly related to the type of microorganisms, which can be identified by its genome. However, due to adaptive aptitude of microorganisms, these values can fluctuate by environmental conditions such as medium composition, temperature, or their cultivation time. Therefore, these values have to be determined for each particular cell-substrate system and at constant experimental conditions such as pressure, temperature, and medium condition.

1.7 Objectives

In this work, utilization of pulp and paper mill wastewater effluents as cultivation media for the sustainable production of microbial lipids as biodiesel feedstock by oleaginous yeast is investigated. In addition, the potential utilization of paper mill sludge as a carbon source for microbial oil production was investigated to make a full use of waste materials from this industry. This process could have the potential of reducing treatment costs in paper mill wastewater treatment by utilizing these effluents as growth medium by oleaginous yeast for the production of high value bioproducts and also eliminates the cost associated for the paper sludge management by this industry. This work is presented in the following chapters:

Chapter II: Lipid production by *Rhodotorula glutinis* from Pulp and paper wastewater for biodiesel production. The objectives for this chapter are:

- To evaluate the lipid accumulation of *R. glutinis* using pulp and paper wastewater as a medium

- To evaluate a potential utilization of different renewable carbon sources such as glucose, xylose, a mixture of glucose and xylose, and glycerol as substrates

Chapter III: Optimization of Biomass and lipid production by *Rhodotorula glutinis* cultured in pulp and paper wastewater

- To find an optimum fermentation condition for *R. glutinis* to enhance biomass and lipid accumulation using design of experiment technique and response surface methodology
- To apply mathematical models to simulate biomass and lipid accumulation to estimate kinetic parameters for design and scale-up the bioreactors

Chapter IV: Effects of inhibitory compounds in lignocellulosic hydrolysate on *Rhodotorula glutinis* growth and lipid accumulation for biodiesel production

- To delineate the effect of three representative lignocellulosic inhibitors (HMF, furfural, acetic acid) on *R. glutinis* growth and lipid accumulation
- To investigate the tolerance level of *R. glutinis* at high concentration of inhibitors in lignocellulose hydrolysate

Chapter V: Cost estimation of microbial oil production

- To estimate the cost of microbial oil production from pulp and paper wastewater
- To investigate the major cost factor of the process
- To make economic suggestions

CHAPTER II

LIPID PRODUCTION BY *RHODOTORULA GLUTINIS* FROM PULP AND PAPER WASTEWATER FOR BIODIESEL PRODUCTION

2.1 Abstract

This study investigated the potential of oleaginous yeast *Rhodotorula glutinis* utilizing pulp and paper wastewater effluents as cultivation media for the sustainable production of microbial lipids as a biodiesel feedstock. *R. glutinis* is an oleaginous yeast, which has the ability to produce significant quantities of intercellular lipids in the form of triacylglycerols. Yeast lipids are a promising potential feedstock for biodiesel production due to similar fatty acid composition to plant oils. The effect of various carbon sources on biomass production, lipid accumulation, substrate utilization, and fatty acid composition using *R. glutinis* in the pulp and paper wastewater media was studied. The pulp and paper wastewater was supplemented with glucose, xylose, and glycerol as carbon sources under nitrogen-limited conditions. The maximum lipid productions of 1.3-2.9 gL^{-1}, which corresponded to the intracellular lipid contents of 8-15% cell dry weight (CDW), were obtained under various carbon substrates. A kinetic study of the batch fermentation was performed in a 3L aerobic batch fermenter to describe the cell growth, lipid accumulation, and substrate utilization process and the kinetic parameters were estimated. The fatty acid profile of oleaginous yeast was rich in palmitic, oleic, and linoleic acids and comparable to vegetable oils. Thus, the results of this study indicated that pulp and

paper wastewater could be used as a fermentation media to produce lipids as biodiesel feedstock if the supplementary carbon sources are added to the wastewater.

2.2 Introduction

The utilization of biodiesel has greatly increased over the past two decades due in part to concerns about the environmental consequences of fossil fuel combustion, the low lubricity quality of ultralow sulfur diesel, and the rapid rises in crude oil prices that can occur. Biodiesel, a mixture of fatty acid methyl esters (FAMEs) derived traditionally from vegetable oils and animal fats, is one of the most prominent renewable energy resources (Nigam and Singh, 2011). The cost of feedstock for producing biodiesel is responsible for almost 80-95% of the total cost of the process (Yuste and Dorado, 2006). Oleaginous microorganisms that can accumulate 20-80% of their dry weight in the form of lipids are considered as alternative raw material for biodiesel production (Ratledge and Wynn, 2002). It has been shown that these microorganisms utilize glucose, xylose, glycerol, food industry waste (Huang et al., 2013), and N-acetylglucosamine (GlcNAc) (Zhang et al., 2011) for the production of lipids.

Glucose is one of the most common carbon substrate for producing microbial biomass and lipids, and has been extensively investigated for use by different oleaginous microorganisms (Mondala et al., 2012; Shields-Menard et al., 2015). However, the use of starch or other corn sugars as a carbon source results in conflicts between the food and biofuel industries and adds significant cost to the final product. Using lignocellulosic base materials, which are representative of the largest potential of fermentable sugars are promising as they can drastically lower the costs of microbial lipid production (Taherzadeh and Karimi, 2007). The depolymerization of cellulose and hemicellulose in

lignocellulosic material results in a carbon source containing glucose and xylose thusly the co-fermentation of mixed sugars must be explored for effective lipid production. Glycerol is another carbon source, which can be used as a substrate for lipid production. Since glycerol is the major by-product of biodiesel manufacturing, its usage as a carbon source for lipid accumulation may provide an additional benefit of offsetting the cost of biodiesel production. Glycerol accounts for 10% of the product output. Approximately 1 kg of glycerol could be obtained for 3.78 liters of produced biodiesel (Papanikolaou and Aggelis, 2002).

Oleaginous microorganisms require high carbon to nitrogen ratios and the fermentation process requires high amounts of water and nutrients in the media to produce biomass with high contents of storage lipids. Various fermentation substrates have been investigated to evaluate the capability of oleaginous microorganisms in terms of growth and product formation, such as starch wastewater, molasses, wastewaters from potato, fruit juice, and lettuce processing, olive oil manufacturing wastewater (Schneider et al., 2012), and municipal wastewater (Hall et al., 2011). Usually, solids that are removed from food industry wastewaters can be used as animal feed or agricultural fertilizer. Pulp and paper wastewater, containing the solids mainly in a form of cellulosic fiber is considered in this study.

The pulp and paper industry is the fifth largest economy in the US and produces a considerable amount of wastewater. Furthermore, the pulp and paper industry can be considered as one of the largest industries in terms of water and energy utilization, consuming between 20,000 and 60,000 gallons of water per ton of product (Ince et al., 2011). Large amounts of wastewater are produced from different steps of the pulp and

paper making process as a result of high water consumption. The general characteristics of the pulp and paper wastewater include high COD, high Biochemical Oxygen Demand (BOD), Suspended Solids (SS), and dark brown in coloration (Poudel et al., 2015).

The present work focuses on the production of microbial lipid to be used as biodiesel feedstock through batch aerobic fermentation of pulp and paper wastewater. Pulp and paper wastewater has been considered as a fermentation medium in order to reduce some of the cost associated with this process. The effects of the utilization of different carbon sources such as glucose, xylose, and glycerol by *Rhodotorula. glutinis* on the lipid productivity were investigated. Kinetic models were applied to describe the processes of microbial growth, product formation, and substrate utilization, which can be further used for the process scale up and bioreactor design in a similar system.

2.3 Materials and Methods

2.3.1 Yeast Inoculum Preparation

R. glutinis (ATCC 15125) was obtained from American Type Culture Collection (ATCC). Sub-cultures of *R. glutinis* (ATCC 15125) was produced by adding an aliquot of *R. glutinis* stock, which was stored at -80°C, into the flask of sugar broth media [1 gL^{-1} Yeast Extract, 1 gL^{-1} $Na_2HPO_4.12\ H_2O$, 1 gL^{-1} KH_2PO_4, 0.4 gL^{-1} $MgSO_4.7H_2O$, 1.6 gL^{-1} $(NH_4)_2SO_4$, 10.0 ml Trace Mineral Solution (3.6 gL^{-1} $CaCl_2.2\ H_2O$, 0.75 gL^{-1} $ZnSO_4.7H_2O$, 0.13 gL^{-1} $CuSO_4.5H_2O$, 0.5 gL^{-1} $MnSO_4.H_2O$, 0.13 gL^{-1} $COCl_2.6\ H_2O$, 0.17 gL^{-1} $Na_2MoO_4.2\ H_2O$ in distilled water), 6.0 ml Iron Solution ($FeSO_4.7H_2O$ in distilled water), and 60 gL^{-1} glucose], were incubated for 4 days using a rotary shaker (New Brunswick Scientific Model I26, Edison, New Jersey) with a rotation speed of ~112 rpm at 30°C. The media compounds were obtained from Fisher Scientific.

2.3.2 Wastewater Collection and Characterization

The pulp and paper wastewater was obtained from the International Paper Mill in Redwood, MS. Multiple samples of wastewater were collected and stored in plastic container. After collection, the wastewater containers were placed on ice for 24 hours prior to conducting the fermentation experiment. The wastewater was characterized prior to being used as a fermentation medium as discussed below. The pH of the wastewater was measured using a bench top pH meter (Accumet Research AR25 Dual Channel pH/Ion Meter, Fisher Scientific). The COD of the wastewater was determined according to EPA standard method (Pokhrel and Viraraghavan, 2004), and residual Ammonium-Nitrogen level was analyzed using ICS 3000 ion chromatograph (Dionex Corp, Sunnyvale, CA, USA). The initial characteristics of the wastewater and its place of origin are provided in Table 2.1. Based on the initial characteristics of the wastewater, low nutrients levels and moderately low COD values were recorded with this wastewater source. Since the carbon to nitrogen ratio dictates the amount of oil accumulated within these microorganisms, the wastewater was supplemented with $60gL^{-1}$ of a carbon source (glucose, xylose, and glycerol), and nutrients ($1\ gL^{-1}\ K_2HPO_4$, $1.5\ gL^{-1}\ Na_2H_2PO_4$ and NH_4Cl) prior to the experiment. The concentration of ammonium chloride as a nitrogen source was calculated to obtain a C:N ratio of 70:1.

Table 2.1 Pulp and paper wastewater initial characteristics (before treatment).

Characteristics	Wastewater
Place of origin	Post paper making process
pH	2.25
COD (mg/l)	1478
Ammonium (mg/l)	3.95
Sulfate (mg/l)	1400

2.3.3 Fermentation Experiment

Our preliminary result in cultivation of *R. glutinis* in pulp and paper wastewater with no addition of sugars resulted in poor growth of microorganisms, which evaluated by almost no production of biomass and lipid accumulation. This could be due to the lack of fermentable sugars present in the wastewater. To investigate the potential utilization of pulp and paper wastewater as fermentation media as a source of water and mineral solutions, the pulp and paper wastewater (2.4 L) was amended with 60 gL^{-1} carbon sources in 4 individual experiments including glucose, xylose, glycerol, and glucose-xylose (2:1) ratio and seeded with a 20% initial inoculum of *R. glutinis* (600 ml) in 3L Bioflo 310 fermenter (New Brunswick Scientific, Edison, NJ) with an initial *R. glutinis* cell concentration of 10 gL^{-1}. The pH of the amended wastewater was adjusted to 6.5 using 1M NaOH solution. The experiment was conducted at the temperature of 25°C. The aeration rate was adjusted at 3 volume of air per volume of medium and per minute (vvm). The agitation rate was set at 300 rpm, and adjusted to maintain a Dissolved Oxygen (DO) level no lower than 60% saturation during the experiment. A polypropylene-based antifoam, 1:10 dilution of nonoil (Sigma-Aldrich, St. Louis, MO) was periodically added to prevent foaming in the vessel. Samples were obtained from the aerobic batch fermenter every 12 h interval during the first 48 h of the experiment and then every 24 hours during the 7 days of incubation time. Control experiment was conducted using *R. glutinis* starter cultured in sugar broth media to compare the potential lipid production by *R. glutinis* using pulp and paper wastewater and the optimal media components.

2.3.4 Analytical Methods

2.3.4.1 Cell Mass Concentration

For each treatment, 30 ml of culture samples were taken and centrifuged at 3000 rpm, at 25°C for 10 minutes using a Sorvall® ST 40 Centrifuge (Thermo Fisher Scientific,Waltham, MA, USA) to separate the supernatant from the cell pellets. Cell pellets were placed in the freezer at -20°C overnight, and then freeze-dried using a Labconco Freezone 2.5 freeze drier followed by gravimetric weight measurements to determine the cell mass concentration. The supernatant was stored in the freezer at -20°C to be used for the analysis of sugar, and nitrogen.

2.3.4.2 Lipid Extraction and Conversion of Lipids to Fatty Acid Methyl Esters

The intercellular lipid content of the freeze-dried samples was determined based on the modified Bligh and Dyer method (Bligh and Dyer, 1959) as described by (Revellame et al., 2012). The lipid content of the samples was expressed by the percentage of lipid in cell dry weight. The dried lipid residues were converted into fatty acid methyl esters (FAMEs) by transesterification using sulfuric acid and methanol as catalyst and reactant, respectively (Christie, 1993). The fatty acid composition in the FAMEs was analyzed using an Agilent 6890 gas chromatograph equipped with a flame ionization detector (GC-FID). Helium was used as carrier gas. The injector temperature of 260°C was used for the injection of the 1 µL Samples. The GC oven temperature was set at 50°C, and then ramped to 250°C after 2 min. The FID temperature was kept at 260°C during the analysis (Revellame et al., 2010). The FAME peaks were identified and quantitated by comparing the sample responses to those generated from a known standard.

2.3.4.3 Determination of Residual Sugars, and Ammonium-Nitrogen

The supernatant samples were analyzed using YSI 2900 Biochemistry analyzer (YSI Inc. Life Sciences, Yellow Springs, OH, USA) equipped with a glucose oxidase, xylose oxidase, and glycerol oxidase membrane probe for the measurement of glucose, xylose, and glycerol, respectively. The residual ammonium-nitrogen level was analyzed using an ICS 3000 ion chromatography (Dionex Corp, Sunnyvale, CA, USA) to monitor the point of nitrogen depletion, and nitrogen level in the wastewater. ICS is equipped with an IonPac CS16 cation exchange analytical column (250 mm × 4 mm) and CG16 guard column (50 mm × 4 mm) and a conductivity detector.

2.3.5 Statistical Analysis

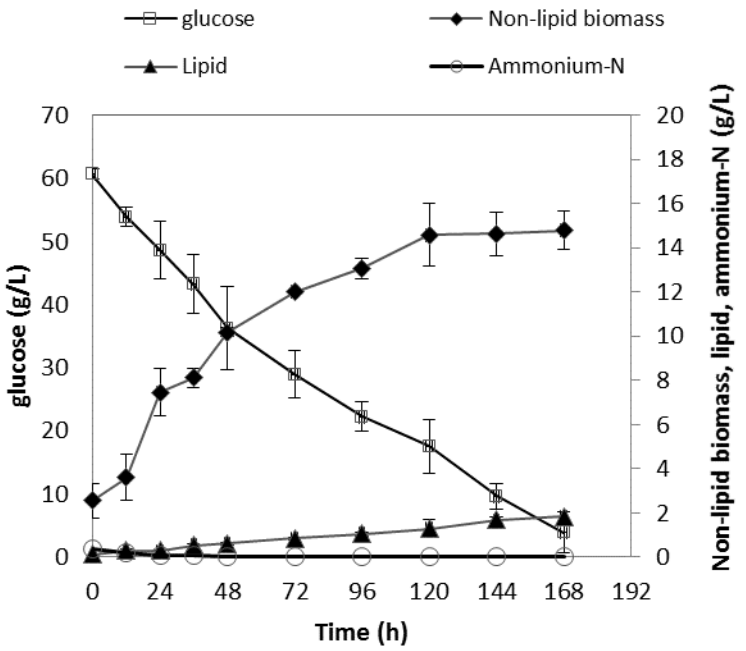
Statistical analysis was carried out using SAS® 9.4 (Copyright © 2015, SAS Institute Inc., Cary, NC, USA) The statistical significance of differences in mean values was analyzed using one-way analysis of variance (ANOVA) with Tukey-Test at 95% confidence intervals.

2.4 Results and Discussion

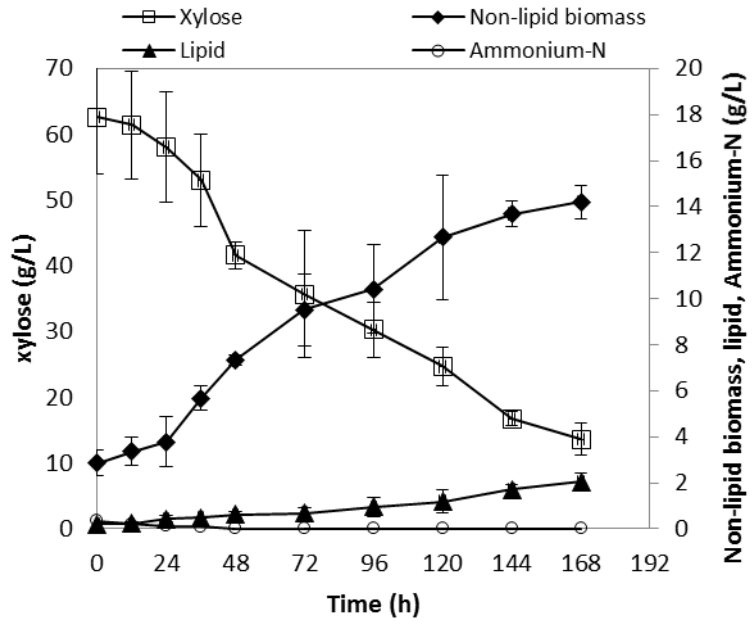
2.4.1 Growth and Lipid Production by *R. glutinis* Using Different Carbon Substrates

It was hypothesized that the oleaginous yeast *R. glutinis* can produce microbial lipids from pulp and paper wastewaters amended with carbon sources, which can be further converted to biodiesel. In this study, carbon to nitrogen ratio of 70:1 with initial nitrogen and carbon source concentrations of 1.3 and 60 gL^{-1} was used, respectively. The fermentation profiles were plotted and presented in Figure 2.1 with the error bars that represent the standard deviation of three independent replicates. Figure 2.1a-Figure 2.1d

show the results of the fermentation profile over the 7 days of fermentation. From Figure 2.1a-Figure 2.1d, it can be seen that fat-free biomass increased significantly within the first 96-120 hours of cultivation and stayed constant after ~120 hours for all the carbon sources except xylose. No lag phase was observed for the growth of *R. glutinis* for all the carbon substrates except xylose. This is most likely due to the fact that samples were not taken during the first 12 hours of incubation when the lag phase would have occurred. In the case of xylose utilization, it can be seen from Figure 2.1b and Figure 2.1c, that growth and lipid accumulation started after 24 hours lag phase. The xylose utilization was observed to lag within the first 24 hours, which could be attributed to the synthesis of enzymes by the oleaginous yeast for xylose utilization. Similar results were observed when co-fermentation of glucose and xylose was used by activated sludge microorganisms (Mondala et al., 2013). A complete depletion of ammonium-nitrogen level was observed after 48 hours of fermentation regardless of the type of substrate initially provided. In all the treatments, growth and lipid accumulation reached the maximum non-lipid biomass and lipid concentration of 12-17 gL^{-1} and 1.3-2.9 gL^{-1}, respectively, which correspond to the intracellular lipid contents of 8-15% CDW. There were no significant differences in biomass production among the treatments using different substrates. However, glycerol grown *R. glutinis* was significantly higher in terms of lipid accumulation among all the treatments. A maximum lipid content of 15±0.92% CDW was obtained when glycerol was used as a substrate, which could be attributed to the highest lipid accumulation. As shown in Figure 2.2, after glycerol, xylose grown *R. glutinis* cells reached the maximum lipid content of 12.6±2.65 % CDW.



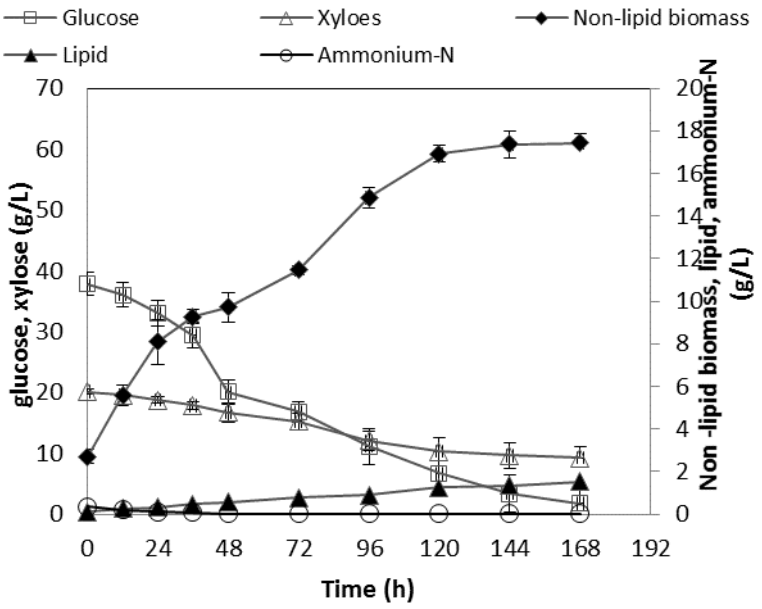
a)



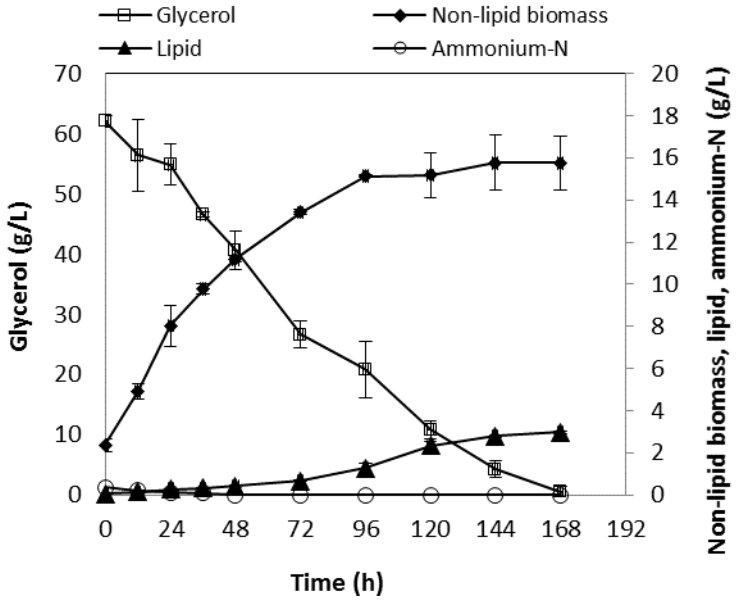
b)

Figure 2.1 Fermentation profile of *R. glutinis* grown on pulp and paper wastewater using different substrates

(a) Pure glucose; (b) Pure xylose; (c) Glucose-xylose (2:1) ratio; and (d) Pure glycerol.



c)



d)

Figure 2.1 (continued)

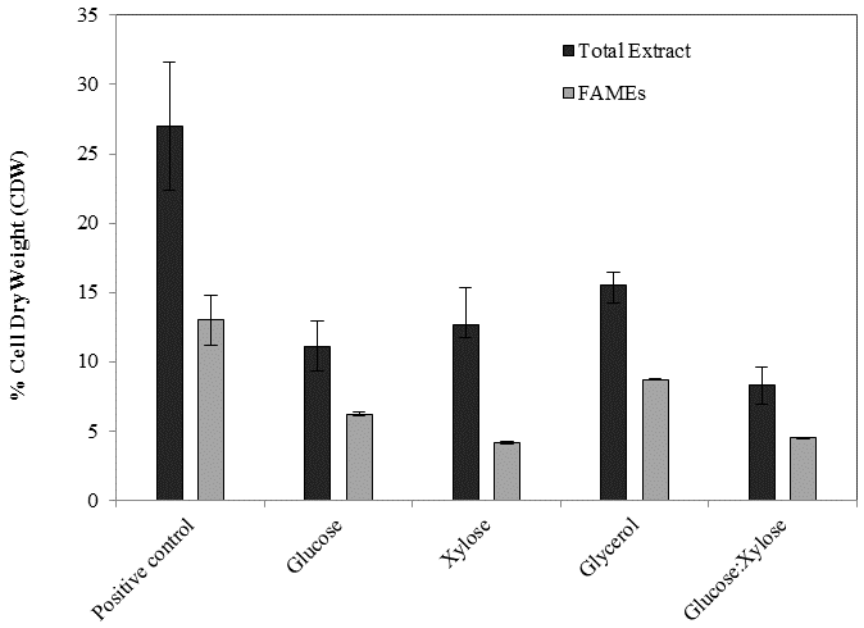


Figure 2.2 Percentage of total lipid extract and FAMEs yield based on cell dry weight (% CDW).

However, in the control experiment under similar fermentation conditions, *R. glutinis* accumulated lipids up to 27±4.6% on a cellular biomass basis with 21±3.12 gL^{-1} biomass production within 6 days, which was significantly higher than all the treatments in terms of biomass and lipid production. Lower lipid content of *R. glutinis* cultivated in pulp and paper wastewater could be due to the presence of organic material and lignin breakdown products in the wastewater, which acts as inhibitory compounds on lipid synthesis. However, in the control experiment, a combination of $(NH_4)_2SO_4$ and yeast extract was used as a nitrogen source, but in the experiment using wastewater as a medium, NH_4Cl was replaced as a nitrogen source to discard the negative effects of sulfate on the final wastewater. It is reported that organic nitrogenous compounds such as yeast extract and peptone have a positive effect on lipid production, but negatively affect

the cell growth; conversely, inorganic nitrogen sources like $(NH_4)_2SO_4$ and NH_4Cl are effective for cell growth, but not favorable for lipid production (Huang et al.,1998). Since nutrient composition can have different effects on the growth and lipid accumulation, the lower lipid content could be as a result of using a different nitrogen source. Thus, further investigations need to be done to achieve a more detailed knowledge about the effects of nutrient compound on the cell growth and lipid accumulation.

2.4.2 Utilization of Different Substrates by *R. glutinis*

R. glutinis was able to deplete the supplied carbon sources below detectable units in 7 days of fermentation except in the treatments where xylose was supplied Figure 2.1. It was observed that considerable amounts of xylose remained in the media after 7 days of cultivation when pure xylose and also xylose in combination with glucose was used. Approximately, 22% of the initial xylose sugar remained in the fermenter when xylose was used as a sole substrate. When substrate combination of glucose-xylose was used, the residual xylose remaining was about 46%. The sequential utilization of glucose followed by xylose was observed Figure 2.1d, which indicates that the consumption of glucose was favored by *R. glutinis*. The sequential utilization of glucose and xylose could be due to synthesizing enzymes by *R. glutinis* in order to metabolize xylose (Mondala et al., 2013). Recent studies indicated that oleaginous yeasts tend to sequentially convert the mixed sugars. The sequential substrate consumption pattern was reported for the yeast *Lipomyces starkeyi* (Zhao et al., 2008). However, a simultaneous utilization of glucose and xylose rather than sequential pattern, no diauxic growth behavior, and no presence of lag phase was reported when oleaginous yeast strain *Trichosporon cutaneum* was used in

a stirred-tank bioreactor (Hu et al., 2011). In this study, it was apparent from the data that *R. glutinis* is following a classic diauxic growth curve.

2.4.3 Kinetic modeling

Monod or Logistic equations can be used to explain the growth process of most microorganisms (Mondala et al., 2012, Zeng et al., 2013, Yang et al., 2011). The logistic equation was selected due to its simplicity for calculating fermentation kinetic parameters. Fat-free biomass was characterized using the logistic model and presented as:

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \tag{2.1}$$

where $\frac{dX}{dt}$ is the rate of cell growth; μ_{max}(h^{-1}) is the maximum specific growth rate; X (gL^{-1}) is the fat-free biomass concentration at any time t (h); $X_{max}$is the maximum value of fat-free biomass in the medium. Assuming that at the beginning of the fermentation (t=0), the fat-free biomass was given by its initial value (X=X_0), integrating Eq. (2.1) becomes:

$$X = \frac{X_0 X_{max} e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}} \tag{2.2}$$

Product formation was modeled by a Luedeking-Piret equation Luedeking and Piret (1959). The rate of lipid accumulation depends on the instantaneous fat-free biomass (X) and fat-free biomass rate ($\frac{dX}{dt}$):

$$\frac{dP}{dt} = nX + m\frac{dX}{dt} \tag{2.3}$$

where $\frac{dP}{dt}$ is the lipid accumulation rate; n and m are empirical constants stand for the non-growth correlation coefficient and growth correlation coefficient. After substituting $\frac{dX}{dt}$ by equation 2.1 in equation 2.3, and integrating assuming at t=0, the concentration of

lipid is equal to its initial concentration ($P = P_0$), the following equation for lipid accumulation was achieved:

$$P = P_0 + mX_0\left(\frac{e^{\mu_{max}t}}{\left[1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}t})\right]} - 1\right) + n\frac{X_{max}}{\mu_{max}} ln\left[1 - \frac{X_0}{X_{max}}(1 - e^{\mu_{max}t})\right] \quad (2.4)$$

The substrate was utilized primarily for cell growth, along with product formation and cell maintenance. The substrate utilization was described by Luedeking-Piret equation Luedeking and Piret (1959), expressing the rate of substrate utilization as a function of instantaneous fat-free biomass rate $\frac{dX}{dt}$, lipid accumulation rate $\frac{dP}{dt}$, and cell maintenance coefficient term:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + K_eX \quad (2.5)$$

where $-\frac{dS}{dt}$ is the total substrate consumption rate; $Y_{X/S}$ (g fat-free biomass produced/ g sugar consumed) and $Y_{P/S}$ (g lipid produced/ g sugar consumed) are the yield coefficients for fat free biomass and lipid, respectively, and K_e refer to the maintenance coefficient. After substituting $\frac{dP}{dt}$ by equation 2.3, and rearranging, equation 2.5 becomes:

$$-\frac{dS}{dt} = \left(\frac{n}{Y_{P/S}} + K_e\right)X + \left(\frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}}\right)\frac{dX}{dt} \quad (2.6)$$

where $\left(\frac{n}{Y_{P/S}} + K_e\right) = \beta$, and $\left(\frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}}\right) = \alpha$

$$-\frac{dS}{dt} = \beta X + \alpha\frac{dX}{dt} \quad (2.7)$$

After substituting $\frac{dX}{dt}$ by equation 2.2 in equation 2.6, and integrating, the equation 2.8 was obtained expressing the substrate concentration:

$$S = S_0 - \left(\frac{n}{Y_{P/S}} + K_e\right)\frac{X_{max}}{\mu_{max}}\left[\left(\mu_{max}t + ln\left(\frac{X_0}{X}\right)\right)\right] - \left(\frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}}\right)(X - X_0) \quad (2.8)$$

where S_0 is the initial substrate concentration at the beginning of the fermentation (t=0).

In this study, the kinetic models expressing cell growth, lipid accumulation, and substrate utilization were fitted to the experimental data for estimation of the model kinetic parameters. The kinetic equations were solved using a nonlinear regression method. Matlab (MATLAB Release 2014a, The MathWorks, Inc., Natick, Massachusetts, United States) was used for data analysis using nonlinear least squares method (Levenberg-Marquardt algorithm) for minimizing residual sum of square of errors (RSSE). The kinetic parameters related to biomass growth (X_0, X_{max}, and μ_{max}), lipid production (m and n), and substrate consumption (S_0, α, and β) were determined and presented in Table 2.2 whereas the experimental results and corresponding fits to the model kinetic equations were illustrated in Figure 2.3.

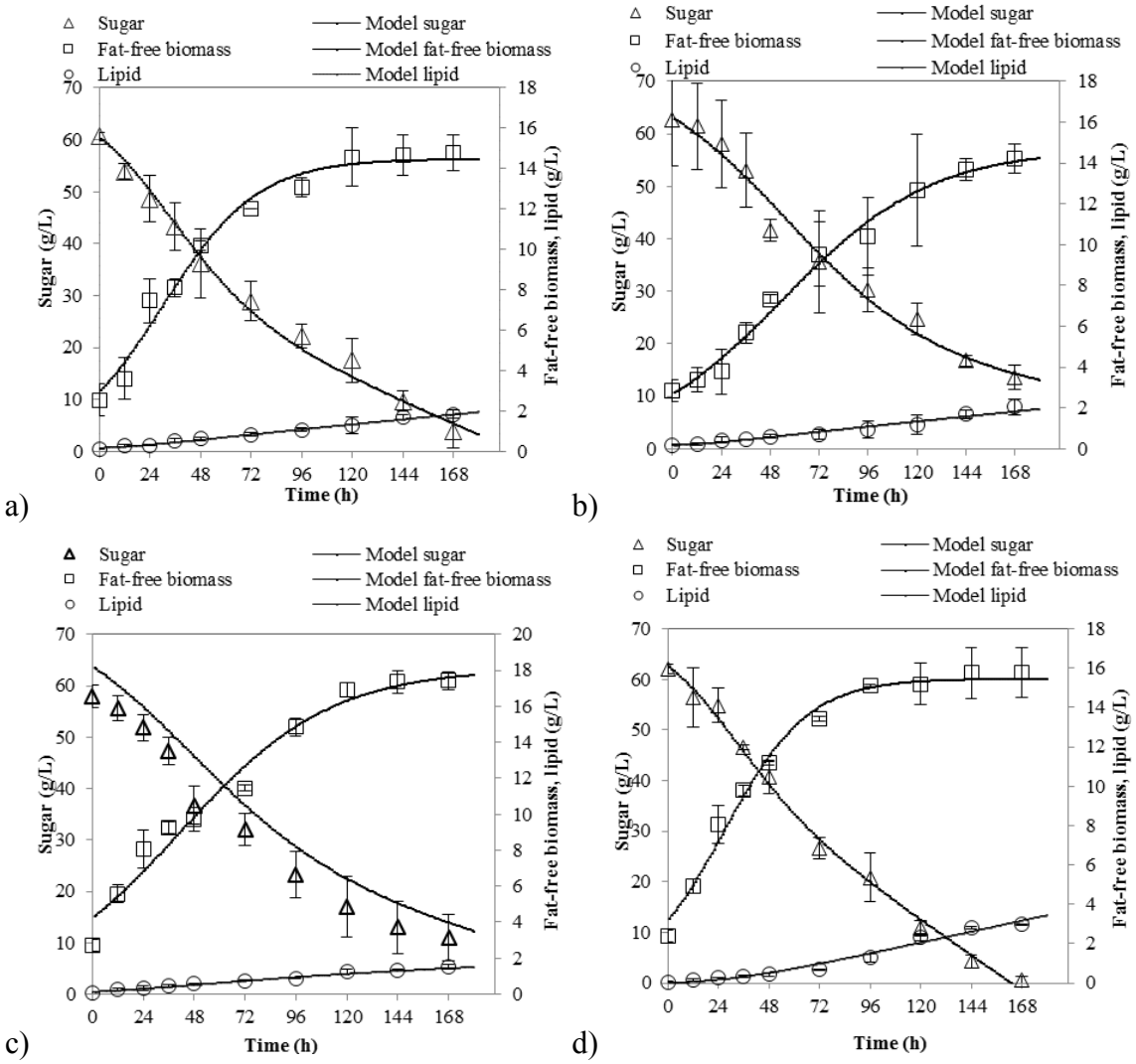


Figure 2.3 Comparison of experimental data and predicted value by the model for fat-free biomass (g/L), lipid (g/L), and substrate utilization (g/L)

(a) Pure glucose; (b) Pure xylose; (c) Glucose-xylose (2:1) ratio; and (d) Pure glycerol.

The values of maximum specific growth rate μ_{max} were in the same range for glucose and glycerol, however, it slightly decreased from the value of about 0.05 to 0.02 (h^{-1}), when xylose used as a single or secondary substrate. From the kinetic parameters regarding to lipid accumulation process (m and n), it can be seen that for all the treatments except glycerol, the constant m is larger than n, which suggest that lipid

production is a mixed growth associated process with a greater contributions from the growth-associated lipid production term. On the other hand, in the case of using glycerol, lipid accumulation process is a mixed growth-associated process with greater contributions from the non-growth-associated lipid production term. The values of coefficients of the determination (R^2) were in the range of 0.97-0.99, which shows a good agreement between the model prediction and the experimental data for non-lipid biomass, lipid production and the substrate utilization.

Table 2.2 The values of kinetic parameters for *R. glutinis* cultivated in pulp and paper wastewater supplemented with different carbon sources.

Parameters	**Substrates**			
	Glucose	**Xylose**	**Glucose/Xylose**	**Glycerol**
Fat-free biomass				
$\mathbf{X_0}$ (g L^{-1})	2.97	2.7	4.26	3.18
$\boldsymbol{\mu_{max}}$ (h^{-1})	0.044	0.027	0.028	0.05
$\mathbf{X_{max}}$ (g L^{-1})	14.51	14.71	18.13	15.47
R^2	0.99	0.99	0.97	0.98
Lipid				
$\boldsymbol{m}$	0.027	0.026	0.05	0.0005
$\boldsymbol{n}$	0.0007	0.0009	0.0003	0.0015
R^2	0.99	0.98	0.98	0.97
Substrate				
S_0	60.68	63.13	63.88	62.74
α	2.84	3.85	2.76	2.087
$\boldsymbol{\beta}$	0.012	0.0031	0.006	0.018
$\mathbf{Y_{X/S}}$	0.42	0.266	0.44	0.48
$\mathbf{Y_{P/S}}$	0.058	0.297	0.1	0.12
$\mathbf{K_e}$	0.000001	0.0001	0.003	0.0057
R^2	0.99	0.98	0.98	0.99

2.4.4 FAMEs Yield and Fatty Acid Profile of Oleaginous Yeast

The fatty acid composition of oleaginous yeasts was greatly dependent on the type of medium used and the growth condition. Some cultivation conditions such as temperature, pH of the medium, the type of the substrate, C:N ratio of the culture, and available oxygen for microorganisms can affect the accumulated lipids and derived fatty acids profile. The results of the characterization of total extracted lipid and FAMEs yield by transesterification of the lipids within 7 days of fermentation were shown in Figure 2.2. The transesterification yield was in the range of 40-60 (w/w) based on extracted lipid for all the treatments.

In this investigation, the fatty acid profile of *R. glutinis* was determined using FAMEs analysis in order to evaluate the potential of using this oleaginous yeast as biodiesel feedstock. Figure 2.4 shows the composition of the produced FAMEs from transesterified *R. glutinis* lipids. According to Figure 2.4, palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) were the main intracellular fatty acids, which accounted for approximately 22-25%, 26-35%, and 23-27% of the total FAMEs for all the substrates, respectively. The palmitic acid (C16:0) level, which was the majority of the saturated fatty acids, did not change considerably by the type of carbon sources and remained in the order of 22-25%. Similar results were observed when other types of oleaginous microorganisms such as *Mortierella isabellina and Cunninghamella echinulata* (Ruan et al., 2012, Papanikolaou et al., 2007, and *Lipomyces starkeyi* (Zhao et al., 2008) were used. The maximum level of monounsaturated fatty acid was achieved in glucose grown *R. glutinis,* which was 34.7±0.85%.

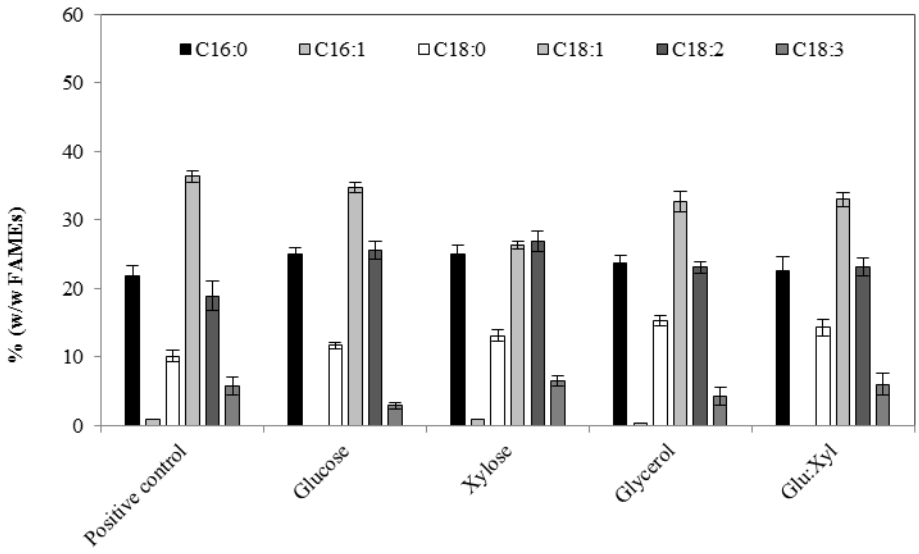


Figure 2.4 Fatty acid composition of transesterified lipids produced by *R. glutinis* grown in pulp and paper wastewater.

The composition of the FAMEs was comparable to the fatty acid profile of the soybean and rapeseed oil, which are the commonly used biodiesel feedstocks in the US and the EU, respectively. Comparing the fatty acid composition of *R. glutinis* in this study with soybean and rapeseed oil indicates that the FAMEs derived from transesterification of lipids from *R. glutinis* were more saturated (Table 2.3). Increased levels of saturated fatty acids improve the quality of the biodiesel such as increasing the cetane number, decreasing the level of nitrogen oxide, and increasing oxidative stability. On the other hand, the unsaturated fatty acids are responsible for improving cold flow property of the biodiesel (Sanford et al., 2009). Thus, the quality of a biodiesel produced from this feedstock could have improved in terms of oxidative stability, cetane number (CN), and nitrogen oxide level (decreased NO_x), which are attributed to the higher levels of saturated fatty acids and lower levels of polyunsaturated fatty acids.

Table 2.3 Comparison of the saturated, monounsaturated, and polyunsaturated fatty acids and their corresponded cetane number, iodine value, saponification value, and higher heating value for the common vegetable oils biodiesel feedstock with *R. glutinis* lipids.

Biodiesel	**Soybean** (Ramos et al., 2009)	**Rapeseed** (Ramos et al., 2009)	**Palm** (Ramos et al., 2009)	**Sunflower** (Ramos et al., 2009)	***R. glutinis*** (This study)
Saturated	14.2	7.1	51	11	37.7±0.6
Monounsaturated	23.2	58.6	39	20	31.9±0.28
Polyunsaturated	57.8	29.6	10	69	29.6±0.15
Cetane number (CN)	45-60	44-65	58-70	49-61.2	57.2±0.15
Iodine value (IV) (g iodine/ 100 g oil)	120.52	108	59	136	92.9±0.3
Saponification value (mg KOH/ g oil)	194.61	197	205	193	195
Higher heating value (MJ/Kg)	39.63	39.73	41	39.45	40±0.004

2.4.5 Prediction of the Quality of the Biodiesel from Fatty Acid Composition

To evaluate the quality of the biodiesel, which can be produced from *R. glutinis* lipids, some fuel properties such as cetane number, iodine value, saponification value, and higher heating value were estimated using available published models. Bamgboye and Hansen showed that the cetane number can be predicted by developing an equation, which relates the cetane number of biodiesel fuels with FAMEs composition of the feedstock used for biodiesel production (Bamgboye and Hansen 2008). One of the properties of the biodiesel that affect the long-term storage of the fuel is oxidative stability of the fuel. The most common method to compare the oxidative stability of the biodiesel fuels is to determine the iodine number or iodine value, which is the indication of a higher numbers of double bonds. Although iodine value may not be the best measurement of oxidative stability since it does not take into consideration the double bond positions for oxidation in fatty acid chain, it can be considered as a rough estimate

for evaluating the biodiesel stability properties. A theoretical model was developed to predict the iodine value and saponification value of biodiesel fuels from the fatty acid profile of the produced FAMEs, so by using the suggested models the properties of the target biodiesel can be estimated without any need of testing these properties (Gopinath et al., 2009). The developed model for prediction of cetane number, iodine value, and saponification value is provided in equations 9 (Bamgboye and Hansen 2008), 10, and 11 (Gopinath et al., 2009), respectively. To predict the higher heating value of the biodiesel, which characterizes the energy content of it, a model was developed by Demirbag (1998) that uses the iodine value and the saponification value of the biodiesel. The proposed model is given as equation 12 (Table 2.3) lists the cetane number, iodine value, saponification value, and the higher heating value of the biodiesels produced from different vegetable oil feedstocks (Gopinath et al., 2009). These biodiesel values were also determined for the *R. glutinis* lipid source, and presented in Table 2.3. Some variations on the iodine value of the *R. glutinis* lipid were observed with a range of 90.4-102.2 g iodine/100 g of oil using different substrate. The cetane number, saponification value, and higher heating value were quite similar using different substrates. Comparing these four biodiesel properties of the *R. glutinis* lipid raw material with the most common plant oils used as biodiesel feedstock indicates that these chemical values were in the range of those observed for the vegetable oils (soybean, rapeseed, palm, and sunflower).

$$Cetane\ number(CN) = 61.1 + (0.088 \times M) + (0.133 \times P) + (0.152 \times S) - (0.101 \times PA) - (0.039 \times O) - (0.243 \times L) - (0.395 \times LL) \quad (2.9)$$

$$Iodine\ value\ (IV) = 35.9 - (0.212 \times P) + (0.660 \times S) + (0.448 \times O) + (1.23 \times L) + (1.73 \times LL) \quad (2.10)$$

$$Saponification\ value\ (SV) = 268 - (0.418 \times P) - (1.3 \times S) - (0.695 \times O) - (0.77 \times L) - (0.847 \times LL) \quad (2.11)$$

where $M = Myristic, P = Palmitic, S = Stearic, PA = Palmitoleic, O = Oleic, L = Linoleic, and\ LL = Linolenic$

$$Higher\ heating\ value\ (HHV) = 49.43 - (0.015 \times IV) - (0.041 \times SV) \quad (2.12)$$

2.5 Conclusion

Cultivation of oleaginous yeast *R. glutinis* in pulp and paper wastewater indicated that this oleaginous microorganism is able to grow in pulp and paper wastewater for producing microbial lipid. The oleaginous yeast used in this study was able to convert pure glycerol and the mixture of glucose and xylose very efficiently that demonstrates the potential utilization of industrial waste glycerol and xylose containing wastes such as lignocellulosic wastes. The fatty acid composition of the oleaginous yeast were similar to the fatty acid profile of vegetable oils which makes its chemical characteristic similar to plant oils suitable for biodiesel production. The cetane number, saponification value, and higher heating value of the derived biodiesel (FAMEs) were quite similar to those observed for the biodiesel produced from common vegetable oils with some improved quality. The applied mathematical model was able to successfully simulate the growth and lipid accumulation process.

CHAPTER III

OPTIMIZATION OF BIOMASS AND LIPID PRODUCTION BY *RHODOTORULA GLUTINIS* CULTURED IN PULP AND PAPER WASTEWATER

3.1 Abstract

The potential of utilizing pulp and paper wastewater effluents as cultivation media for the sustainable production of microbial lipids as biodiesel feedstock by oleaginous yeast *Rhodotorula glutinis* was investigated. In this study, response surface methodology (RSM) with a Box–Behnken design with total of four variables and 3-levels of variables, was used to optimize the biomass and lipid production. The effects of four independent variables as glucose concentration, nitrogen concentration, inoculum concentration, and fermentation time on the actual responses were evaluated. The developed statistical models estimated the maximal biomass growth and lipid productivity of 11.96 and 3.9 g/L, respectively. A kinetic study of the batch fermentation was performed in a 3L aerobic batch fermenter to describe the lipid accumulation process. The fermentation equipped with aeration system and pH control resulted in a lipid content of 44±9.97%, which was about 65% and 121% improvement in biomass and lipid production, respectively, compared to the shake flask experiment.

3.2 Introduction

Biodiesel is one of the alternative fuels that can be derived from vegetable oils and animal fats (Nigam & Singh, 2011). However, this environmental friendly fuel

cannot economically compete with traditional petroleum fuels due to the high cost of its feedstock (Ma & Hanna, 1999). Microbial oils produced from oleaginous microorganisms are attracting interests to be used in the production of renewable biodiesel (Uçkun Kiran et al., 2013). It has been demonstrated that their fatty acid composition is similar to that of vegetable oils (Azeem et al., 1999), which makes them a promising feedstock for biodiesel production (Li et al., 2007). The pulp and paper industry is one of the largest industries in terms of water and energy utilization, consuming between 20,000 and 60,000 gallons of water per ton of product (Ince et al., 2011). This industry produces considerable amounts of wastewater through its different processing steps. Pulp and paper processes can be categorized into wood preparation, pulping, pulp washing, pulp bleaching, and papermaking (Ince et al., 2011). During mechanical and chemical pulping processes, organic and inorganic wastes are commonly disposed to wastewaters (Pokhrel & Viraraghavan 2004). A biological treatment of organic wastes results in by-products that are commonly referred to as biosolids. Large amounts of biosolids are produced daily in wastewater treatment plants. Of the operating costs for treating wastewaters, biosolids management accounts for 60% of the total cost (Canales et al., 1994). Thus, using waste biosolids in a value-added manner is of interest as an alternative for treatment processes such as landfilling, anaerobic digestion, and incineration. Pulp and paper mills generate about 45 kg of waste biosolids per ton of pulp produced (Campbell et al., 1991). Waste sludge from the pulp and paper wastewater treatment plant is a byproduct of mechanical/biological treatment of organic wastes, which is mainly composed of cellulose fibers. Paper sludge contains a very low amount of lignin. The cellulose content of waste paper sludge could vary between 50-80%,

depending on its original source. Cellulose can be converted to glucose through the acid hydrolysis or enzymatic hydrolysis methods (Pallapolu et al., 2011). Thus, the waste paper sludge can be potentially used as a source of carbon (glucose) for the production of microbial oil. The large volume of wastewater discharge and its high chemical oxygen demand creates potential for environmental pollution if left untreated. In this work, utilizing pulp and paper wastewater effluents as fermentation medium is of interest as a source of water and nutrients for the production of microbial lipids.

The process of lipid accumulation by oleaginous microorganisms requires a growth medium containing an excess of the carbon source with limited nutrients, typically nitrogen(Papanikolaou & Aggelis 2011; Christophe et al., 2012). At a low carbon to nitrogen (C/N) ratio, the carbon source is distributed for cellular production; therefore, high lipid concentration could be achieved as a result of a high number of cells with low lipid content. However, at a high C/N ratio, insignificant microbial growth with high lipid content is detected. Although high lipid content is achieved, the lipid accumulation could be low as a result of low number of cells. Therefore, for enhanced lipid production it is important to determine the optimal composition of the medium and the process fermentation condition that optimizes cell concentration as well as lipid concentration.

Response surface methodology (RSM) is a mathematical and statistical technique that is commonly used in combination with factorial design methods for designing experiments, fitting models, and optimization. For a novel and complex system with an unknown mechanism, RSM technique can be a useful tool for optimizing and understanding the performance of a system. RSM is a powerful technique that is able to

evaluate the relationship between responses (objective function) and factors (design variables). Recently, RSM has been extensively applied to optimizing process parameters in combined systems (Liu et al., 2015; Revellame et al., 2011; Sivamani et al., 2015). In this work, a Box–Behnken design of experiment method was adopted, which can effectively reduce the number of experiments without a negative effect on the accuracy of the model and optimization. Several shake flask experiments were conducted to optimize the production of biomass and lipid using pulp and paper wastewater as a medium and glucose as a model compound for cellulose fiber hydrolysate by the oleaginous yeast *R. glutinis*. It has been shown that *R. glutinis* has the ability to produce significant quantities of intercellular lipids in a form of triacylglycerols when it is cultivated in sugars such as glucose and xylose (Jenkins et al., 2015). In our previous work, we have demonstrated the potential of *R. glutinis* to grow in pulp and paper wastewater effluents for the sustainable production of microbial lipids (Amirsadeghi et al., 2015). This oleaginous yeast was able to handle the harsh environment, which contains toxic compounds in pulp and paper industrial wastewater effluents.

The main objective of the current study was to apply a design of experiment combined with RSM optimization techniques to enhance the biomass and lipid accumulation in *R. glutinis*. The potential of utilizing pulp and paper wastewater as fermentation medium and cellulosic biomass residues as a source of fermentable sugars were evaluated for the production of microbial lipid to serve as biodiesel feedstock. Kinetic models that describe the process were applied in order to determine kinetic parameters. The models could be applied in similar systems for microbial lipid production and used as a useful implement for designing bioreactors and large-scale

biodiesel production. The characterization of the lipid extracts was performed regarding its potential suitability for biodiesel production.

3.3 Materials and Methods

3.3.1 Microorganism and media preparation

R. glutinis ATCC 15125, obtained from American Type Culture Collection (ATCC), was used for the production of lipids. The stock culture was grown in liquid Yeast Mold (YM) media (3 g/L Yeast Extract, 3 g/L Malt Extract, 5 g/L peptone, 10 g/L Dextrose), and maintained with 30% (v/v) glycerol at -80 °C until used. One ml of the culture was transferred to a 2 L flask containing 500 ml of culture medium [1 g/L Yeast Extract, 1 g/L $Na_2HPO_4.12\ H_2O$, 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4.7H_2O$, 1.6 g/L $(NH_4)_2SO_4$, 10.0 ml Trace Mineral Solution (3.6 g/L$CaCl_2.2\ H_2O$, 0.75 g/L $ZnSO_4.7H_2O$, 0.13 g/L $CuSO_4.5H_2O$, 0.5 g/L $MnSO_4.H_2O$, 0.13 g/L $COCl_2.6\ H_2O$, 0.17 g/L $Na_2MoO_4.2\ H_2O$ in distilled water), 6.0 ml Iron Solution ($FeSO_4.7H_2O$ in distilled water), and 60 g/L glucose]. The medium compounds were obtained from Fisher Scientific. The flask was incubated at 30 °C and 120 rpm for 4 days.

3.3.2 Medium optimization through RSM

The oleaginous potential is affected by several factors including growth medium components (Gao et al., 2013) and fermentation process variables. The C/N ratio, initial seed concentration, operating temperature, pH, agitation, aeration rate, and a fermentation time, are several factors that can affect the biomass and lipid accumulation of oleaginous microorganisms. A Box–Behnken design with total of four variables (k=4) including initial glucose concentration (x_1), initial nitrogen concentration (x_2), the inoculum

concentration (x_3), and fermentation time (x_4) was selected. The initial carbon and nitrogen concentration is related to the C/N ratio, which has been shown to play a significant role in the growth process of microorganisms (Saenge et al., 2011b; Mondala et al., 2012). Inoculum concentration can accelerate the rate of growth and sugar consumption rate, while an optimum fermentation time would have a profound effect on the fermentation cost and sustainability of the whole process. The synergy between four independent variables was determined with respect to their effects on the biomass (Y_1) and lipid production (Y_2) as responses. Four independent variables were studied at three different levels, low (-1), medium (0), and high (+1) to determine the response pattern, which is shown in Table 3.1. According to this design, the experimental plan contains 27 trials, which were conducted with three replications at the central point. The experiments were conducted in 1L baffled Erlenmeyer flasks. The pulp and paper wastewater was used as fermentation medium to reduce the fermentation cost. The process flow diagram for the pulp and paper wastewater treatment is shown in Figure 3.1. The wastewater treatment plant has both aerobic and anaerobic wastewater treatment system. The wastewater effluents have been purified anaerobically to remove high COD and then led to the aerobic basin coupled with surface aerators. The pulp and paper wastewater used in this work, was originated from the anaerobic pond digester with an initial pH of 8.59, and an initial ammonium concentration of 2.83 mg/L. The glucose and ammonium sulfate were autoclaved separately and loaded to makeup their concentration into the desired level to obtain the initial concentrations of 40-80 and 0.6-1.4 g/L, respectively. The pH of the medium was adjusted to 6.5 prior to the experiments, and then the flasks were placed in the rotary incubator with shaking speed of 120 rpm. The seeding levels of 10-20%

(v/v) was used as inoculum in 200 mL medium and incubated at 30 °C for 5-9 days. To determine the correlation between four independent variables, the second order polynomial equation was fitted to the data from the 27 trials. The low, middle, and high levels of each variable, the experimental design, the respective experimental results, and its corresponded predicted values are given in Table 3.2.

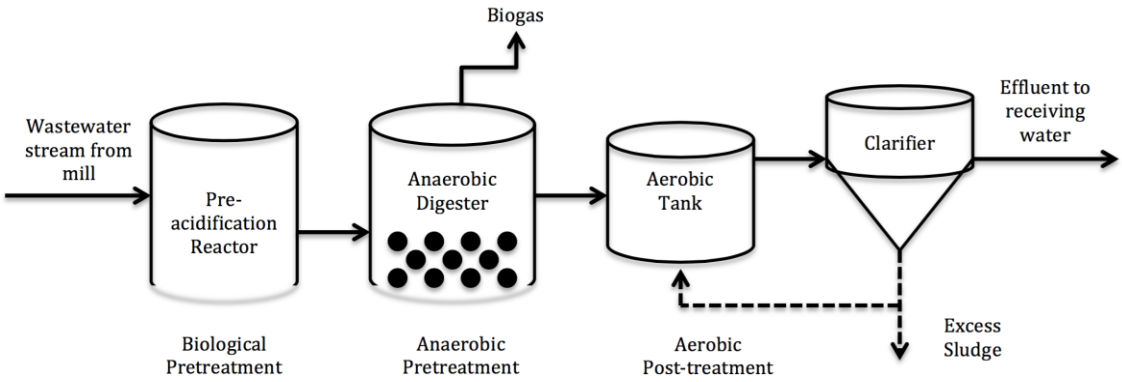


Figure 3.1 A simplified process flow diagram for the pulp and paper wastewater treatment plant.

Table 3.1 Minimum and maximum levels of 4 factors in terms of coded and uncoded symbols

Factor	**Symbols**		**Code level**		
	Coded	**Uncoded**	**-1**	**0**	**1**
Initial carbon (g/L)	x_1	X_1	40	60	80
Initial Nitrogen (g/L)	x_2	X_2	0.6	1	1.4
Inoculum concentration % (v/v)	x_3	X_3	10	15	20
Fermentation time (day)	x_4	X_4	5	7	9

Table 3.2 Box-Benhken experimental design matrixes and the response values

Trial	**Code level**				**Biomass (g/L)**		**Lipid productivity (g/L)**	
	x_1	x_2	x_3	x_4	Experimental	Predicted	Experimental	Predicted
1	-1	-1	0	0	8.14	8.1231	1.15	0.7386
2	-1	1	0	0	8.426	7.7283	2.029	1.91
3	1	-1	0	0	8.679	8.4207	2.994	3.1338
4	1	1	0	0	9.104	9.1647	2.615	3.0472
5	0	0	-1	-1	11.166	10.9576	2.909	2.9824
6	0	0	-1	1	10.692	10.3924	2.325	2.48
7	0	0	1	-1	10.866	11.209	3.153	3.0188
8	0	0	1	1	10.87	11.1218	2.875	2.8224
9	-1	0	0	-1	8.14	8.57	1.844	1.8399
10	-1	0	0	1	8.072	8.6562	1.591	1.5475
11	1	0	0	-1	10.2	9.8494	3.748	3.6631
12	1	0	0	1	9.307	9.1108	3.381	3.2576
13	0	-1	-1	0	9.828	9.8381	1.886	1.8574
14	0	-1	1	0	10.491	10.4529	2.496	2.5132
15	0	1	-1	0	9.866	10.1371	3.012	2.8662
16	0	1	1	0	10.28	10.5031	2.689	2.5892
17	-1	0	-1	0	8.968	9.0707	1.637	1.8458
18	-1	0	1	0	10.76	10.3585	1.478	1.8478
19	1	0	-1	0	10.611	10.7351	3.686	3.4246
20	1	0	1	0	10.808	10.4281	3.902	3.8014
21	0	-1	0	-1	9.544	9.2271	1.998	2.1612
22	0	-1	0	1	9.241	8.8609	1.782	1.9032
23	0	1	0	-1	9.259	9.3617	2.808	2.795
24	0	1	0	1	9.036	9.0755	2.409	2.3542
25	0	0	0	0	11.489	11.4053	3.481	3.4202
26	0	0	0	0	11.018	11.4053	3.175	3.4202
27	0	0	0	0	11.709	11.4053	3.605	3.4202

The general equation for the second-degree polynomial equation, which is based on the multiple linear regressions, is shown as equation 3.1. This Equation takes into account the main, quadratic, and interaction effects for the independent variables.

$$Y = \beta_0 + \sum \beta i \; xi + \sum \beta ij \; xi \; xj + \sum \beta ii \; xi^2 \qquad (3.1)$$

where *Y* is the predicted response, *xi* and *xj* stand for the input variables which affect the response variable (Y), β_o is the intercept, βi is the linear coefficient, βij is the linear-by-linear interaction effect between *xi* and *xj* and βii is the squared interaction effect.

Statistical analysis of the model was accomplished using the analysis of variance (ANOVA) to determine the significance of the predictive model and the regression coefficient terms. For each variable, the response surface curves and contour plots (3D) for the quadratic models were provided. Statistical analyses, numerical optimization, 3D response surface and contour plots were performed using SAS® Software, a statistical analysis software package (SAS® System for Windows version 9.3 SAS Institute Inc., Cary, NC, USA (Copyright© 2013).

3.3.3 Fermentation in bioreactor

The batch fermentation experiments were conducted in a 3L Bioflo 310 fermenter that contained 2 L of optimized media obtained from RSM study. The pH of the media was controlled at 5 using 1M NaOH solutions. The temperature was controlled at 30 °C and the aeration rate was adjusted at 3 volume of air per volume of medium and per minute (vvm). The agitation was set at 300 rpm and adjusted to maintain a dissolved oxygen (DO) level no lower than 60% saturation during the experiment. A polypropylene-based antifoam, 1:10 dilution of nonoil (Sigma-Aldrich, St. Louis, MO) was periodically added to prevent foaming in the vessel.

3.3.4 Kinetic modeling

A mathematical model was used to describe the behavior of oleaginous yeast *R. glutinis* in pulp and paper wastewater. Monod or Logistic equations can be used to explain the growth process of most microorganisms. In this study, the logistic equation was selected due to its simplicity for calculating fermentation kinetic parameters

(Mondala et al., 2012; Yang et al., 2011). Fat-free biomass was characterized using a logistic model and presented as:

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \tag{3.2}$$

where $\frac{dX}{dt}$ is the rate of cell growth; μ_{max}(1/h) is the maximum specific growth rate; X (g/L) is the fat-free biomass concentration at any time t (h); $X_{max}$is the maximum value of fat-free biomass in the medium. Assuming that at the beginning of the fermentation (t=0), the fat-free biomass was given by its initial value (X=X_0), integrating Eq. (6) becomes:

$$X = \frac{X_0 X_{max} e^{\mu max t}}{X_{max} - X_0 + X_0 e^{\mu max t}} \tag{3.3}$$

Product formation was modeled by a Luedeking-Piret (Luedeking & Piret, 1959; Mondala et al., 2012) equation. The rate of lipid accumulation depends on the instantaneous fat-free biomass (X) and fat-free biomass rate ($\frac{dX}{dt}$):

$$\frac{dP}{dt} = nX + m\frac{dX}{dt} \tag{3.4}$$

where $\frac{dP}{dt}$ is the lipid accumulation rate; n and m are empirical constants stand for the non-growth and growth correlation coefficient. Considering 3.4, for $m = 0$ and $n \neq 0$, the product formation is non-growth associated; for $m \neq 0$ and $n = 0$, the product formation is connected to the microbial growth. In this case, the mode of product formation is partially associated to the microbial growth ($m \neq 0$ and $n \neq 0$). However, a significant fraction of lipid is generated during the stationary phase. After substituting $\frac{dX}{dt}$ by Equation 3.3 in Equation 3.4 and integrating, the following equation for lipid accumulation was achieved:

$$P = P_0 + mX_0\left(\frac{e^{\mu_{max}t}}{\left[1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}t})\right]} - 1\right) + n\frac{X_{max}}{\mu_{max}} ln\left[1 - \frac{X_0}{X_{max}}(1 - e^{\mu_{max}t})\right] \quad (3.5)$$

The substrate was utilized primarily for cell growth, along with product formation and cell maintenance. The substrate utilization was described by Luedeking-Piret equation, expressing the rate of substrate utilization as a function of instantaneous fat-free biomass rate $\frac{dX}{dt}$, lipid accumulation rate $\frac{dP}{dt}$, and cell maintenance coefficient term:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + K_eX \quad (3.6)$$

where $-\frac{dS}{dt}$ is the total substrate consumption rate, $Y_{X/S}$ (g biomass produced/ g sugar consumed) and $Y_{P/S}$ (g lipid produced/ g sugar consumed) are the yield coefficients for fat-free biomass and lipid, respectively, and K_e refer to the maintenance coefficient. After rearranging, Equation 3.6 becomes:

$$-\frac{dS}{dt} = \left(\frac{n}{Y_{P/S}} + K_e\right)X + \left(\frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}}\right)\frac{dX}{dt} \quad (3.7)$$

where $\left(\frac{n}{Y_{P/S}} + K_e\right) = \beta$, and $\left(\frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}}\right) = \alpha$

$$-\frac{dS}{dt} = \beta X + \alpha\frac{dX}{dt} \quad (3.8)$$

After substituting $\frac{dX}{dt}$ by Equation 3.3 in Equation 3.7, and integrating, the Equation 3.9 was obtained expressing the substrate concentration:

$$S = S_0 - \left(\frac{n}{Y_{P/S}} + K_e\right)\frac{X_{max}}{\mu_{max}}\left[\left(\mu_{max}t + ln\left(\frac{X_0}{X}\right)\right)\right] - \left(\frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}}\right)(X - X_0) \quad (3.9)$$

where S_0 is the initial substrate concentration at the beginning of the fermentation (t=0).

3.3.5 Analytical methods

For each treatment, 30 ml of culture samples were taken and centrifuged at 3000 rpm for 10 minutes using a Sorvall® ST 40 Centrifuge to separate the supernatant from the cell pellets. Cell pellets were placed in the freezer at -20 °C overnight, and then freeze-dried using a Labconco Freezone 2.5 freeze-drier. Then, the biomass concentration was determined by gravimetric weight measurements. The supernatant was stored in the freezer at -20 °C and used for the analysis of sugar at a later time. Extraction of lipids from the produced biomass was carried out according to the Bligh and Dyer method (Bligh & Dyer 1959). The dried lipids were obtained by evaporating the chloroform in the lipid-dissolved chloroform extracts using a TurboVap LV. After the complete evaporation of chloroform, the residual lipid was weighed using an analytical balance. The microbial lipids were transesterified to fatty acid methyl esters (FAMEs) following Christie method of transesterification using methanol as a reactant and 2% H_2SO_4 (v/v%) as a catalyst at 60 °C for 2 hours. After neutralization with 2% (wt) $KHCO_3$ amended with 5% NaCl solution, the FAMEs were extracted using toluene (Christie, 1993). The fatty acid composition in the FAMEs was analyzed using an Agilent 6890 gas chromatograph equipped with a flame ionization detector (GC-FID). Helium was used as carrier gas. The injector temperature of 260 °C was used for the injection of the 1 μL Samples. The GC oven temperature was set at 50 °C, and then ramped to 250 °C after 2 min. The FID temperature was kept at 260 °C during the analysis (Revellame et al., 2010). The FAME peaks were identified and quantitated by comparing the sample responses to those generated from a known standard. Total reducing sugar concentration

was determined using YSI 2900 Biochemistry Analyzer equipped with a glucose oxidase membrane probe.

3.4 Results and Discussion

3.4.1 Development of the regression model

This work investigates the process of the microbial growth and lipid accumulation through their growth phase and secondary metabolism. Various initial sugar (40-80 g/L) and nitrogen (0.6-1.4 g/L) concentrations resulting in various C/N (50-250) ratios were investigated. To develop a model, the actual responses resulted from the 27 runs were fitted to the second order polynomial equation. Mathematical models were suggested showing the effect of each factor and their interaction on the biomass and lipid production. The second-order polynomial model for biomass (Y_1), and lipid production (Y_2) as a function of coded factors are shown as follows:

$$Y_1 = 11.4053 + 0.4336\,x_1 + 0.0872\,x_2 + 0.2452\,x_3 - 0.1631\,x_4 - 1.5654\,x_1^2 + 0.2847\,x_1\,x_2 - 0.3987\,x_1\,x_3 - 0.2062\,x_1x_4 - 1.4807\,x_2^2 - 0.0622\,x_2\,x_3 + 0.02\,x_2x_4 + 0.3082\,x_3^2 + 0.1195\,x_3x_4 - 0.7933\,x_4^2 \quad (3.10)$$

$$Y_2 = 3.4202 + 0.8831\,x_1 + 0.2712\,x_2 + 0.0947\,x_3 - 0.1747\,x_4 - 0.4697\,x_1^2 - 0.3145\,x_1\,x_2 + 0.0937\,x_1\,x_3 - 0.0285\,x_1x_4 - 0.7431\,x_2^2 - 0.2332\,x_2\,x_3 - 0.0457\,x_2x_4 - 0.2206\,x_3^2 + 0.0765\,x_3x_4 - 0.374\,x_4^2 \quad (3.11)$$

The results of the analysis of variance (ANOVA) for the suggested models are shown in Table 3.3. A very low probability value and its corresponding large F-value denote that the models are highly significant. The P-values of the model for biomass and lipid production were 0.0003 and < 0.0001, respectively, which shows that the developed models are very significant. The significance of each coefficient of the models was

checked by the P-value and F-value of the model coefficients for each variable. Smaller P-values and larger F-values indicate that the coefficient terms are more significant.

Table 3.3 Analysis of variance (ANOVA) for the quadratic biomass model

Source	**DF**	**SS**	**MS**	**F-value**	**P-value**
Model	14	30.9095	2.2077	8.8158	0.0003
x_1	1	2.2558	2.2558	9.0078	0.011
x_2	1	0.0914	0.0914	0.3655	0.5567
x_3	1	0.7221	0.7221	2.884	0.1152
x_4	1	0.3191	0.3191	1.2744	0.281
$x_1 x_2$	1	0.3242	0.3242	1.295	0.2773
$x_1 x_3$	1	0.636	0.636	2.5396	0.137
$x_1 x_4$	1	0.17	0.17	0.6793	0.4259
$x_2 x_3$	1	0.0155	0.0155	0.0619	0.8077
$x_2 x_4$	1	0.0016	0.0016	0.0064	0.9376
$x_3 x_4$	1	0.057	0.057	0.2281	0.6415
x_1^2	1	13.0716	13.0716	52.1947	< .0001
x_2^2	1	11.6927	11.6927	46.6887	< .0001
x_3^2	1	0.507	0.507	2.0246	0.1802
x_4^2	1	3.3562	3.3562	13.4018	0.0033
Lack-of-fit	10	2.756	0.2755	2.2114	0.3514
Pure error	2	0.2491	0.1245		
Total	26	33.9147			
R^2=0.92	Adj-R^2=0.81				

Table 3.4 Analysis of variance (ANOVA) for the quadratic lipid model

Source	**DF**	**SS**	**MS**	**F-value**	**P-value**
Model	14	14.7354	1.0524	14.0633	< .0001
x_1	1	9.358	9.358	125.037	< .0001
x_2	1	0.8835	0.8835	11.8042	0.0049
x_3	1	0.1078	0.1078	1.442	0.253
x_4	1	0.3664	0.3664	4.8962	0.047
$x_1 x_2$	1	0.3955	0.3955	5.2862	0.0403
$x_1 x_3$	1	0.0351	0.0351	0.4696	0.5061
$x_1 x_4$	1	0.0031	0.0031	0.0433	0.8384
$x_2 x_3$	1	0.2175	0.2175	2.9077	0.1139
$x_2 x_4$	1	0.0082	0.0082	0.1119	0.7438
$x_3 x_4$	1	0.0233	0.0233	0.3128	0.5863
x_1^2	1	1.1773	1.1773	15.73	0.0019
x_2^2	1	2.9458	2.9458	39.3617	< .0001
x_3^2	1	0.2598	0.2598	3.4713	0.0871
x_4^2	1	0.7452	0.7452	9.9587	0.0083
Lack-of-fit	10	0.8	0.08	1.6333	0.4387
Pure error	2	0.098	0.0489		
Total	26	15.6335			
R^2=0.94 Adj-R^2=0.87					

The coefficient of determination R^2 is defined as a measurement of the degree of fit. The values of R^2 of 0.91 and 0.94 for biomass and lipid production indicate a good agreement between the experimental data and the predicted values. This implies that the

models can explain up to 91~94 % of the variations in biomass and lipid production and about 6~9% of the total variations could not be justified by these equations. Furthermore, the values of adj-R^2 of 0.81 and 0.87 were suggested, which is the indication of the precision of the prediction. The acceptable values of coefficients of variance (CV) of 5.1 and 10.44 were observed, which shows a good degree of precision and reliability of the models. A CV value higher than 15 shows the lower reliability of the experiments. The lack-of-fit values were not significant for both biomass and lipid productivity with p-values of about 0.34 and 0.44, respectively, indicating that the polynomial models were adequate for predicting biomass and lipid production.

3.4.2 Effects of process parameters on optimization and model accuracy check

Further statistical analysis showed that the initial glucose concentration, initial nitrogen concentration, and fermentation time had a significant effect on lipid production, while initial glucose concentration was the only significant factor for biomass production ($P < 0.05$). Sriwongchai et al., showed that the lipid accumulation for the oleaginous bacterium *Rhodococcus erythropolis* grown on glycerol, was influenced by initial glycerol concentration, nitrogen concentration, and incubation time (Sriwongchai et al., 2012).

The effect of inoculum concentration was insignificant for both responses in the studied ranges. The terms of $x_1\,x_3$, x_1x_4, $x_2\,x_3$, and x_2x_4, indicating the interaction effects were also insignificant for both biomass and lipid production. The only significant interaction effect was $x_1\,x_2$, which had a significant effect on the lipid production. After evaluation of the significance of each parameter, the models can be modified by excluding the terms that do not significantly affect the biomass and lipid production. The

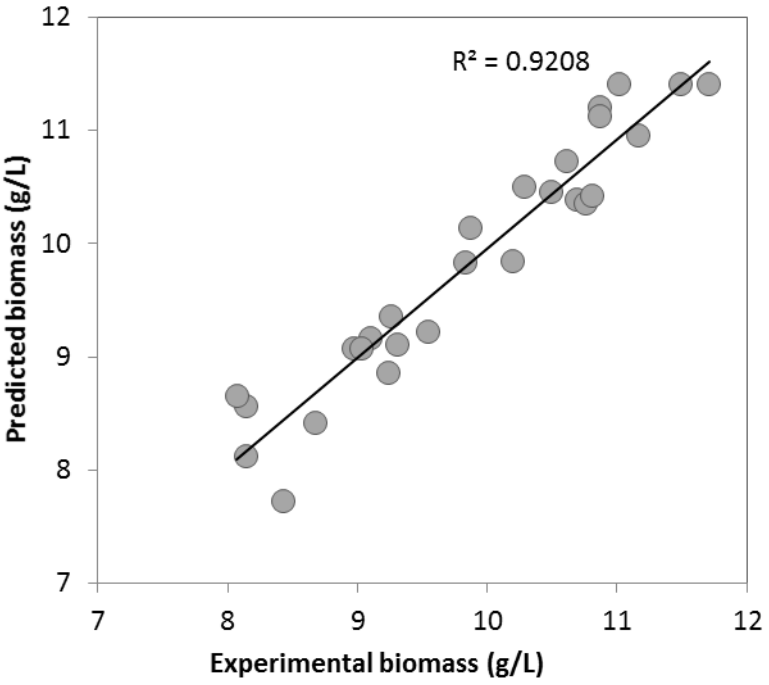
final equations for describing the relationship between the biomass (Y_1) and lipid production (Y_2) as a function of independent variables considering only the significant effects ($p < 0.05$) are shown in Equation 3.12 and Equation 3.13, respectively:

$$Y_1 = 11.6793 + 0.4336\,x_1 + 0.0872\,x_2 - 0.1631\,x_4 - 1.6682\,x_1^2 - 1.5833\,x_2^2 - 0.8961\,x_4^2 \quad (3.12)$$

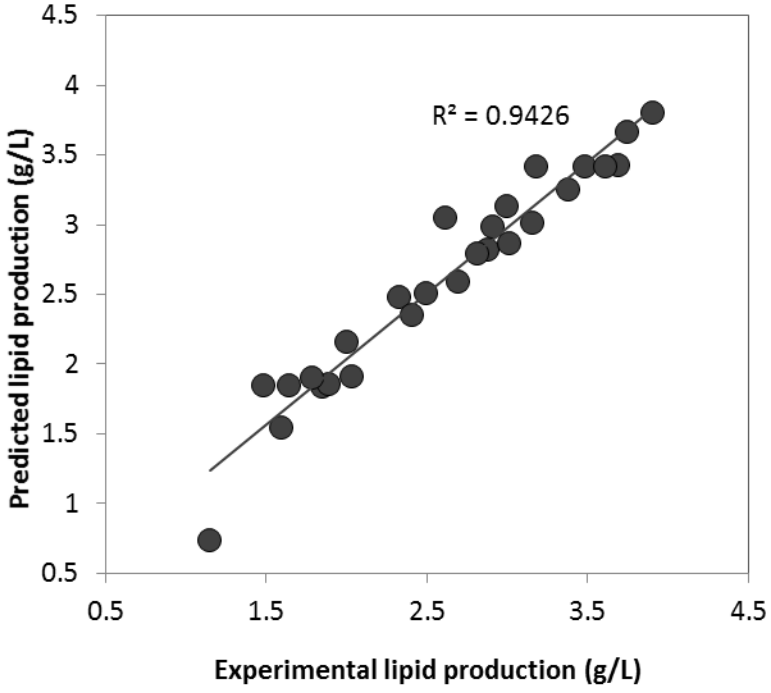
$$Y_2 = 3.224 + 0.8831\,x_1 + 0.2712\,x_2 - 0.1747\,x_4 - 0.3963\,x_1^2 - 0.3145\,x_1\,x_2 - 0.6695\,x_2^2 - 0.3003\,x_4^2 \quad (3.13)$$

For the modified models, the R^2 values of 0.81 and 0.87 for the biomass and lipid production indicates that theoretical values obtained from statistical models, and the experimental data are reasonably in good agreement. Although the R^2 values have decreased from 0.91 to 0.81 and from 0.94 to 0.87 for the biomass and lipid production, respectively, it still shows a good fit of regression. However, the adj-R^2, which is more important for determining the degree of fit of the regression model have not changed significantly. The adj-R^2 was approximately constant (0.8) for the biomass production and a slight decrease from 0.87 to 0.86 was observed for lipid production.

A number of statistical evidences such as low p-value, high F-value, the determination coefficient (R^2), adjusted R^2, non-significant lack of fit, and coefficient of variance were used to determine the accuracy of the suggested model. To visualize an excellent agreement between the experimental and predicted values, the results of the biomass and lipid production were compared by plotting the predicted responses versus experimental responses. Figure 3.2a and Figure 3.2b show the linear relationship between the predicted and experimental biomass and lipid production, respectively.



(a)



(b)

Figure 3.2 Comparison of predicted and experimental results a) biomass (g/L) and b) lipid production (g/L)

3.4.3 Response surfaces and contour plots

The relationship between the biomass and lipid production and these four factors are illustrated in 3D response surface plots. The optimum values for the chosen variables were obtained by solving Eq. (2) and (3). Response surface plots visualize the tendency of each variable and their interaction to affect the biomass and lipid production. Each plot represents the effects of two factors within their considered ranges by holding the other two factors fixed to the zero level. The shapes of the contour plots identify the nature and significance of the interactions between independent variables. Figure 3.3 and Figure 3.4 show the 3D response surface and contour plots for the optimum conditions of biomass and lipid production, respectively. Figure 3.3a shows that the maximum biomass was obtained at moderate glucose and nitrogen concentrations of 60 and 1 g/L, respectively. Conversely, the lipid production increased with increasing glucose concentration, up to the highest concentration of 80 g/L and increasing initial nitrogen concentration up to 1 g/L at center point (Figure 3.4).

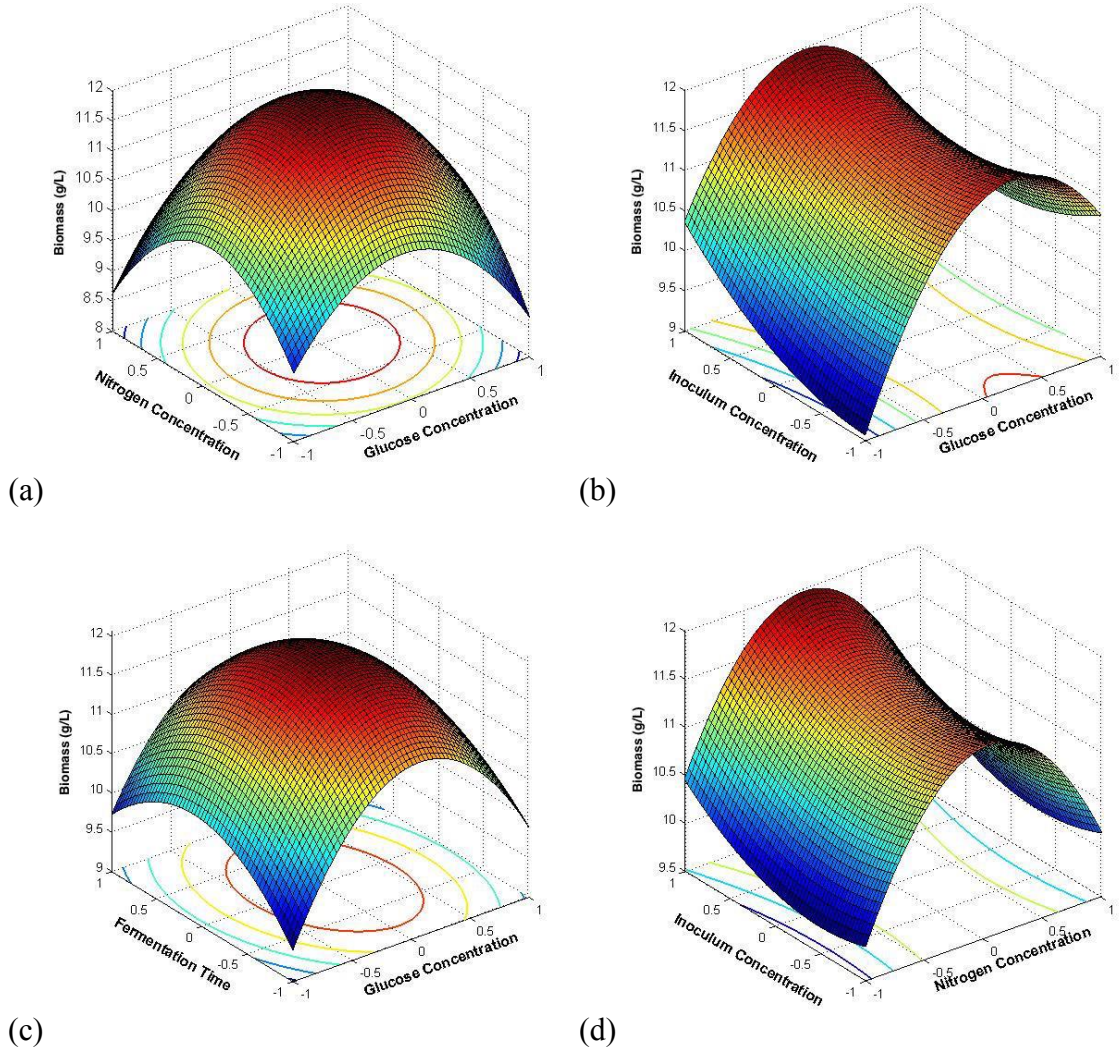
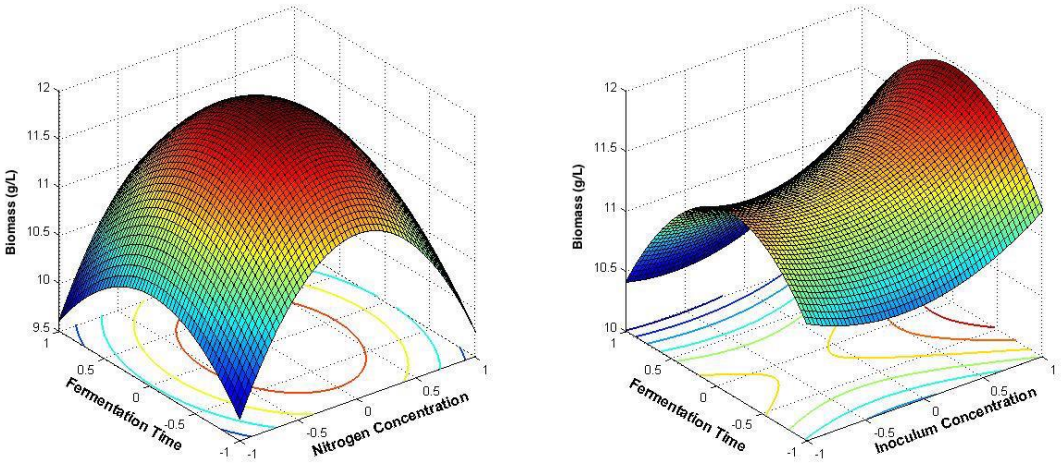


Figure 3.3 Response surface and contour plots showing the binary interaction between two factors on biomass production (g/L).

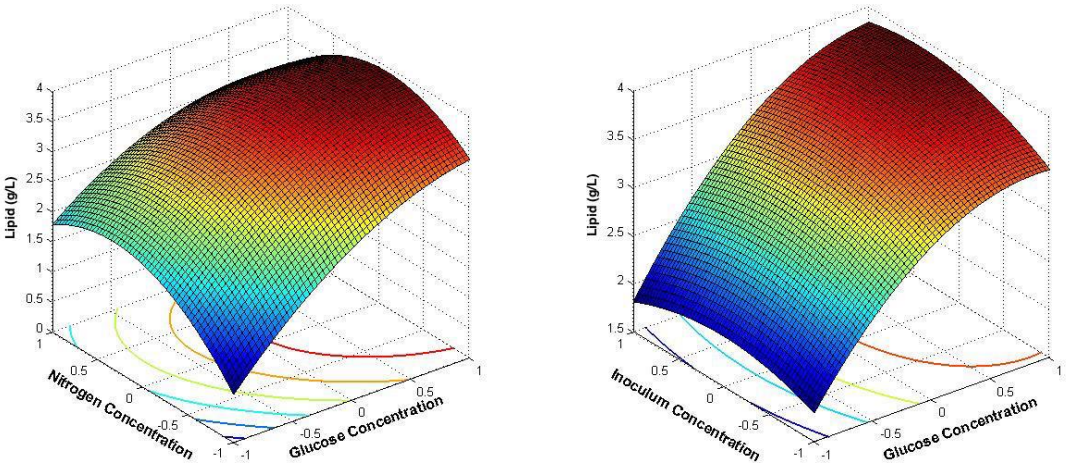
(a) carbon and nitrogen concentration, (b) carbon and inoculum concentration, (c) carbon concentration and fermentation time, (d) nitrogen and inoculum concentration



(e) (f)

Figure 3.3 (continued)

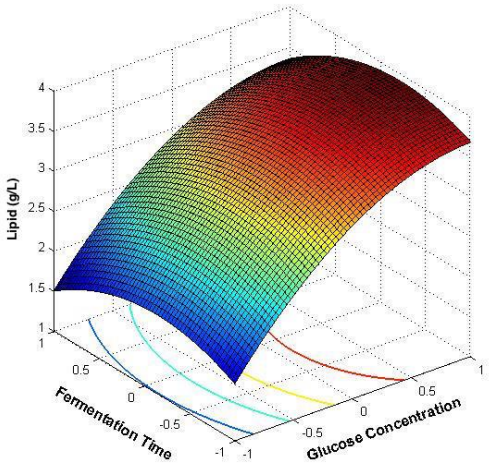
(e) nitrogen concentration and fermentation time, (f) inoculum concentration and fermentation time.



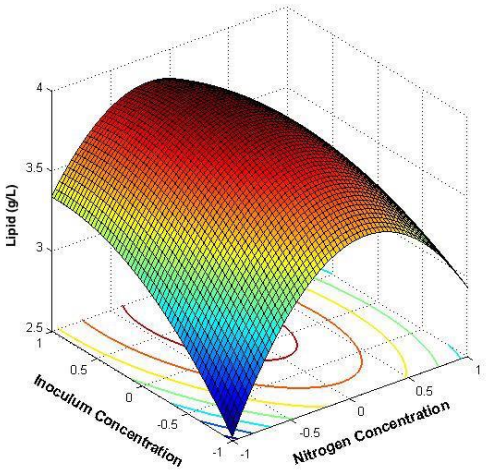
(a) (b)

Figure 3.4 Response surface and contour plots showing the binary interaction between two factors on lipid production (g/L).

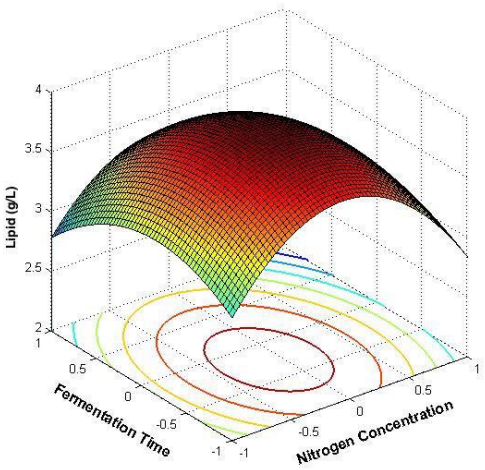
(a) carbon and nitrogen concentration, (b) carbon and inoculum concentration



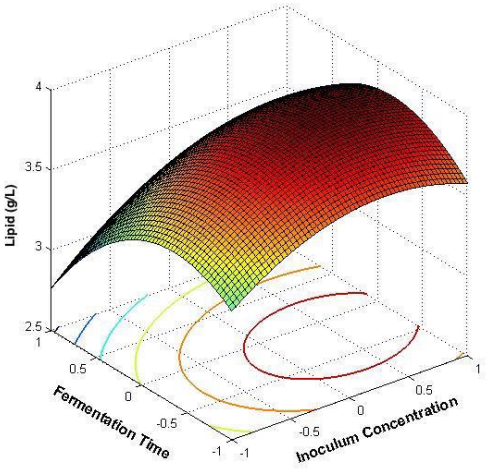
(c)



(d)



(e)



(f)

Figure 3.4 (continued)

(c) carbon concentration and fermentation time, (d) nitrogen and inoculum concentration, (e) nitrogen concentration and fermentation time, (f) inoculum concentration and fermentation time.

3.4.4 Condition optimization and confirmation tests

The optimization function in SAS was used to obtain the optimal conditions for the production of biomass and lipids. The optimum condition for biomass was at an initial glucose concentration of 60.3 g/L, a nitrogen concentration of 1 g/L, an inoculum concentration of 20% (v/v), and a fermentation time of 6.8 days. However, the optimum

condition for the lipid production was different from biomass production. The maximum lipid production was at a glucose concentration of 80 g/L, a nitrogen concentration of 1 g/L, an inoculum concentration of 17% (v/v), and at a fermentation time of 6.5 days. Under these optimum conditions, the suggested model estimated the maximum biomass and lipid production of 11.96 and 3.9 g/L, respectively. In order to validate the predicted optimal responses by the optimization study, additional experiments were performed in triplicate under these optimal conditions. The average produced biomass and lipid production were 10.91 and 3.65 g/L, respectively, which was moderately close to the predicted value with a relative error of 9.6 and 6.7%, respectively. Thus, the confirmation test for verification of optimization condition shows a very good agreement between the experimental and predicted values, which indicates that RSM is a powerful technique for the optimization of the medium component and fermentation process. Several researchers have demonstrated application of response surface methodology for optimization of medium and fermentation condition. Huang et al., showed that C/N ratio of 165, inoculum concentration of 11%, initial pH of 7.6, and fermentation time of 9 days was the optimum condition for improving lipid production from bagasse hydrolysate with *Trichosporon fermentans,* which lead to lipid accumulation of 15.8 g/L (Huang et al., 2012). Saenge and colleagues showed when oleaginous yeast *Rhodotorula glutinis* cultured in palm oil mill effluent, among three investigated factor the C/N ratio contributed the significant effect on the biomass and lipid production. The optimum C/N ratio for the biomass was 140, while it showed 180 for the lipid accumulation (Saenge et al., 2011a). Lower C/N ratio for maximum biomass, and higher values of C/N ratio for optimum lipid was also observed in our study.

3.4.5 Kinetic study of *R. glutinis* growth

The pH of the fermentation medium (Xia et al., 2011) and aeration (Saenge et al., 2011b) can significantly affect the cell growth and lipid accumulation. To further enhance the growth and lipid production, the batch fermentation was performed in a 3L aerobic batch fermenter with aeration system and pH controlled at 5. The fermentation experiment using initial glucose and nitrogen concentration of 80 and 1 g/L with 17% inoculum concentration showed the maximum biomass and lipid production of 18.07±0.68 and 8.1±0.9 g/L after 6 days of fermentation, respectively, which corresponds to a lipid content of 44.9±9.97%. This was about 65 and 121% improvement in biomass and lipid production compared to the shake flask experiment without pH control. The result of biomass growth, lipid production, sugar consumption, and lipid content based on cell dry weight (% CDW) are provided in Figure 3.5. Fermentation results on an optimum C/N ratio of 152 showed that the rate of lipid production increased after 96 hours of fermentation as interpreted from the slope of the graph for lipid accumulation versus time. The overall lipid accumulation rate of 0.049 g/l/h was observed. All sugar was consumed within the 6 days of fermentation with total sugar consumption rate of 0.39 g/l/h. The results in this study using pulp and paper wastewater as a medium are comparable with *Rhodococcus rhodochrous* grown on glucose (Shields-Menard et al., 2015) in terms of lipid content (43%) and lipid productivity (0.02 g/l/h).

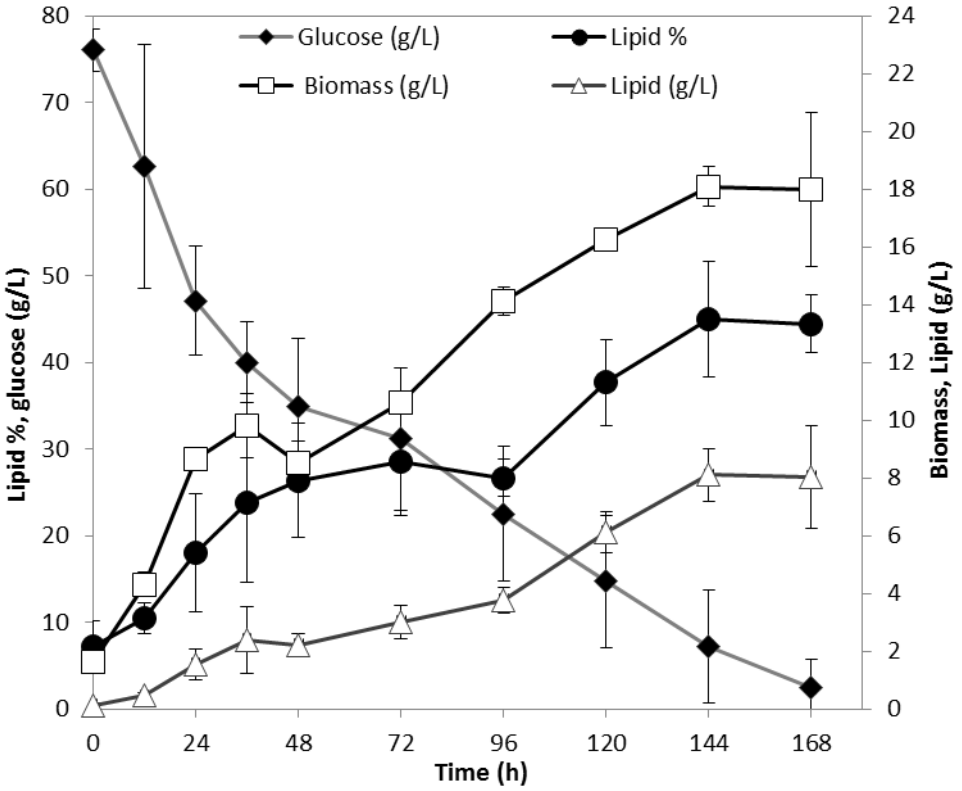


Figure 3.5 Fermentation profile of *R. glutinis* cultivated in pulp and paper wastewater Including biomass (g/L), lipid production (g/L), lipid content (%), and glucose consumption (g/L).

In this study, the kinetic models (Equations 3.3, 3.5, and 3.9) were fitted to the experimental data of biomass production, lipid accumulation, and substrate utilization for estimation of the model kinetic parameters. The kinetic equations were solved using a nonlinear regression method. Matlab (MATLAB Release 2014a, The MathWorks, Inc., Natick, Massachusetts, United States) was used for data analysis using nonlinear least squares method (Levenberg-Marquardt algorithm) for minimizing residual sum of square of errors. The kinetic parameters were determined and compared with values reported in the literature for some oleaginous microorganisms and presented in Table 3.5 whereas the experimental results and corresponding fits to the model kinetic equations were

illustrated in Figure 3.6. The correlation coefficients (R^2) for the models describing fat-free biomass, lipid production, and substrate consumption were 0.92, 0.98, and 0.98, respectively.

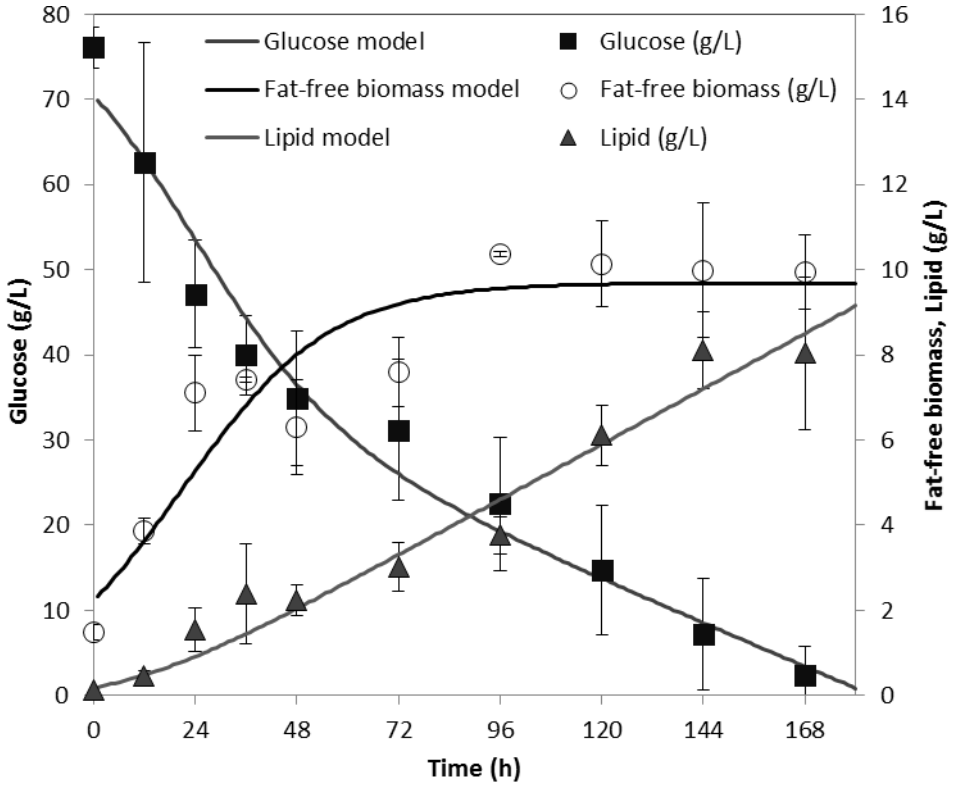


Figure 3.6 Comparison of experimental data and predicted value by the model for fat-free biomass (g/L), lipid (g/L), and glucose utilization (g/L)

Table 3.5 Kinetic parameters values of *R. glutinis* cultivated on pulp and paper wastewater in batch cultures

Parameters	**This study (*R. glutinis*)**	***Chlorella minutissima*** (Yang et al., 2011)	**Activated sludge** (Mondala et al., 2012)
Fat-free biomass			
X_0 (g L^{-1})	2.24	0.7926	1.15
μ_{max} (h^{-1})	0.057	0.0144	0.046
X_{max} (g L^{-1})	9.69	7.42	6.66
R^2	0.92	-	0.959
Lipid			
m (g g^{-1})	0.08	0.1125	0.08
10^3 n (g g^{-1} h^{-1})	5.6	0.0000	0.0977
R^2	0.98	-	0.997
Substrate			
S_0	70.46	10.19	-
α (g g^{-1})	4.931	-	5.03
10^3 β (g g^{-1} h^{-1})	21.96	-	0.483
$Y_{X/S}$(g g^{-1})	0.216	0.8629	0.167
$Y_{P/S}$(g g^{-1})	0.255	0.097	0.038
R^2	0.98		0.975

3.4.6 Production of biodiesel from yeast lipids feedstock

The fatty acid composition of *R. glutinis* was determined using FAMEs analysis in order to evaluate the potential of using this oleaginous yeast as biodiesel feedstock. The analysis of the FAMEs was carried out by transesterification of the lipids and saponifiable fraction of the lipid extracts, which represents the total fatty acid methyl

ester (FAMEs) or biodiesel yield was calculated. A maximum biodiesel yield of 58% based on total extracted lipid was obtained, which was about 26% of the cell dry weight (CDW). Table 3.5 presents the fatty acid profile of biodiesel produced from *R. glutinis* lipids and their contents. The data shows that the long chain fatty acids were the major fatty acids including palmitic, oleic, and linoleic acids. Concerning the fatty acid composition of the lipids produced by *R. glutinis*, it is in agreement with the information in the literature for this strain (Gonzalez-garcia et al., 2013; Easterling et al., 2009; Li et al., 2008). The similar fatty acid profile of yeast lipids to that of soybean (Christophe et al., 2012) and rapeseed oils (Azeem et al., 1999), which are the commonly used biodiesel feedstock in the US and the EU, indicates that the yeast lipids have potential to be used as a feedstock for biodiesel production.

The physicochemical properties of the biodiesel are characterized by chemical structure of its fatty acids(Knothe 2005). Thus, prediction of the quality of the biodiesel can be implemented by analysis of the fatty acid of microbial oil. To that aim, statistical models can be useful to predict the biodiesel properties based on its fatty acid profile. Table 3.7, summarizes some of these physicochemical characteristics including low calorific value (LCV), cetane number (CN), kinematic viscosity (μ), flash point (FP) and cold filter plugging point (CFPP) using previous published statistical models(Pinzi et al., 2011). These properties were comparable to rapeseed (Christophe et al., 2012) and palm oil(Azeem et al., 1999) biodiesel and also fulfilled European standard for biodiesel EN 14214 (Leiva-Candia et al., 2014).

Table 3.6 Fatty acid composition of biodiesel derived from *R. glutinis* lipids cultivated in pulp and paper wastewater.

Distribution of fatty acids	**%**
Palmitic acid (C16:0)	30.23±0.76
Stearic acid (C18:0)	0.38±0.34
Oleic acid (C18:1)	53.37±1.68
Linoleic acid (C18:2)	12.28±0.59
Linolenic acid (C18:3)	3.74±0.02

Table 3.7 Predicted properties of biodiesel using yeast oil

Oil origin	**Chain length**	**Unsaturation degree**	**Low calorific value, LCV** (Kj/Kg)	**Cetane number, CN**	**Kinematic viscosity, μ** (mm^2/s)	**Flash point, FP** (°C)	**Cold filter plugging point,** CFPP (°C)
EN 14214			-	>51	3.5-5	>120	-
Rapeseed (Leiva-Candia et al., 2014)	17.72	1.3	37,442.56	56.93	4.89	166.88	-3.16
Palm (Leiva-Candia et al., 2014)	17.03	0.61	37,442.56	69.87	5.46	162.16	8.23
R. toruloides AS21389 (Xu et al., 2012)	17.09	0.66	37,449.83	68.75	5.41	162.54	7.07
Lipomyces starkeyi AS2. 1560 (Gong et al., 2012)	17.09	0.59	37,474.44	70.55	5.52	164.36	9.18
Cryptococus curvatus TYC-19 (Takakuwa and Saito, 2010)	17.92	1.54	37,5110.35	52.99	4.7	167.95	-5.53
This study (*R. glutinis*)	17.39	0.89	37,508.93	64.54	5.2	166.31	3.23

3.5 Conclusions

Pulp and paper wastewater can be used as a cultivation media for the production of microbial lipids using oleaginous yeast *R. glutinis*. Improved biomass and lipid

production have been accomplished by optimization of fermentation parameter using RSM. This study indicates that RSM can be applied successfully for model development and prediction of the optimum condition for the biomass and lipid production in batch fermentation using *R. glutinis* in pulp and paper wastewater. Fermentation equipped with an aeration system and pH control resulted in 65% and 121% improvement in biomass and lipid Production. The applied mathematical model was able to successfully simulate the growth and lipid accumulation process.

CHAPTER IV

EFFECTS OF INHIBITORY COMPOUNDS IN LIGNOCELLULOSIC HYDROLYSATE ON *RHODOTORULA GLUTINIS* GROWTH AND LIPID ACCUMULATION FOR BIODIESEL PRODUCTION

4.1 Abstract

Lignocellulosic hydrolysates, which are produced from pretreatment of lignocellulosic materials, are considered as a feasible approach for cost-effective lipid production using oleaginous microorganisms. However, the depolymerization of cellulose and hemicellulose in lignocellulosic biomass results in the production of inhibitory byproducts that can considerably decrease the fermentability of lignocellulosic sugars. In this study, the effects of three representative lignocellulose inhibitors including furfural, 5-hydroxymethylfurfural (HMF), and acetic acid on the growth and lipid accumulation of *Rhodotorula glutinis* were explored. The synergetic effect of inhibitors was investigated and the tolerance level of *R. glutinis* was evaluated. This study reports that *R. glutinis* can grow in pulp and paper wastewater media in the presence of model inhibitory compounds while producing lipids. A mathematical model based on Haldane's kinetic model, which takes into account substrate inhibition was applied to describe the biochemical behavior of oleaginous yeast. The model was in satisfactory agreement with

experimental data to predict the cell growth, lipid production, and substrate utilization. The kinetic parameters were estimated.

4.2 Introduction

A first generation biorefinery, which is manufactured based on the conversion of food resources to biofuel has generated a serious argument and led to a development of second generation biofuels with focuses on utilization of inexpensive non-edible lignocellulosic waste material. Microbial lipophilic compounds, referred to as single-cell oils (SCO) being produced from oleaginous microorganisms, are of potential industrial interest due to their triacylglycerol structure, which can be used as biodiesel feedstock (Li et al., 2007). Oleaginous microorganisms, are capable of producing storage lipids, rich in polyunsaturated fatty acids are attractive source of oils for the production of biodiesel due to their ability to utilize various agro-industrial wastes (Leiva-Candia et al., 2014). Although, the production of microbial oil has been investigated for decades, its improved production from lignocellulosic material as a promising feedstock for production of biofuel has been studied recently. Lignocellulosic biomass has a complex polymeric structure, which is composed of cellulose, hemicellulose, and lignin. Therefore, pretreatments must be applied to extremely recalcitrant lignocellulosic biomass to break down its polymeric structure to fermentable sugars. The relative sugar proportion converted from the pretreatment of lignocellulose depends on their biomass sources and the operating conversion conditions (Huang et al., 2012).

The current production cost of biodiesel is not economically sustainable, and it is mainly due to the cost of its feedstock (Wang et al., 2014). In order to reduce the cost of biofuels attributed to feedstock fermentation, utilization of inexpensive and abundant

lignocellulosic biomass obtained from agricultural wastes (Huang et al., 2009; Mondala et al., 2015; Tsigie et al., 2011; Yu et al., 2011), forestry residues (Leu et al., 2013), or municipal solid wastes (G hanavati et al., 2015), for TAGs production needs to be implemented and this has been presented in a few studies.

However, existing pretreatment methods such as thermal and chemical pretreatments generate undesirable byproducts that interfere with the fermentation products. A variety of by-products mainly organic acids and aldehydes would be generated during the pretreatment, which can negatively affects the growth and metabolism of microorganisms in the down stream fermentation process. Organic acids such as acetic acid from de-acetylation of hemicelluloses, and aldehydes such as furfural and 5- hydroxymethylfurfural (HMF), from decomposition of pentoses and hexoses, respectively are reported as a major inhibitory byproduct of lignicellulosic degradation. Formic acid was generated from degradation of furfural and HMF, and levulinic acid was produced from break down of HMF (Yu et al., 2011). The tolerance level of microorganisms to inhibitors is species dependent; thus, a strong microorganism capable of utilizing lignocellulosic biomass, which can tolerate fermentation inhibitors while producing lipids, is essential for effective biofuel production. In order to overcome their inhibitory results, it is necessary to understand the effects that they can make on the growth and product formation of microorganisms.

During last decades *Rhodotorula glutinis* has been used for microbial oil production using wide variety of carbon substrates (Amirsadeghi et al., 2015; Mast et al., 2014; Saenge et al., 2011a; Schneider et al., 2013). It has been reported that this oleaginous yeast can utilize glucose, xylose, glycerol (Easterling et al., 2009; Saenge et

al., 2011a), N-acetylglucosamine (Zhang et al., 2011) and obtain high lipid contents up to 70% under optimum cultivation condition. In addition, utilization of wastewaters (Saenge et al., 2011b; Schneider et al., 2013) and low-cost substrates (Huang et al., 2013) as a fermentation media to cultivate this oleaginous yeast has been investigated in several studies. The cost advantages of using wastewater as growth medium include providing a nutrient enriched growth medium, which is practically free of cost. Although this oleaginous yeast has been used for lipid production from lignicellulosic biomass, the effects of inhibitory compounds on its growth and lipid synthesis has not been well studied to determine its tolerance level for utilizing lignocellulosic substrate in a fermentation system.

In this study, the effect of acetic acid, furfural, and 5- hydroxymethylfurfural on growth and lipid accumulation of *R. glutinis* using glucose as the primary carbon source in pulp and paper wastewater as cultivation medium was explored. The purpose of this study is to simulate fermentation of sugars in the presence of these inhibitors without any pretreatment and determine the tolerance level of oleaginous yeast *R. glutinis* towards it. Fermentation kinetics and yield parameters in the presence of fermentation inhibitors and glucose-xylose co-substrate were obtained using established models to aid in the interpretation of the data and for their potential use in large-scale bioreactor design. The effect of inhibitors on fatty acid composition of storage lipid was then determined in order to evaluate the suitability of the microbial oil feedstock for biodiesel production.

4.3 Materials and methods

4.3.1 Microorganism and media preparation

Oleaginous yeast *R. glutinis* (ATCC 15125) stock culture was purchased from American Type Culture Collection (ATCC) and grown in liquid Yeast Mold (YM) media (3 g/L Yeast Extract, 3 g/L Malt Extract, 5 g/L peptone, 10 g/L Dextrose), and kept in 30% (v/v) glycerol at -80 °C until used. The starter culture was produced by inoculation of an aliquot of *R. glutinis* stuck culture into the 2 L flask containing 500 ml of sugar broth medium comprising [1 g/L Yeast Extract, 1 g/L $Na_2HPO_4.12\ H_2O$, 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4.7H_2O$, 1 g/L $(NH_4)_2SO_4$, 10.0 ml Trace Mineral Solution (3.6 g/L$CaCl_2.2\ H_2O$, 0.75 g/L $ZnSO_4.7H_2O$, 0.13 g/L $CuSO_4.5H_2O$, 0.5 g/L $MnSO_4.H_2O$, 0.13 g/L $COCl_2.6\ H_2O$, 0.17 g/L $Na_2MoO_4.2\ H_2O$ in distilled water), 6.0 ml Iron Solution ($FeSO_4.7H_2O$ in distilled water), and 60 g/L glucose]. The flask was incubated using a rotary shaker (New Brunswick Scientific Model I26, Edison, New Jersey) with a rotation speed of ~120 rpm at 30 °C for 4 days. The media compounds were obtained from Fisher Scientific (USA).

4.3.2 Batch Fermentation

The batch fermentation was conducted in 2 L baffled flasks containing 500 ml of wastewater medium in a rotary shaker at the temperature of 30 °C and 120 rpm. The results from previous chapter showed that the maximum lipid production were obtained at a constant carbon-to-nitrogen ratio (C:N) of 150:1. Glucose (80 g/L) and ammonium sulfate (1 g/L) were added as carbon and nitrogen sources, respectively to obtain the (C:N) of 150:1. Three concentration levels of acetic acid 2 g/L (33 mmol/L), 5 g/L (83 mmol/L), 10 g/L (167 mmol/L), furfural 0.2 g/L (2.1 mmol/L), 0.5 g/L (5.3 mmol/L), 1

g/L (10.4 mmol/L), and 5- hydroxymethylfurfural 0.5 g/L (3.9 mmol/L), 1 g/L (7.93 mmol/L), and 1.5 g/L (11.9 mmol/L) were tested. Single inhibitors with their corresponding concentrations were added into the cooled and sterile media by pipetting. The control runs were conducted with no inhibitors. The initial pH of the media was then adjusted to 6.5 using sterile 1M NaOH. The media was inoculated by 17 % (v/v) of the *R. glutinis* seed culture as it was shown to be optimum.

To study the inhibitory effects of combined inhibitors, two different model lignocellulosic hydrolysate including combination of inhibitors 1: (0.2 g/L furfural, 1.5 g/L HMF, and 5 g/L acetic acid, 60 g/L glucose, 20 g/L xylose, 1 g/L ammonium sulfate) and combination 2: (0.5 g/L furfural, 1.5 g/L HMF, and 10 g/L acetic acid, 60 g/L glucose, 20 g/L xylose, 1 g/L ammonium sulfate) were chosen. The pulp and paper wastewater was collected and used as cultivation medium as described in a previous publication (Amirsadeghi et al., 2015).The furfural and 5- hydroxymethylfurfural (HMF) were purchased from Sigma-Aldrich (USA). The Acetic acid, ammonium sulfate, and other chemicals were purchased from Fisher Scientific (USA).

4.3.3 Sequential inoculum preparation for fermentation experiment

To perform kinetic study of *R. glutinis* fermentation, the fermentation experiments was conducted in a 3L Bioflo 310 bioreactor that contained 2 L of wastewater medium. The two model lignocellulosic hydrolysate as we described earlier with their corresponding concentrations was introduced to the medium without additional phosphorous or mineral sources. The control experiment containing glucose and xylose co-substrate with their corresponding concentration (60 g/L glucose, 20 g/L xylose) with no inhibitors were performed.

To prepare the inoculum for fermentation runs, two sequential batch cultivation was performed in a 1 L and 2 L baffled flasks using the previously described sugar broth medium in the first step and sugar broth medium using 3:1 glucose to xylose ratio in the second step, in order to obtain a cell rich inoculum and to enable the microorganisms to adapt to both major sugar components of the lignocellulose hydrolyzate (glucose and xylose) in the second step. Each flask was incubated at 30 °C and 120 rpm for 4 days and consequently 20% (v/v) inoculum was transferred to the bioreactors. To prevent acetic acid inhibition and potential utilization of acetate as a carbon source, the pH of the medium was initially adjusted to 6.5 and controlled at 5 using 1M NaOH solutions to avoid acidic environment for *R. glutinis* cells. The temperature was controlled at 30 °C and the aeration rate was adjusted at 3 volume of air per volume of medium and per minute (vvm). The agitation was set at 300 rpm and adjusted to maintain a dissolved oxygen (DO) level no lower than 60% saturation during the experiment. A polypropylene-based antifoam, 1:10 dilution of nonoil (Sigma-Aldrich, St. Louis, MO) was periodically added to prevent foaming in the vessel. The total incubation time was seven days and samples were collected at regular time intervals (24 hours) and then analyzed according to the methods outlined below.

4.3.4 Analytical methods

Biomass was harvested by centrifugation of 30 ml of samples at 3000 rpm for 10 minutes using a Sorvall® ST 40 Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and the supernatant separated from the cell pellets. The cell pellets were freeze-dried using a Labconco Freezone 2.5 freeze-drier (Labconco, Kansas City, Missouri) and the biomass concentration was determined by gravimetric methods. The supernatant was

stored in the freezer -80 °C for the analysis of sugar. Extraction of lipids from the dried biomass was carried out according to the Bligh and Dyer method using methanol, chloroform, and water (Bligh and Dyer, 1959). The dried lipids were obtained by evaporating the chloroform in the lipid-dissolved chloroform extracts using a TurboVap LV (Caliper Life Sciences, Hopkinton, Massachusetts). After the complete evaporation of chloroform, the residual lipid was weighed using an analytical balance. To convert the dried lipids into fatty acid methyl esters (FAMEs), transesterification method was applied using sulfuric acid (2% v/v) and methanol as a catalyst and reactant, respectively at 60 °C for 2 hours according to Christie method of transesterification (Christie, 1993). The fatty acid composition in the FAMEs was analyzed using an Agilent 6890 gas chromatograph equipped with a flame ionization detector (GC-FID). Helium was used as carrier gas. The injector temperature of 260 °C was used for the injection of the 1 μL Samples. The GC oven temperature was set at 50 °C, and then ramped to 250 °C after 2 min. The FID temperature was kept at 260 °C during the analysis (Revellame et al., 2010). The FAMEs peaks were identified and quantitated by comparing the sample responses to those generated from a known standard. The supernatant samples were filtered with 0.45 μm syringe filters just before analysis. Total reducing sugar concentration was determined using YSI 2900 Biochemistry Analyzer (YSI Inc. Life Sciences, Yellow Springs, OH, USA) equipped with a glucose oxidase membrane probe.

4.3.5 Mathematical modeling

A cell growth of microorganisms in a batch reactor can be described by the Malthusian model, which is also known as exponential law. The balance equations for the fat-free biomass, is stated as below:

$$\frac{dX}{dt} = \mu X \tag{4.1}$$

where X is the fat free biomass concentration (g/L), μ is the specific growth rate (1/h) and t is the fermentation time (h). Similarly, the rate of lipids accumulation is also proportional to the mass of cells:

$$\frac{dL}{dt} = q_L X \tag{4.2}$$

where L is the lipids concentration (g/L), q_L is the specific lipid accumulation rate in biomass (g lipids/ g fat-free biomass-h).

Finally, the balance equation for the substrate is:

$$\frac{-dS}{dt} = (\mu \frac{1}{Y_{X/S}} + q_L \frac{1}{Y_{L/S}})X \tag{4.3}$$

where S is the substrate (sugar) concentration (g/L), $Y_{X/S}$ is the yield coefficient of the fat free biomass (g fat-free biomass produced/g sugar consumed), and $Y_{L/S}$ is the yield coefficient for lipid production (g lipids produced/g sugar consumed).

A mathematical model based on Haldane's kinetic model, which incorporates terms for single limiting substrate with substrate inhibition was applied to describe both the specific growth rate (μ), and specific lipid accumulation rate (q_L). The Haldane or Andrew's model has a similar form as shown below:

$$\mu = \mu_{max} \frac{S}{K_S + S + \frac{S^2}{K_{i1}}} \tag{4.4}$$

$$q_l = q_{l\,max} \frac{S}{K_{lS} + S + \frac{S^2}{K_{i2}}} \tag{4.5}$$

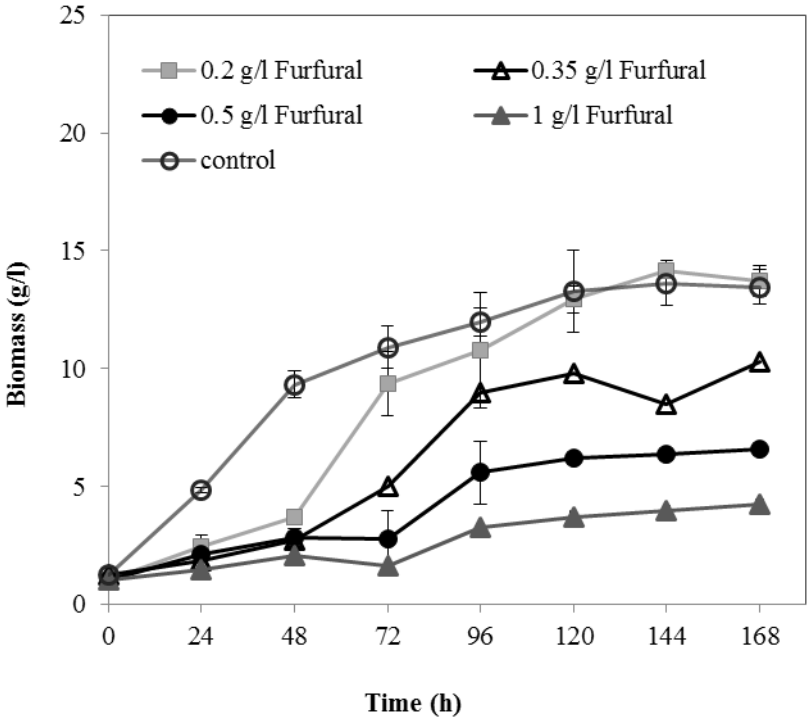
In the above equations, μ_{max} is the maximum specific growth rate (1/h), $q_{l\,max}$ is the maximum specific lipid accumulation rate (g lipids/ g fat-free biomass-h), K_S, K_{lS}, are the

saturation constants (g/L), K_{i1}, K_{i2}, are the inhibition constants related to fat-free biomass and lipids (g/L), respectively. The kinetic parameters μ_{max}, $q_{l\,max}$, K_S, K_{lS}, K_{i1}, K_{i2}, were determined from the experimental data using non-linear regression using Matlab (MATLAB Release 2014a, The MathWorks, Inc., Natick, Massachusetts, United States).

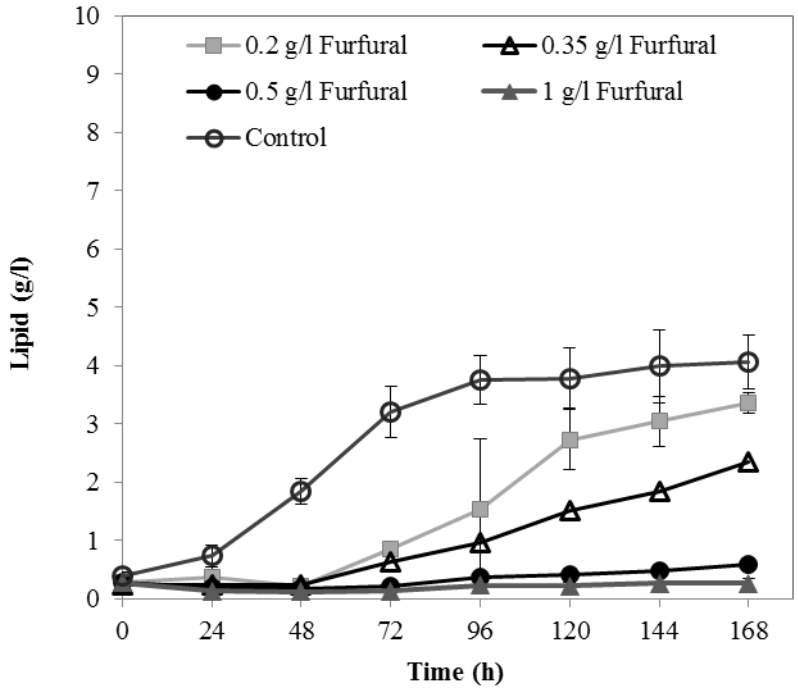
4.4 Results and Discussion

4.4.1 Effects of furfural, HMF, and acetic acid on biomass and lipid accumulation

To investigate the effect of aldehydes and acetic acid on the growth and lipid accumulation of *R. glutinis* various concentrations of furfural, HMF, and acetic acid were used. Figure 4.1, Figure 4.2, and Figure 4.3 depict the effects of furfural, HMF, and acetic acid on *R. glutinis* cell behavior, respectively. In the absence of inhibitors (control run), the cell dry weight (CDW) and lipid accumulation of *R. glutinis* were 13.46 g/L, and 4 g/L, respectively, which lead to a lipid content of 30%. When furfural concentrations were increased, the cell biomass gradually decreased compared to the control experiment and at furfural concentration of 0.5 g/L, a dramatic drop in both biomass and lipid was observed. At 0.5 g/L furfural, the CDW and lipid production decreased to 6.59 g/L (Figure 4.1a) and 0.58 g/L (Figure 4.1b), respectively.

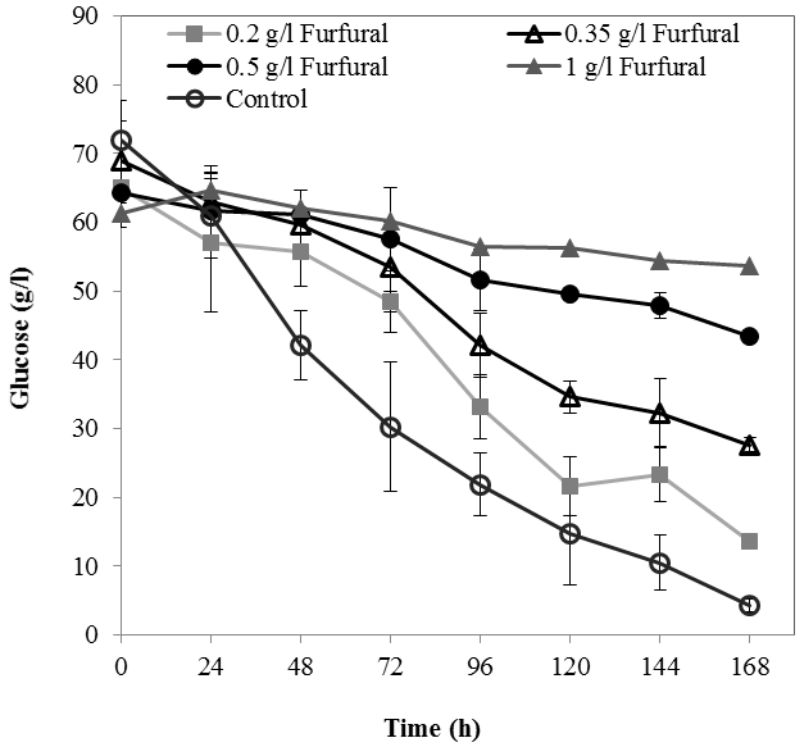


(a)



(b)

Figure 4.1 Cell biomass (CDW) (a, g/L), lipid accumulation (b, g/L), glucose consumption (c, g/L) of *R. glutinis* grown in glucose in a presence of furfural.



(c)

Figure 4.1 (continued)

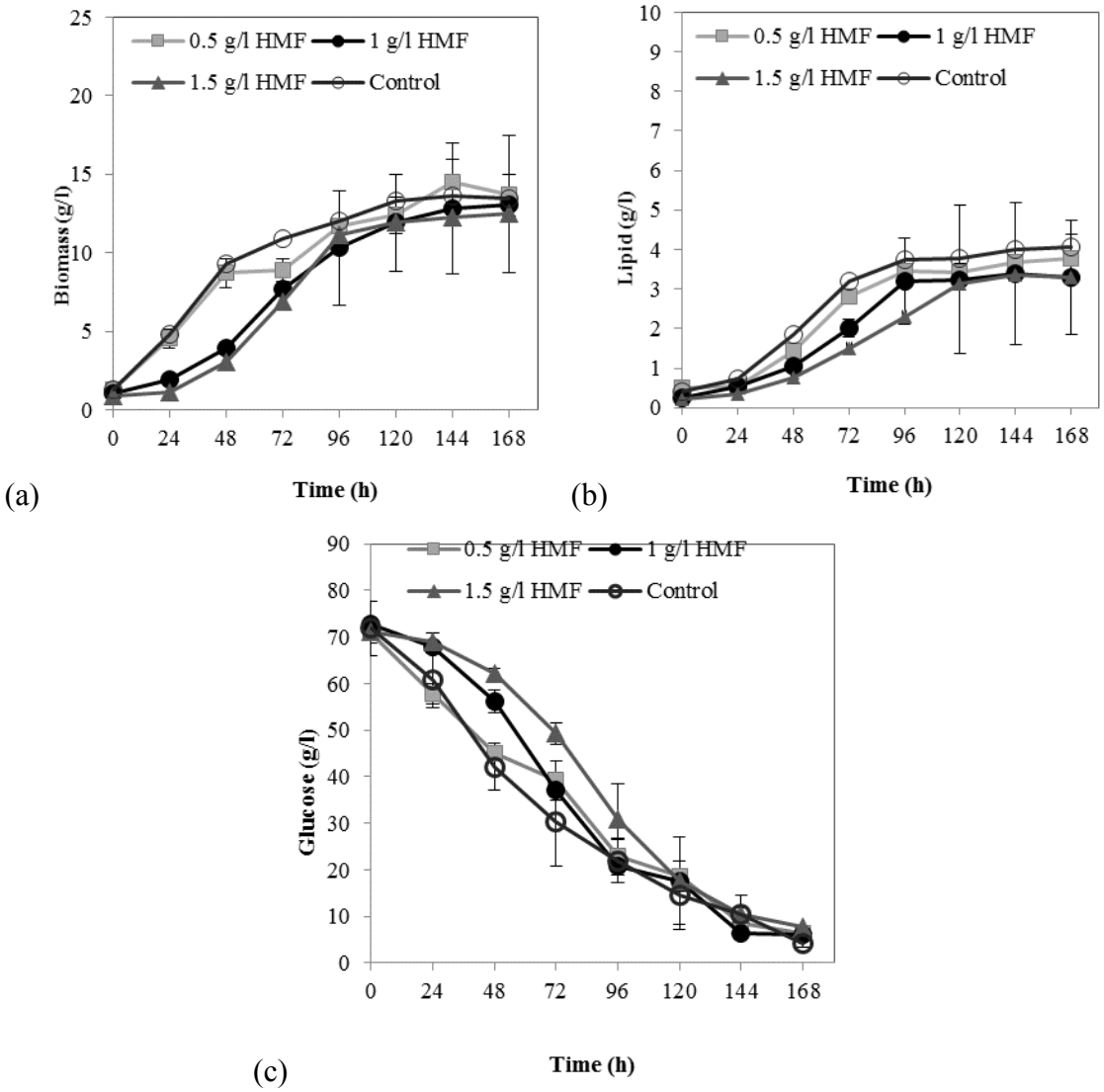


Figure 4.2 Cell biomass (CDW) (a, g/L), lipid accumulation (b, g/L), glucose consumption (c, g/L) of *R. glutinis* grown in glucose in a presence of HMF.

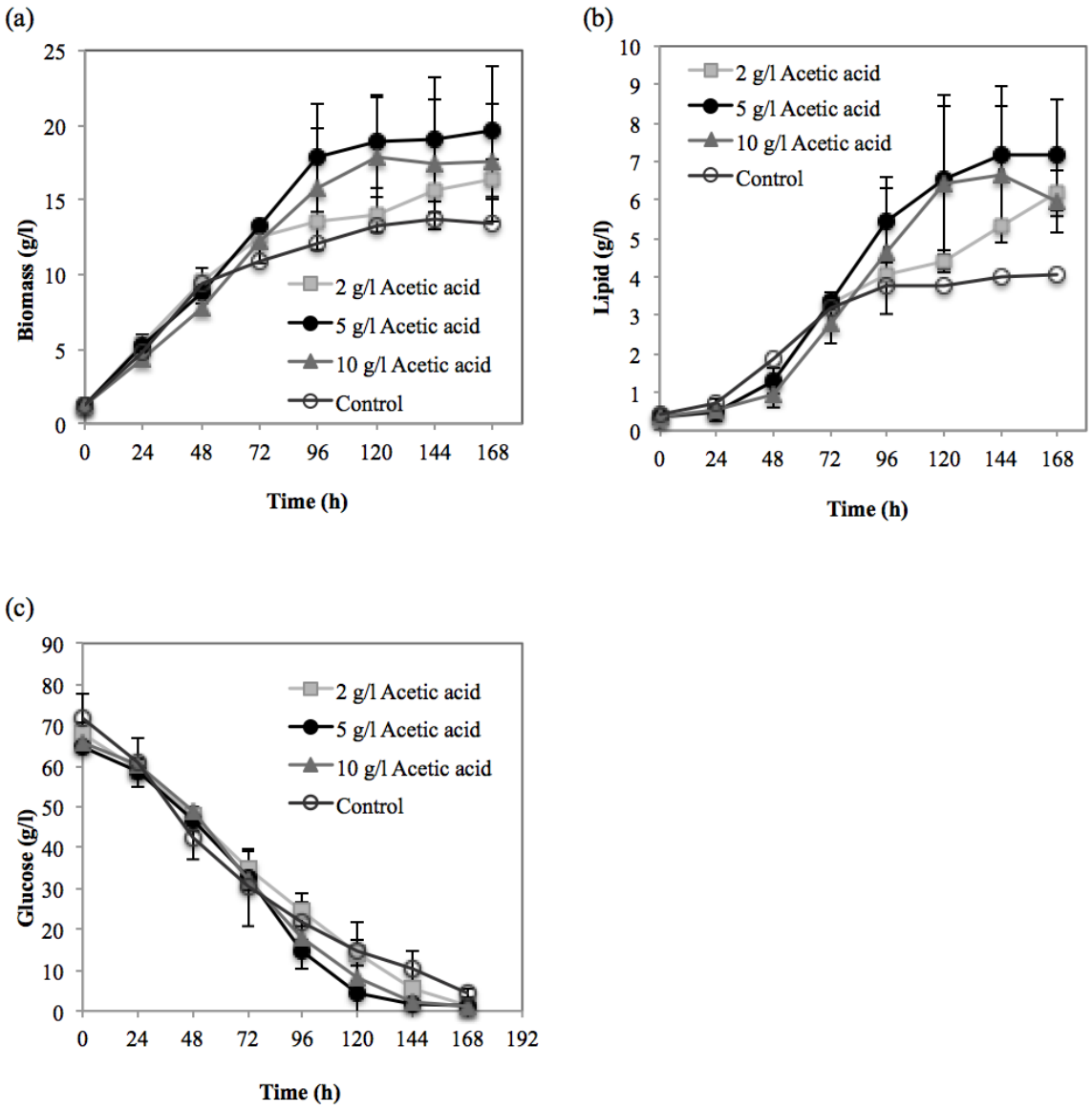


Figure 4.3 Cell biomass (CDW) (a, g/L), lipid accumulation (b, g/L), glucose consumption (c, g/L) of *R. glutinis* grown in glucose in a presence of acetic acid

At 0.2 g/L furfural, a short lag period (0 – 48 h) in biomass production was observed, overlapping with the onset of the exponential growth phase. However, increasing furfural concentration resulted in a longer lag period (0 – 72 h) in biomass

production at 0.5 g/L and 1 g/L furfural. Similar behavior of the cells in terms of appearance of the lag phase was observed for glucose uptake, indicating significant inhibition in glucose utilization at higher furfural concentration levels (Figure 4.1c).

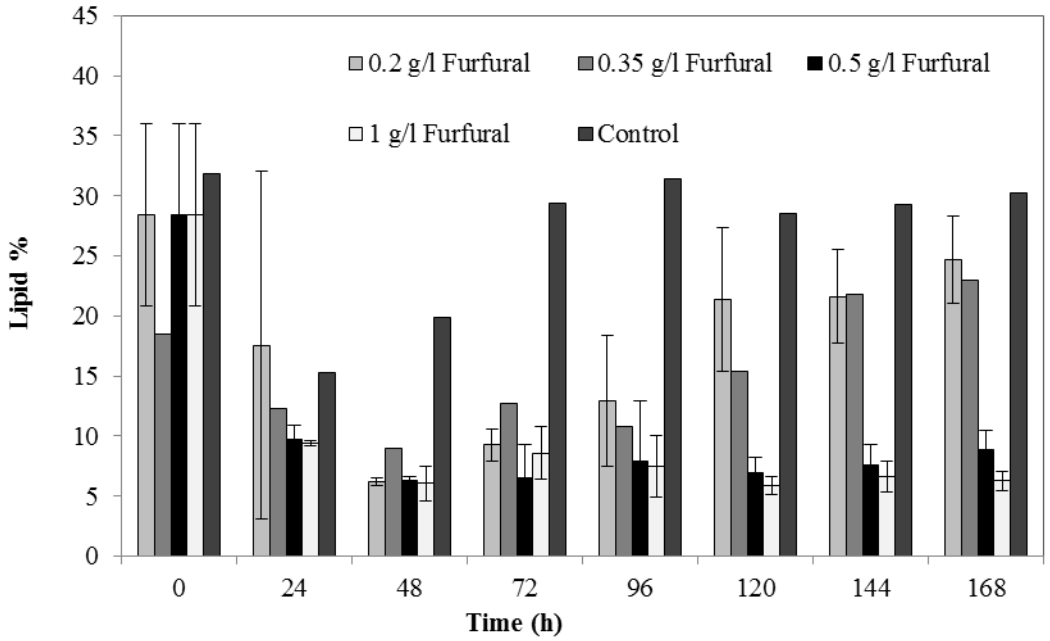
Several previous studies have reported the inhibitory effects of furfural, a main byproduct from acid pretreatment of lignocellulosic biomass on the bioethanol production in yeast ethanol fermentation (Gutierrez-padilla and Karim, 2005; Schell et al., 2003). Parawira and Tereke showed that furfural blocked the aldehyde dehydrogenase activity and caused the lag phase in the growth of *S. cerevisiae* (Parawira and Tekere, 2011). However, in the fermentation of *Trichosporon fermentants*, Huang et al. reported that furfural inhibited the activity of glycolytic enzymes, an important lipid synthesis enzyme and malic enzymes, a key enzyme responsible for providing NADPH for lipid metabolism (Huang et al., 2012). Thus, it is expected that the inhibition effect of furfural on lipid accumulation be due to channeling NADPH for furfural oxidation and inhibiting malic enzyme. Different studies on the effect of lignocellulosic hydrolysis inhibitors on various oleaginous microorganisms and their growth process shows that their tolerance ability is species dependent. At 0.1 g/L furfural, for *Rhodosporidium toruloides* (Hu et al., 2009), the biomass and lipid content was decreased by 45.5% and 26.5%, respectively, while at the same furfural concentration, *Cryptococcus curvatus* (Yu et al., 2014) was decreased the CDW and lipids only by 5.1% and 6.4%, respectively. Our results showed that *R. glutinis*, can tolerate 0.2 g/L furfural and produce the same concentration of biomass (13.46 g/L) and decreased lipid accumulation by 16% compared to control. Indeed, the sugar consumption was decreased with increasing furfural

concentration and almost completely inhibited when furfural concentration was increased to 1.5 g/L (Figure 4.1c).

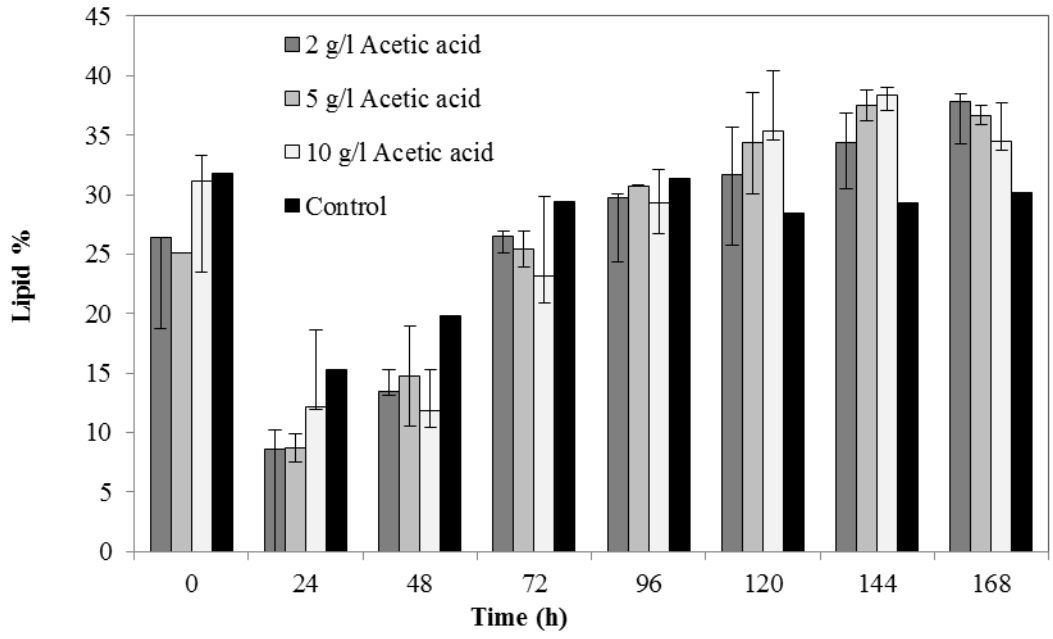
HMF is the dehydration product of hexoses from cellulose, which is produced during lignocellulosic hydrolysis and it is known as a toxic compound for some oleaginous microorganisms. HMF commonly influences the energy metabolism of microorganisms by interrupting electron transfer and affects the glycolysis cycle (Hu et al., 2009). Our results indicated that *R. glutinis* has considerably higher tolerance to HMF than furfural. As shown in Figure 4.2, HMF with concentration of 0.5-1.5 g/L had no negative impact on cell growth and slightly decreased lipid accumulation. According to the experimental results, the lipid accumulation decreased by 5.5% and 17.5% in the presence of 0.5 g/L and 1.5 g/L HMF, respectively. Although HMF has shown slight inhibitory effect on *R. glutinis* lipid accumulation, the rate of lipid production decreased as it can be observed from the slope of the curves in Figure 4.2b. Glucose utilization profiles (Figure 4.2c) were similar for the control and all the HMF treatments, with roughly the same overall glucose uptake rates. A minor inhibitory effect of HMF was also established in previous studies; Hu et al. reported a slight inhibitory effects of HMF on lipid accumulation of oleaginous yeast *Rhodosporidium toruloides* up to 14.7 mM concentration of HMF (Hu et al., 2009). Ruan et al, also showed that HMF had no negative effect on the growth and lipid synthesis of *M. isabellina* until HMF concentration reached to 2.5 g/L (Ruan et al., 2015).

Acetic acid is produced by hydrolysis of acetyl groups in hemicellulose during the decomposition of lignocellulose. Acetic acid is known as one of the fermentation inhibitors; however, its toxicity on fermentation products depends on the microorganism

species and the concentration of its undissociated form, which is related to concentration and pH. At acidic pH, the undissociated form of acetic acid is able to diffuse into cell membrane and dissociates in the cytoplasm, consequently reduces the intracellular pH. Our results indicated that *R. glutinis* had a strong ability to tolerate acetic acid, and in fact its stimulatory effect was observed under acetic acid concentration of 5 g/L and 10 g/L, which resulted in 31% and 22% increase in *R. glutinis* biomass production, respectively. However the positive impact of acetic acid on the *R. glutinis* lipid accumulation is more significant than on its biomass production, which resulted in 44% and 33% increase in lipid accumulation at 5 g/L and 10 g/L acetic acid, respectively. In particular, according to our experimental results, 5 g/L acetic acid is the optimum concentration to obtain high biomass and lipid accumulation in *R. glutinis* fermentation. As it can be seen in Figure 4.3c, glucose was utilized more quickly in the presence of acetic acid compared to the control run. At 5 g/L acetic acid almost all the glucose was consumed at day 5, while the production of biomass and lipid was steadily increased until day 7. Thus, it can be indicated that *R. glutinis* can use acetic acid as a building block to improve cell performance and increase lipid synthesis as lipid content reached to 37% (Figure 4.4b) in the presence of 5 g/L acetic acid compared to 30% lipid content at control. Moreover, excess acetic acid resulted in increased C/N ratio, the most important factor that extensively triggers lipid accumulation process. It was demonstrated that adjusting the pH of the media to 6.5 in our study, resulted in conversion of undissociated form of acetic acid into its dissociated form, consequently decreased the concentration of its undissociated form and made it difficult for acetic acid to cross the plasma membrane and exhibit toxicity effect to the cell performance.



(a)



(b)

Figure 4.4 Lipid content (%CDW) of *R. glutinis* at different concentrations of (a) furfural and (b) acetic acid during fermentation time.

Taken together, studying the inhibitory effects of furfural, HMF, and acetic acid as major inhibitors present in lignocellulosic biomass on the performance of oleaginous microorganism fermentation revealed that cell growth is more sensitive to those toxic compounds than lipid synthesis process. Furfural imposed as major inhibitory compound on cell growth and lipid biosynthesis.

4.4.2 Inhibitory effect of model lignocellulosic hydrolysate on *R. glutinis* fermentation profile

The lignocellulosic hydrolysate usually contains multiple inhibitors that may depict additive inhibitory effects on microbial fermentation. To that aim, we further designed experiments using model lignocellulosic hydrolysate composed of mixture of three major lignocellulosic inhibitors along with glucose as a primary substrate and xylose as a secondary substrate to look into the possible synergetic inhibition effect of combination of those inhibitors. The inhibitor combination 1: (0.2 g/L furfural, 1 g/L HMF, and 5 g/L acetic acid) and combination 2: (0.5 g/L furfural, 1 g/L HMF, and 10 g/L acetic acid) was chosen to study the kinetics of biomass production, lipid synthesis, and carbon utilization. Since inhibition greatly depends on various factors such as microorganism species, the mode of fermentation, and the number of inhibitors and their synergetic function, the tolerance level of microorganism in terms of inhibitors concentration is not quite precise. Ruan and colleagues reported that once four inhibitors combination including 0.5 g/L furfural, 0.1 g/L HMF, 0.025 g/L ferulic acid, and 0.025 g/L coumaric acid along with glucose, xylose, and acetate as carbon sources were present, there was stronger inhibition for the lipid biosynthesis of *Mortierella isabellina* and its cell performance as described by longer lag phase, lower lipid production and

substrate utilization compared to control (Ruan et al., 2015). Zhao et al. also showed a synergetic inhibition of four representative inhibitors (furfural, acetic acid, formic acid, and vanillin) on cell growth and sugar utilization of *R. toruloides* (Zhao et al., 2012). Figure 4.5 and 4.6 show the fermentation profile when model lignocellulosic hydrolysate combination 1 and 2 were used as a substrate, respectively. The control experiment containing sugars with no inhibitors is also shown in Figure 4.7.

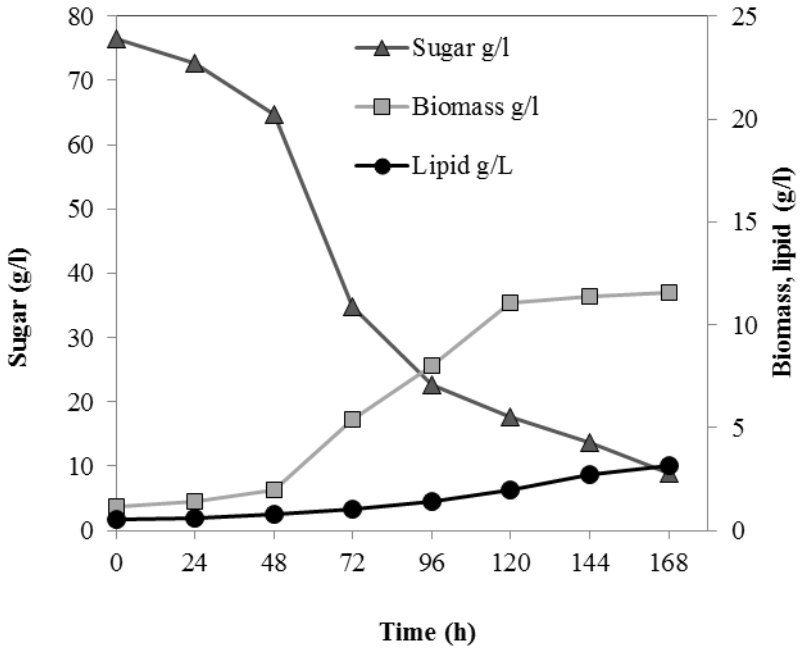


Figure 4.5 Fermentation profile for inhibitor combination 1 (0.2 g/L furfural, 1 g/L HMF, and 5 g/L acetic acid) under shake flask cultivation

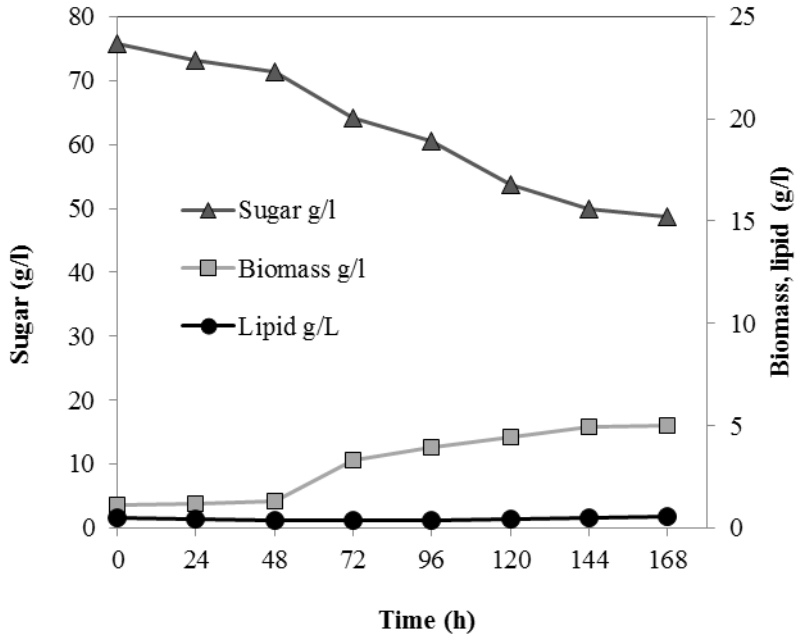


Figure 4.6 Fermentation profile for inhibitor combination 2 (0.5 g/L furfural, 1 g/L HMF, and 10 g/L acetic acid) under shake flask cultivation

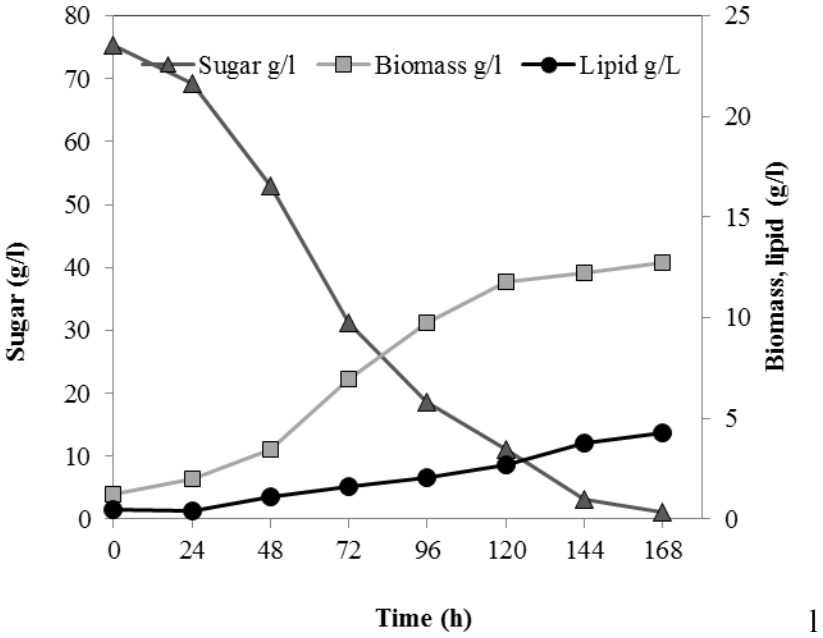


Figure 4.7 Fermentation profile of control (no inhibitors present) under shake flask cultivation

Our results showed that the synergetic inhibition dramatically decreased the biomass production and sugar utilization. They revealed evident inhibition even at combination 1, which was composed of lower concentration of inhibitors. For example when furfural was solely present in the medium 0.2 g/L, the cell biomass production was 13.5 g/L, which was almost similar to the control. However, the 0.2 g/L furfural decreased *R. glutinis* growth by 14% when other inhibitors are co –present. The synergetic inhibition effect even became more severe at higher concentration of inhibitors. When inhibitor combination 2 was applied, cell biomass only reached to the maximum value of 5 g/L compared to 6.59 g/L when 0.5 g/L furfural was the only inhibitor present and it was about 24% decrease in biomass production. From our results, it can be established that existence of mix inhibitors in the fermentation medium exhibited a noticeable inhibition on cell growth, while lipid accumulation was almost stayed intact. Similar results were also found for other oleaginous microorganisms such as *R. toruloides Y4* (Hu et al., 2009).

4.4.3 Kinetics of *R. glutinis* fermentation on the model lignocellulosic hydrolysate

The kinetic experiments containing the same concentration of inhibitors as combination 1 and 2 were conducted in a 3 L aerobic batch reactor in order to take advantage of more efficient aeration system, and controlled temperature and pH. To decrease the inhibitory effects of lignocellulosic hydrolysis byproducts on yeast growth function, increasing the inoculum size has shown a positive influence for improving cell biomass and also accelerating bioconversion of inhibitors into their less toxic biological form (Yu et al., 2014). To increase the turbidity of the inoculum, which results in

increasing initial cell biomass concentration, the sequential cultivation method was applied. The result of the fermentation in 3L aerobic batch bioreactor for inhibitor combination 1 and 2 are shown in Figure 4.8 and 4.9, respectively.

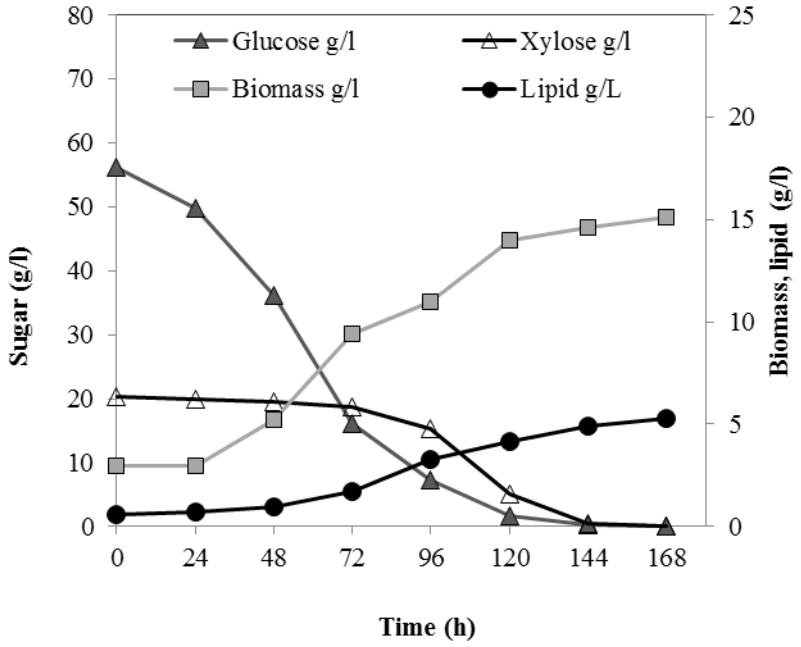


Figure 4.8 Fermentation profile of inhibitor combination 1 (0.2 g/L furfural, 1 g/L HMF, and 5 g/L acetic acid) in 3L aerobic batch fermenter

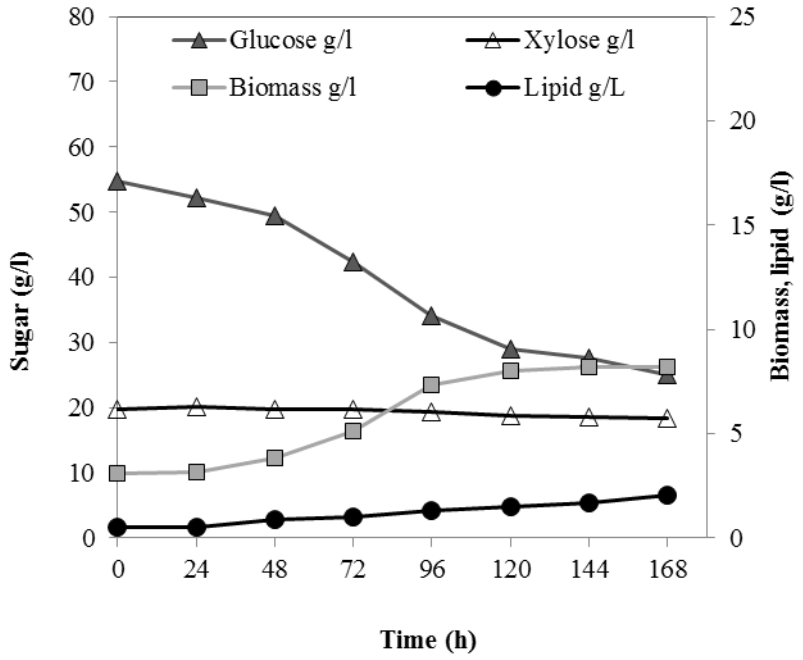


Figure 4.9 Fermentation profile for inhibitor combination 2 (0.5 g/L furfural, 1 g/L HMF, and 10 g/L acetic acid) in 3L aerobic batch fermenter

As a result of the sequential cultivation of seed culture, the initial cell biomass increased from 1.2 g/L to 3 g/L, which was about 150% increase in cell dry weight (CDW). Additionally, in order to facilitate the assimilation of acetic acid and improve its potential stimulatory effects on the biomass and lipid accumulation, the pH of the fermentation medium was controlled at 5. The basic environment in the aqueous phase could improve the equilibrium to shift toward the formation of acetate. The bioreactor results revealed noticeable improvements in both biomass and lipid production. For inhibitors combination 1, the cell mass production increased from 11.56 g/l to 15 g/l while the lipid accumulation has improved from 3.15 g/l to 5.2 g/l. In the shake flask experiment of inhibitor combination 2, the fermentation result showed a biomass and lipid production of 5 g/l and 0.55 g/l, respectively. However, the fermentation experiment

in 3 L aerobic batch fermenter using sequential seed cultivation method improved the biomass production and lipid accumulation to 8.2 g/l and 2 g/l, respectively.

The kinetic study of *R. glutinis* fermentation using model lignocellulosic biomass was performed to study the cell behavior in the presence of combined inhibitors. A mathematical model based on Haldane's kinetic model (Abuhamed et al., 2004; Economou et al., 2011), which takes into account substrate inhibition was applied into the experimental data for the fermentation of model lignocellulosic hydrolysate as a substrate. Figure 4.10 and 4.11 illustrate the experimental results of the biomass production, lipid accumulation and sugar consumption and corresponding fits to the model kinetic equations for the inhibitor combination 1 and 2, respectively. The kinetic parameters were determined and presented in Table 4.1.

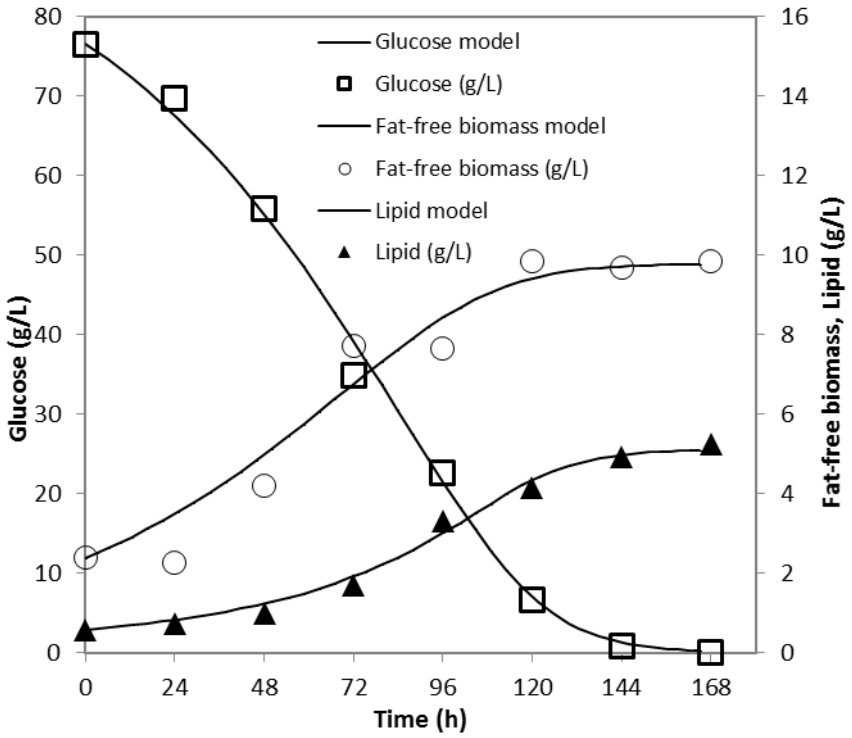


Figure 4.10 Comparison of experimental data and predicted value by the model for fat-free biomass (g/L), lipid (g/L), and glucose utilization (g/L) in inhibitor combination 1

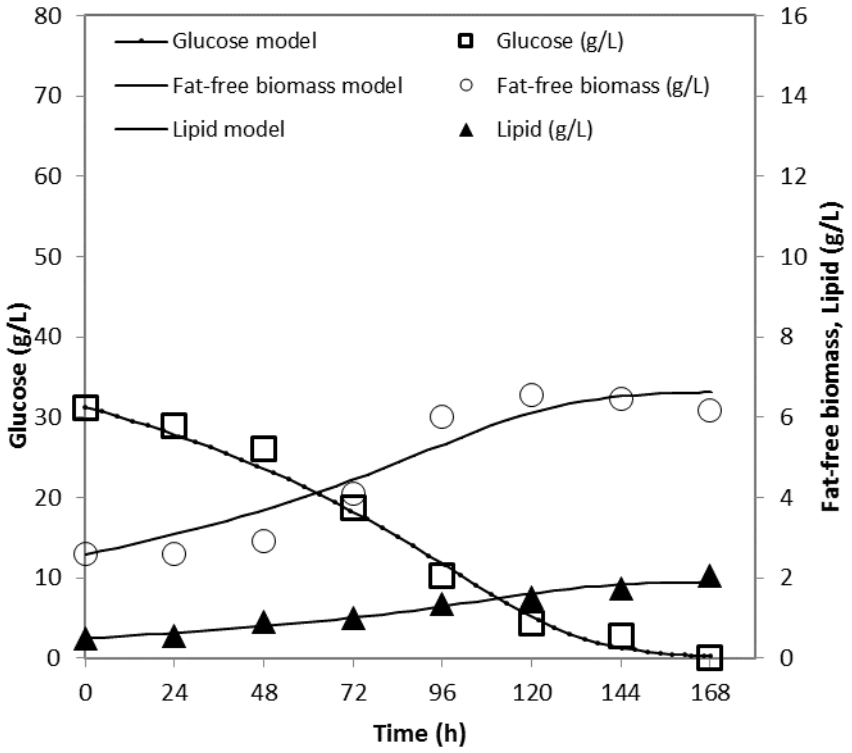


Figure 4.11 Comparison of experimental data and predicted value by the model for fat-free biomass (g/L), lipid (g/L), and glucose utilization (g/L) in inhibitor combination 2

Table 4.1 Summary of kinetic parameters for inhibition combination 1 and 2

Parameters	Inhibitor combination 1	Inhibitor combination 2
Fat-free biomass		
μ_{max} (h^{-1})	0.056	0.028
K_S (gL^{-1})	148.75	26.39
$K_{i1}(gL^{-1})$	160.49	15.1
Lipids		
$q_{l\,max}$ (gL^{-1})	0.027	0.0068
K_{lS} (gL^{-1})	28.84	9.73
K_{i2} (gL^{-1})	11.23	12.96
Substrate		
$Y_{X/S}$	0.14	0.22
$Y_{l/S}$	0.17	0.1

Inhibition combination 1 (0.2 g/L furfural, 1 g/L HMF, and 5 g/L acetic acid) and inhibitor combination 2 (0.5 g/L furfural, 1 g/L HMF, and 10 g/L acetic acid)

4.5 Individual and synergetic effects of lignocellulosic inhibitors on the FAMEs profile

To evaluate the effect of individual inhibitors as well as selected combined inhibitors on the fatty acid profile of intracellular lipids of *R. glutinis*, lipids were transesterified into FAMEs and its composition were determined (Table 4.2). Regardless the presence of inhibitors and its type and concentration level, the fatty acid profile of all treatments were dominated by oleic acid (C18:1), and palmitic acid (C16:0) except for the 1 g/L furfural, which the level of linoleic acid (C18:2) was higher than oleic and palmitic acid. Increasing furfural concentration continuously decreased the level of palmitic and oleic acid while increasing the content of stearic acid (C18:0). At 1 g/L furfural, the levels of stearic acid reached to the levels of palmitic and oleic acid, resulting in increasing the saturation levels of fatty acid. This observation was similar to the study of the effects of inhibitors on oleaginous fungus *M. isabellina* (Ruan et al., 2015).

Table 4.2 Fatty acid composition of biodiesel produced by *R. glutinis* lipids in presence of lignocellulosic inhibitors.

Oil origin	**Myristic acid (C14:0)**	**Palmitic acid (C16:0)**	**Stearic acid (C18:0)**	**Oleic acid (C18:1)**	**Linoleic acid (C18:2)**	**Linolenic acid (C18:3)**	**Others**
R. glutinis in 2 g/l acetic acid	2.25	35	8	36.35	12	3	3.4
R. glutinis in 5 g/l acetic acid	1.86	33	10.41	36.67	12.47	3.06	2.53
R. glutinis in 10 g/l acetic acid	1.88	33.7	10.86	36.3	12.32	3.08	1.86
R. glutinis in 0.2 g/l furfural	2.26	33.2	10.19	36.6	9.21	-	8.54
R. glutinis in 0.35 g/l furfural	1.98	31.89	11.95	33.78	12.87	-	7.53
R. glutinis in 0.5 g/l furfural	1.38	29.72	14	30.78	18.43	1.56	5.13
R. glutinis in 1 g/l furfural	-	21.86	21.85	21.72	27.42	4.9	2.25
R. glutinis in model lignocellulosic hydrolysate combination 1	1.79	30.76	10.27	36.82	10.87	1.65	7.84
R. glutinis in model lignocellulosic hydrolysate combination 2	-	24.74	13.56	29.2	16.69	1.92	13.89
R. glutinis Control	1.97	35.21	8.12	40.78	9.67	1.97	2.28

Although acetic acid showed a stimulatory effect on *R. glutinis* growth, its fatty acid composition remained almost unaffected, which could be an evidence for presence of other classes of lipids that needs to be further studied. For each combination inhibitions, the distribution of main fatty acids did not change considerably; however, at commination 2, the levels of palmitic and oleic acid was lower, while the levels of stearic acid and linoleic acid was higher compared to combination 1 (Table 4.2). Despite the alterations in fatty acid composition affected by different inhibitors, it has been observed

that *R. glutinis* mainly produced long chain fatty acids with 16 and 18 carbon atoms, which is similar to vegetable oils and demonstrates its suitability for biodiesel production. Moreover, for each inhibitor treatment, estimation of low calorific value (LCV), cetane number (CN), kinematic viscosity (μ), flash point (FP), iodine value, and cold filter plugging point (CFPP) using chemical structure of its fatty acids were shown in Table 4.3. The corresponded specification based on European standard for biodiesel EN 14214 (Leiva-Candia et al., 2014) also listed. Our results showed that these properties were comparable to rapeseed (Christophe et al., 2012) and palm oil (Azeem et al., 1999) biodiesel. These results suggesting that *R.glutinis* can utilize lignocellulosic biomass with existing inhibitors for biodiesel feedstock production.

Table 4.3 Predicted properties of biodiesel produced by *R. glutinis* lipids from lignocellulosic hydrolysate in presence of various inhibitors.

Oil origin	Chain length	Unsaturation degree	Low calorific value, LCV (Kj/Kg)	Cetane number, CN	Kinematic viscosity, μ (mm^2/s)	Flash point, FP (°C)	Cold filter plugging point, CFPP (°C)
EN 14214			-	>51	3.5-5	>120	-
Rapeseed	17.72	1.3	37,442.56	56.93	4.89	166.88	-3.16
Palm	17.03	0.61	37,442.56	69.87	5.46	162.16	8.23
R. glutinis in 2 g/l acetic acid	16.59	0.89	37,197.48	65.69	5.03	146.94	2.33
R. glutinis in 5 g/l acetic acid	16.81	0.69	37,295.21	66.31	5.15	152.62	3.57
R. glutinis in 10 g/l acetic acid	16.91	0.7	37,349.09	66.94	5.23	155.94	4.55
R. glutinis in 0.2 g/l furfural	15.7	0.7	36,819.93	65.06	4.67	128.7	-0.75
R. glutinis in 0.35 g/l furfural	15.92	0.55	36,909.20	64.99	4.75	132.49	-0.21
R. glutinis in 0.5 g/l furfural	16.42	0.59	37,114.34	64.93	4.92	142.43	1.07
R. glutinis in 1 g/l furfural	16.28	0.69	36,959.16	59.55	4.53	135.2	-4.34
R. glutinis in model lignocellulosic hydrolysate combination 1	15.90	0.63	36881.27	63.97	4.67	131.3	-1.3
R. glutinis in model lignocellulosic hydrolysate combination 2	15.0	0.68	36425.82	59.33	4.05	116.54	-7.7

4.5.1 Conclusions

The oleaginous yeast *R. glutinis* showed excellent tolerance to lignocellulosic biomass inhibitors such as HMF, and acetic acid. However, furfural was a strong inhibitor for the yeast growth and also delayed the substrate consumption and cell growth process as its concentration increased. Considering furfural as a major inhibitory compound during acid hydrolysis of lignocellulosic biomass, future studies must focus on methods of lignocellulose hydrolysis to lower its concentration to the level that can be managed by *R. glutinis* with no major changes to its lipid productivity and fatty acid composition. Therefore, future work can be focused on removing furfural during lignocellulose hydrolysis, applying current methods for detoxification of furfural or developing a new cost-effective detoxification method to eliminate furfural from hydrolysate.

CHAPTER V

DESIGN AND COST EVALUATION OF MICROBIAL OIL PRODUCTION

5.1 General Discussion

The utilization of pulp and paper wastewaters from different processes of the mill for microbial lipid production has been studied in previous chapters. The main results of the study have been presented in the form of scientific articles in chapters II-IV. The results from those chapters are briefly summarized in this chapter to study the primarily developed hypotheses and also to make economic suggestions for further research in this area. The present chapter also aims to assess the potential of microbial biodiesel production as a future transportation fuel by providing cost estimates of the microbial oil process.

It has been specified in several previous studies that some modifications in terms of applied technologies needs to be done to decrease the cost of microbial biodiesel in order to make its cost competitive to vegetable oils biodiesel. To that aim, it is inevitable to establish different costs that arise in different processes, to detect the major cost factors and also to calculate various cost related economic parameters such as capital cost, the manufacturing cost and finally the break-even price of microbial oil biodiesel.

Industrial feasibility of microbial oil production from oleaginous yeast is strongly reliant on the utilization of feedstock and on the fermentation step where considerably higher efficiencies and microbial oil production are attained. To that point, as mentioned

before, to decrease the cost of fermentation including the water and carbon feedstock we intended to use existing pulp and paper wastewater treatment facility as a source of water and nutrients to reduce the overall production costs of the oil. Several studies have investigated and published different economic assessments of the algae oil for biodiesel production. The algae oil production have some similarities to those of microbial oil production in terms of its process steps such as biomass separation, drying and lipid extraction; however, there exist significant variation in terms of its production cost, which depends on the underlying assumptions. Since autotrophic algae use CO_2 as carbon source, and open pond systems are commonly used for the cultivation process, there are cost related variances to microbial oil production. While demonstration of large-scale algae biofuel production have been established recently and algae companies have had many achievements in the commercial production, the production of microbial oil needs some modification to meet industrial satisfaction. Thus, a simplified cost estimation of the suggested microbial oil production process has been carried out. This investigation helps to draw a realistic sketch of the present cost situation and also to develop recommendations to reduce the cost associated to this process. Figure 5.1 and Figure 5.2 illustrate a process flow diagram for the existing biodiesel production from algae oil as an example for commercialized microbial oil production and the proposed process from yeast oil production, respectively.

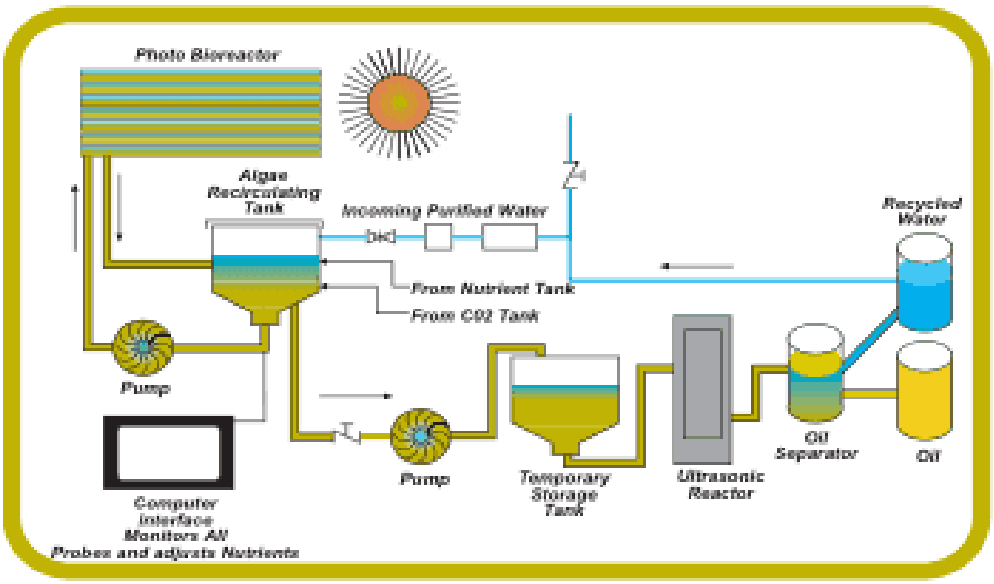


Figure 5.1 A process flow diagram of algae cultivation and algal oil production using ultrasonication

(courtesy of hielscher ultrasonics gmbh)

For algae cultivation, series of tubular and flat photobioreactors has been developed. The application of LED panels emitting light in the PAR part of spectrum leads to reach a maximum growth rate of algae. *Chlorella Vulgaris* with initial density of 0.146 g/L was initially inoculated and subsequently the cell density of 7.3 g/L was achieved in 7 days.

After a complete growth of algae cell, when the cells are mature for oil production treatment, the cell disruption method was applied, which is significant regarding the release of the complete intracellular material. Both ultrasound and microwave-assisted methods was applied, which resulted in significant improvements in algal extraction process, with higher productivity, reduced extraction times as well as reasonable costs and negligible further toxicity. An ultrasonic system for cell disruption and extraction are integrated into the setup process to ensure very efficient process attaining a full liberation

of intracellular matter and thus higher yields in shorter time. The algae cells are destroyed prior to extraction, however, most of the time and most specifically for the case of biofuel production, the cell destruction itself leads to release of the final product, and the separation process is only required to achieve the end product.

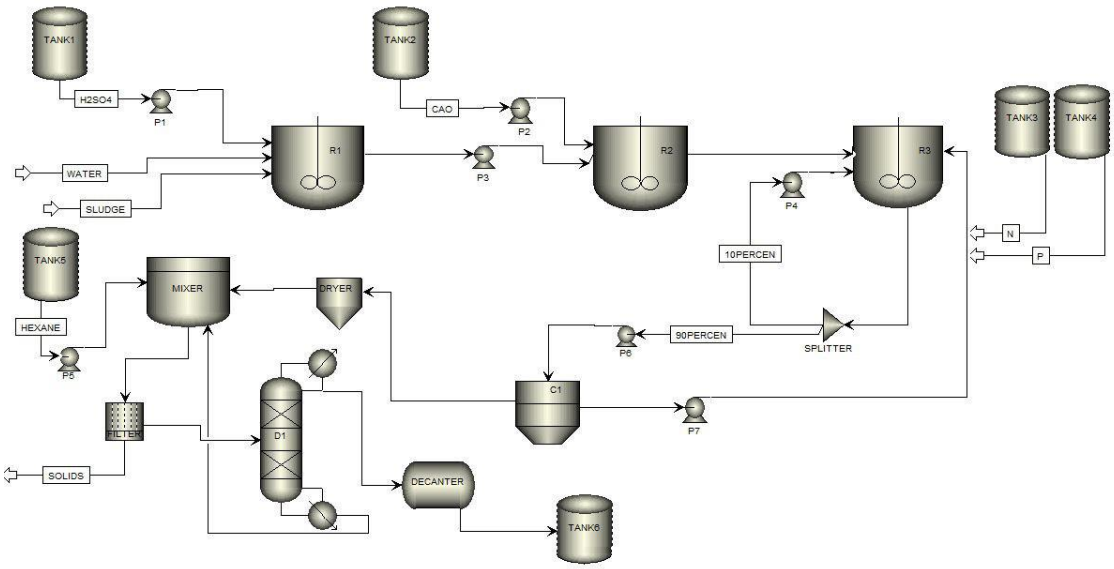


Figure 5.2 Schematic of the proposed process flow diagram for microbial oil production from pulp and paper wastewater

The purpose of this chapter is to estimate the cost of microbial oil production within cultivation of the oleaginous yeast in pulp and paper wastewater as fermentation media and using cellulosic waste material (paper mill sludge). The experimental results from this study along with several assumptions from the open literature have been employed for the design and economic evaluation for the production of microbial oil or biodiesel. The fermentation of glucose-based media from pulp and paper mills wastewater using the yeast strain *Rhodotorula glutinis* has been considered.

5.2 Microbial oil production Processes

The overall process consists of several steps that include three main sections: acid hydrolysis, fermentation, and lipid extraction (Figure 5.3). The feedstock for acid hydrolysis step is the primary paper mill sludge generated from pulp and paper wastewater treatment plant. The microbial oil that is produced from this process is projected to biodiesel manufacturing for fuel production.

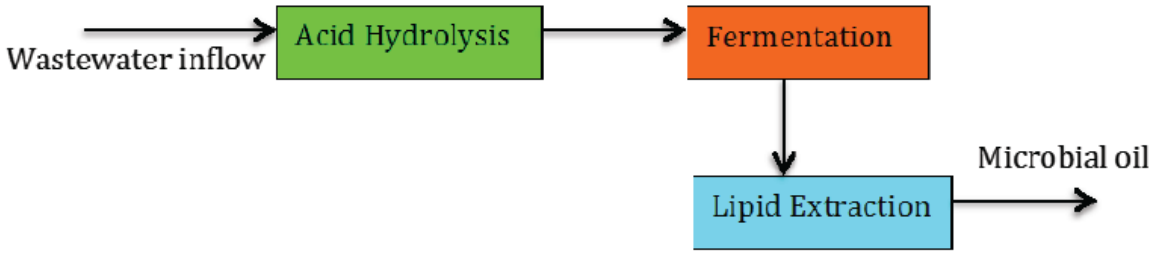


Figure 5.3 Main processes of microbial oil production using paper sludge from pulp and paper wastewater

5.2.1 Pulp and paper wastewater treatment plant

A pulp and paper wastewater treatment plant, the wastewater discharge from pulp and paper mill enters the wastewater treatment facility. A typical pulp and paper process wastewater treatment is channeled for primary treatment followed by biological treatment. The primary treatment is usually a mechanical-chemical pretreatment such as sedimentation, floatation or filtration, which consists of two parallel settling tanks, equipped with mixing and flocculation chambers to protect the following biological treatment. The pulp and paper industry employs a variety of effluent treatment techniques. The preferred process for a specific case varies in terms of the quality of the effluent that is going to be treated. Among all the treatment technologies, sedimentation

is the simplest and most cost-effective approach for separating solids from the liquid phase. In pulp and paper wastewater plant, specifically for effluents with high fiber concentrations, sedimentation is equipped with sets of lamella-shaped passages. The settled sludge is removed from the sedimentation tank, which is called primary sludge, which is composed of wood fibers (cellulose, hemicellulose, and lignin). The primary sludge contains very low amount of lignin (less than 10%), which makes it a better substrate for biocrude (oil) production than waste activated sludge (Poudel et al., 2015).

Biological wastewater treatment is followed by primary treatment and designed to degrade dissolved pollutants in effluents by implementation of microorganisms to utilize those pollutions as nutrients to live and reproduce.

Biological treatment includes two aerated lagoons. After removal of the suspended solids, the wastewater is treated in the aeration basin coupled with surface aerators. The resulting stream flows from the primary and secondary settling tanks to a drying system, where the solids are correctly eliminated. The product of the aerobic biological treatment is called activated sludge, which contains microbial biomass, cell-decay products and non-biodegradable lignin precipitates.

Primary sludge typically contains low amount of nutrients, especially N, and have high C:N ratios. However, secondary sludge has higher concentrations of N and P and lower C:N ratios compared to primary sludge, because N and P are usually added to the aeration tank to improve biological degradation. In our proposed microbial oil production process, the aeration tank is where oleaginous microorganisms are introduced to utilize the dissolved carbon, nitrogen, phosphorous, and other nutrients available in the wastewater for growth and production of lipids. The primary and secondary sludges go

through the dewatering step to get the dry sludge. Figure 5.4 shows the simplified classical flow diagram of the wastewater treatment plant for pulp and paper mills.

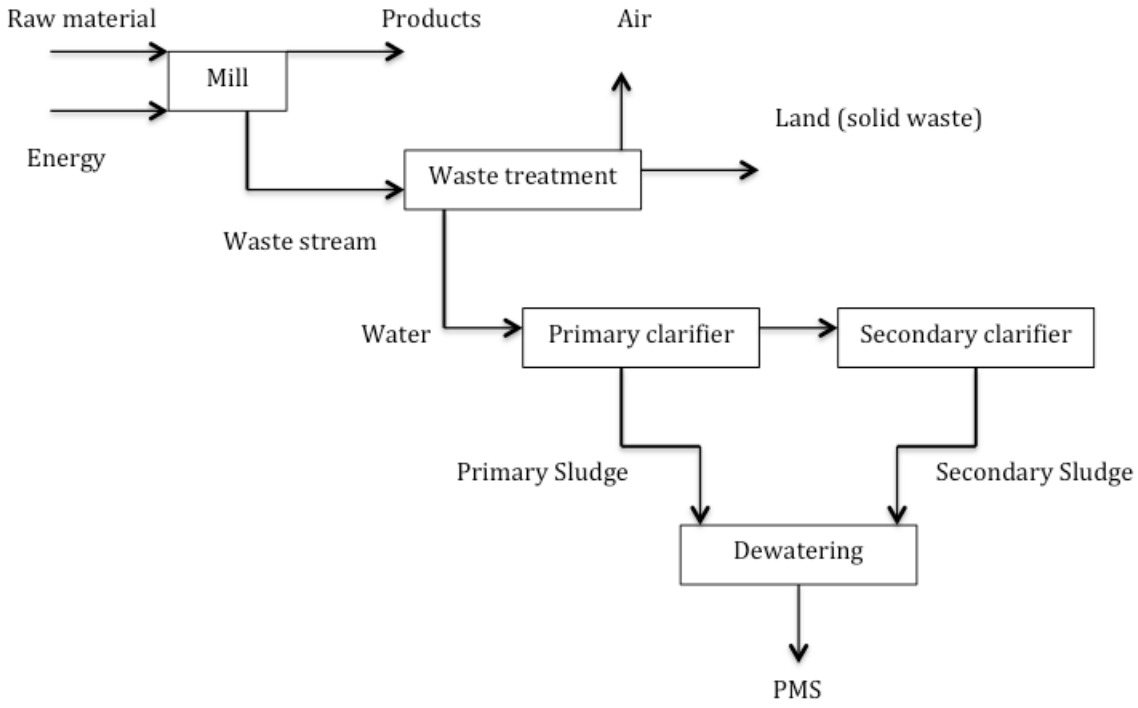


Figure 5.4 Pulp and paper wastewater treatment plant process diagram

The pulp and paper wastewater inflow to the wastewater treatment facility is assumed to be 0.5 Mgal/day with solid content of 5%. For many years the belt press and the screw press have been used as the standard sludge dewatering strategies in the paper industry. The moisture content of the primary paper mill sludge lies between 50% to 70%, whereas the content of cellulosic fibers is about 80% and the rest is inorganic. After drying its solid content has reported to be between 70 - 80 %, then PMS are grinded to decrease its particle size and subsequently continues through special mechanical treatment, which is called unraveling in order to increase its surface area. This stage is

essential in the whole process because in this step cellulose fibers are released from the inorganic matrix, which allows the solid matters to float on the surface of the fluid phase. Figure 5.5 shows the raw and treated paper sludge.

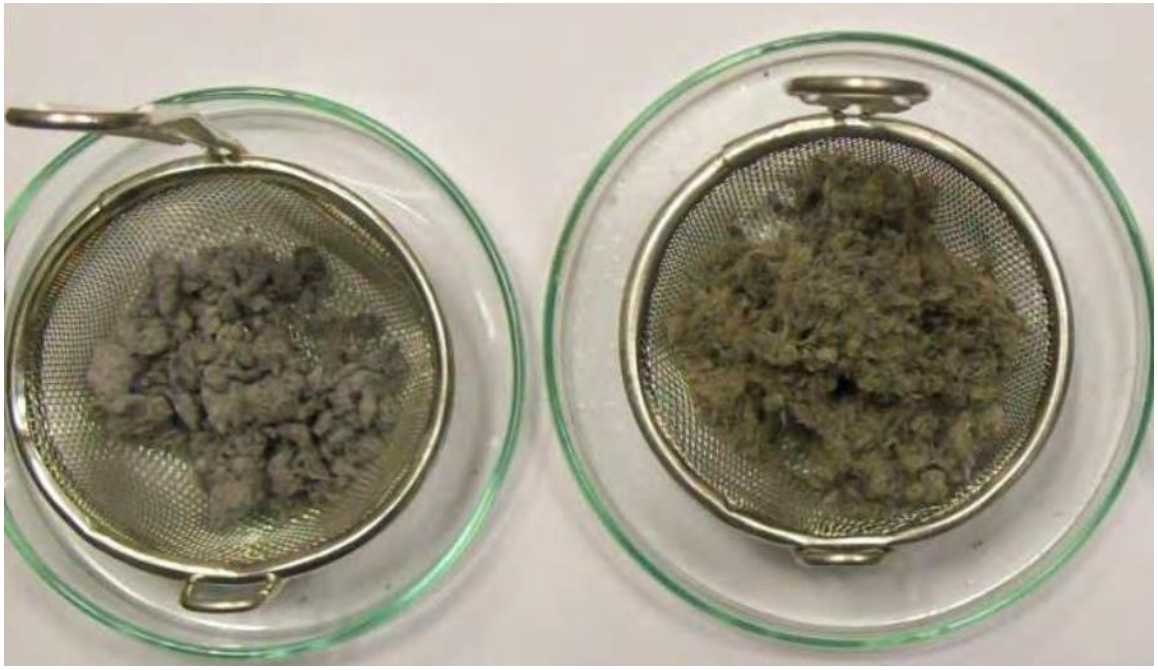


Figure 5.5 Paper mill sludge (PMS); (left) raw PMS, (right) unraveled PMS

Figure 5.6 shows a block flow diagram including underlying assumptions for the wastewater treatment step, which the PMS has been produced. This part of the process has not been considered in our cost estimation since it is part of the wastewater treatment process in pulp and paper mills. However the material balance was carried out to calculate the amount of sludge being produced through the pretreatment process.

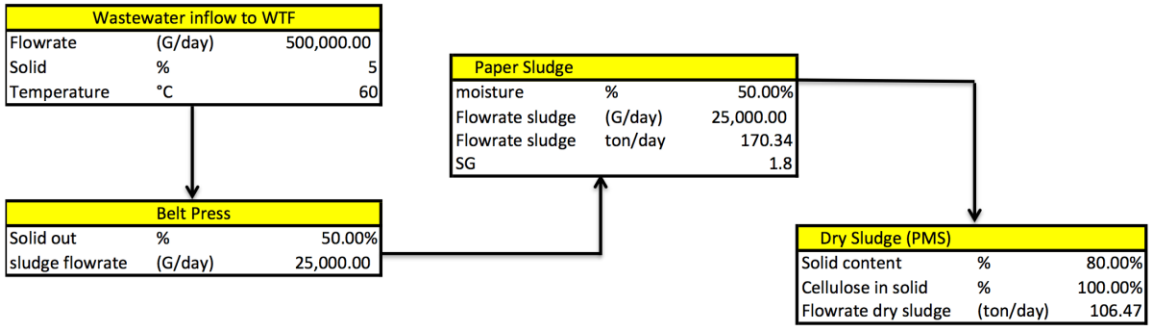


Figure 5.6 Block flow diagram for the wastewater treatment plant with PMS as a product

5.2.2 Acid hydrolysis

A portion of the pulp and paper inflow to the wastewater treatment facility is used to cover the water required in the acid hydrolysis reactor and for the fermentation, the rest returns to the wastewater treatment plant to be recycled to the mill for pulp and paper production. The water needed for the hydrolysis reactor needs to be heated up to carry out the hydrolysis at 90 °C. To reduce the energy required for the hydrolysis process, we used the wastewater originated from pulp wash process, which stays at 60 °C. Figure 5.7 illustrates the process steps involved in biocrude production to be used in biodiesel manufacturing.

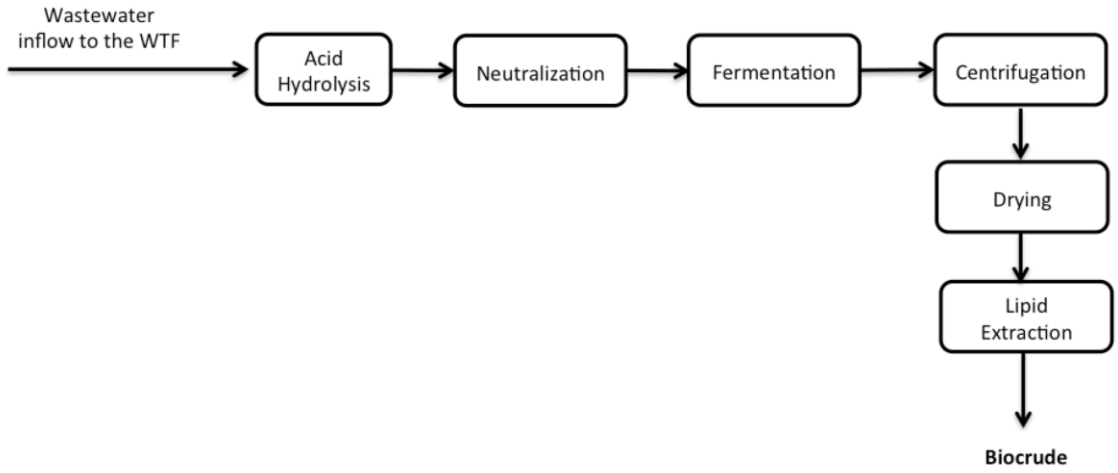


Figure 5.7 A simplified proposed process steps of biocrude production from pulp and paper mill wastewater

Utilization of lignocellulosic sugars resulted from hydrolyzing PMS, which is primary paper sludge (dried primary sludge) from wastewater treatment plant was implemented as the first step in our entire process to support carbon required for microbial growth. The process design and assumptions for acid hydrolysis was followed by concentrated acid hydrolysis adapted from BluFire Renewables, an established company, which has developed many improves in well-known concentrated acid hydrolysis. The dry sludge (PMS) is assumed to be 100% cellulose with 80% solid content. Considering 5% solid in pulp and paper effluent, the amount 106 (ton dry sludge/day) is estimated to be produced at pulp and paper wastewater treatment plant. Cellulose to glucose conversion efficiency of 90% was assumed, while the hydrolysis was carried out at acid to biomass ratio of 1.35 at 90 °C. A 30% sulfuric acid is used for 0.67 hours reaction time. A 143 ton/day of sulfuric acid is needed to hydrolyze 106 ton of

dry sludge/year, while the water requirement is about 80,423.60 ton/year, which counts for 17% of the wastewater effluent.

The acid hydrolysis sugar stream goes through a naturalization reactor. Neutralization of acidic streams involves raising the pH, usually by adding an alkaline chemical, such as lime. Lime treatment was adapted using quicklime (CaO), which is commonly used for large-scale treatments due to the cost associated process and alkali requirement. Lime is normally hydrated (slaked) and fed to the process as slurry and then dissolves to increase the pH. The naturalization efficiency of 90% was assumed and the reaction time of 4 hours was estimated (based on experiment). The fist two following equations reveal the reactions take place in the reactor, while the third reaction shows a reaction of sulfuric acid and lime:

$$CaO + H_2O \Rightarrow Ca(OH)_2 \quad (5.1)$$

$$Ca(OH)_2 \Rightarrow Ca^{2+} + 2OH^- \quad (5.2)$$

$$Ca(OH)_2 + H_2SO_4 \Rightarrow CaSO_4.2H_2O \quad (5.3)$$

A common by-product of lime neutralization is gypsum. Gypsum is widely used as a fertilizer, and as the main constituent in many forms of plaster, cement, blackboard chalk and wallboard. Other industrial application of gypsum includes: as an additive in turbid water, particularly ponds, to settle dirt and clay particles without injuring aquatic life, production of surgical and orthopedic casts, as a color additive for drugs and cosmetics, as a primary ingredient in toothpaste, and to be used as food additive as a dietary source of calcium, to condition water used in brewing beer, to control the tartness and clarity of wine, and as an ingredient in canned vegetables, flour, white bread, ice cream, blue cheese, and other foods. The selling price of gypsum in its raw form is

estimated to be $10/ton to be used as cement raw material. Figure 5.8 shows underling assumptions and information to calculate the flow rates and the size of the reactor for the hydrolysis process.

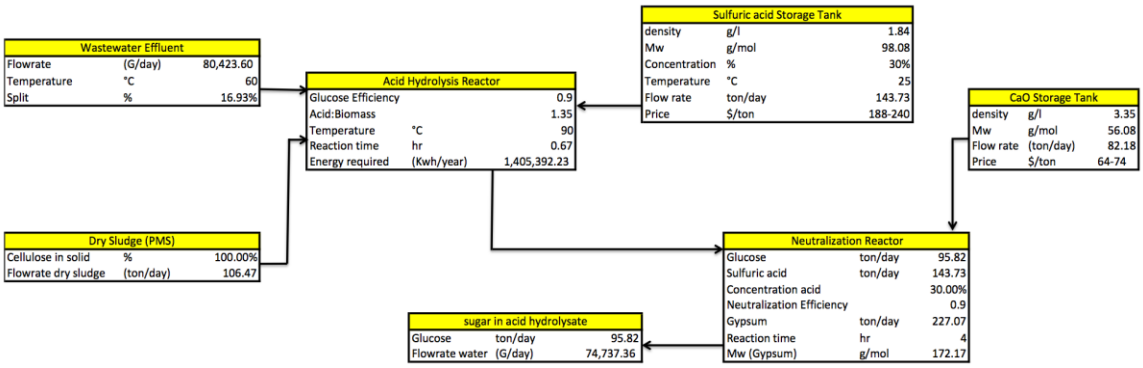


Figure 5.8 Block flow diagram of the hydrolysis process

5.2.3 Fermentation

After naturalization process, the sugar hydrolysate, which is mainly glucose is used as carbon supplement in the fermenter. The airlift bioreactor with an internal airlift loop was used for the fermentation of glucose using oleaginous yeast *Rhodotorula glutinis*. In the airlift fermenters, circulation is forced by the motion of gas through a central tube. The fluid recirculating through the headspace, where excess air and the by-product CO_2 separate. Figure 5.9 shows a design of airlift fermenter.

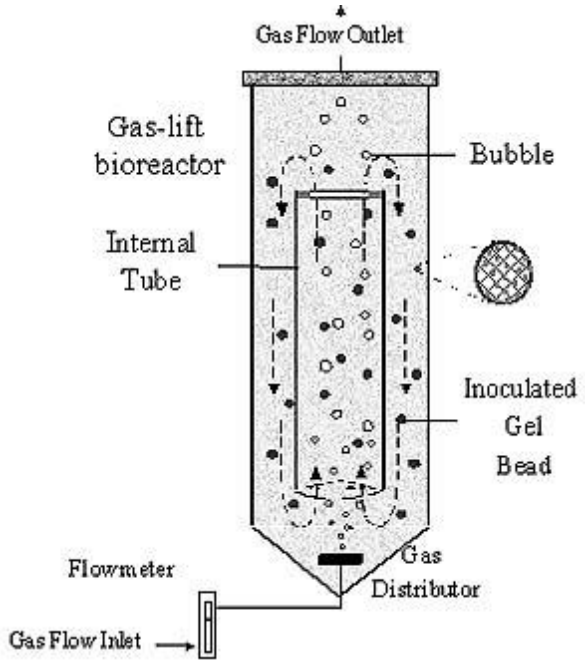


Figure 5.9 Airlift bioreactor

Following the results from chapter 3, based on the optimization results for maximum lipid production the concentration of glucose is assumed to be 80 g/L with lipid content of about 40%. The assumption of 99% of sugar consumption rate is reasonable based on our experimental results and model prediction. The operation oxygen concentration is assumed to be 2, while the COD for glucose is calculated to be 1.0677 g O_2/g glucose. The effect of inhibitory products on the cell growth and lipid production is not considered in our calculations.

The exiting stream from the fermenter is divided into two splits, one is called recycled seed culture, which is 10% of the total exiting stream recycled to the fermenter and the rest goes to the centrifuge process to get the concentrated biomass. A flow disc track centrifuge is used to recover the moisture content and to achieve a concentrated

biomass. The centrifuge works at 1.1592 kWh/ton processed. The moisture content of the concentrated biomass is assumed to be 75%. The recovered water effluent from centrifuge then leads to a seed tank, where the nutrients (nitrogen and phosphorous) and oleaginous yeast are added to the tank. The biomass paste is further dewatered and dried using a spray dryer. The 95% dry solid is assumed, which contains 40% lipid. A process flow diagram for the fermentation and post fermentation processes including centrifugation and drying is illustrated in Figure 5.10.

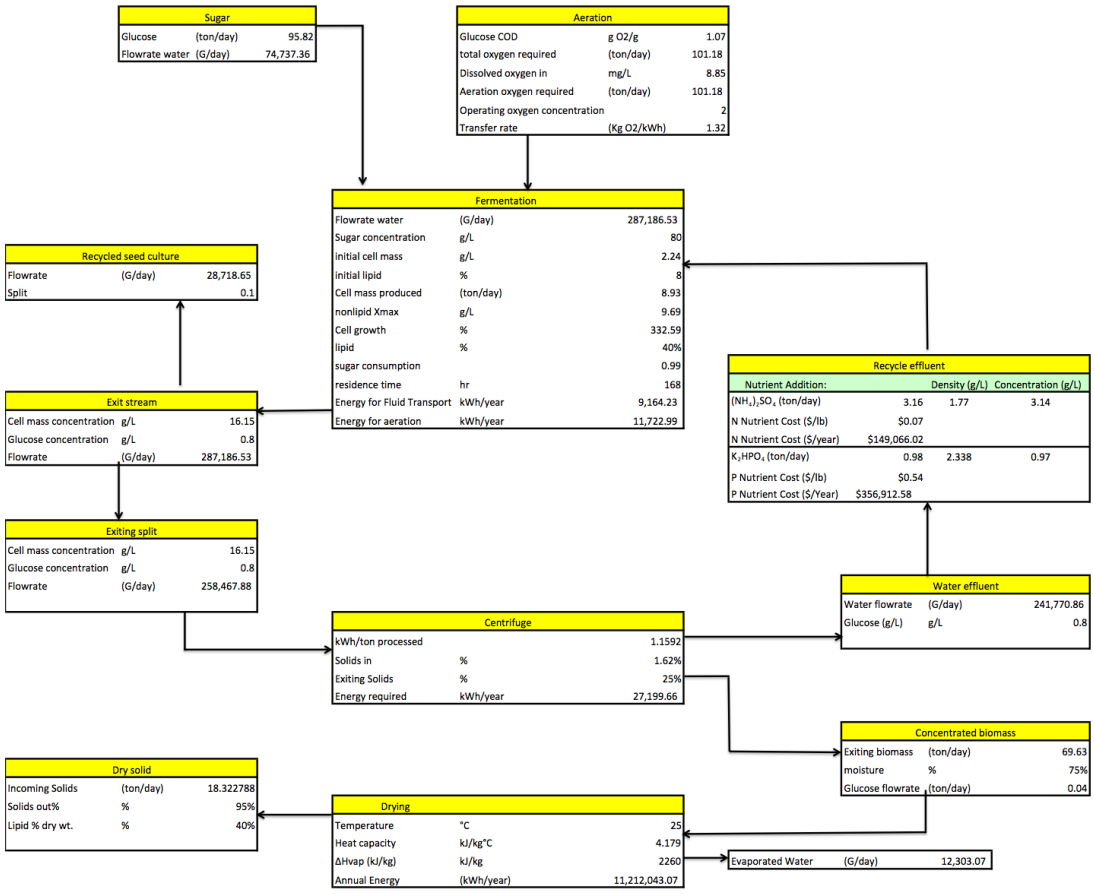


Figure 5.10 Block flow diagram of the fermentation and down stream processes

5.2.4 Lipid Extraction

The dry biomass with 40% lipid content follows to the extraction process using hexane as a solvent equipped with solvent recovery system. As the first step of the extraction, the dry biomass and solvent (hexane) are mixed and pumped to the filtration unit, where the dry solids are removed. The resulting fluid from filtration process is distilled to achieve lipids tryglyceride and the solvent was recovered to be recycled to the process. In a decanter the solvent was separated from lipids. The heavier lipids settled at the bottom are recovered and stored in the storage tank. The process of lipid extraction using hexane as solvent was adapted from Pokoo-Aikins et al., (2010), who evaluated the cost of lipid extraction from swage sludge with different solvents. The total production cost (TPC) of extraction was adapted and integrated in the cost calculation of microbial lipid production.

It is estimated that 6.89 ton/day of biocrude is produced which gives an annual biocrude production of 645,264 gal/year. The final process of transesterification of oil to biodiesel is not considered in our cost estimation since the process of microbial oil to biodiesel is similar to the conversion of vegetable oils to biodiesel. Figure 5.11 shows a process block flow diagram for the extraction unit.

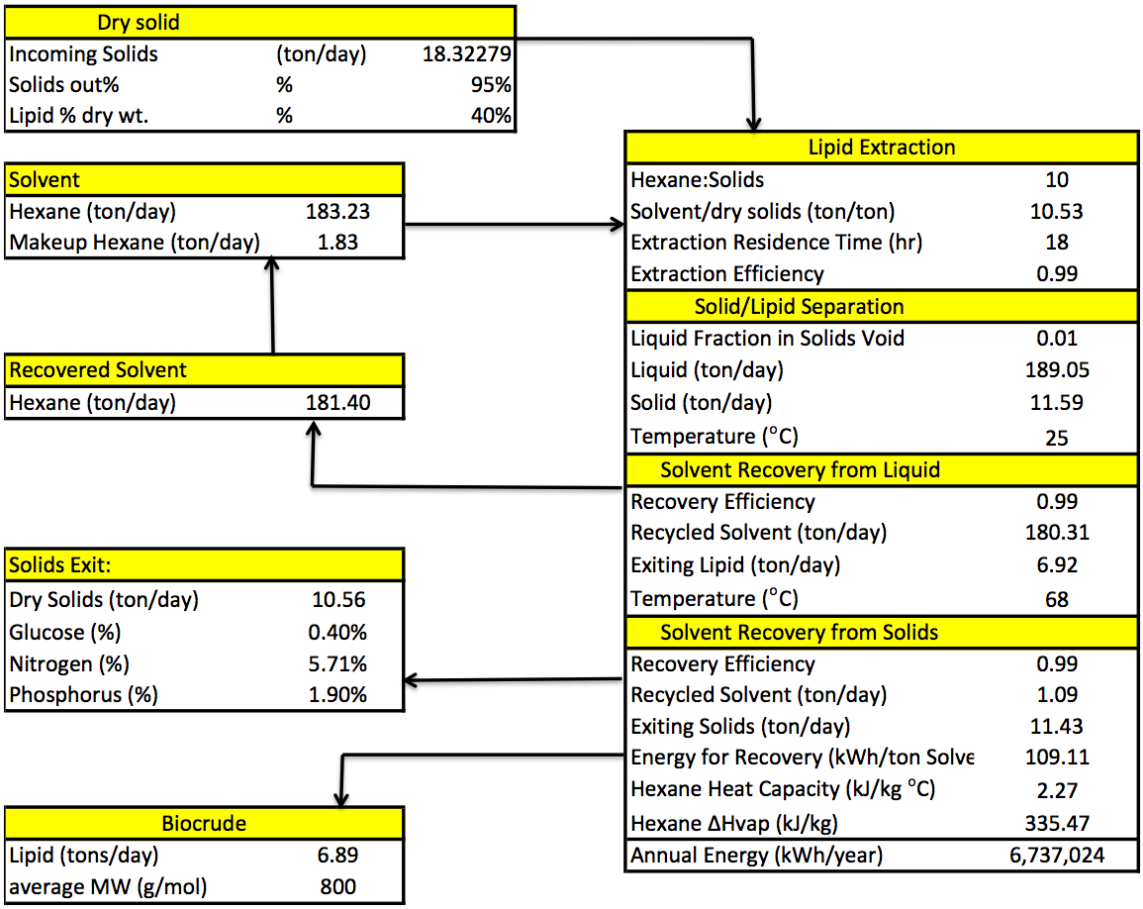


Figure 5.11 Block flow diagram for oil extraction process

5.3 Cost Analysis

For the selection and sizing of the different equipment, the total operating time of 8,088 hours was assumed. The biomass yield was set at 9.69 g/L with an average lipid content of 40%. Assuming a total of 14 fermenters with a working volume of 80%, the fermenters for lipid production were sized at 680 m^3. The fermenter are sparged with air, and equipped with temperature-controlled system.

The residual biomass after extraction of oil contains 5.71% of nitrogen and 1.9% of phosphorous and therefore can be used as a soil fertilizer with selling price of

$128/ton. It has been shown that considerable amounts of post extraction dry biomass (3,557 ton/year) were produced. In addition to that, 1,836,571 tons of gypsum is produced annually as by-product of acid hydrolysis unit, which can be sold at $10/ton. A list of materials of consumption, the products of the process along with their selling prices, and some cost associated assumptions that are used for the cost estimation of the proposed process are provided in Table 5.1.

Table 5.1 Calculation assumptions

Oil Selling Price ($/gallon)	$2.00
Fertilizer Selling Price ($/ton)	$128.00
Gypsum Selling Price ($/ton)	$10.00
Sulfuric Acid ($/ton)	$128.00
CaO (Quicklime) ($/ton)	$74.00
Hexane ($/gallon)	$4.00
$(NH_4)_2SO_4$ ($/lb)	$0.07
K_2HPO_4 ($/lb)	$0.54
Electricity ($/kwh)	$0.085
Heating Utilities ($/MMBTU)	$3.50
Yearly Hours of Operation	8088

An economic analysis is completed using the experimental data from previous chapters. Equipment sizing and cost was estimated for the oleaginous microorganism cultivated in pulp and paper wastewater and for the production of microbial oil from the biomass.

An electricity demand of 1,405,392.23 kwh/year for the hydrolysis reactor, 20,887.22 kwh/year for fermentation including the energy for aeration and for the fluid transport, 27,199.66 kwh/year for centrifugation was calculated, which leads to a total electricity demand of 1,453,479 kwh/year. A heating energy of 47,821 MMBTU/year is estimated for drying, which leads to a total annual energy cost of $167,374.70/year. The

energy required for the extraction is not considered since the electricity cost of extraction process is incorporated in the total production cost adapted from the extraction process reported by Pokoo-Aikins et al., (2010).

The cost of water is discarded since we have used wastewater in our suggested process. The wastewater assumed 100% freshwater and free of cost. Table 5.2 shows the calculation of the various cost parameters including a total capital investment cost, total annual production cost, and total manufacturing cost.

Table 5.2 Annual cost estimation for the process of microbial oil production from pulp and paper wastewater

A. Total Capital Investment Cost, TCC (TCC=FCC+WCC)			**27,333,282.94**
A.1. Equipment Cost, EC		15,494,180.00	
1-Acid hydrolysis reactor	118,000.00		
2-Nuatrilization reactor	296,000.00		
3-Fermenters	11,300,000.00		
4-Centrifuge	905,400.00		
5-Spray Dryer	640,580.00		
6-Pumps	251,200.00		
7-Storage tanks	1,983,000.00		
8- Others	774,709.00		
A.2. Contingencies and Fees, CFC (18% EC)	2,788,952.40		
A.3. Total Basic Module Cost, TBMC (EC+CFC)	18,283,132.40		
A.4. Auxiliary Facility Cost, AFC (30% TBMC)	5,484,939.72		
A.5. Fixed Capital cost, FCC (TBMC+AFC)	23,768,072.12		
A.6. Working Capital, WCC (15% FCC)	3,565,210.82		
B. Total Annual Production Cost, TPC (TPC=DOC+IOC+GE+DEPC)			**$23,438,615.65**
TPC acid hydrolysis, fermentation, harvesting and drying			
B.1. Direct Operating Cost, DOC		$13,543,751.53	
1-Sulfuric Acid	$6,210,042.96		
2-CaO	$2,049,350.61		
3-Nutrients (NH4)2SO4	$149,066.02		
4- Nutrients K2HPO4	$356,912.58		
5-Electricity	$1,076,569.38		
6-Operating Labor	$1,586,010.00		
7-Supervisory and Clerical Labor (15% operating labor)	$237,901.50		
8-Maintenance and repair (6% FCC)	1,426,084.33		
9-Operating Supplies (15% maintenance and repair)	$213,912.65		
10-Laboratories Charges (15% Labor)	$237,901.50		
B.2. Indirect Operating Cost, IOC		$1,426,967.44	
1- Overhead, Packing and Storage (60% of Labor)	$951,606.00		
2-Local Taxes (1.5% FCC)	356,521.08		
3-Insurance (0.5% FCC)	118,840.36		
B.3. General Expenses, GE		$5,308,951.50	
1- Administrative expenses (25% Overhead)	$237,901.50		
2- Distribution and selling (≈ 10 % of TMC)	$3,380,700.00		
3- Research and development (≈ 5 % of TMC)	$1,690,350.00		
B.4. Depreciation, DEPC (10% FCC)		2,376,807.21	
TPC extraction and processing			$782,137.96
C. Total Manufacturing Cost, TMC (TMC=TPC-CBP)			**$22,217,996.66**
C.1. Credit of by-product, CBP		$1,220,618.98	
Gypsum	$765,238.24		
Fertilizer	$455,380.74		
D. $US/ gal of oil produced			**$21.08**

It is shown that the proposed process leads to microbial oil production at $21/ gal of oil produced. Koutinas et al showed that when glucose-based renewable resources are

used as carbon source for an annual production capacity of 10,000 t microbial oil and no cost of glucose (assumption of utilizing waste or by products of a developed integrated biorefineries in present industries), the expected cost of microbial oil is $3.4/kg. However, when the cost of glucose was considered as materials of consumption for price of $400/t, the microbial oil production cost is estimated to be $5.5/kg oil (Koutinas et al., 2014). Indirect transesterification of extracted microbial oil for biodiesel production revealed more cost competitive process comparing with the direct conversion of dried yeast cells. Under this assumption, the projected cost of biodiesel production is $5.9/kg biodiesel (Koutinas et al., 2014).

Our proposed process using waste paper sludge as a renewable source of carbon showed the microbial oil production of $6.5/kg of oil is produced, which is higher than the price calculated for the process when glucose was purchased at $400/t. Looking at the hydrolysis process, the annual consumption cost of $11,893,785/ year is calculated for the hydrolysis process, which includes the price for sulfuric acid, quick lime, and the energy required for the hydrolysis. A major cost factor for the hydrolysis is the cost of sulfuric acid, which counts for 52% of the complete hydrolysis process. The acid hydrolysis process with acid recovery system is required to reduce the cost associated with acid requirement. Cheng et al., (2008) showed that the acid hydrolysis of sugarcane bagasse hemicellulose in an acid recycle process equipped with detoxification of hydrolysate by electrodialysis treatment, increased the reducing sugar concentration in the sugarcane bagasse hydrolysate and recovered the acid. They have demonstrated that performing the treatment by electrodialysis, removed 90% of acetic acid in hydrolysate and the recovery ratio of sulfuric acid was 88%. Two cycles of acidic treatments

increased the reducing sugar concentration from 28 to 63.5 g/l in sugarcane bagasse hydrolysate (Cheng et al., 2008). However, those systems needs to be evaluated in terms of cost associated to the process.

In terms of the energy required for the hydrolysis, it is also shown to be a large fraction along with electricity demand for the fermentation. The electricity demand of 1,405,392.23 kwh/year was estimated for the hydrolysis process. While, using the pulp wash wastewater at a temperature of 60 °C for the hydrolysis process reduced the annual energy consumption required for the hydrolysis by 47%, there still exist a potential to find a better alternative methods of hydrolysis such as the low temperature cellulose hydrolysis, as an energy-efficient and cost-effective approach for the biorefinery of lignocellulosic biomass (Zhang et al., 2012).

To reduce the whole production cost, a major cost factors of the complete process needs to be recognized in order to find the less expensive alternatives. The equipment cost for the fermentation process obviously shows the main cost factor in the process. However, replacing airlift bioreactors has significantly reduced the energy required for the mixing. Mixing in fermentation processes is essential to provide uniformity of the pH, improve the contact between the substrate and microbial culture, and avoid fouling and foaming in the system. Although conventional mixing using stirred tanks may give higher biomass yields, when the process energy requirement is being concern, these processes become economically unviable. Therefore, reducing the energy required for mixing in fermentation is one of the most challenging goals in large-scale bioenergy production. In the present study, we have proposed the use of an airlift bioreactor with an internal airlift loop as an alternative to traditional stirred tanks. The internal airlift loop

reactor is equipped with a tube or a plate to provide the conduit (channel) inside the reactor to provide circulating liquid inside the reactor. Other than improved mixing system, airlift reactors provides adequate time for gas–liquid to contact and do not cause shear damage to cells, which is essential for cell yeast production (Al-Mashhadani et al., 2015). Although airlift bioreactors have several advantages over the fermentation with traditional mixing systems in terms of the energy required for agitation, still the equipment cost counts it as one of the major cost factors of the entire process.

Regarding to an alternative less expensive processes, looking at similar processes to microbial oil production is helpful to find an alternative cultivation system in order to decrease the cost of the process. Studying the cultivation of autotrophic algae shows that two types of cultivation modes are commonly used in algae growth are open pond fermentation and closed cultivation in photobioreactors (PBR). Photobioreactors are mainly similar to the fermenters that displayed in our proposed lipid production process. However, economic assessments of feasibility of the cultivation of microalgal in photobioreactors have revealed that the use of PBRs cause higher production cost for microalgal biodiesel and therefore its application has been limited in the production of high-value nutraceutical products (Greenwell et al., 2010).

5.4 Environmental Advantages

In terms of the cost associated to the wastewater treatment, the cost that has been saved for the pulp and paper wastewater treatment and sludge management has not been considered in our calculations for the annual production cost. Generally, the cost of wastewater treatment counts for 10% of the entire process. In addition, sludge management has been a challenge in pulp and paper industries. Global production in the

pulp and paper industry is estimated to increase by 77 % by 2020 (Licon and Trebse, 2012). It has been reported that the majority of waste produced from paper production and recycling is paper mill sludge (PMS), which is a by-product of up to 23.4 % per a unit of produced paper. The global production of PMS was expected to increase in the next 50 years by 48-86 % beyond the present level. Currently, possible methods for paper mill sludge management are: landfilling, land spreading, composting, and application of PMS for energy production, cement production and etc. However, it is reported that more than 69 % of the generated PMS is currently landfill disposed. In terms of the negative effect of landfilling on the environment, Buswell and Mueller (1952) reported that theoretically, 1 ton of PMS in landfill releases approximately 2.69 tons of CO_2 and 0.24 ton of CH_4 into the environment. Utilization of paper mill sludge as land spreading material must be used very carefully due to a harmful C:N ratio, high ion exchange capacity and possible Cr toxicity (Norris and Titshall, 2011). Implementation of composting has also similar problem as land spreading in terms of very high C:N ratio. Composting of PMS demands a large land area and requires additional costs for storage and processing (Gea et al., 2005).

5.5 Conclusion

In this work, a study of the economics of microbial oil from oleaginous yeast using pulp and paper wastewater for the subsequent biodiesel production is presented. The main cost generators are the capital cost of fermenters that are essential in order to produce the microbial biomass and oil and the cost of hydrolysis for the conversion of cellulosic waste biomass to glucose feed for the fermentation. The majority of the hydrolysis cost arises due to the cost of sulfuric acid. The cost of electricity consumption

associated with the acid hydrolysis reactor also counts as major cost factor. An effective alternative acid hydrolysis method combined with acid recovery system is essential to make the process economically feasible. Isolation and adaptation of robust oleaginous yeast strains that can produce higher quantities of lipids with lower glucose consumption in lower cultivation time and innovative fermenter designs are needed in order to reduce the cost of fermentation and bring about the step changes necessary to make commercialization of the microbial oil production technologies possible. However, the exploitation of food waste streams instead of industrial waste streams could result in cost-competitive production of microbial oil at least for oleochemical synthesis. Valorization of by-product streams seems imperative in order to reduce microbial oil or biodiesel production costs.

5.6 Recommendations

- The acid hydrolysis process with acid recovery system
- Development of novel methods of hydrolysis at milder treatment condition (lower temperature)
- Isolation and adaptation of robust oleaginous yeast strains that can produce higher quantities of lipids with lower glucose consumption in lower cultivation time
- Integration of the microbial oil production from yeast with algae oil systems

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APPENDIX A

MATLAB CODE FOR CALCULATION OF KINETIC PARAMETERS

A.1 Malthusian Model

```
function [flag]=hyroModel()
%This function finds the best parameters to fit the batch model
%Simulation to the data by minimizing the sum of squared errors (SSE)
clc; clear all; flag=0;
EXP=xlsread('inputs.xlsx',2,'B3:G10');
t=EXP(:,1); X=EXP(:,5); S=EXP(:,6); L=EXP(:,3); COV = 0.9; num = 8;

%        muMAX KS    Ki1   qLMAX KLS    Ki2   YXS   YLS
initial=[0.2   80.0  150.0 0.06  94.0   110.0 0.2   0.09]; %initial guess
lb = (1-COV)* initial;
ub = (1+COV)* initial;
%*************************************************************************
Y=[X;S;L]; data=[Y]; knobs=[t;X;S;L];

options=optimset('algorithm','interior-point','Display','off',...
    'MaxIter',1E6,'MaxFunEval',1E6);
kinetic_params = fmincon(@myfit,initial,[],[],[],[],lb,ub,@const,...
    options,knobs,data,num);

%%
{'Parameter','Value';
    'muMAX=',[kinetic_params(1)];
    'KS='   ,[kinetic_params(2)];
    'Ki1='  ,[kinetic_params(3)];
    'qLMAX=',[kinetic_params(4)];
    'KLS='  ,[kinetic_params(5)];
    'Ki2='  ,[kinetic_params(6)];
    'YXS='  ,[kinetic_params(7)];
    'YLS='  ,[kinetic_params(8)]}

X0=knobs(num+1); S0=knobs(2*num+1); L0=knobs(3*num+1); Ci0=[X0 S0 L0];
[ti,Ci]=ode45(@(ti,Ci) batch(ti,Ci,kinetic_params),[0 168],Ci0);

hold on
figure(1)
subplot(2,2,1)
plot(ti,Ci(:,1),'-r','LineWidth',3); hold on
plot(t,X,'ro','MarkerEdgeColor','r','MarkerSize',10)
```

```
subplot(2,2,2)
plot(ti,Ci(:,2),'b','LineWidth',3); hold on
plot(t,S,'r*','MarkerEdgeColor','b','MarkerSize',10)
subplot(2,2,3)
plot(ti,Ci(:,3),'k','LineWidth',3); hold on
plot(t,L,'r+','MarkerEdgeColor','k','MarkerSize',10)
%%
xlswrite('data-plot.xlsx',ti,1,'B4:B50');
xlswrite('data-plot.xlsx',Ci(:,1),1,'C4:C50');
xlswrite('data-plot.xlsx',Ci(:,2),1,'E4:E50');
xlswrite('data-plot.xlsx',Ci(:,3),1,'D4:D50');

function SSE=myfit(params,input,actual_output,num)
Ymodel=0;
timevec=input(1:num); X0=input(num+1); S0=input(2*num+1); L0=input(3*num+1);
Y0=[X0 S0 L0];

%Call ODE solver
options=odeset('RelTol',1E-3,'AbsTol',1E-4);
[t,that]=ode45(@(t,Data) simul(t,Data,params),timevec,Y0,options);

Ymodel=[Ymodel;[that(:,1);that(:,2);that(:,3)]];
Fitted=Ymodel(2:length(Ymodel));
Error_Vector=Fitted-actual_output;

SSE=sum(Error_Vector(1:num).^2)/sum(actual_output(1:num).^2) + ...
    sum(Error_Vector(num+1:2*num).^2)/sum(actual_output(num+1:2*num).^2) + ...
    sum(Error_Vector(2*num+1:3*num).^2)/sum(actual_output(2*num+1:3*num).^2);

function f = simul(t,Data,params)
%Define parameters ******************************************************
muMAX=params(1); KS =params(2); Ki1=params(3); qLMAX=params(4);
KLS  =params(5); Ki2=params(6); YXS=params(7); YLS  =params(8);

%Define Data
X=Data(1); S=Data(2); L=Data(3);

if (S<0) S=0; end;
%Define mu and qL - eq(5) and eq(6)
mu = muMAX * S / (KS  + S + S^2/Ki1 );
```

```
qL = qLMAX * S / (KLS + S + S^2/Ki2 );

%Equation (1)to(3)
rX = mu * X;
rS = -1*(mu/YXS + qL/YLS) * X;
rL = qL * X;

f=zeros(3,1);
f(1)=rX; f(2)=rS; f(3)=rL;

function y = batch(ti,Ci,Fitted_Params)
muMAX=Fitted_Params(1); KS=Fitted_Params(2); Ki1=Fitted_Params(3);
qLMAX=Fitted_Params(4); KLS=Fitted_Params(5);Ki2=Fitted_Params(6);
YXS=Fitted_Params(7);   YLS=Fitted_Params(8);

X=Ci(1); S=Ci(2); L=Ci(3);
%Define mu and qL
mu = muMAX * S / (KS  + S + S^2/Ki1 );
qL = qLMAX * S / (KLS + S + S^2/Ki2 );
%Equation (1)to(3)
rX = mu * X;
rS = -(mu/YXS + qL/YLS) * X;
rL = qL * X;

y=zeros(3,1);
y(1)=rX; y(2)=rS; y(3)=rL;

function [c,ceq] = const(params,Input,actual_output,num)
% Inequality constraints
c = [];
% Equality Constraints
        ceq = [];
```