Economical Lipid Production by *Lipomyces starkeyi*

A Thesis

Presented to the

Graduate Faculty of the

University of Louisiana at Lafayette

In Partial Fulfillment of the

Requirements for the Degree

Master of Science

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Spring 2016

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ACKNOWLEDGMENTS

First, I must thank my major advisors and mentors, Dr. Ramalingam Subramaniam and Dr. Rakesh Bajpai, for all the support, encouragement, and love over the years. I am grateful to my committee members, Drs. Mark Zappi, Rafael Hernandez, Andrei Chistoserdov, and Wu Xu for providing their assistance, expert opinion, and guidance throughout the project. I would also like to thank Dr. Stephen Dufreche and Mr. William Holmes for their immense cooperation and valuable guidance in operating laboratory instruments. My gratitude goes to fellow students, lab mates, and staff for their help and support. Lastly, I would like to thank my parents, brothers, sisters, and my wife for their love, support, and encouragement.

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LIST OF ABBREVEATIONS

$(NH_4)_2SO_4$	Ammonium Sulfate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
C:N	Carbon to Nitrogen ratio
$CaCl_2$	Calcium Chloride
$CoCl_2$	Cobalt Chloride
$CuSO_4$	Copper Sulfate
FAMEs	Fatty Acids Methyl Esters
FAS	Fatty acid synthase
$FeSO_4$	Ferrous Sulfate
g	Gram
g/L	grams per liter
GC	Gas Chromatography
H_2O	Water
HPLC	High Pressure Liquid Chromatography
ICDH	Isocitrate dehydrogenase
KCl	Potassium Chloride
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium Iodide
MeOH	Methanol
mg	milligrams
$MnSO_4$	Manganese Sulfate

Na_2MoO_4	Sodium Molybdate
NaOH	Sodium Hydroxide
TAGs	Triglycerides
TCA	Tricarboxylic acid
v/v	Volume/Volume
w/v	Weight/Volume
wt	Weight
YM	yeast, malt extract

CHAPTER 1: INTRODUCTION

1.1 Background

The global demand for fossil fuels is increasing in the transportation, industrial, electrical, commercial, defense, and residential sectors. Crude oil is the world's largest energy source in the transportation sector, and the energy consumption in this sector is expected to grow 45% globally between 2007 and 2035. In the U. S., the energy consumption in the transportation sector is expected to rise 29% during the same period. In 2014, about 136.78 billion gallons of gasoline were consumed in the United States, an average of about 374.74 million gallons/day (US Energy Information Administration EIA, 2015).

The United States Environmental Protection Agency has recently started targeting sources of greenhouse gases under the Clean Air Act. Greenhouse gases are predominantly made up of carbon dioxide, which is produced by power plants, large scale industries, and vehicles that use fossil fuels. In 2013, 38% of total CO_2 emissions in the U. S. came from power plants using fossil fuels; the transportation sector contributed another 32% of total U. S. CO_2 emissions. Renewable alternatives to fossilized fuels are being explored to reduce the emissions of greenhouse gases. In the transportation sector, bioethanol and biodiesel are being used as fuel additives, and their production was about 15.6 billion gallons in 2013.These are currently produced mainly from food sources, such as corn and soybeans. Studies by the U. S. Department of Energy (DOE) suggests that renewable resources in the U. S. can be used to produce roughly 60 billion of gallons of renewable fuels by 2030, replacing 30% of gasoline consumed (ACORE, 2007).

Renewable fuels from microbial lipids have a great potential in supplementing fossil fuels. Several microbes (called oleaginous) are capable of accumulating lipids in excess of 20% of

their dry mass. These are often algae, yeast, and bacteria. Some of them can accumulate as much as 70% of their dry cell weight as lipids and are appropriate candidates for microbial lipid production. Notable oleaginous yeasts are *Rhodotorula glutinis, Rhodosporidium toruloides, Mortierella isabellina, Cryptococcus curvatus,* and *Lipomyces starkeyi,* which can convert primarily glucose as well as xylose to lipids (Dai et al., 2007; Zhao et al., 2008; Yu et al., 2011; Huang et al., 2013; Zeng et al., 2013). *Lipomyces starkeyi* has the ability to use a wide range of sugars, including glucose, starch, xylose, L-arabinose, and D-cellobiose to produce lipids (Oguri et al., 2012).

Lipomyces starkeyi is a good choice for economical lipid production since it can accumulate more than 70% of its dry cell weight as lipids, and it consumes its intracellular lipids at a considerably slower pace when compared to other microbial species during shortage of carbon supply. *L.starkeyi* showed less than 20% of internal lipid consumption in a 36 hours' starvation period while *R. toruloides, C. curvata,* and *T. cutaneum* used about 40% of total lipid (Holdsworth et al., 1988)

The main barrier for commercial production of microbial lipids is their production costs (Fisher et al., 2008; Jin et al., 2014; Zhang et al., 2008). The major cost contributing factors in production of microbial lipids are the medium components, processing (filtration and drying), and lipid recovery from cells. Medium cost comprises of carbon substrate and other essential nutrients, mainly phosphate.

1.2 Lignocellulosic Biomass Availability and Issues in Lipid Production

Lignocellulosic biomass from agricultural and forestry residues is an abundant source of sugars for production of microbial lipids. Annually, 20 billion metric tons of this biomass are available globally, including U.S. production of over 1 billion tons (Perlack et al., 2011;

Hadar, 2013). At the same time, a considerable amount of R & D work has been done in the past 40 years towards the recovery of fermentable sugars from lignocellulosic biomass. Assuming 40% fermentable sugars recovered from lignocellulosics (Humbird, 2011), microbial lipid yield of 0.25 g/g from sugars (Ratledge, 2002), and 90% efficiency of lipid extraction from cells, one can expect as 26 billion gallons from agricultural and forestry wastes available in U.S. Still there are major technological challenges in producing microbial lipids from lignocellulosics. These challenges arise from the fact that a mixture of sugars, generally glucose, xylose, arabinose, are present in hydrolysates of lignocellulosics, and when multiple sugars are present in fermentation media, the microorganisms often exhibit a very complex pattern of substrate use and product formation. Additionally, a number of byproducts (mainly furan derivatives, organic acids, and aldehydes) are formed during the commonly used acid hydrolysis of lignocellulosics.

These toxic compounds inhibit cellular growth by affecting their sugar uptake rate and simultaneously decrease the rate of product formation. Lowering the concentration of the toxic compounds can reduce the inhibitory effects but it depends on the type of microorganism and hydrolysates. For instance, furfural concentration at 0.5, 1.0, and 2.0 g/L inhibited *Scheffersomyces stipitis,* (one type of ethanol producing yeast) growth by 25%, 47%, and 99 %, respectively (Delgenes et al., 1996). In another study, there was no effect on microbial growth at 0.25 g/L of furfurals but had effect on ethanol yield and productivity at 1.4 g/L furfural concentration using *S. stipitis* (Nigam et al., 2001). In the case of oleaginous species, *R. toruloides*, furfural has strong inhibitory effect for the cellular growth and lipid production at or above concentration of 0.096 g/L (Hu et al., 2009).

High density cultivation requires a high concentration of substrate and in case of a lignocellulosic substrate, the concentration of byproducts also becomes higher. Though *Lipomyces starkeyi* is a desirable microbe for producing lipids, there is no published report in literature that characterizes the effect of byproducts of lignocellulosic hydrolysates on growth and product formation by the microorganism.

1.3 Phosphate Cost Factor in Microbial Lipid Production

In terms of medium costs, phosphate is one of the costly ingredients in microbial lipid production. It is an essential element and it is mostly incorporated into nucleic acids, phospholipids, and coenzymes (Wu et al., 2010). High carbon to phosphorus (C/P) ratios has been shown to cause lipid accumulation in yeasts Candida 107 (Gill et al., 1977) and *Rhodotorula glutinis* (Granger et al., 1992). Phosphate-limited culture of *Rhodotorula toruloides* Y4 produced lipid content of 63.7% and total lipid 12.1 g/L while producing lipid yields of 0.21 g/g substrate. Hence, optimization of phosphate concentration could be effective in reducing lipid production costs.

1.4 Extraction Cost Factor in Microbial Lipid Production

The high cost of extracting neutral lipids is a major obstacle for the commercialization of microbial lipid products. The toughness of yeast cell walls requires a lysis step at the beginning of lipid extraction process to release intracellular lipids. High-yielding, low-energy lysis methods are demanded for efficient commercial-scale extraction. Physical methods include high-pressure homogenization, solid shear, ultrasonic ruptures, freeze-thawing, and extrusion; chemical/biological methods include use of organic solvents, acid/basic hydrolysis, surfactants (detergents), enzymatic treatment, and autolysis (Probst Kyle, 2014). Criteria for selecting the proper solvent for extraction of lipid include high selectivity and

extraction efficiency for the neutral lipids, non-reactivity with the lipid of interest, and easy separability (Halim et al., 2012). Hexane, cyclohexane, heptane, benzene, ether, and acetone, are commonly used solvent for extraction of lipid (Harun et al. 2010; Horst et al. 2012). These methods require large quantities of solvents that are expensive and toxic. Therefore, they are not preferred for industrial-scale extraction.

In solvent extraction, at lab scale, the most common and effective methods for extracting oil from wet materials include those developed by Folch (1957) and Bligh and Dyer (1959), which use biphasic systems of the nonpolar organic solvent chloroform and the polar solvents methanol and water. Hexane is used widely in large scale for the extraction of vegetable oils, such as soybean (Zhang et al., 2003; Koc et al, 2010).

1.5 Objectives

In the light of information presented above, the objective of this research work as follows:

- Characterize the effect of byproducts from acid hydrolysis of lignocellulosics on cell growth and lipid accumulation
- Study the effect of phosphate concentration on cell growth and lipid production and find the optimum concentration of phosphates in the medium
- Evaluate different solvents for lipid extraction process from wet as well as freeze dried cells

CHAPTER 2: LITERATURE REVIEW

2.1 United States' Biofuel Production and Consumption

In 2014, about 136.78 billion gallons of gasoline were consumed in the United States, as an average of about 374.74 million gallons/day (U. S. Energy Information Administration, EIA, 2015). Current bioethanol and biodiesel production comprises about 15.6 billion gallons at the same period where contribution of bioethanol is about 10% and for biodiesel it is only 1 percent. The goal of U. S. Department of Energy (DOE) to displace 30% of gasoline consumption at 60 billion gallons per year is feasible by lignocellulosic biomass resources (ACORE 2007; Jacobson et al., 2009).

The available biofuels from renewable feedstocks are ethanol, biodiesel, and the emerging green diesel. Currently, ethanol is being used as a blend of 10-15% with gasoline, which is called E15, defined by the Environmental Protection Agency, and can be used in vehicles 2001 and newer models. It is produced primarily from corn starch using yeast. Ethanol can also be produced from fermentable carbohydrates derived from cellulose and hemi cellulose of lignocellulosic plant.

Biodiesel are the fatty acid methyl esters produced by reacting vegetable oils or animal fats in the presence of methanol or ethanol. The other renewable diesel, often called green diesel, is chemically the same as petrodiesel and can be generated using the same feedstock of biodiesel in existing petroleum refineries by hydrotreating process. In this process, hydrogen is used to convert the triglycerides into hydrocarbon (Yoon J, 2009). Both the alternative diesels have complete compatibility with petroleum diesel with high energy density, low specific gravity, excellent storage stability, and very low combustion emissions (Kalnes et al., 2008).

At present, the feedstocks for biodiesel and green diesel are vegetable oil and recycled greases. These feedstocks are not sufficient to meet the projected demand of biofuels. Lignocellulosic biomass based carbohydrates could be in use to produce microbial lipid that can be converted to biodiesel or green diesel. Table 2.1 presents the properties of petroleum diesel, biodiesel, and green diesel.

Table 2.1: Properties of Petroleum diesel and Biodiesel

Fuel Property	**Diesel**	**Biodiesel**	**Renewable Diesel (Green Diesel)**
Fuel Standard	ASTM D975	ASTM D6751	ASTM D975
Energy content, Btu/ gal	~129,050	~118,170	~123,000
Kinematic Viscosity, @ 40 0C	1.3–4.1	4.0–6.0	
Specific Gravity, kg/l @ 15.5 0C	0.85	0.88	0.78
Oxygen, by difference wt. %	0	11	0
Sulfur, ppm	<10	<1	<1
Cloud Point, ^{0}C	-35 to 5	-3 to 15	-20 to 20
Cold flow properties	Baseline	Poor	Excellent
Oxidative stability	Baseline	Poor	Excellent
Cetane Number	40-55	48–65	75-90

Source: U.S. Department of Energy: Biodiesel handling and user guide, 4th edition, 200; Kalnes et al., 2008

The U. S. Energy Information Administration (U. S. EIA) report presented in Table 2.2 shows the annual U. S. biodiesel production in 2014. Table 2.3 represents the current sources of biodiesel being produced. It shows that the feedstocks are mainly from food sources and there is no current commercial biodiesel production from microbial lipids. Extensive research is proceeding to find renewable and non-edible feedstocks for microbial fermentation towards lipid production.

Table 2.2: U.S. Biodiesel production capacity and production (U.S. EIA, 2015)

Year	Annual production capacity (Million gallons)	Annual B100[ϕ] production (Million gallons)
2013	2116	1359
2014	2130	1270 (Total producers: 98)

ϕ B100 is the industry designation for pure biodiesel

Table 2.3 U.S. feedstocks for biodiesel production (U.S. EIA, 2015)

	Feedstock	**Million pounds**
Vegetable Oils	Canola Oil	1046
	Corn Oil	970
	Palm Oil	63
	Soybean Oil	4802
Animal fats	Poultry	173
	Tallow	355
Recycled feed	White grease	427
	Yellow grease	1074
	Alcohol	941
	Total Biodiesel	9851

2.2 Microbial Lipids: Biochemistry of Lipid Accumulation

Microorganisms belonging to several different families, such as microalgae, bacillus, and fungi (molds and yeasts), possess the ability to produce and accumulate a large fraction of their dry mass as lipids. Those with lipid content in excess of 20% are classified as 'oleaginous.' Lipids produced by oleaginous microorganisms are considered as promising candidates for biofuels production because of their fatty acid composition is similar to that of

vegetable oils. Oleaginous microorganisms have many advantages for production of lipids, such as short life cycles, less labor requirement, less demand on space, venue, season and climate, and ease of scale up.

The major biological functions of lipids include cell signaling, energy storage, and the primary constituent of the cell membrane. All living cells accumulate lipids, either as a building material of membranes, or as energy reserves. Oleaginous microorganisms produce a significant fraction of their lipids as triacylglycerols (TAG) or neutral lipids, which makes them valuable sources of feed stocks in the production of biodiesel.

The induction of lipid accumulation depends on nutrient limitation, usually nitrogen, in the presence of excess carbon in the culture medium, referred to as the C/N ratio. After nitrogen exhaustion, the cell ceases the growth cycle but continues to assimilate the carbon source, resulting in storage as lipids (Ratledge, 2002). Previous studies showed that manipulation of C/N ratio in *Rhodortula glutinis* cultivated in batch mode could result up to a three-fold increase in lipid production (Granger et al., 1992). Nitrogen limitation adjusts the primary metabolism of the yeast to efficiently produce lipids because it is a key ingredient of important macromolecular components such as DNA, RNA, protein, etc. (Evans et al., 1984). In the absence of nitrogen sources, the production of such molecules is drastically reduced, and thus growth cycle is halted. To offset the lack of nitrogen, the enzyme AMP deaminase is activated to breakdown AMP to IMP and an ammonium ion. At the onset of nitrogen exhaustion, oleaginous cells show up to a five-fold increase in AMP deaminase activity compared to cells under normal condition (Ratledge et al., 1985).

$AMP + H_3O^+ \rightarrow$ Inosine-monophosphate + $NH4^+$

The ammonium ion can be used as a nitrogen source to maintain cell function, but is insufficient to continue the growth cycle. The drastic reduction in both energy requirements and AMP concentration results in a reduction of tricarboxylic acid (TCA) cycle activity, and the enzyme Isocitrate dehydrogenase (IDH) in particular, which is dependent on AMP for its activity in yeast (Ratledge, 2004; Papanikolaou et al., 2011). IDH is the enzyme responsible for the conversion of isocitrate to α-ketoglutarate in the TCA cycle. This decrease in IDH activity results in an accumulation of isocitrate in the mitochondrial matrix, whose concentration remains in equilibrium with citrate due to the action of the isomerase aconitase. As citrate concentration in the matrix increases, the flux through the citrate-malate shuttle increases, resulting in increased cytoplasmic citrate concentration (Ratledge, 2004).

Another key enzyme involved in lipid biosynthesis in oleaginous yeasts is ATP-citrate lyase (ACL) (Boulton et al., 1981). ACL is a cytosolic protein that catalyzes the cleavage of citrate using a Complex-A (CoA) carrier to form oxaloacetate and acetyl-CoA. ACL is considered the key enzyme for the oleaginicity of microorganisms, as it is absent in non-oleaginous microorganisms (Ratledge, 2004). The oxaloacetate can then be converted to malate by malate dehydrogenase, which can be transported back into the mitochondrial matrix, or converted to pyruvate. The acetyl-CoA serves as the precursor of lipid biosynthesis, since it supplies the first two carbon atoms for fatty acid (FA) synthesis. Acyl-CoA carboxylase (ACC) is a biotin-dependent multi-domain enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA. Once malonyl-CoA is synthesized, it is transferred to malonyl-CoA:ACP transacetylase (MAT), one of the fatty acid synthase (FAS) multi-enzymatic complex subunits, to form malonyl-ACP. A similarly primed acetyl-ACP can then be added to the malonyl-ACP by β-ketoacyl-ACP synthase, which catalyzes

the condensation reaction resulting in an elongated chain and the release of carbon dioxide (Beopoulos et al., 2009; Liang et al., 2013).

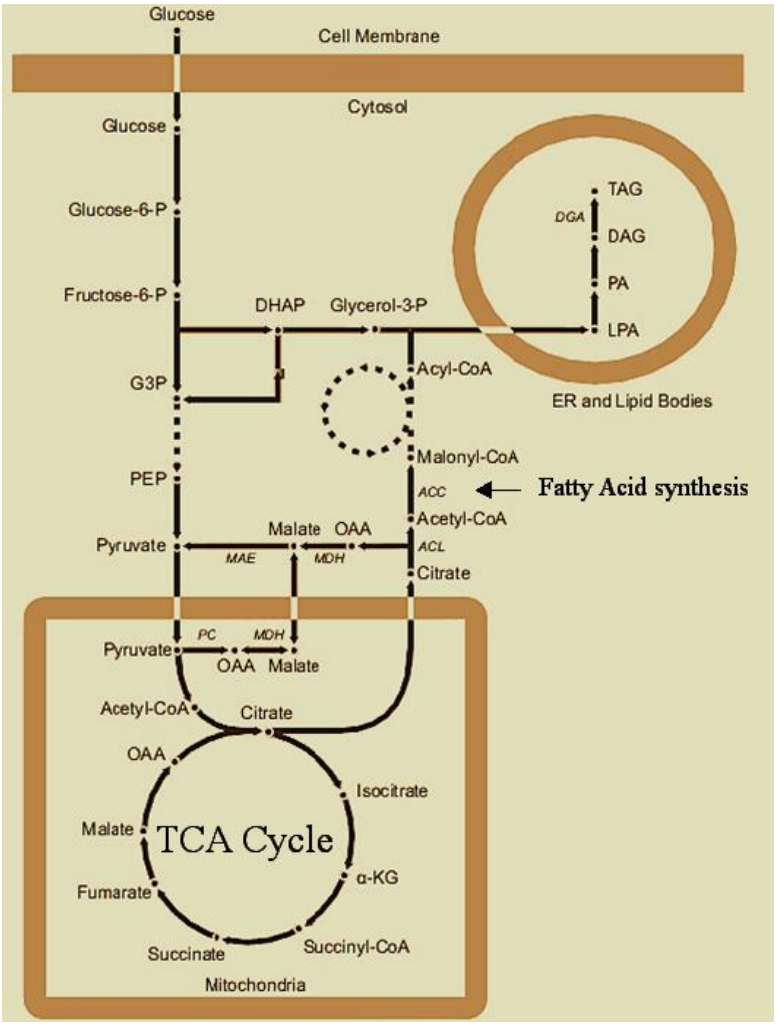


Figure 2.2.1: overall lipid production pathway in yeast (Tai et al., 2013)

This cycle is repeated to produce long chain fatty acids (LCFA), such as palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). Completed FA chains are then transported to the endoplasmic reticulum (ER) where they are used to produce TAG.

Each cycle of FA acyl chain elongation necessitates two molecules of NADPH. This NADPH is generated principally by the activity of the NADP-malic enzyme (NADP-ME). It is an oxidoreductase that catalyzes the reduction of $NADP^+$ to NADPH and the oxidation of malate to pyruvate and carbon dioxide, in the presence of a bivalent cation. The three enzymes ACL, ACC, and ME are believed to play a crucial role in determining the potential for lipid accumulation and in regulating the process (Ratledge, 2002; Beopoulos et al., 2009). When ME was inhibited in *Mucor circinelloides* and *Mortierella alpine*, lipid accumulation was halted (Wynn et al. 2001). In a recent study, Hamid et al. (2011) reported that at low ME and ACL activities, lipid accumulation ceased at 48 hours in *Cunninghamella sp* 2A1 as compared to *Yarrowia lipolytica* (Makri et al. 2010). However, Malic enzyme was not observed in a recent study on proteomic analysis of *Lipomyces starkeyi* (Liu et al., 2010). Instead, GND2p, 6-phosphogluconate dehydrogenase, was upregulated drastically. GND2p catalyzes an NADPH-regenerating reaction in the pentose phosphate pathway. Upregulation of GND2p was also observed with the oleaginous yeast *R. toruloides Y4* (Liu et al., 2011). Therefore, proteomic data suggested that the pentose phosphate pathway is likely functioning as an alternative reducing equivalent producer to drive the lipid accumulation process.

The first step of TAG synthesis is the acylation of glycerol-3- phosphate (G3P) with an acyl-CoA to form lysophosphatidate (LPA), which is catalyzed by acyl-CoA: glycerol-sn-3-phosphate acyl-transferase (GPAT). The LPA is then further condensed, catalyzed by lysophosphatidate acyl-transferase (LPAT), with another acyl-CoA to produce phosphatidate (PA). Afterwards, PA can be dephosphorylated by phosphatidic acid phosphatase (PAP) to produce diacylglycerol (DAG). At last, synthesis of TAG is catalyzed by acyl-CoA: diacylglycerol acyl-transferase (DGAT), which incorporates the third acyl-CoA into DAG.

This enzyme is also known as an important regulator for this pathway. It was observed that the deficits in TAG synthesis are associated with a striking accumulation of DAG, confirming DAG as a critical metabolic branch point in the Kennedy pathway for glyceride and glycerophosphatide synthesis. Overexpression of DGAT would commit more DAG to TAG formation rather than phospholipid formation (Tai et al., 2013, Liang et al. 2013)

2.3 Factors Influencing Microbial Lipid Production

Different nutritional and cultural conditions influencing the cell growth, lipid accumulation, and lipid composition has been summarized below.

Different carbon sources, such as ethanol, sewage sludge, xylose, and glucose have been used to grow oleaginous yeast, *L. starkeyi*, for microbial lipid production (Yamauchi et al., 1983; Angerbauer et al., 2007; Zhao et al., 2008). The growth of *L.starkeyi* in 1-L fed batch culture was studied with ethanol as carbon source and obtained cell dry mass of 153 g/L with lipid content of 54% in 140 hours with lipid yield of 0.21 (g lipids/ g ethanol) (Yamauchi et al., 1983). Angerbauer et al. (2007) studied growth of *Lipomyces starkeyi* in 1-L batch fermentation on sewage sludge and achieved 9.5 g/L cell mass growth and 68% of lipids content in 190 hours of fermentation. Zhao et al. (2008) studied the growth of *Lipomyces starkeyi* on mixed sugars of glucose and xylose (2:1 w/w) as carbon source and they observed the cell density as 19 g/L with 61% of lipid content after 180 hours of cultivation. The effect of nutrients on the cell growth and lipid content using different carbon sources including D-glucose, D-mannose, D-cellobiose, and L-arabinose were studied with different oleaginous species such as *Lipomyces mesembrius, Lipomyces doorenjongii, Lipomyces kockii, Lipomyces tetrasporus, Lipomyces kononenkoae, Lipomyces lipofer,* and *Lipomyces starkeyi.* Of these strains, *L. starkeyi* showed the maximum ability to convert all types of

sugars into lipids (Oguri et al., 2011). The deficiency of ammonium, phosphate, potassium, magnesium, sulfate, zinc, calcium, ferrous, and manganese in the culture medium showed decreased total cell number. These are the essential element for normal growth of *L. starkeyi*. Manganese and zinc adequacy increased the total cell number and lipid, respectively. The concentration of sodium, chloride, copper, bromide, iodide, molybdenum oxide and biotin had almost no effect on the cell mass, lipid content, and lipid yield of *Lipomyces starkeyi* (Naganuma et al., 1985).

Oxygen demand during the lipid accumulation stage depends on the oleaginous yeast species and the culture conditions used. It was observed that under conditions of low dissolved oxygen, increased amounts of saturated FAs mainly palmitic acid (C16:0) and stearic acid (C18:0), were detected, and further increasing the oxygen content produced greater degrees of unsaturated FAs, specifically oleic acid (C18:1) and linoleic acid (C18:2) (Davies et al., 1990). The lipids in anaerobically grown yeast are characterized by a high percentage of C8:0 to C14:0 acids in the glycerides fraction and low level of unsaturated fatty acids in the phospholipid and sterols fraction. On the other hand, aerobically grown cells have around 80 to 90 % of their fatty acids (i.e. C16:0 and C18:1) associated with glycerides (TAG) and phospholipids (Rattray et al., 1975; Ratledge & Hall, 1977; Pan & Rhee, 1986a; Probst K, 2014). In concise, oxygen demand during lipid production was lower than during the cellular growth stage (Ratledge & Hall, 1977; Pan & Rhee, 1986a).

The effect of temperature on the growth and lipid content of *Lipomyces starkeyi* was studied in a 10L fermenter by Suutari et al. (1993). The maximum specific growth rate of 0.15 hr^{-1} and lipid content of 55% was obtained at growth temperature of 28°C. Lipid accumulation was faster with the temperature of 28°C than with the temperature of 16-18°C. The effect of

temperature on fatty acid composition was observed in the same study. The oleic acid composition increased from about 52 to over 60% but palmitic acid decreased from about 33 to 26%when the temperature decreased from 28 to 15 °C (Suutari et al. 1993). However high cell growth and lipid accumulation, were reported at the temperature of 30°C with *Lipomyces starkeyi* (Yamauchi et al., 1983; Evans and Ratledge, 1984).

Naganuma et al. (1974) studied the effect of pH on cell growth of *Lipomyces starkeyi* on glucose in shake flasks. Cells were grown on glucose medium with initial pH of 6.7, 6.5, 6.0, and 5.0 in different experiments. Experiments with initial pH of 6.5, 6.0 and 5 showed dropping of pH values while increasing the cellular growth. The pH came down to 2.2 in all fermentations with initial pH 6.5, 6.0 and 5.0. In medium of pH 6.7, optical density increased slightly in the range of 0.02-0.05 within the first 20 h and then remained constant. With pH of 6.5 growths was faster compared to cells grown in glucose medium of pH 6.7, but was lower compared to pH 6.0 and 5.0. The medium of pH 6.0 and 5.0 showed the growth pattern almost identical with high growth rate. Most of the research conducted on *L. starkeyi* for growth and lipid studies has used pH range of 5.0 to 6.0 (Yamauchi et al., 1983; Suutari et al., 1993; Angerbauer et al., 2007; Wild et al., 2010).

One of the most important factors in TAG accumulation is the carbon-to-nitrogen (C/N) ratio. As the ratio increases, excess carbon becomes available for lipid production. Too high of a ratio (i.e., limited nitrogen) will limit cellular growth, biomass production, and even result in significant secondary metabolite formation; thus, optimal C: N ratios depend on the production method, fermentation conditions and yeast species used (Beopoulos et al., 2009). Most researchers have reported that C: N ratios vary from 50-150 in lipid production by oleaginous yeasts (Ratledge, 2010; Ageitos et al., 2011). For example, Angerbauer et al.

(2008) reported that *L. starkeyi* produced a lipid content of 68% (dry mass basis) when grown with the C: N ratio of 150 compared with 40% lipid content (dry mass basis) with the C: N ratio of 60.

Economical lipid production can be achieved by increasing cellular lipid contents along with higher volumetric lipid productivity. High lipid productivity can be reached by fed batch, repeated fed batch and continuous fermentations. Fed-batch fermentation is a common technique in lipid production due to its ease of nutrient control during cellular growth and lipid accumulation phases (Yamauchi, 1983; Beopoulos et al., 2011; Zhang et al., 2011; Zhao et al., 2011). Lipid productivity of 0.59 g/L/h and 54% of lipid content was obtained with *Lipomyces starkeyi grown* on ethanol using fed-batch cultures (Yamauchi et al., 1983). A lipid productivity of 0.88 g/L/h was reported using the fed-batch culture of *Rhodotorula glutinis* aerated with oxygen-enriched air (Pan et al., 1986). The oleaginous yeast *Rhodosporidium toruloides* Y4 grown on glucose substrate under fed-batch fermentation resulted lipid productivity of 0.54 g/L/h and cellular lipid content of 67.5%, respectively (Li et al. 2007). Another fed-batch cultivation via a two stage operation (nutrient rich medium and glucose only medium) in a fermenter using *Lipomyces starkeyi* produced a lipid productivity of 1.6 g/L/h with the lipid content of 65% (Lin et al., 2011).

Zhao et al. (2011) reported a lipid productivity 0.55 g/l/h and lipid content 61.8% of *Rhodosporidium toruloides* Y4 grown on glucose by using repeated fed-batch feeding strategies. High cellular concentrations and high lipid productivities were obtained when the glucose concentration was maintained at 5 g/L rather than 30 g/L. This result indicates that controlled substrate loading is an important factor in fed-batch operation. Previous work at UL Lafayette (Yvonne, M., 2011) reported lipid productivity and lipid content of 0.16 g/L/h,

and 75%, respectively, using *Lipomyces starkeyi* on sweet potato starch by repeated fed-batch cultivation.

Continuous chemostat operations can be used as an effective fermentation technique for lipid production (Ykema, 1988; Brown et al., 1989; Alvarez et al., 1992; Papanikolaou & Aggelis, 2002). The yeast *Apiotrichum curvatum* grown in a continuous culture system on glucose reached a lipid productivity of 0.42 g/L/h and lipid content of 31.9% (w/w) (Hassan et al., 2009). Alvarez et al. (1992) used a dilution rate of 0.04/h to achieve a lipid productivity of 0.24 g/L/h with *R. glutinis*. So, higher lipid productivity can be achieved by controlling substrates using appropriate method of fermentation. The Table 2.4 represents the cell mass production, lipid fraction, feed stock and experimental conditions for lipid production by oleaginous yeast. It shows that *Lipomyces starkeyi* possess the highest lipid fraction and they are able to grow on multiple sugars.

Table 2.4: Cell growth, lipid fraction and fermentation conditions of different oleaginous yeasts (Ageitos et al., 2012)

	Cell mass, g/L	% lipid, g/g	T ^{0}C	Time, hr	Carbon source	References
Apiotrichum curvatum UfaM3	15	45.6	30	-	Glucose	Hassan et al. 1993
A. curvatum Ufa25	15	40	30	150	Whey	Ykema et al. 1989
A. curvatum ATCC20509	85	35	30	70	Lactic-permeate	Ykema et al. 1988
A. curvatum ATCC20509	15.1	47	32	145	Lactose MM	Park et al. 1990
C. curvata	10.6	27	28	72	Glucose	Heredia and Ratledge, 1988
	8.2	30	28	72	Xylose	
Cryptococcus curvatus	91	33.3	28	75	Glycerol	Meesters et al. 1996b
C. curvatus	118	25	28	50	Glycerol	Meesters et al. 1996
C. curvatus ATTC 20509	18.4	49	30	96	Lactose+ fish oil	Iassonova et al. 2008
C. terricolus	16	39	25	184	Glucose	Boulton and Ratledge 1984
L.starkeyi	20.5	61.5	30	120	Glucose+ Xylose	Zhao et al. 2008
L. starkeyi DSM 70295	13.3	56.3	30	220	Glucose, sludge	Angerbauer et al. 2008
L. starkeyi AS 2. 1390	18	30	28	96	Glucose	Li et al. 2005
	20.9	20.5	28	96	Xylose	
	14.2	24.9	28	96	L-arabinose	
L. starkeyi	18.2	76	30	120	Glucose	Zhao et al. 2008
R. toruloides Y4	151.5	48	30	600	Glucose	Li et al. 2007
	106.5	67.5	30	134	Glucose	
	6.9	42	28	96	Glucose	Li et al. 2005

	Cell mass, g/L	% lipid, g/g	T ^{0}C	Time, hr	Carbon source	References
R. toruloides AS 2. 1389						
	7.2	26.8	28	96	Xylose	
	4.8	16.8	28	96	L-arabinose	
R. glutinis NRRL y-1091	185	40	-	-	Glucose, O_2 enriched air	Pan et al. 1986
R. glutinis AS 2. 703	5	30.2	28	96	Glucose	Li et al. 2005
	6.9	28	12	96	Xylose	
	4.3	4.9	28	96	L-arabinose	
R. toruloides Y4	127.4	61.8	30	140	Glucose	Zhao et al. 2010
Trichosporon cutaneum AS 2. 571	3.2	65.6	28	96	Glucose	Li et al. 2005
	4.2	13.4	28	96	Xylose	
	5.6	8.2	28	96	L-arabinose	
Yarrowia lipolytica	8.7	40	28	240	Industrial lipids + glycerol	Papanikolaou and Aggelis 2002
R. glutinis IIP-30	17.2	39	30	120	Molasses+glucose + sucrose	Johnson et al. 1995
Y. lipolytica ACA-DC 50109	15	44	28	120	Animal fats	Papanikolaou et al. 2001

T: Temperature

2.4 Structure and Composition of Lignocellulosic Biomass

The major lignocellulosic feedstocks that can be used as a source of sugars are agricultural residues, (stalks, leaves, and husks of the plant, corncobs, rice straw, wheat straw) forest materials (slash, pre-commercial thinnings, solid tree residue) and cellulosic components of

separated food and yard waste from food processing industry. Fast growing trees and grasses (including switchgrass, miscanthus, energy cane, giant reed, and napier grass) can play a vital role in the emerging technology of biofuels production (Schnepf & Yacobucci, 2013.). The feedstocks should produce sustainably on land that would not be competitive to food crops. The short pathway of lignocellulosic biomass conversion to fuels has been presented in Figure 2.4.1.

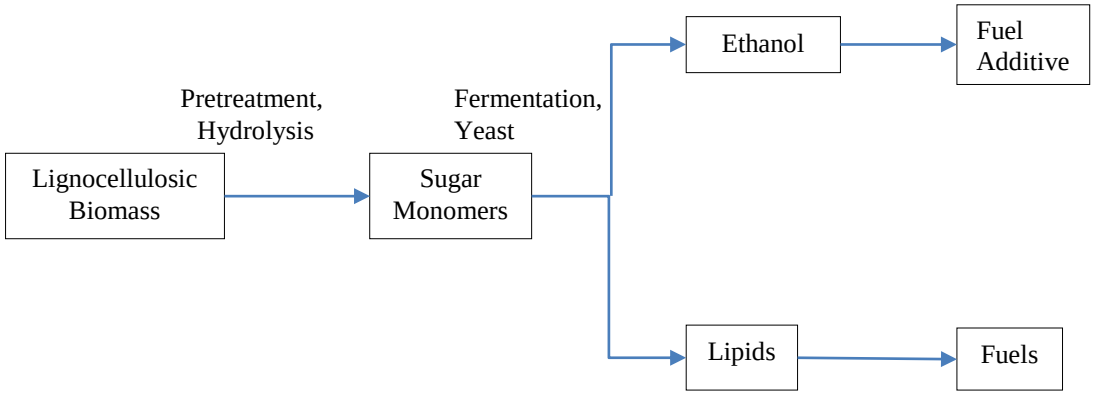


Figure 2.4.1: Pathway of lignocellulosic to fuels

The key components of lignocellulosics are cellulose, hemicellulose, and lignin. These polymers are closely associated with each other forming the complex structure of the plant biomass. Cellulose is the major components of the complex skeleton surrounded by hemicellulose and lignin (Mussatto et al., 2010). The Figure 2.4.2 demonstrates the simple orientation of lignin, hemicellulose, and cellulose.

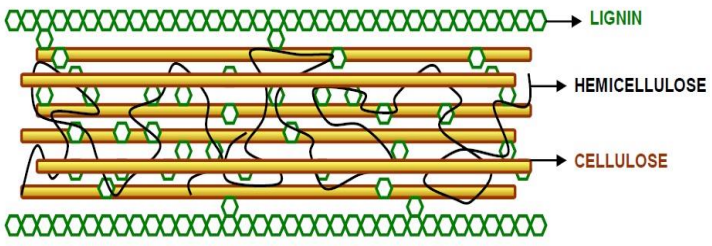


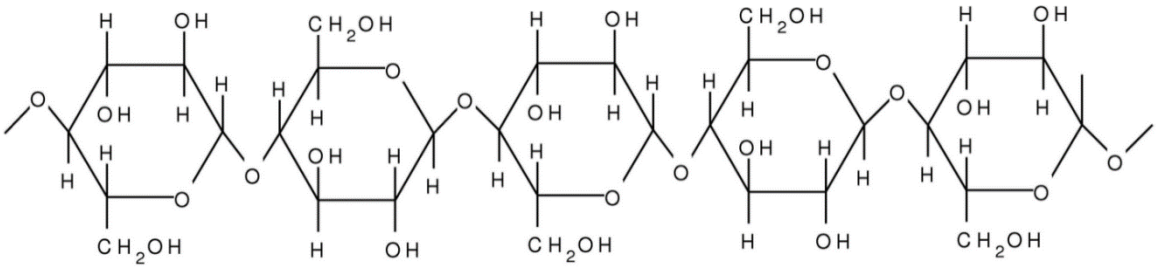
Figure 2.4.2: Basic structure of lignocellulosic biomass (Mussatto et al., 2010)

The overall composition of carbohydrates in lignocellulosic biomass is about 40-50% cellulose, 25-30 % hemicelluloses and 15-20 % lignin on dry basis (Menon and Rao, 2012). However, the actual composition of carbohydrates depends upon the source of biomass. Lignocellulosic compositions from different source of lignocellulosics are presented in Table 2.5 (Saha, 2003; Zhu et al., 2010; Shafei et al., 2015)

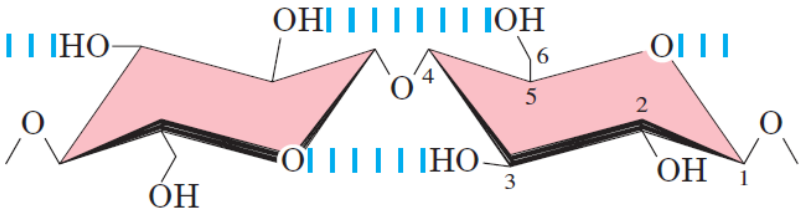
Table 2.5: Compositions of lignocellulosic biomass from different sources

	Composition (% dry basis)		
	Cellulose	Hemicellulose	Lignin
Softwood			
Spruce	40–46	21–31	27–29
Pine	40–46	18–29	25–30
Douglas	44	21–27	28–32
Hardwood			
Oak	45	24	24
Eucalyptus	45–48	13–19	27–31
Birch	41–49	21–32	21–22
Poplar	34–44	19–22	23–25
Maple	44–46	17–23	23–24
Aspen	46–50	18	18–23
Agricultural residues			
Switchgrass	36–43	12–25	23–28
Wheat	33–50	24–36	9–17
Rice	28–47	19–25	10–25
Corn	34–36	22–29	7–20.2
Sugarcane	40–41	27–38	10–20

Cellulose is a high molecular weight linear homopolysaccharides of cellobiose (consists of two glucose molecules) linked together by β-l- 4-glycosidic bonds. Cellobiose is the unit component of cellulosic chain having the hexose, D-glucose as the main sugar component (Figure 2.4.3 a, b).



(a)



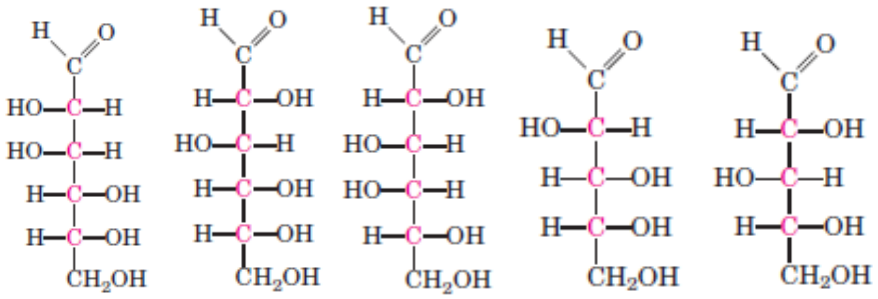
(b)

Figure 2.4.3: (a) structure of cellulose; (b) Cellobiose with two glucose unit

The long-chain cellulose polymers are bonded together by hydrogen bonds within hydroxyl groups and oxygen atoms among adjacent chains. Hydrogen bonding and van der Waals forces cause the cellulose to be packed into microfibrils and being arranged in parallel stacking to form bundle of microfibrils or crystalline cellulose (Somerville, 2006; Ha et al., 2010; Li et al., 2014). Cellulosic microfibrils create highly ordered crystallinity (about two third of the total cellulose); this complicated structure makes cellulose less degradable and high resistance towards chemical and biological pretreatment for sugar extraction (Taherzadeh et al., 2008; Mussatto et al., 2010).

Hemicellulose, the second most abundant polysaccharide in lignocellulosics, is a linear and branched heteropolymer of pentoses (D-xylose, D-arabinose) and hexoses (D-mannose, D-glucose, and D-galactose) as well as some other sugar acids including acetic acid, ferulic acid

and D-glucuronic acid. In Figure 2.4.5, the monomer sugars derived from hemicellulose are presented in linear structure. The most abundant hemicelluloses are xylans and glucomannans; hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans (Ji et al., 2008). Xylose is the primary building block for heteropolysaccharide xylan of hemicellulose and the composition of hemicellulose depends on the type of plants. For example, xylan is dominant, typically about 30%, in hardwoods and agricultural plants. The branches of xylan differ from species to species and can also contain arabinose, glucuronic acid, or the 4-O-methyl ether, acetic, ferulic, and p-coumaric acids (Ji et al., 2012). Hemicelluloses are differed from cellulose by composition of sugar units of shorter chains, by a branching of lateral chains with different sugars, and being amorphous which build their structure more degradable by hydrolysis than cellulose. Hemicellulose is also hydrophilic and serves as support for cellulose microfibrils. The degree of polymerization (DP) of hemicellulose is typically in the range of 50 to 300 while the DP



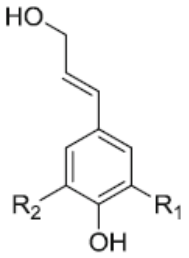
D- Mannose D- Glucose D-Galactose D- Arabinose D- Xylose

Figure 2.4.4: Monomers of Hemicellulose

Lignin is an amorphous, cross-linked, and three dimensional complex aromatic phenolic polymers. It consists of three phenyl propane units called guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units, and their respective precursors are three phenyl propionic alcohol

components (monolignols), namely, coniferyl, sinapyl, and p-coumaryl alcohols (Hu et al.,2012).

In general, softwood lignin is almost exclusively composed of guaiacyl units (G lignin), with a small quantity of p-hydrophenyl units (H lignin), whereas hardwood lignin contains both guaiacyl and syringyl units (G and S lignin) with a small proportion of p-hydrophenyl units as well. The Figure 2.4.5 represents the structure of lignin in a common form. From this common structure three building blocks of lignin can be formed by replacing R_1 and R_2 with corresponding functional group.



Coniferyl alcohol/ guaiacyl: R_1= OCH_3, R_2=H
Sinapyl alcohol/ syringyl: R_1=OCH_3=R_2
P-coumaryl alcohol/ p-hydrophenyl: R_1=H=R_2

Figure 2.4.5: Three building blocks of lignin

Additionally, lignin derived from grass and herbaceous crop contains all the three units (G, S, and H lignin) along with p-hydroxycinnamic acids (p-coumaric acid, ferulic acid, and sinapic acid). Lignin is relatively hydrophobic and covalently linked to hemicelluloses, and it fills the spaces in the cell wall between cellulose and hemicelluloses (Klinke et al., 2004; Pu et al., 2010, Hu et al., 2012,). As a structural component lignin provides a structural role of the matrix in which cellulose and hemicellulose is embedded and it protects water impermeability to xylem vessels of the plant, and form a barrier against microbial attack (Mussatto et al., 2010). Lignin also contains methoxyl, phenolic, hydroxyl and aldehyde

groups in the side chain with low solubility in most solvents (Nanda et al., 2013). In general, softwoods have higher lignin content than hardwoods; so, hardwoods have a greater amount of cellulose and hemicellulose than softwoods (Demirbas, 2006; Nanda et al., 2013). Lignin, due to its complex molecular structure, makes the lignocellulosics extremely resistant to chemical and enzymatic treatment to extract fermentable sugars.

2.4.1 Pretreatment and Byproducts of Lignocellulosic Biomass

Due to the recalcitrant nature of lignocellulosics, they need to be pretreated (e.g. milling, grinding, extrusion, and pressing to reduce the size and crystallinity) before further processing to extract the sugar monomers from cellulose and hemicellulose for microbial fermentation. The conversion of lignocellulosic biomass to fermentable sugars requires: (1) delignification to liberate cellulose and hemicellulose from the complex with lignin and (2) depolymerization of the carbohydrate polymers to release free sugars (Nanda et al., 2013). Hydrolysis, usually catalyzed by acids or cellulase enzymes, is the prime step to convert the lignocellulosic components to fermentable sugars. Lignocellulosic hydrolysis depends on porosity of the materials, fiber crystallinity of cellulose, and amount of lignin present in the biomass. The hydrolysis process consists of removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity (McMillan et al., 1994; Kumar et al., 2009; Saratale and oh, 2012).

An ideal lignocellulosics hydrolysis method should have (1) high hydrolysis efficiency, (2) limited formation of byproducts from hydrolysis process, and (3) cost effectiveness (Kumar et al., 2009). There are various methods that have been developed in recent years for the lignocellulose hydrolysis; the most common methods are briefly described below in Table 2.5.

Table 2.5: Various physical, chemical and biological treatment methods of lignocellulosic biomass (Kumar et al., 2008; Zhang et al., 2009; Saratale et al., 2012; Nanda et al., 2013)

Treatment method	Source	Advantages	Disadvantages
Physical treatment	Autohydrolysis: Steam pressure, steam explosion, hydrothermolysis, steam and mechanical sheer, pyrolysis, dry heat expansion, moist heat expansion	Causes hemicellulose degradation and lignin transformation; cost-effective	Destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms
	Irradiation: Gamma, electron beam, photooxidation	Gamma, electron beam, photo-oxidation Increase accessible surface area	High cost
Chemical treatment	Acids: Dilute or concentrated sulfuric acid, dilute or Concentrated hydrochloric acid, nitric, phosphoric, acetic	Remove hemicelluloses and lignin; increase accessible surface area	High cost; equipment corrosion; formation of inhibitory substances
	Alkali: Sodium hydroxide, ammonium hydroxide	Hydrolyzed hemicellulose to xylose and other sugars; alters lignin structure	Long residence times required; irrecoverable salts formed and incorporated into biomass; difficulty in recovering bases
	Oxidizing Agents: Peracetic acid, sodium hypochlorite, sodium chlorite, hydrogen peroxide	Increase accessible surface area, removes lignin and hemicellulose to small extent	Expensive and not effective for biomass
	Solvents: Organosolv: Methanol, ethanol, butanol, phenol, ethylamine, hexamethylenediamine, ethylene glycol,	Hydrolyzes lignin and hemicelluloses	Solvents need to be removed from the reactor, high cost

Treatment method	Source	Advantages	Disadvantages
Biological treatment	Cellulolytic Microorganism: Bacteria, Fungi and Actinomycetes	Degrades lignin and hemicelluloses; low energy requirements	Rate of hydrolysis is very low; Use of reducing sugar by microorganisms for their growth limits the application
	Cellulolytic Enzymes: Endoglucanases (endo-l,4-[3- D-glucan-4-glucanohydrolase, EC 3.2.1.4) Exoglucanases (exo-l,4-[3-D-glucan-4-cellobiohydrolase, EC 3.2.1.91) β-glucosidases (β-d-glucoside glucohydrolase; EC 3.2.1.21)	Increase accessible surface area, cause formation of less inhibitory compounds Hydrolysis of cellulose into fermentable sugars for the production of biofuels. Little energy requirement and mild reaction conditions, high yield of sugars, and high hydrolysis efficiency	Due to enzyme cost, process becomes expensive
	Hemicellulose degrading enzymes: Endoxylanases (1,4-[3-D-xylan xylanohydrolase, EC 3.2.1.8) Exoxylanase (1,4-[3-D-xylan xylohydrolase, EC 3.2.1.37) Xylosidase (1,4-[β-D-xylan xylohydrolase, EC 3.2.1.37) α-L-arabinofuranosidase (EC 3.2.1.55)	Depolymerization of hemicellulose to monomeric sugars for biofuels and other valuable chemicals production. Increase accessible surface area; does not cause formation of inhibitory compounds; Little energy requirement and mild reaction conditions, high yield of sugars, and high hydrolysis efficiency	Due to enzyme cost process becomes Expensive
	Lignin degrading enzymes:	Useful biological tool for the degradation of lignin. For the delignification of wood	Due to enzyme cost process becomes Expensive

Treatment method	Source	Advantages	Disadvantages
	Lignin peroxidase (ligninase, EC 1.11.1.14) Manganese peroxidase (EC1.11.1.13) Laccases (benzenediol: 02 oxidoreductase, EC 1.10.3.2)	and agricultural residues to increase the digestibility. Increase accessible surface area; cause formation of less inhibitory compounds	

Figure 2.4.6 shows the distorted structure of lignocellulosic biomass after treatment with physical and chemical or biological methods.

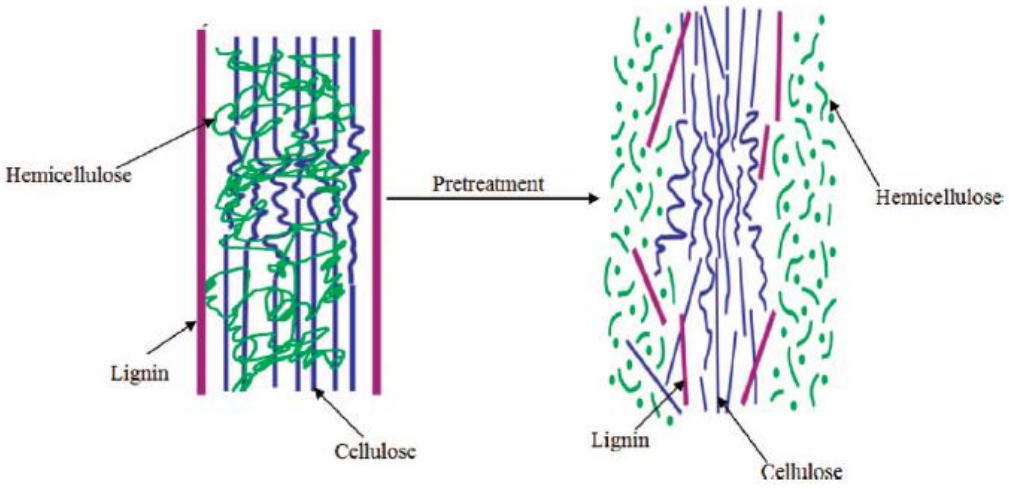


Figure 2.4.6: Lignocellulosics break down after pretreatment (Kumar et al, 2009)

The key factors of acid hydrolysis, are acid concentration, temperature and residence time (Mussatto et al., 2010). The acid hydrolysis with concentrated H_2SO_4 or HCl (10– 30%) at high temperatures and pressures of about 160^0C about 10 bar (Sun et al., 2002; Kumar et al., 2009) are more capable to break down the structure of celluloses. But this process produces byproducts which are inhibitory to microorganisms.

Dilute acid (1-4%), usually H_2SO_4, HCl, HNO_3 and medium temperature (120^0 to 160^0) is able to hydrolyze hemicelluloses more effectively than cellulose and lignin to its monomers, mainly xylose and arabinose (Saha et al.2003; Mussatto et al., 2006). The long chains of

hemicellulose can be broken down by the acid catalysis to form shorter chain oligomers and subsequent sugar monomers. The advantages of using dilute acid treatment is the generation of less toxic byproducts and less corrosion problems in hydrolysis tanks (Carvalheiro et al., 2008).

Steam explosion is used for lignocellulosic biomass hydrolysis. In this method, the biomass is heated using high-pressure saturated steam (0.69-4.83 MPa, 160-260^{0}C) for a short period (from seconds to few minutes). Steam condenses under high pressure, thereby wetting the material, and then the pressure is suddenly reduced, which makes the material undergo an explosive decompression (Carvalheiro et al., 2008; Kumar et al., 2009). Limitations of steam explosion include an incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms (Sun et al., 2002).

Autohydrolysis is a process similar to the steam explosion, but in this case, the explosion does not occur. This process uses compressed liquid hot water (200 ^{0}C, pressure > saturation point) and the acids resulting from hydrolysis of acetyl and uronic groups, originally present in hemicelluloses, catalyze hydrolysis of hemicellulose, lignin and carbohydrates. This process is able to hydrolyze hemicellulose in minutes, with high yield, low byproducts formation and no significant lignin solubilization (Carvalheiro et al., 2008).

Enzymatic hydrolysis is also able to break down the cellulose chain and it has gained attention as a replacement of harsh, concentrated acid hydrolysis. The advantages of enzymatic pretreatment are milder conditions with higher pH (about 5) and lower temperature (less than 50 ^{0}C). Moreover, this process requires less energy consumption, less environmental impact, and no corrosion problems, and more importantly, it provides high

yield of glucose extraction with low formation of toxic byproducts (Wen et al., 2004; Mussatto et al., 2010).

Enzymatic hydrolysis of cellulose is conducted by a mixture of several cellulase enzymes, among which the following three plays a major role. (1) 1-4-endoglucanase (EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber creating free chain ends; (2) -1-4-exoglucanase or cellobiohydrolase (EC 3.2.1.91.), which degrades the molecule further by removing cellobiose units from the free chain ends; (3) glucosidase or cellobiase (EC 3.2.1.21.), which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988; Sun et al., 2002; Mussatto et al., 2008).

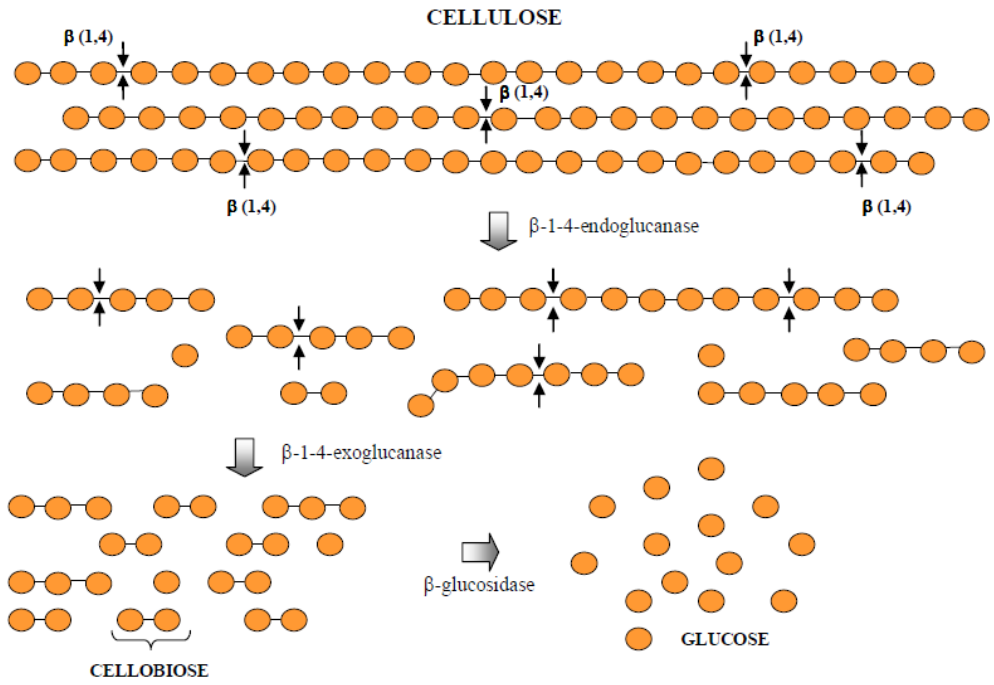


Figure 2.4.7: Break down of cellulose by cellulase enzymes (Mussatto et al., 2010)

In Figure 2.4.7, cellulose has been cleaved to glucose unit by cellulase enzymes. A wide range of microorganisms, both bacteria and fungi, can produce cellulases from the hydrolysis

of lignocellulosic materials including *Clostridium*, *Bacillus*, *Trichoderma*, *Aspergillus (*Sun et al., *2002;* Varga et al., 2002*).*

Three main enzymes involved in the lignin biodegradation are, namely lignin peroxidase, manganese peroxidase, and laccase. These enzymes have gained large attention by their industrial applications in pulp and paper industries, for biochemical pulping and decolorization of plant effluent. Biological or enzymatic treatments cannot be applied directly on the raw materials because lignin hinders the attack of enzymes to the material cell wall. Therefore, pretreatment is crucial for the enzymatic hydrolysis of lignocellulosic biomass to promote a partial removal of lignin and hemicellulose, so that the cellulose fibers become more accessible to the enzymes. It is important to emphasize that the selection of a treatment method affects the cost and performance in the subsequent hydrolysis and fermentation stages.

Biological treatments based on the use of brown, white and soft-rot fungi being considered a cheap and effective method of delignification. Degradation of lignin by fungi such as *Phanerochaete chrysosporium, Trametes versicolor, Trametes hirsuta* and *Bjerkandera adusta*, allow better access to the cellulose and hemicellulose components and considered as an effective biological detoxification. Main problems in using biological methods are that fungi may also attack cellulose and hemicellulose, in addition to slower rate of hydrolysis (Sun et al., 2002).

Alkaline treatments, ozonolysis, peroxide treatments, and organic solvents (Table 2.5) are some of the methods usually employed for lignin removal from lignocellulose biomass. Such methods are effective for lignin solubilization but in most of cases, part of the hemicellulose

is also hydrolyzed (Mussatto et al., 2010). NaOH, Ca $(OH)_2$ or NH_3 are most commonly used for alkali treatments. Among these, NaOH is the most used for delignification of agricultural residues (Iglesias et al., 1996). The alkali treatment causes swelling leading to an increase in internal surface area, decrease in the degree of polymerization, decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Iglesias et al., 1996). As a consequence, the lignin is separated in the form of liquor; rich in phenolic compounds that represents the process effluent (Mussatto et al., 2007). The disadvantage of this technique is that it also degrades part of the hemicellulose. Hydrogen peroxide treatment uses alkaline solutions at temperatures higher than 100 ºC, which promote a fast decomposition of H_2O_2. As a consequence, reactive radicals such as hydroxyl radicals (HO^-) and superoxide anions (O^{2-}) are produced, which are responsible for lignin degradation. This technique is commonly used in paper and pulp industries for bleaching and delignification purposes (to improve the brightness of pulp as it reacts with colored carbonyl containing structures in the lignin). However, delignification by this method on a large scale would be costly.

Treatment with organosolvents involves the use of an organic liquid (for example, methanol, ethanol, acetone, ethylene glycol or triethylene glycol) and water, with or without addition of catalysts such as oxalic, salicylic, and acetylsalicylic acid. This mixture hydrolyzes lignin bonds and lignin-carbohydrate bonds, as well as carbohydrate bonds in the hemicellulose components. Lignin is dissolved as a result of the solvent action and the cellulose remains in the residual solid material (Sun et al., 2002; Taherzadeh et al., 2008). Ozone treatment is another way of reducing the lignin content of lignocellulosic materials. Lignin attacks as a scavenger during this pre-treatment because it consumes most of ozone during the

degradation of the carbohydrate content. As a consequence, low ozone amounts are available for cellulose degradation. However, this treatment may also attack the cellulose and hemicellulose components besides the lignin molecule. (Nigam et al., 2009).

The limitations of existing pretreatment processes include incomplete separation of cellulose and lignin, formation of byproducts that inhibit the microbial growth, high use of chemicals and energy. Based on their origin, the byproducts are usually divided in three major groups: weak acids, furan derivatives, and phenolic compounds. These compounds limit efficient use of the hydrolysates for lipid production by fermentation. In the Figure 2.4.8, inhibitory byproducts formed from different components of lignocellulosic biomass are shown.

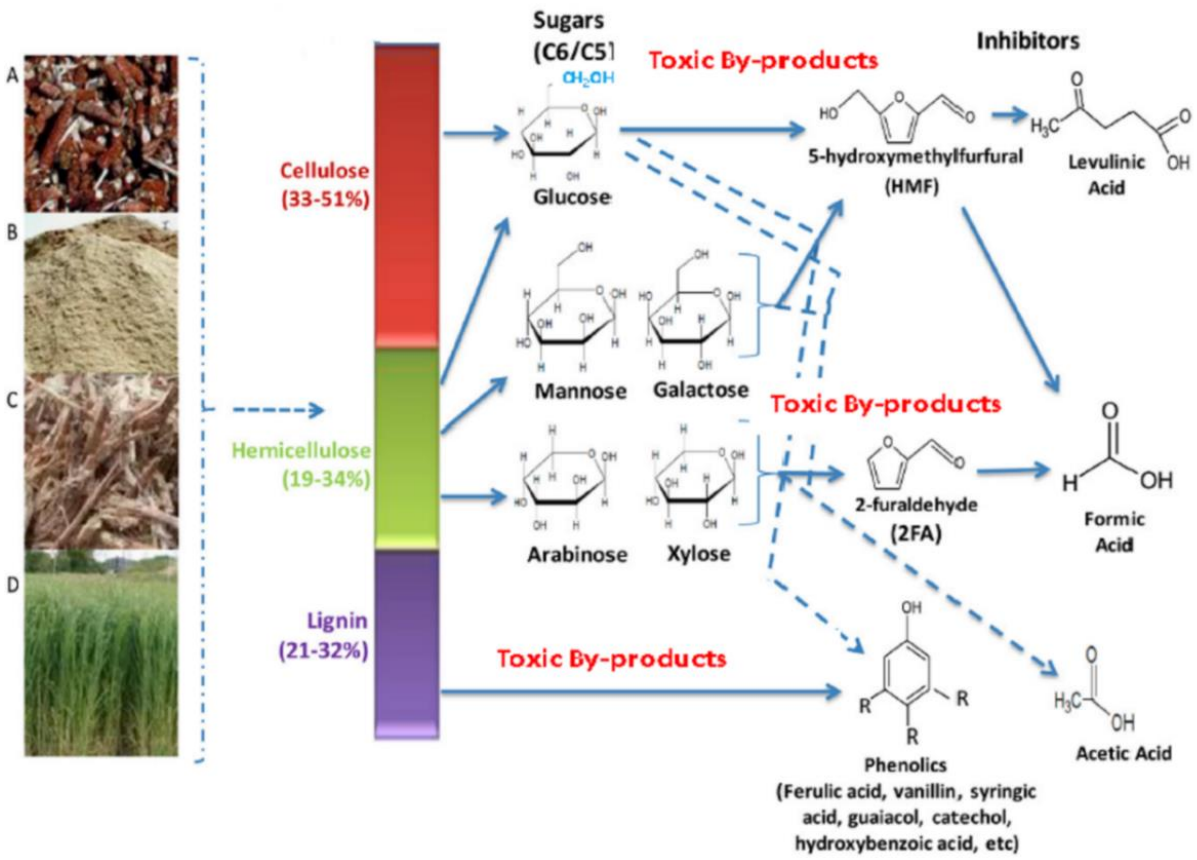


Figure 2.4.8: Products from acid hydrolysis of lignocellulosic biomass (Ibraheem et al.,2013)

The byproducts of lignocellulosic biomass hydrolysates are furan derivatives such as furfural and 5-hydroxymethylfurfural (HMF), phenolic compounds, weak organic acids (levulinic, formic, and acetic acid) (Huang et al. 2011). Furfurals are generated during xylose degradation while HMF is generated during hexose degradation. Phenolics generated from lignin breakdown, may exist in three different forms: acid, ketone, and aldehyde. The type of toxic compounds and their concentration in hydrolysates depend on both the raw material and the method of treatments. For instance, furfural concentration from wheat straw hydrolysates is 0.15 g/L (Nigam et al., 2001) while the concentration is 0.5 g/L from rice straw hydrolysates (Huang et al., 2009). Acetic acid concentration from wheat straw is 2.7 g/L (Nigam et al., 2001) and the concentration from rice straw is 1.4 g/L (Huang et al., 2009). The different byproducts formed from different sources of lignocellulosic biomass and from different treatment methods are presented in Table 2.6.

Table 2.6: List of byproducts and their concentration, and sugar composition from lignocellulosics hydrolysates from different treatment methods

References	Source of lignocellulosic	Pretreatment method	Sugar concentration, g/L	Concentration of byproducts from hydrolysates, mg/L
Zhu e al.,2011	Corn stover	2% H_2SO_4, 100 ^0C	Glucose: 7 Xylose: 51 Arabinose: 8	Acetic Acid: 4700 Furfural: 570 HMF: 3200
Alriksson et al., 2011	Spruce	4% H_2SO_4, 200 ^0C	Glucose: 82.9 Xylose: 9.1 Mannonse:26.5 Arabinose: 2.8 Galactose: 9.8	Acetic Acid:3100 Furfural: 1200 HMF: 1600
	Sugar cane bagasse	4% H_2SO_4, 200 ^0C	Glucose: 86.3 Xylose: 14.5 Mannonse:0.5 Arabinose: 0.2 Galactose: 9.8	Acetic Acid:3600 Furfural: 900 HMF: 3000

References	Source of lignocellulosic	Pretreatment method	Sugar concentration, g/L	Concentration of byproducts from hydrolysates, mg/L
Zeng et al.,2013	Wheat straw	2% H_2SO_4, 121 ^{0}C	Glucose: 3 Xylose: 18 Arabinose:2	Acetic acid: 2000 Furfural: 350 HMF: 40
Rumbold et al., 2009	Sugar cane bagasse	2 % H_2SO_4, 160 ^{0}C	Glucose:15 Xylose: 6.2 Arabinose: 0.8	Acetic acid: 2400 Furfural: 410 HMF: 70
	Wheat straw	2 % H_2SO_4, 160 ^{0}C	Glucose:15 Xylose: 6.6 Arabinose: 1.1	Acetic acid: 1300 Furfural: 270 HMF: 60
	Corn stover	2 % H_2SO_4, 160^{0}C	Glucose:15 Xylose: 5.7 Arabinose: 1.1	Acetic acid: 2300 Furfural: 510 HMF: 100
Koo et al., 2011	Yellow poplar wood	50% Ethanol, 1% H2SO4, 120 ^{0}C,140 ^{0}C	At 120^{0}C-140^{0}C Glucose:9-22 Xylose: 13-55	At 120^{0}C- 140^{0}C Acetic acid: 900-2400 Furfural: 1.7-35.2 HMF: 16.2-56.5
Huang et al., 2011	Corn stover	Steam explosion, 204 ^{0}C	Glucose: 147.5 Xylose: 13.42 Mannonse:0.93 Arabinose: 0.26 Galactose: 0.96	Acetic acid: 7800 Furfural: 710 HMF: 560 Vanillin:4000
Heer and Sauer,2008	Wheat straw	0.08N H_2SO_4, steam explosion, 19.5 bar	-	Furfural: 480-680 HMF:277 Vanillin: 122
	Spruce	2.5% SO_2, 215 ^{0}C	-	Furfural: 1100 HMF: 2140 Vanillin:152
Almeida et al., 2007	Willow	Dilute acid	-	Vanillin: 430 PHB: 100
	Wheat straw	Wet oxidation	-	Acetic acid: 1600 PHB: 21 Vanillin: 32 Syringaldehyde:24
	Spruce	Dilute acid	-	Acetic acid: 2400 HMF: 2000 Vanillin: 120 Syringaldehyde:107

References	Source of lignocellulosic	Pretreatment method	Sugar concentration, g/L	Concentration of byproducts from hydrolysates, mg/L
Zha et al.,2012	Sugar cane bagasse: PA	2% H_2SO_4, 121 ^0C	Glucose: 107.3 Xylose: 62.9 Arabinose: 4.2 Galactose: 1.5	Acetic acid: 241 Furfural: 27 HMF: 80 Vanillin:300 PHB:400
	Oak dust	2% H_2SO_4, 121 ^0C	Glucose: 90.9 Xylose: 42.3 Mannonse:4.2 Arabinose: 1.8 Galactose: 2.5	Acetic acid: 7994 Furfural: 431 HMF: 55 Vanillin:100 PHB: 100
Yu et al., 2011	Wheat straw	2% H_2SO_4	Glucose: 3.7 Xylose: 19.6 Arabinose: 4.7 Galactose: 1.2	Furfural: 440 HMF: 50
Chen et al., 2009	Corn stover	2.5% H_2SO_4	Glucose: 86.65 Xylose: 36.39	Acetic acid: 1158 Formic acid: 256 Furfural: 320 HMF: 1000 Vanillin:61 PHB: 103

2.4.2 Inhibitory Effects on Cell Growth

The effects of inhibitors on microorganisms for lipid production are under investigation in recent years. A recent work on *Mortierella isabellina* (oleaginous yeast) grown on xylose showed significant inhibition when concentrations of syringaldehyde, vanillin, and PHB in the medium were higher than 1.0, 1.5, and 2 g/L, respectively. They also observed that the effect of furfural and HMF up to 2.0 g/L (maximum concentration used in the experiment) was insignificant. Weak acids of acetic acid, formic acid, and levulinic acid showed no effect up to 6 g/L. The interesting finding was that the presence of acetic acid and formic acid at concentrations up to 3 g/L enhanced the cell growth and lipid production (Zeng et al.2013). Yu et al. (2011), reported the effect of Furfural, and HMF on oleaginous yeasts strain,

Cryptococcus curvatus grown on glucose. The experiments resulted no significant effect on cell growth and lipid production on HMF at concentration of up to 3 g/L; biomass and lipid content were dropped only by 6% and 8%, respectively, compared to the control samples. At the same time, with 1 g/L furfural concentration the cell growth and lipid production were dropped by 72% and 62%, respectively. Oleaginous yeast, *Rhodortula toruloides* Y4 was examined for the effect of lignocellulosic inhibitors. The results indicate that the PHB and vanillin were highly inhibitory above 1.22 g/L and 1.82 g/L, respectively. Furfural showed significant effect even at 0.1 g/L by dropping the cell mass, lipid content by 45% and 26%, respectively. For the same species, syringaldehyde reduced the cell growth and lipid content by 20% and 10% at 2g/L and HMF had no effect in cell mass and lipid up to 1.8 g/L (Hu et al., 2009). Huang et al. (2013), reported the cultivation of *Lipomyces starkeyi* on corncob hydrolysates (xylose: 36.3 g/L, glucose 2 g/L) which was pretreated by sulfuric acid and detoxified by overliming and activated charcoal. The final concentration of inhibitors was 0.06, 0.3, 0.04 g/L for furfural, HMF, and acetic acid, respectively (other inhibitors were not identified). 17.2 g/L biomass and 47% lipid content (8.1 g/L lipid) was observed after 8 days' fermentation. In investigating the effects of phenol (from 0.1 to 4 g/L) on xylose consumption by *Candida guilliermondii*, it was observed that phenol at concentrations below 0.1 g/L had neither effect on xylose consumption nor cell growth but at higher concentrations it was strongly inhibitory (Villa et al.,1998).

The mechanisms of the toxicity of byproducts on cell growth are not well-known because there is few research available on the mechanisms of inhibitors on bacteria and ethanol producing yeast; not to oleaginous microorganisms. The toxicity of furfural and 5-HMF results the inhibition of essential glycolytic and fermentative enzymes to central metabolic

pathways (such as pyruvate, acetaldehyde and alcohol dehydrogenases) (Modig et al., 2002). Furfural and HMF compromise membrane integrity leading to extensive membrane disruption or leakage, which eventually reduces cell replication rate, ATP production, and lipid production (Zaldivar et al., 1999). Formic acid and levulinic acid are formed as byproducts during acid degradation of 5-HMF (Hadi et al., 1989). Formic acid is more toxic than levulinic acid due to its smaller molecular size and undissociated form which facilitates its membrane permeability (Almeida et al., 2007). Formic acid inhibits the synthesis of macromolecules, as well as DNA synthesis and repair (Cherrington et al., 1990).

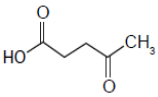
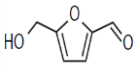
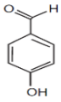
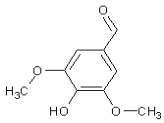
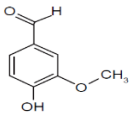
Phenolic compounds have been reported to be more toxic, even at low concentrations, than furfural and HMF (Philip et al., 2009). Phenolic compounds include acids (ferulic acid, vanillic acids, 4- hyydroxybenzoic acid and syringic acid), alcohols (guaiacol, catechol and vanillyl alcohol) and aldehydes (vanillin, syringaldehyde and 4- hydroxyl benzaldehyde) (Zimmermann et al., 1990). These compounds are known to alter the permeability and lipid/ protein ratio, which thus increases cell fluidity, leading to cell membrane disruption, dissipation of proton/ion gradients and compromising the ability of cellular membranes to act as selective barriers (Heipieper et al., 1994). This membrane disruption, allows the release of proteins, RNAs, ATP, and ions out of the cytoplasm, consequently causing reduced ATP levels (Heipieper et al., 1994). Furthermore, they enhance the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), super oxides (O^{2-}) and super hydroxyl (OH^-) that interact with proteins/ enzymes, which results in their denaturation, damage of cytoskeleton and other hydrophobic intracellular targets, cause DNA mutagenesis, and induce programmed cell death (Mikuláasová et al.,1990). While the mechanism and extent of cytotoxicity of lignocellulose inhibitory compounds generally differ, they all result in gross

physiological/metabolic changes in the microorganisms which concomitantly result in decreased cell viability and fermentation efficiency. One of the major determining factors of toxicity of these inhibitors is their hydrophobicity potentials which represents the extent to which a compound can accumulate in the cytoplasm of cell. Table 2.7 shows the hydrophobicity potentials of the aforementioned inhibitory compounds. The value of hydrophobicity derives from partition coefficient in terms of Log P; the ratio of the concentrations of the unionized compound in water and a non-polar solvent. In other words, it is called the measurement of lipophilicity; the higher value of logP of a compound indicates more absorption capacity by cells (Hansh et al., 2003). For higher hydrophobicity value, the compound can readily translocate into the cell across the non-polar cell membrane, and consequently, turns to higher inhibitory compound (Hansh et al., 2003). The logP values of compounds were obtained from http://www.chemspider.com.

Several methods such as physical (membrane-mediated detoxification, evaporation), chemical (over-liming, calcium hydroxide, neutralization, ion-exchange resins, activated charcoal column, and extraction with ethyl acetate), biological (microbial and enzymatic), and in situ microbial detoxification, have been applied to remediate fermentation inhibitors (Chandel et al., 2012). But still these available processes are not capable to reduce the concentrations of byproducts to the required level for microbial growth.

Table: 2.7: Properties of byproducts from lignocellulosic hydrolysates

Inhibitor	Structure	IUPAC Name	Molecular formula	Molecular weight	LogP
Acetic acid		Acetic acid	$C_2H_4O_2$	60.05	-0.32
Formic acid		Formic acid	CH_2O_2	46.03	-0.54

Inhibitor	Structure	IUPAC Name	Molecular formula	Molecular weight	LogP
Acetic acid		Acetic acid	$C_2H_4O_2$	60.05	-0.32
Levulenic acid		5-hydroxy-5-methyl-2-tetrahydrofuranone	$C_5H_8O_3$	116.12	1.34
HMF		5-(hydroxymethyl)-2-furaldehyde	$C_6H_6O_3$	126.11	-0.37
Furfural		Furan-2-carbaldehyde	$C_5H_4O_2$	96.08	0.41
4-Hydroxy benzaldehyde		4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.12	1.392
Syringaldehyde		4-Hydroxy-3,5-dimethoxybenzaldehyde	$C_9H_{10}O_4$	182.17	0.863
Vanillin		4-Hydroxy-3-methoxybenzaldehyde	$C_8H_8O_3$	152.15	1.188

2.5 Phosphate in the Medium

Phosphorus is indispensable to life because of its role in diverse cellular functions, including cell membrane, nucleic acid, bioenergetics, and information transfer. Organisms need certain amount of phosphate to grow fast; usually Phosphate constitutes about 1% of microbes (w/w). In cell membranes, phosphate is found as phospholipids which contain ~4% P by mass (sterner et al., 2002). Inside the cell, phosphate is connected with the intracellular processes of bioenergetics molecules of ATP, ADP, and AMP. Although these molecules are extremely phosphate rich (ATP is 18% phosphate by mass) but the contribution of ATP/ ADP/AMP to overall cellular biomass is relatively minor (0.1%). Nevertheless, a continuous

supply of PO_4 to recharge AMP to ADP to ATP is clearly essential in maintaining the vitality of any living thing (Elser, 2012). Phosphate is also present in the processing of genetic information; phosphate atoms exist in nucleic acids (DNA and RNA) about 9% by mass. Most of the phosphate involved in genetic transactions is associated with ribosomes and the variability in the phosphate content of living things reflects growth related demand for construction of ribosomal RNA.

The biomass composition of the Gram-negative bacterium, Escherichia coli (Stephanopoulos et al.1998) consists of about 55% protein (with minor phosphate), 20% RNA (about 3.5% (w/w) phosphate), 9% lipids mainly glycerophospholipids (about 2% phosphate), 6% cell wall constituents (very minor phosphate), 4% free metabolites (phosphate as pH buffer H_3PO_4, as activation group (e.g., glucose-6-phosphate), and as pyrophosphate), 3% DNA (about 3.5% phosphate), and 3% storage polymer (no phosphate). Early studies showed that high carbon to phosphorus (C/P) ratio could lead to high lipid accumulation in yeasts *Candida* 107 (Gill et al., 1977) and *Rhodotorula glutinis* (Granger et al., 1993a). Phosphate-limited culture of *Rhodotorula toruloides* Y4 produced lipid content of 63.7% and lipid concentration 12.1 g/L while producing lipid yield on substrate 0.21 g/g (Wu et al., 2010). The following Table 2.8 presents phosphate concentration used by oleaginous microorganisms using different carbon sources. The lowest phosphate in this table is 1 g/L used by *R. toruloides* Y4 and *L. starkeyi* (Li et al., 2005; Zhao et al. 2010).

Table 2.8: Phosphate concentration in different cultivation media available in literature

	DW, g/L	% lipid	Carbon source	References	Phosphate Source
A. curvatum UfaM3	15	45.6	Glucose	Hassan et al. 1993	KH_2PO_4: 2.5 g/L $NaHPO_4$: 2 g/L
A. curvatum ATCC20509	15	35	Sweet whey permeate(lactose)	Ykema et al. 1988	KH_2PO_4: 7 g/L Na_2HPO_4: 2 g/L
Cryptococcus curvatus	91	33.3	Glycerol	Meesters et al. 1996b	KH_2PO_4: 2.7 g/L Na_2HPO_4:0.95 g/L
C. albidus var. aerius IBPhM	-	63.4	Ethanol	Evans et al. 1983	KH_2PO_4: 7 g/L Na_2HPO_4:2 g/L
C. albidus var. albidus CBS 4517	26.7	46.3	Glucose	Hansson and Dostalek 1986	KH_2PO_4: 3 g/L
C. curvatus ATCC 20509	118	25	Glycerol	Meesters et al. 1996	KH_2PO_4: 2.7 g/L
C. terricolus	16	39	Glucose	Boulton and Ratledge	KH_2PO_4: 7 g/L Na_2HPO_4: 2 g/L
Lipomyces starkeyi	15	70	Glucose	Boulton and Ratledge, 1983b	KH_2PO_4: 7 g/L Na_2HPO_4: 2 g/L
Lypomyces lipofer	-	51.5	Ethanol	Evans et al. 1983	KH_2PO_4: 7 g/L Na_2HPO_4: 2 g/L
L.starkeyi	20.5	61.5	Glucose+ Xylose	Zhao et al. 2008	KH_2PO_4: 7 g/L Na_2HPO_4: 2 g/L
L. starkeyi DSM 70295	13.3	56.3	Glucose	Angerbauer et al. 2008	KH_2PO_4: 12.5 g/L Na_2HPO_4: 1 g/L
L. starkeyi AS 2. 1390	18	30	Glucose	Li et al. 2005	KH_2PO_4: 1 g/L
	20.9	20.5	Xylose		
	14 2	24.9	L-arabinose		
Rhodosporidium toruloides	18.2	76	Glucose	Zhao et al. 2008	KH_2PO_4: 2.5 g/L
R. toruloides Y4	151.5	48	Glucose	Li et al. 2007	KH_2PO_4: 1 g/L
	106.5	67.5	Glucose		
R. toruloides AS 2. 1389	6.9	42	Glucose	Li et al. 2005	KH_2PO_4: 1 g/L
	7.2	26.8	Xylose		
	4.8	16.8	L-arabinose		

	DW, g/L	% lipid	Carbon source	References	Phosphate Source
R. toruloides Y4	127.4	61.8	Glucose	Zhao et al. 2010	KH_2PO_4: 1 g/L
R. glutinis IIP-30	17.2	39	Molasses+glucose +sucrose	Johnson et al. 1995	KH_2PO_4: 1.26 g/L Na_2HPO_4: 0.75 g/L
R. glutinis NRRL y-1091	185	40	Glucose Oxygen enriched Air	Pan et al. 1986	KH_2PO_4: 12.5 g/L Na_2HPO_4: 1 g/L
R. glutinis AS 2. 703	5	30.2	Glucose	Li et al. 2005	KH_2PO_4: 1 g/L
	6.9	28	Xylose		
	4.3	4.9	L-arabinose		
Yarrowia lipolytica LGAM S(7)1	8.7	40	Industrial lipids (stearin) +glycerol	Papanikolaou and Aggelis 2002	KH_2PO_4: 7 g/L Na_2HPO_4: 2.5 g/L
Y. lipolytica ACA-DC 50109	15	44	Animal fats	Papanikolaou et al. 2002	KH_2PO_4: 7 g/L Na_2HPO_4: 2.5 g/L
Zygolipomyces lactosus	-	66.5	Ethanol	Evans et al. 1983	KH_2PO_4: 7 g/L Na_2HPO_4: 2 g/L

2.6 Extraction of Lipids

The different types of lipids inside the oleaginous cells show varying degrees of extraction depending on the type of solvents used. For neutral lipids, nonpolar solvents are effective and for phospholipids, polar solvents are effective. Neutral lipids are hydrophobic and have weak van der Waals attraction between the molecules. Some neutral lipids are found as a complex with polar lipids. This complex is in strong hydrogen bonding with proteins in the cell membrane. The van der Waals interaction between non-polar organic solvent and neutral lipids is too weak to disrupt the membrane-associated lipid-protein complexes. This tends to resist the diffusion of non-polar solvents such as hexane or chloroform, if such a solvent is employed straightaway. But a polar organic solvent such as methanol or iso-propyl alcohol

can disrupt such lipid-protein associations through hydrogen bonds with polar lipids. However polar solvents are not effective by themselves to extract the neutral lipids. Hence a mixture of polar and nonpolar solvents with specific composition or a series of solvents may be necessary for effective extraction. With polar and nonpolar solvent mixture, the polar solvent may diffuse inside the cell first and facilitate the diffusion of nonpolar solvent into the cell and the nonpolar solvent interacts with neutral lipid molecules forming an organic solvent-lipid complex. This complex elutes out of the cell because of the concentration gradient. (Halim et al. 2012, Kannan et al., 2014, Medina et al. 1998). Hexane, cyclohexane (Harun et al. 2010), and heptane (Horst et al. 2012) are commonly used for solvent extraction. Benzene, ether, acetone (Harun et al. 2010), and other polar co-solvents such as methanol and chloroform (Bligh and Dyer 1959) have also been used.

Folch method (Folch et al. 1957) and Bligh-Dyer method (Bligh and Dyer 1959) employing methanol-chloroform mixtures are generally used as analytical methods. These methods require large quantities of solvents that are expensive and toxic. Therefore, they are not preferred for industrial-scale extraction. In Soxhlet- hexane extraction process, the solvent is heated, percolated, and repeatedly refluxed to extract lipids. This method, however, provides low lipid yields (Halim et al. 2010; Mercer and Armenta 2011).

At present, Bligh and Dyer method has been modified by adding more steps into the original method for cell well rupture by sonication and washing of lipid-chloroform phase to get better recovery of lipid was employed (Manirakiza et al, 2000; Burja et al, 2007). Another process for lipid extraction from tissue with low toxic solvent system, hexane-isopropanol was reported by Hara et al. (1978). It has number of advantages over chloroform-methanol solvent system such as health hazard, separation of two phases and cost. Hexane extraction is

presently the most economical option for extracting lipids. Hexane extraction facilities are widely used for vegetable oil extraction (Lundquist et al. 2010). Another solvent, methyl tertiary butyl ether (MTBE) and methanol was used by Matyash et al. (2008) for effective lipid extraction. MTBE is an organic compound, commonly used as an additive with gasoline, is less dense, makes the lipid containing organic layer on the upper phase and cause the extraction process simple and rapid.

Drying of the biomass before extraction is the main challenge with solvent extraction. The solvent requirement is much higher (by an order of a magnitude) if cells are not dried. The Aquatic Species Program reported that direct solvent extraction was unlikely to be feasible for wet biomass (Sheehan et al. 1998). The following tables Table 2.9 and Table 2.10 present the properties of common solvents and different methods of cell preparation before solvent extraction.

Table 2.9: Cost and properties of different solvents

Property	Chloroform	Methanol	Hexane	IPA*	MTBE#
Price ($/L)	0.97	0.28	0.66	0.79	0.70
Dielectric constant (25 ^{0}C)	4.81	32.7	1.89	19.92	2.6
Molecular Weight (g/mol)	119.38	32.1	86.19	60.09	88.14
Density (Kg/m^3, 25 ^{0}C)	1450	792	655	786	740
Boiling point (25 ^{0}C)	61.2	64.7	68	82.6	55.2
Solubility in water (g/100g, 20 ^{0}C)	0.8	Miscible	0.014	Miscible	4.8

**IPA- Iso propyl alcohol # MTBE- Methyl tertiary butyl ether*

Table 2.10: Cell wall lysis methods, Garcia (1999) and Jacob (1992)

Method	Lysis Principle	Advantages	Disadvantages
High pressure homogenization	Liquid shear: pressurized cell suspensions are forced through a small valve and impact a wall at high velocity	Robust, industry use, continuous, handles concentrated cell solutions	Maintenance costs with valve wear/blockage, used for larger volumes (not useful for laboratory purposes)
Ultrasonic (sonication)	Sound waves generated by an oscillator produce cavitation forces that disrupt cell membranes	Low energy, can be used simultaneously with other methods, useful at both small and large scale	Heat generation, difficult to use on microbes with tough cell walls (fungi, microalgae), loud
Freeze-thaw	Ice crystals puncture and disrupt the cell wall	Works best as a conditioning scheme before using another lysis method (e.g. bead milling)	Slow, energy intensive for large sample volumes, works well for laboratory purposes
Method	Lysis Principle	Advantages	Disadvantages
Organic solvents	Solubilize components of the cell wall and membrane	Can be used as a preservative, often serves as the extraction medium (e.g. chloroform)	Destruction of other cellular compartments that release degradation enzymes, some are toxic
Detergents	Solubilize cell membrane lipids and proteins	Fast acting, inexpensive, works well in combination with other methods, various types for specific applications	May degrade proteins and lipids, cell wall polysaccharides are resistant
Enzymes	Hydrolyze cell wall polysaccharides (glucanases, zymolyase, chitinases)	Low energy, mild conditions, no need for specialized equipment	Expensive, specific to certain cell wall components, enzyme stability can

CHAPTER 3: MATERIAL AND METHODS

3.1 Material and Methods:

3.1.1 Microorganism

Lyophilized cells of yeast strain, *Lipomyces starkeyi* NRRL Y-11557, were obtained from the, Department of Agriculture laboratory, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, Illinois, United States. This strain was contaminated after two years of research, and the same strain, NRRL Y-11557 (ATCC 58680), was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The lyophilized cells were revived using yeast mold (YM) medium (composition: malt extract 3 g/L, yeast extract 3 g/L, Dextrose 10 g/L, and peptone 5 g/L). The cells were then cultivated on YM-agar slants (YM medium with 2% agar) at 30 ^{0}C in an Isotemp Standard Lab Incubator (Fisher Scientific, U.S.A.) for 48 hours and then stored at -80 ^{0}C with 50% (v/v) glycerol/dH_2O.

For experiments, colonies of *Lipomyces starkeyi* were propagated every month on agar slants at 30°C for 48 hours (composition of agar medium: 20 g/L soluble potato starch or 22 g/L D(+) glucose , 5 g/L yeast extract, 10 g/L peptone, and 20 g/L agar (Uzuka et al., 1975b). These slants were stored at 4 ^{0}C. *Lipomyces starkeyi* cells containing lipid globules are shown in Figure 3.1.

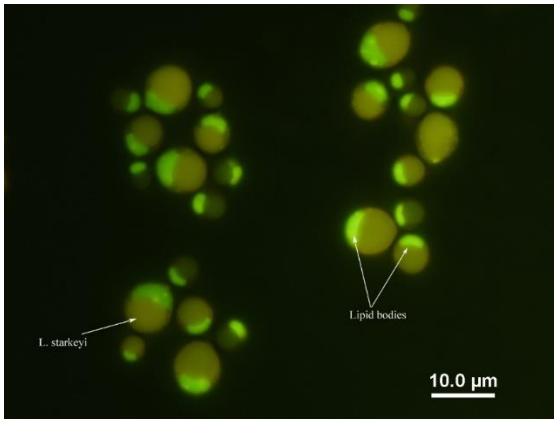


Figure 3.1: *Lipomyces starkeyi* cells under microscope

3.1.2 Chemicals

Chemicals used in this work were obtained from different sources as listed below.

Table 3.1: List of chemicals used

Chemicals	Supplier
D-(+)-Glucose	Sigma Aldrich, St. Louis, MO
sweet potato starch	
Biotin	
Inositol	Acros, New Jersey, USA
Calcium Pantothenate	
Para hydroxyl benzaldhyde	
Hydroxy methyl Furfural	
$(NH_4)_2SO_4$	Baddley Chemicals, Inc, Baton rouge, LA
$FeSO_4$	
$MgSO_4 \cdot 7\ H_2O$	
Syringaldehyde	Alfa Aesar, England
$Na_2HPO_4 \cdot 7\ H_2O$	Fisher scientific, Pittsburgh, PA
Vanillin	
Furfural	
$Na_2HPO_4 \cdot 7\ H_2O$	
$CaCl_2$	MP Biochemicals, LLC, Solon, Ohio
KH_2PO_4	Fluka Chemika, Germany
$ZnSO_4 \cdot H_2O$	
Chloroform	Fisher scientific
Methanol	
Hexane	
Iso Propanol	
Methyl Tertiary butyl ether	

3.2 Cultivation of Cells

3.2.1 Inoculum Preparation

Fresh agar slants were streaked with loopful cells from refrigerated agar slants. The new slants were incubated at 30ºC for 48 hours. In the next step, loop of cells was used to inoculate 125 mL, sterilized seed culture - 1 medium of same composition of fermentation medium in a 500 mL baffled flask. The Seed Culture -1 was incubated at 30^0C in an orbital shaker at140 rpm for 48 hours before inoculating Seed Culture - 2 (identical to seed culture - 1) medium. The Seed Culture - 2 was incubated for 30 hours under the same conditions as Seed Culture 1 and it was used to inoculate the actual experiments culture medium. A 2% (v/v) inoculum was used for all experiments. The seed culture was examined under a microscope to check for any possible contamination before inoculating the experimental flasks.

3.2.2 Growth Media

Fermentation media were prepared for experiments in shake-flasks as well as in fermenter with compositions of: 27.27 g/L starch or 30 g/L D (+) glucose, 0.5 g/L $CaCl_2 \cdot 2H_2O$, 0.5 g/L $(NH_4)_2SO_4$, 7 g/L KH_2PO_4, 2.5 g/L $Na_2HPO_4 \cdot 7H_2O$, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.0082 g/L $FeSO_4$, 0.01 g/L $ZnSO_4 \cdot H_2O$, 1 mL/L Vitamin solution, and 1 mL/L trace element. The vitamin solution was prepared with concentrations of 3 µg/L Biotin, 3000 µg/L Inositol, and 600 µg/L Calcium Pantothenate. This vitamin's concentration was equivalent to the concentration of biotin, inositol, and calcium pantothenate available in 1.5 g/L yeast extract. Trace elements solution was prepared with concentrations of $CoCl_2.6H_2O$ at 9.41 mg/L, $MnSO_4$ at 0.007 mg/L, and $CuSO_4$ at 10 mg/L.

The medium used for growing *Lipomyces starkeyi* was the same as that of Yamauchi et al. (1983) and Suutari et al. (1993) except that 27.27 g/L starch or 30 g/L glucose was used as carbon source instead of ethanol. Starch was dissolved in water at 70 ^{0}C. $CaCl_2$ was added in starch solution to keep starch from precipitating out after cooling. Nutrients were dissolved in the starch solution and pH was adjusted to 5.5 using hydrochloric acid (1M) and NaOH (1M) prior to autoclaving. All media were sterilized at 121°C, 15 psig for 20 minutes in an electric-heated vertical steam autoclave (LA 3100L model, China). The autoclaved medium was cooled to room temperature before inoculation.

3.2.3 Experimental Description for Study of the Effect of Inhibitors

Para-hydroxy-benzaldehyde (PHB), vanillin, syringaldehyde, furfural, and 5-hydroxy methyl furfural (HMF) were used individually in the glucose media (noted under section 3.2.2). The concentrations range studied in this work are the typical concentrations of these inhibitors produced in acid hydrolysis of lignocellulosics. The experimental concentrations of the inhibitors are presented in Table 3.2.

Table 3.2: Concentrations of inhibitors used individually in experiments

Inhibitors	g/L		
Furfural	0.05	0.1	0.2
Vanillin	0.25	0.5	1
Syringaldehyde	0.5	1	2
Hydroxy Methyl Furfural (HMF)	0.25	0.5	1
Para Hydroxy Benzaldehyde (PHB)	0.25	0.5	1

3.2.4 Experimental Description for Phosphate Optimization Using Shake Flask

Phosphate optimization experiments were performed using batch fermentation in 500 mL Erlenmeyer baffled shake-flasks using specified medium and 27.27 g/L of the soluble sweet potato starch as carbon source. All shake-flask fermentations were incubated at 30°C in a

temperature controlled incubator shaker at 140 rpm. Figure 3.2 displays the experimental setup for shake flask experiments.

A Wide range of phosphate concentration from 9.5 g/L to 0.11 g/L was used. The medium composition listed in 3.2.2 Growth Media, was used as 'control', represented as '1-X', phosphate concentration in it was 7 g/L KH_2PO4 and 2.5 g/L Na_2HPO_4. This medium was used so far in the UL Bioprocessing Research Laboratory for production of microbial oil using *Lipomyces starkeyi* growing on soluble sweet potato starch, and it differed from the medium composed of yeast extract by substituting it with optimal concentrations of biotin, inositol, and calcium pantothenate. Phosphate concentrations were varied from $\frac{X}{10}$ (0.95 g/L) to $\frac{X}{80}$ (0.11 g/L) in the experiments. Every element other than phosphate was maintained at the levels present in 1-X experiments. Seed cultures 1 and 2 were prepared with $\frac{X}{20}$ g/L phosphates (i.e. 0.350 g/L KH_2PO_4 and 0.125 g/L Na_2HPO_4) in the medium and the level of inoculum was 2% (v/v).The seed culture used $\frac{X}{20}$ phosphate, so that it would contribute less supply of phosphate to fermenting medium. Since phosphate in seed culture (age 30 hours) must have been used up, there should have been little contribution of additional phosphate to the medium via seed culture. Phosphate concentration was not measured in the fermenting medium. The results are presented in Figure 4.2.1. All the samples were taken after 40-h fermentation based on preliminary studies.

Figure 3.2: Shake Flask experiment

3.3 Sample Analysis

3.3.1 Cell growth

During the fermentation process, cell growth was measured in terms of optical density using a DR 5000 UV-Vis Spectrophotometer (DR5000, Hach Company, U.S.A.) from the beginning to the end of experiment in a specified interval of time (six to twelve hours). The sample was diluted in such a way that the measured optical density stays below 0.60 to have linearity. Optical density was measured at 550 nm using distilled water as a blank to zero the spectrophotometer. All the measurements were conducted in duplicate.

3.3.2 Cell Dry Weight Measurement

Cell dry weight was measured at the end of each experiment, from the final culture medium by filtering 5-mL sample through a pre-weighed 0.45µm Durapore Membrane Filter (Millipore). The filter cake was washed twice with ample of DI water through the filter. The filter paper was dried in an Isotemp Standard Lab Oven (Isotemp 637G, Thermo Fisher Scientific, Pittsburgh, PA) at 80°C for 24 hours. After 24 hours, the filter paper was weighed again and the final weight was subtracted from the initial filter paper weight to obtain the dry weight of cells. From the same sample used for filtration, optical density was measured from

0% to 100% concentration with necessary dilutions. Optical density and dry weight measurements were plotted and the slope obtained was used to convert the measured optical density during the course of fermentation into dry weight. Similar data obtained from all the experiments of this work were used to get the average slope. This average slope was used to have the best correlation of cell dry weight and optical density. The dry weight versus optical density calibration curve is given in figure 3.3.

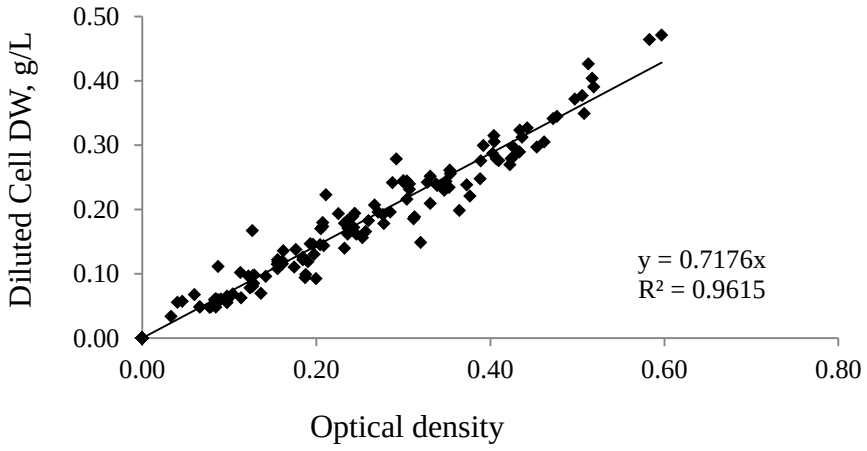


Figure 3.3: Calibration of cell dry weight of *Lipomyces starkeyi*

3.3.3 Lipid Analysis

Lipids in the cells were measured by Nile red fluorescence method (Kimura et al., 2003) using Shimadzu spectrofluorophotometer (RF-1501, Shimadzu Corporation, Japan) for all the samples taken at periodical interval. For the measurement, 100µL of culture medium was mixed with 2000µL of 10 mM potassium phosphate buffer with 0.15M KCl (pH 7.0; PBS) in a fluorescence cuvette. The emission spectrum in a wavelength region of 500 to 700 nm for the cell suspension without Nile red was recorded at the exciting wavelength of 488nm. 10-µl Nile red solution was then added and mixed well. Five minutes later, the cell suspension in a cuvette was mixed well by an upside down inversion just before measurement in order to

avoid cell sedimentation and the spectrum in the same wavelength region was recorded again. Spectra were corrected by subtracting the spectra before and after the Nile red addition. The fluorescence intensity corresponding to the lipid amount was determined at the peak of the corrected spectrum. The cell broth was diluted in such a way that the measured lipid fluorescence stays below 1000 units (maximum recordable units with the instrument). At the end of each fermentation, from the final culture medium, lipid content was measured by fluorescence method with 0% to 100% of cell concentration. The lipids were extracted from the freeze-dried cells of the same final culture medium by Accelerated Solvent Extractor (ASE300 from Dionex, Sunnyvale CA, USA) using chloroform and methanol (2:1 ratio). Extracted lipid from the final culture medium was calibrated with fluorescence at 100% to 0% cell concentrations and plotted. Similar data obtained from all the experiments of all studies were used to get the average slope in order to avoid variation in lipid calculation from fluorescence. The obtained average slope was used to convert all fluorescence measurements of each sample to lipid concentration in g/L. The combined lipid concentration versus fluorescence calibration curve is given in Figure 3.2.

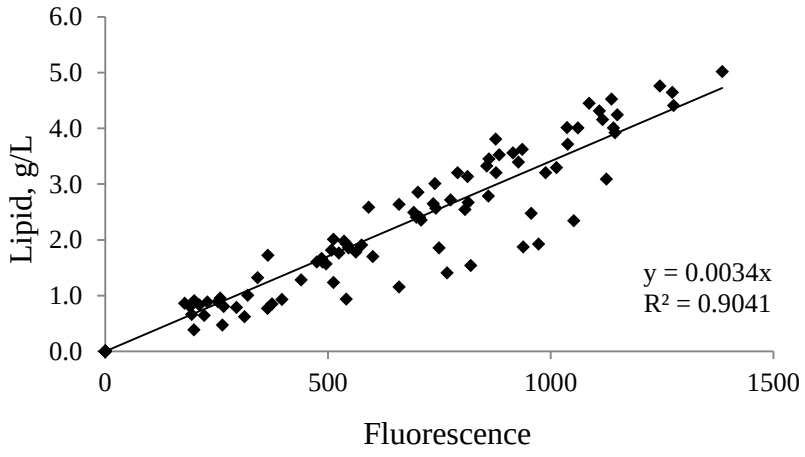


Figure 3.2: Calibration of Lipid of *Lipomyces starkeyi*

3.3.4 Fatty Acid Analysis

Transesterification of lipids was carried out for fatty acid methyl ester (FAME) analysis. This was conducted by adding 2-mL ACS-grade methanol containing 2 % (v/v) H_2SO_4 to the lipid samples in 40-mL glass vial. The vial was incubated at 60 °C in a water bath for 2 hours. The vial was cooled and the reaction was quenched by adding 5-mL DI water containing 3% $NaHCO_3$ and 5 % NaCl. 2 x 2 mL of internal standard (100-ppm BHT and 200-ppm 1, 3-Di chloro benzene in toluene) was added and the vial was vortexed. The top layer containing the FAMEs was pipetted into an 8-mL vial with a pinch of sodium sulfate and vortexed with 2-mL internal standard. The vial was vortexed and kept standing overnight. 1 mL of top layer was drawn and diluted with 3 mL internal standard. This diluted liquid of 1.5 mL was transferred to GC-vial for analysis. Table 3.3 shows the GC operating conditions in this experiment.

Table 3.3: GC operating conditions

	Set point
Injector Temperature	260°C
Detector Temperature	260°C
Split ratio	30:1
Oven Equilibration Time	0.5 minutes
Oven Ambient Temperature	25°C
	Oven program
Set point	50°C
Initial Time	2 minutes
Level 1 rate	10 (°C/min)
Final Temperature	250°C
Final Time	8 minutes

Samples after transesterification were separated and analyzed using an Agilent 6890 Gas Chromatograph. The gas chromatograph was equipped with a Stabilwax column (Restek, U.S.A). The peaks were detected using a FID detector. GC operating conditions are presented in Table 3.1. The data were analyzed, integrated, and interpreted using Agilent Chemstation.

3.3.5 Glucose Analysis

Filtrates obtained from fermentation culture medium were processed on a Variance Prostar 210 Liquid Chromatography (Walnut Creek, CA) and an Agilent ultra-amino column (Agilent Tec. U.S.A.). Operating conditions for HPLC are presented in Table 3.4. The peaks were detected using a UV Detector (199 nm) and analyzed using Variance Star Work Station.

Table 3.4: HPLC operating conditions

Column Temperature:	40^0C
Gradient:	Isocratic
Flow rate:	0.75 mL/min
Mobile phase:	Acetonitrile/Water 83%/17% (v/v)
Injection Volume:	20 µL

3.4. Methods for Lipid Extraction

There are various techniques for lipid extraction from biological tissues or biomasses in practice. The following methods were used in this work.

3.4.1 Lipid Extraction with Chloroform and Methanol from Wet Cells

20 mL of fermentation culture medium was collected in a corning tube for centrifugation. Centrifugations were conducted in pre-weighed 50mL corning centrifuge tubes at 7000 rpm for 10 minutes and at 4^0 C (Biofuge stratus model from Thermo Electron Corporation, Germany).

The cells were washed two times by re-suspending the cells in 10 mL deionized water and using a vibromixer followed by centrifugation and discarding the supernatant. The harvested cells were suspended in methanol (5+5 mL methanol / 2 g wet cell or 1 g dry cell) in a test tube. In the first step 5 mL methanol was added to the centrifuge tube followed by 20 seconds of vortex and transferred the contents to a pyrex vista glass tube. Centrifuge tube was rinsed two more times with the remaining methanol and collected all the cells in the same pyrex tube. For cell disruption, 5-g of glass beads were added to the glass tube and cells were disrupted continuously at 60% amplitude for 10 minutes in a Digital Sonifier 450 equipped with a micro horn tip (Sonifier 450, Branson, U.S.A.). The glass tube was covered with an ice-water bath to prevent heating of cell suspension during sonication. After cell disruption, 20-mL of chloroform was added to the suspension to give a ratio of chloroform/methanol 2:1 (v/v). The suspension was stirred for 1 hour using a flat-bed stirrer at room temperature (flask was covered with aluminum foil paper and wrap it with parafilm to avoid loosing of any solvent) followed by filtering through 0.45μm membrane filter with a vacuum filtration. The filter cake (containing the cell debris and glass beads) was washed with 10-mL chloroform/methanol (2:1; v/v). The extract (filtrate) was transferred to 250-mL glass beaker, mixed with 10-mL of 0.034% $MgCl_2$ solution, and stirred for 10 min. The extract was centrifuged in pyrex glass vials at 3000 rpm for 5 min. The upper aqueous layer was discarded and the organic phase was washed with 10-mL of 2N KCl/methanol (4:1 v/v). The suspension was centrifuged again at 3000 rpm for 5 min. The upper aqueous layer was discarded and the organic phase was washed with 10-mL of artificial upper phase (chloroform/methanol/water; 3:48:47 by volume). The suspension was centrifuged again at 5000 rpm for 5 min. The upper aqueous layer was discarded, including the protein layer that

formed at the phase boundary, and the organic phase was repeatedly washed with 10-mL of the artificial upper phase until the phase boundary became clear. The organic phase was transferred to a pre-weighed aluminum foil boat for 24 hours under air vent hood (Thermo Fisher Scientific, U.S.A). The final weight of the boat was measured after solvent evaporation and lipid content was calculated (Folch et al., 1957).

3.4.2 Lipid Extraction with Chloroform

Chloroform was used to extract lipid from wet cells and dried cells. Methanol amount used in the previous section was replaced with chloroform while keeping the other steps unchanged.

3.4.3 Lipid Extraction with Hexane-Isopropanol

Lipid was extracted from wet cells and dry cells by using Hexane-Isopropanol solvent system with a ratio of 3:2 (v/v). The harvested cells were suspended in 20 mL isopropanol in a pyrex vista tube followed by adding 10-gram glass beads for cell disruption under sonication for 10 minutes at 60% amplitude. This procedure was similar to chloroform methanol solvent system. After sonication, 30 ml of hexane was added to the disrupted cells and transferred to a flask and then stirred for one hour. The contents were filtered through 0.45μm membrane filter with a vacuum filtration. The filter cake was washed with 10 mL Hexane: Isopropanol (3:2) mixture. Filtrate was collected in a 125 mL flask and added 36 mL of aqueous Na_2SO_4 (1 gm anhydrous Na_2SO_4: 15 mL DIW). This mixture was centrifuged for 5 min at 3000 rpm. Lipid rich upper layer was collected in a pre-weighed aluminum foil boat and dried under air vent hood. The final weight of the boat was measured after solvent evaporation and lipid content was calculated.

3.4.4 Lipid Extraction with Methyl Tertiary Butyl Ether (MTBE) and Methanol

MTBE was used for lipid extraction from wet cells and dried cells. In this method 0.1 gm dried cells or 0.5 gm wet cells were suspended in 18 mL methanol in a tube. The tube was vortexed and mixed with 60 mL MTBE in a 125 mL flask for incubation at room temperature for one hour. Sonication was conducted after incubation in the same flask for 10 minutes at 60% amplitude. The flask was further incubated for 10 minutes after adding 15 mL water. The flask contents were centrifuged at 3600 rpm for 10 rpm. The lipid rich top layer was collected in a pre-weighed aluminum foil boat. The bottom layer was centrifuged again at same rpm by adding 24 mL MTBE: Methanol: H_2O (10:3:2.5 v/v). The final weight of the boat was measured after solvent evaporation and lipid content was calculated.

3.4.5 Lipid Extraction with Hexane

0.2 gm wet cells or 1 gm dry cells and 5 gm glass bead were suspended in 10 mL hexane in a Pyrex vista tube. Sonication was performed for 10 min in at 60 % amplitude. The contents were centrifuged at 5000 rpm for 10 min and the top layer was collected into another glass tube. Again 5 mL Hexane was added to glass tube with pellet and perform sonication for 5 min. After sonication the contents were centrifuged and collected the top layer again in the collection glass tube. The top layer was mixed with 6 mL sodium sulfate and centrifuged again at 3000 rpm for 3 min. the organic phase was collected in a pre-weighed aluminum foil boat. The final weight of the boat was measured after solvent evaporation and lipid content was calculated.

3.4.6 Lipid extraction by Accelerated Solvent Extractor (ASE)

Wet cells were frozen at -80^{0}C and then lyophilized in a 1-L benchtop freeze dry system (Labconco freeze dryer) before processing them using ASE extractor. In order to extract the lipids from cells, known quantity (1to 2 g) of freeze dried cells were placed into a 40-mL ASE-cell and processed in the ASE 300 Solvent Extractor (Dionex, U.S.A.) using the following protocol. ASE operating conditions are presented in Table 3.5. Hexane, Chloroform, Chloroform/Methanol, MTBE and Hexane/IPA are the solvent systems used in this study.

Table 3.5: ASE operating conditions

Solvent:	Hexane, Chloroform, Chloroform/Methanol, MTBE, Hexane/IPA,
System Pressure:	1500 psi
Oven Temperature:	100^{0}C
Sample Size:	1 gm freeze dried cells
Oven Heat-up Time:	6 minutes
Static Time:	5 minutes
Static Cycles:	3
Flush Volume:	60 % of extraction cell volume
Nitrogen Purge:	150 psi for 60 seconds

CHAPTER 4: RESULTS AND DISCUSSIONS

Experiments were conducted in shake flasks to (a) characterize the effect of byproducts of lignocellulosic hydrolysis on cell growth and lipid accumulation by *Lipomyces starkeyi*, (b) establish the effect of initial phosphate concentration in the medium on cell growth and lipid production, and (c) study extraction of lipids from wet cells as well as freeze dried cells using different solvents. The experimental conditions and results are presented and discussed in this chapter.

4.1 Effect of Inhibitors on Cell Growth and Lipid Production

Para-hydroxy-benzaldehyde (PHB), vanillin, syringaldehyde, furfural, and 5-hydroxy methyl furfural (HMF) were identified as the more toxic byproducts in acid hydrolysates of lignocellulosics. The concentrations range studied in this work are the typical concentrations of these inhibitors produced in acid hydrolysis of lignocellulosics. These were added individually in cultivation media in shake flasks at concentrations identified below to study their effect on *Lipomyces starkeyi* growth and lipid production.

4.1.1. Effect of Para-Hydroxy-Benzaldehyde (PHB)

PHB concentrations used in this study were 0.25 g/L, 0.5 g/L, 1 g/L, and 1.5 g/L. The usual concentration range of PHB in acid hydrolyzates from lignocellulosic biomass published in literature ranged from 0.40 to 1 g/L (Table 2.6). Profiles of concentration of cell dry weight (DW), intracellular lipid content as percentage of cell dry weight and glucose concentrations are shown in Figure 4.1.1(a-c). Error bars in the figures represent standard error with two standard deviations from the mean.

a.

b.

c.

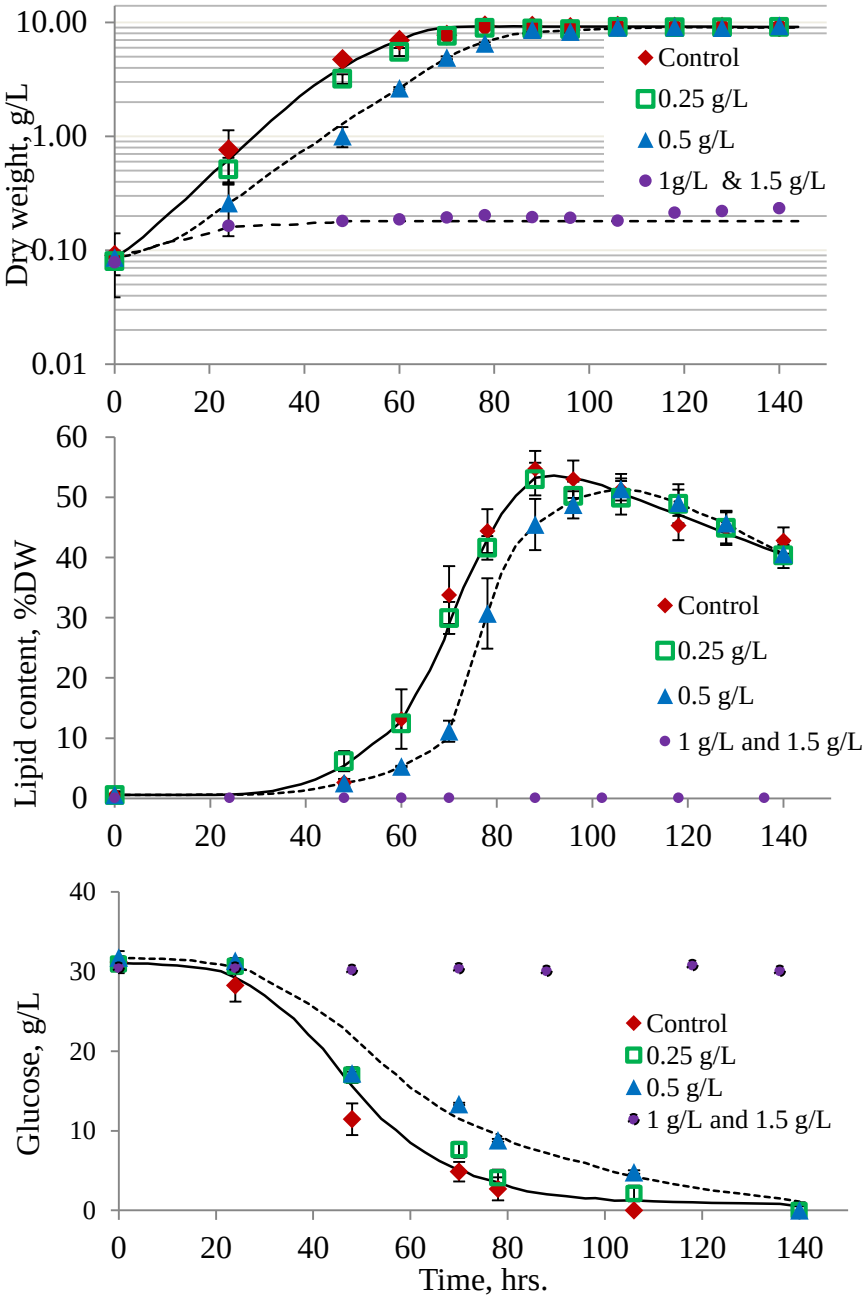


Figure 4.1.1: Effect of PHB on cell mass, lipid production, and glucose consumption by *Lipomyces starkeyi*

The solid line represents the control and 0.25 concentrations which were very similar, the narrow dotted line was fitted to the 0.5g/L concentration, and the wide dotted line presents 1 g/L and 1.5 g/L concentrations which were very identical. Table 4.1 represents values of the

growth factors of the cells. Specific growth rate was calculated from the exponential phase of cell growth and they are presented in the Appendix.

Table 4.1: Effect of PHB on *L. starkeyi*

PHB Conc. (g/L)	Specific Growth rate (h^{-1})	Maximum dry weight (g DW/L) (Mean ± 95% CI)	Time for Max DW (h)	Lag Period (h)	Maximum lipid fraction (% DW) (Mean ± 95% CI)
0	0.09	9.33± 0.26	78	0	54.7± 2.93
0.25	0.085	9.12± 0.21	106	0	53.1± 2.72
0.5	0.068	9.06± 0.15	118	0	51.3 ± 2.51
1	0	0.25± 0.002	140	140	0
1.5	0	0.24± 0.006	140	140	0

* CI – Confidence Interval

These results suggest that PHB concentration of 0.25 g/L has almost no impact on cell growth (maximum specific growth rate 0.085 h^{-1}) and lipid fraction in cells, as well as on the consumption of glucose. At higher concentration of 0.5 g/L PHB, growth of cells is adversely impacted (maximum specific growth rate down to 0.068 h^{-1}); the time profiles of intracellular lipid fraction and glucose concentration follow the pattern of cell growth as well. PHB at ≥1 g/L is highly inhibitory for *Lipomyces starkeyi* ATCC58680 cells, suppressing its growth almost completely and resulted no lipid production. This severe effect occurred because of the maximum disruption of the cell membrane and inhibition of the key enzymes associated in glycolysis and TCA cycle of cell metabolisms. Intracellular ATPs and NAD(P)Hs are also reduced in presence of phenolics (Boral et al., 2014).

In control flasks as well as in flasks containing 0.25 g/L and 0.5 g/L PBH (Figure 4.1.1a), exponential growth of cells ended when cell density of ~ 5 g DW/L was achieved and in each case, lipid production started in earnest around the time exponential growth ended. The protocol for preparation of seed cultures ensured almost no lag phase for the cells, although

the level of inoculum concentrations (2% v/v) were quite low; as a result, the cultivation periods were quite long.

The profiles of intracellular lipid content and glucose levels also followed those of cell dry weight. The maximum cell DW concentrations and % lipids in cells reached almost the same levels (considering standard errors with 95% confidence interval) in both PHB concentrations and control (0, 0.25, and 0.5 g/L) (except those severely inhibited), albeit at different times. In fact, the concentration of glucose in media at the start of rapid lipid accumulation is also same in both PHB concentrations (0, 0.25, and 0.5 g/L) suggesting that the trigger for excessive accumulation of lipids by the *Lipomyces starkeyi* cells is not impacted by the presence of PHB. In essence, PHB impacts only the constitutive pathways but not the lipid pathway. Intracellular lipids were re-consumed by the cells once all the glucose in medium was gone.

4.1.2. Effect of Vanillin and Syringaldehyde (SYR)

Vanillin concentrations used in this study were 0.25 g/L, 0.5 g/L, 1 g/L, and 1.5 g/L. Syringaldehyde concentrations used in the study were 0.5, 1.0, and 2.0 g/L. This range is typical of the concentrations of vanillin produced in acid hydrolysis of lignocellulosics. The typical concentration range for vanillin reported in literature is 0.03 g/L to 4 g/L and for syringaldehyde the range was widened to 0.03 to 2 g/L (Table 2.6). The impact of these chemicals on cell growth, lipid production, and glucose consumption is presented in Figures 4.1.2 (a-c) and 4.1.3 (a-c). Error bars again represent two standard deviations of observation from the mean. Table 4.2 and 4.3 represents the summarized results of the associated growth factors of the cells.

Table 4.2: Effect of Vanillin on *L. starkeyi*

Furfural Conc. (g/L)	Specific Growth rate (h^{-1})	Maximum dry weight (g DW/L) (Mean ± 95% CI)	Time for Max DW (h)	Lag Period (hr.)	Maximum lipid fraction (% DW) (Mean ± 95% CI)
0	0.09	10± 0.27	80	0	44.4± 2.9
0.25	0.09	8.89± 0.44	88	0	43.3± 2.7
0.5	0.07	8.2± 0.16	104	0	42.6± 1.87
1	0	0.25± 0.03	104	104	0
1.5	0	0.28± 0.02	104	104	0

* CI – Confidence Interval

Table 4.3: Effect of Syringaldehyde on *L. starkeyi*

Furfural Conc. (g/L)	Specific Growth rate (h^{-1})	Maximum dry weight (g DW/L) (Mean ± 95% CI)	Time for Max DW (h)	Lag Period (hr.)	Maximum lipid fraction (% DW) (Mean ± 95% CI)
0	0.09	10± 0.27	80	0	44.4± 2.9
0.5	0.09	8.46± 0.26	96	0	39.41± 2.35
1	0.08	7.6± 0.24	104	0	38.56± 1.95
2	0.08	0.25± 0.04	104	104	0

* CI – Confidence Interval

Vanillin concentrations of 1.0 g/L and higher, and of Syringaldehyde concentration ≥ 2 g/L resulted in complete inhibition of cell growth. Vanillin up to 0.25 g/L and syringaldehyde up to 0.5 g/L had no impact on cell growth or lipid production (considering 95% band width). Presence of higher concentrations of vanillin or syringaldehyde in medium increased the adjustment period for the cells, but did not impact the maximum specific growth rate (0.08 h^{-1}) of cells. Although the initiation of lipid accumulation was delayed, neither the lipid accumulation rate nor the maximum lipid fractions were impacted by the presence of inhibitors. Glucose consumption was also only delayed, suggesting that the impact of low concentrations of these inhibitors can be overcome by pre-cultivating cells in medium containing the chemicals.

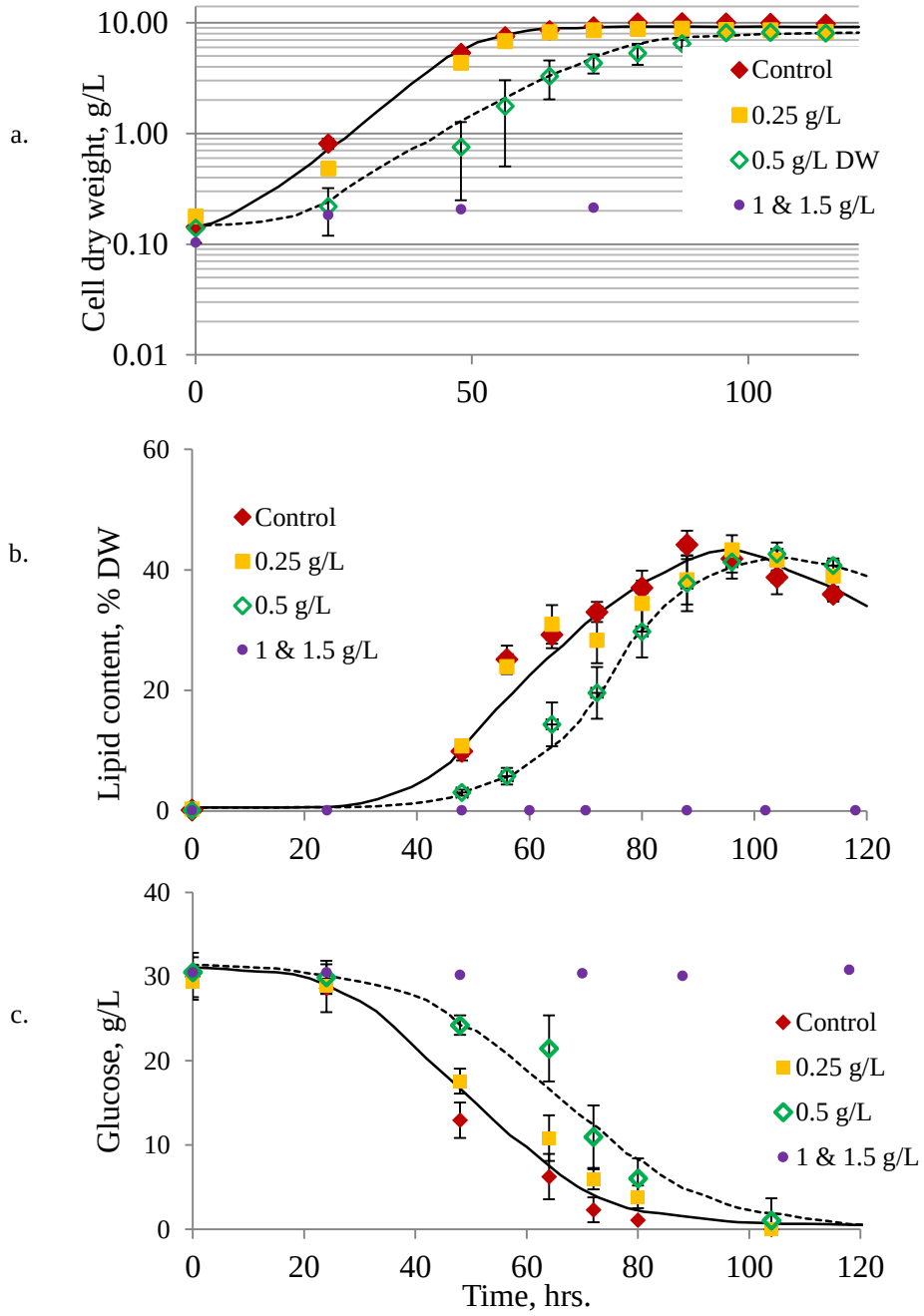


Figure 4.1.2: Effect of Vanillin on cell mass, lipid production, and glucose consumption by *Lipomyces starkeyi.*

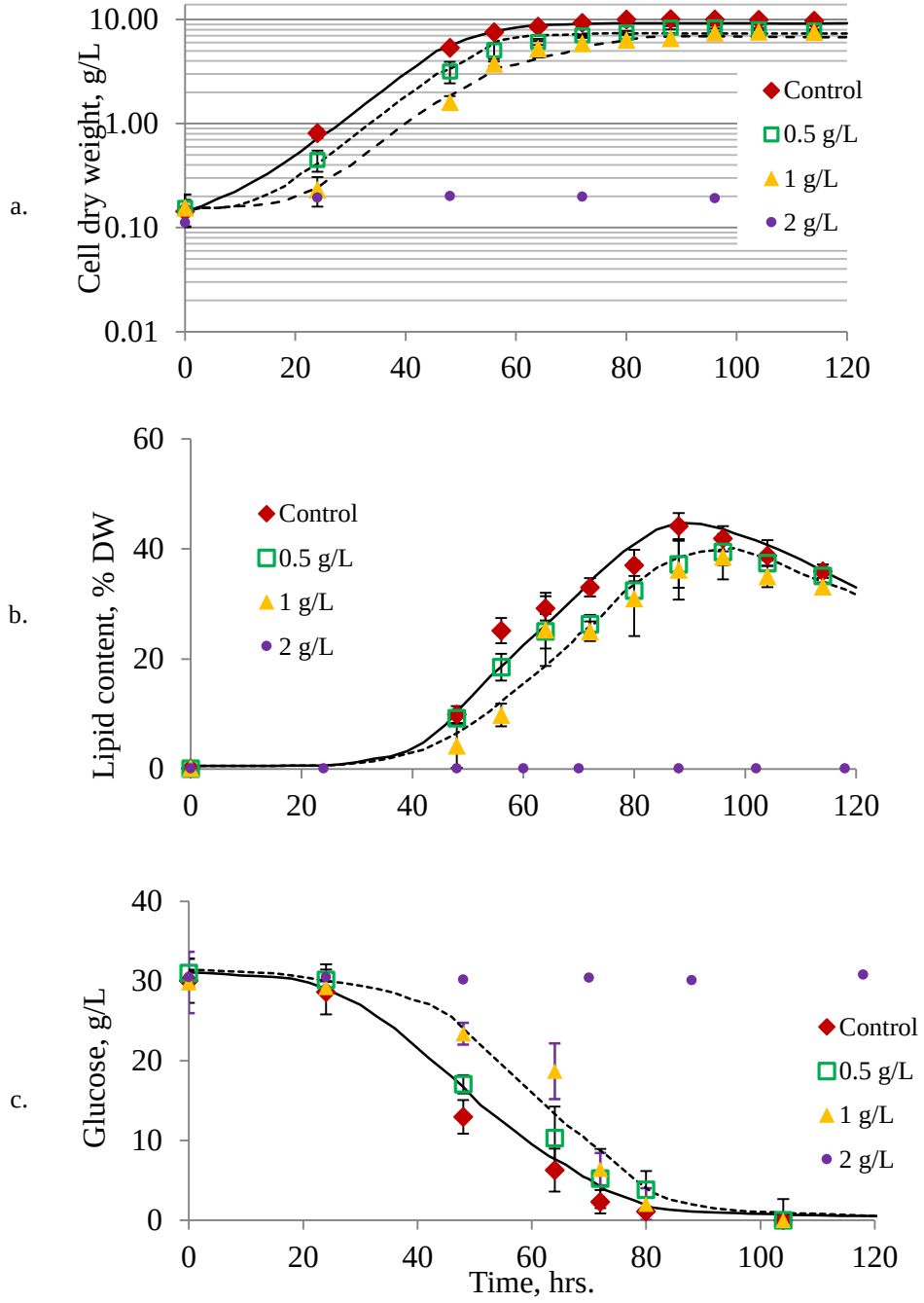


Fig 4.1.3: Effect of Syringaldehyde on cell mass, lipid production, and glucose consumption by *Lipomyces starkeyi.*

4.1.3 Effect of Furfural

Furfural concentrations used in this study were 0, 0.05, 0.1, 0.2 and 0.4 g/L while their concentration range found in literature from 0.03 to 1.2 g/L. The results have been plotted in Figure 4.1.4(a-c).

a.

b.

c.

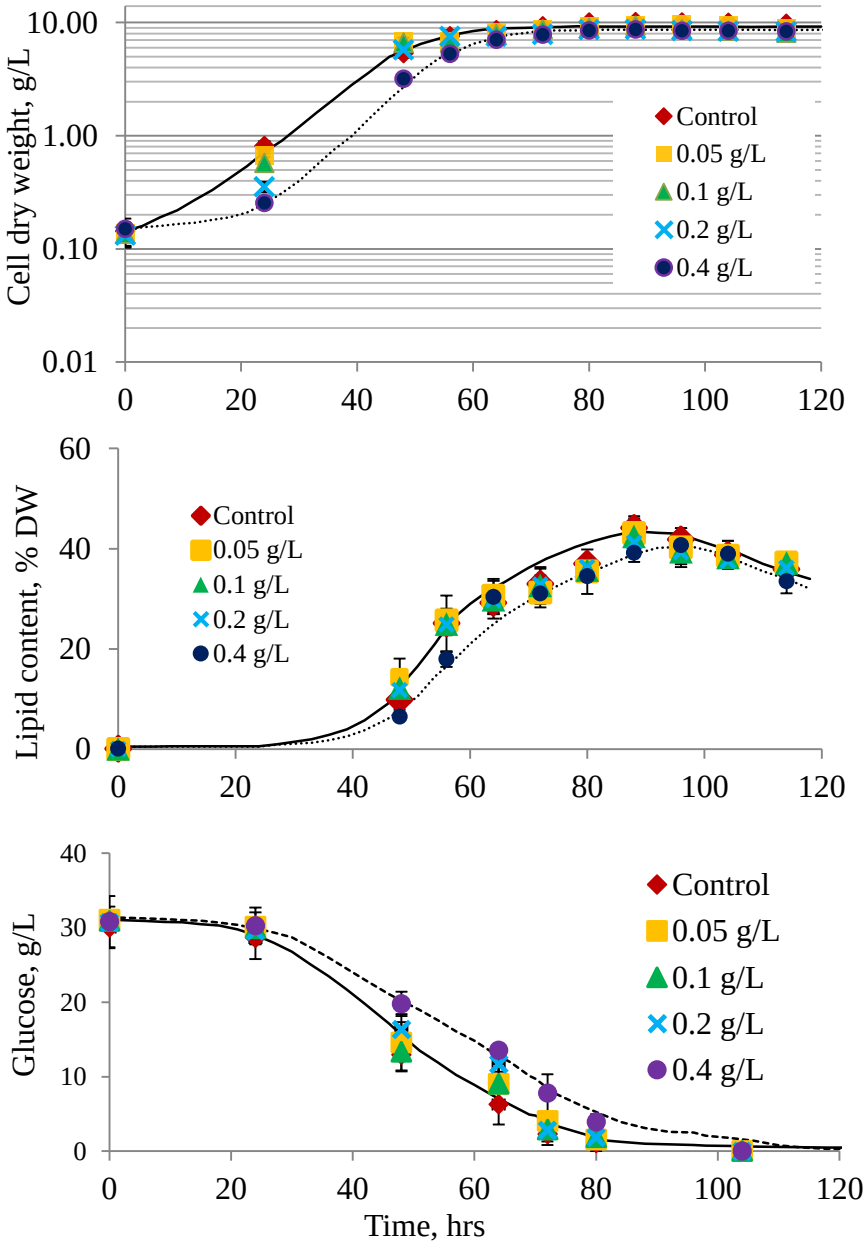


Figure 4.1.4: Effect of Furfural on cell mass, lipid production, and glucose consumption by *Lipomyces starkeyi*

These results suggest that furfural is only slightly toxic for cellular processes at concentrations up to 400 mg/L with increasing concentrations causing reductions in the specific growth rates of the cells. This is confirmed by a summary of the critical growth parameters estimated from results in Figure 4.1.4 presented in Table 4.4 below.

Table 4.4: Effect of Furfural on *L. starkeyi*

Furfural Conc. (g/L)	Specific Growth rate (h^{-1})	Maximum dry weight (g DW/L) (Mean ± 95% CI)	Time for Max DW (h)	Lag Period (hr.)	Maximum lipid fraction (% DW) (Mean ± 95% CI)
0	0.09	10± 0.27	80	0	44.4± 2.9
0.05	0.09	9.6± 0.35	88	0	43.1± 2.8
0.1	0.09	9.4± 0.31	88	0	42.5± 2.8
0.2	0.08	8.7± 0.5	88	0	41.2± 2.16
0.4	0.08	8.7± 0.17	96	20	39.1 ± 1.83

* CI – Confidence Interval

The net effect of furfural on lipid production can be seen more dramatically when lipid production is observed in terms of lipid production per unit culture medium volume: even at furfural concentrations of 0.2 and 0.4 g/L, these numbers were 3.05 g/L and 3.11 g/L, compared to 3.96 g/L in control flasks.

4.1.5 Effect of 5-Hydroxy-methyl-furfural (HMF)

5-Hydroxy-methyl-furfural (HMF)concentrations used in this study were 0.25 g/L, 0.5 g/L, 1 g/L and 1.5 g/L. Profiles of concentration of cell dry weight, intracellular lipid content as percentage of cell dry weight and glucose concentrations are shown in figure 4.1.5(a-c). The critical results are presented in Table 4.5.

Table 4.5: Effect of HMF on *L. starkeyi*

HMF Conc. (g/L)	Specific Growth rate (h^{-1})	Maximum dry weight (g DW/L) (Mean ± 95% CI)	Time for Max DW (h)	Lag Period (h)	Maximum lipid fraction (% DW) (Mean ± 95% CI)
0	0.09	9.33± 0.27	78	0	54.7± 2.93
0.25	0.09	9.07± 0.18	88	0	53.4± 4.1
0.5	0.08	9.26± 0.13	118	0	56.5± 3.1
1	0.07	8.96± 0.32	128	0	45.5± 1.94
1.5	0.06	8.05± 0.17	140	20	27.25± 3.2

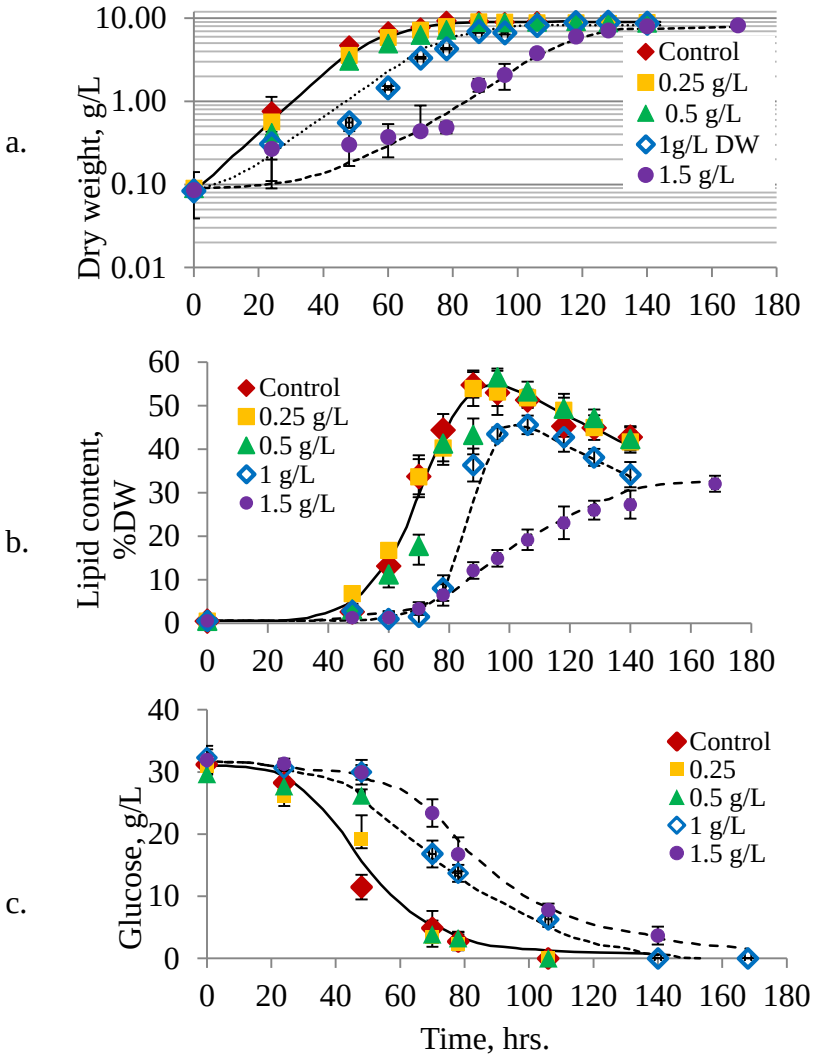


Figure 4.1.5: Effect of HMF on cell mass, lipid production, and glucose consumption by *Lipomyces starkeyi.*

Similar to the effect of furfural, HMF concentration of 0.25 g/L and 0.5 g/L did not impact on cell growth and lipid fraction in cells, as well as on the consumption of glucose. At higher concentration of 1 to 1.5 g/L of HMF, growth of cells was adversely effected.

The specific growth rates were calculated from Figure 4.1.5(a) and the numbers were $0.09h^{-1}$ in flasks containing up to 0.5 g/L HMF, 0.05 h^{-1} in presence of 1 g/L HMF, and 0.045 h^{-1} in flasks containing 1.5 g/L HMF. Maximum cell concentration achieved was same in all concentrations of HMF but in different times of fermentation.

Intracellular lipid content increased rapidly once again when cell concentration of around 5 g DW/L was achieved and the maximum lipid fraction was 55% in flasks with HMF ≤0.5 g/L. But higher HMF concentration results in significantly lower maximum lipid fraction in cells (43% with 1 g/L HMF and 36.4% with 1.5 g/L HMF). The intracellular lipids were re-consumed once all the glucose in medium was gone.

4.1.6 Summary of the Effect of Inhibitors on Cell Growth and Lipid Production of Lipomyces Starkeyi

Five lignocellulosic acid hydrolysis derived byproducts were used individually with regular medium to find the effect on lipid production of *Lipomyces starkeyi* at different concentrations and they were described in previous sections. The maximum lipid fractions and cell mass obtained are plotted in the Figure 4.1.6 to summarize the effect of the hydrolysis-by products. Error bars represent the expected observations within two standard deviations from the mean.

These observations suggest that the concentrations of PHB and vanillin should be limited to ≤ 0.5 g/L in broth and that of HMF and syringaldehyde ≤1 g/L. Furfural should either be completely avoided or limited to ≤0.4 g/L. This result conforms the hydrophobicity of the

inhibitors present in the Table 2.7. In the table, the hydrophobicity value from higher to lower sequence showed as PHB>Vanillin>Syringaldehyde>Furfural>HMF; which indicated PHB and vanillin were the most lethal inhibitors comparing to others. The same sequence has been observed in this study.

There are very few reported results are available to compare the cell growth and lipid production of *L. starkeyi.* Chen et al. (2009) reported that Furfural at 0.5 g/L impacted the cell growth and reduced the cell mass and lipid production significantly while 1 g/L of HMF reduced the cell growth by 10% but there was no impact in lipid production. From the same report, Vanillin at 1 g/L impacted the cell growth severely while at 0.5 g/L, the cell growth and lipid production decreased by 18% and 24% and PHB showed no effect up to 0.5 g/L in *L.starkeyi.*

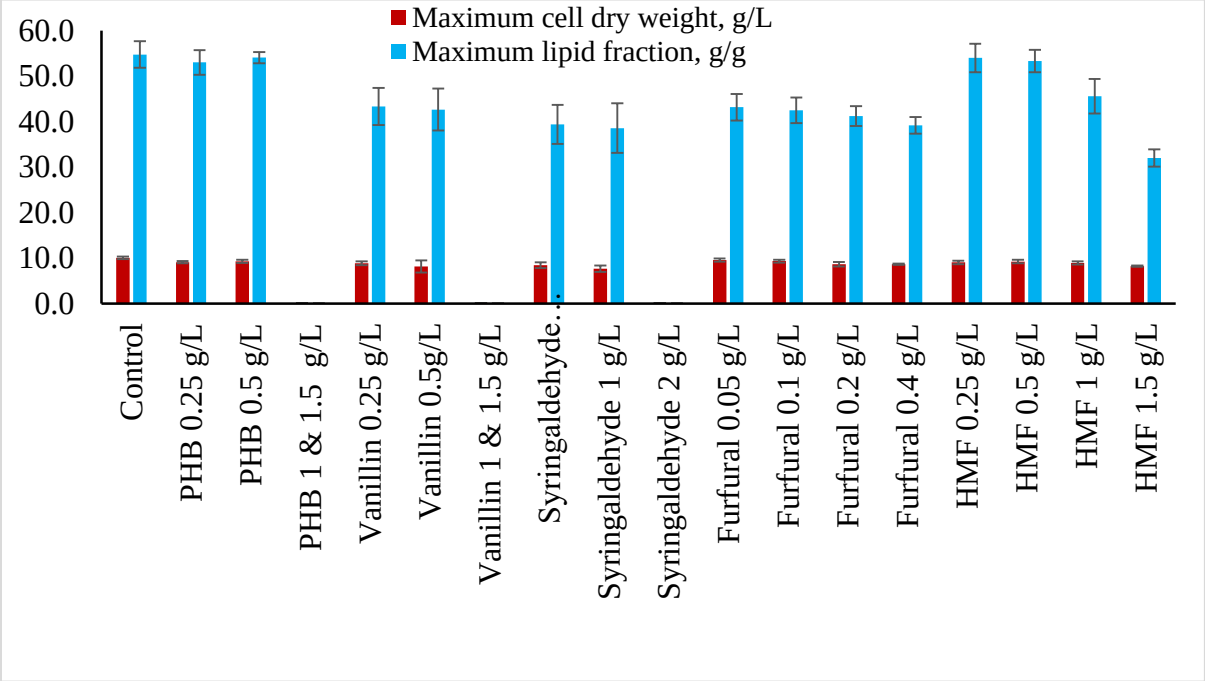


Figure 4.1.6: Comparison of inhibitors in terms of maximum cell growth and lipid production by *Lipomyces starkeyi*

In another work, *Lipomyces starkeyi* was grown on hydrolysates from *Arundo donax* (giant cane) and *Sorghum vulgaris* (grass species) separately after dilute H_2SO_4 treatment. The concentrations of furan and phenolics were less than 1 g/L while acetic acid was ≈5 g/L in both hydrolysates. Cell dry weight observed was around 5-7 g/L and lipid content was 6-19% with the fermentation time of 150 hours (Pirozzi et al., 2013).

Pirozzi et al. (2015) investigated more on *Lipomyces starkeyi* using acid hydrolysates of Arundo donax. The concentrations of acetic acid, furfural, 5-HMF, vanillin, and PHB present in the Arundo donax hydrolysates were 6.22, 0.1, 0.73, 0.035, 0.056 g/L, respectively. *L.starkeyi* grown on these inhibitors resulted cell mass 9.99 g/L, and lipid fraction 19.7% by dry weight in 100 hours of cultivation which represents the competence of *L. starkeyi* in acid hydrolysates containing inhibitors. The results from this study with Furfural, 5-HMF, and vanillin are in agreement with that of Chen et al. (2009).

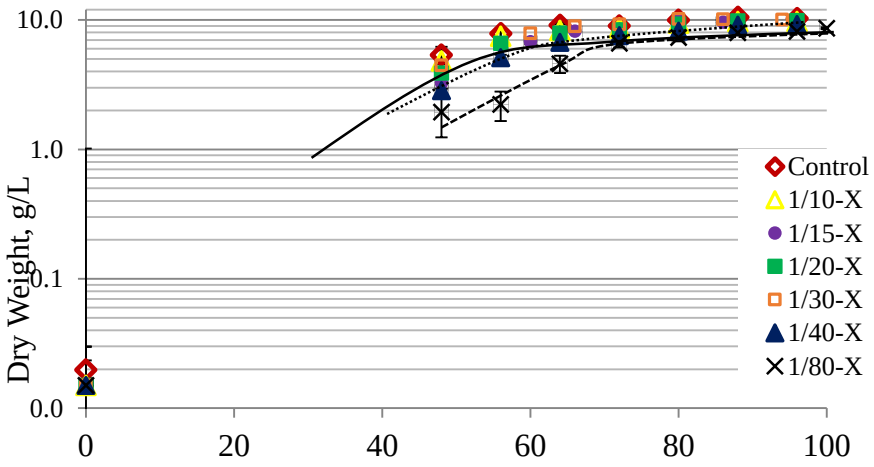
This study did not investigate whether *Lipomyces starkeyi* cells consume these hydrolysis byproducts or not. Their fate would be established as it will impact the total amounts that can be put in the fermenters during fed-batch operation.

4.2 Effect of Phosphate Concentration on Cell Growth and Lipid Production

There is no information available in literature about the effect of phosphate on *Lipomyces starkeyi.* A recent study showed that phosphorous limitation has positive effect on lipid accumulation of *Rhodosporidium toruloides,* even in the presence of rich nitrogen sources (Wu et al., 2010). These authors used KH_2PO4 as a source of phosphate from 0 g/L to 3.6 g/L. At 3.6 KH_2PO_4 g/L lipid production and lipid yield were 3.9 g/L and 21.2%, respectively. Outstanding lipid yield (62%) and lipid production (12.1g/L) were achieved with no added phosphate; phosphorous was transferred only from seed culture (The seed

culture medium contains1.84 g/L KH_2PO_4 and 5% v/v seed was used). In both cases cell biomass was 19 g/L (Wu et al., 2010). Hence, a number of experiments were conducted in shake flasks to find the optimum phosphate concentration and to reduce the medium costs without compromising high lipid yield. A Wide range of phosphate concentration from 9.5 g/L (1-X) to 0.11 g/L ($\frac{1}{80}$-X) was used.

a



b

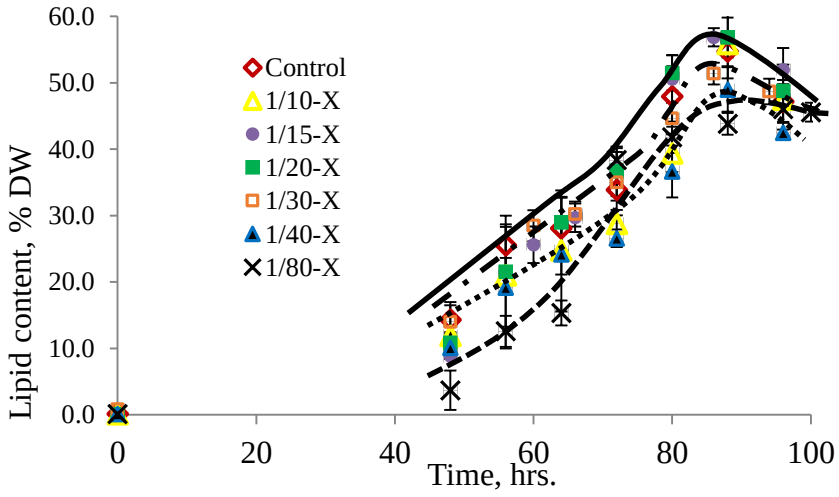


Figure 4.2.1: Cell dry weight and lipid contents (%) from flask experiments using variable concentrations of Phosphate

4.2.2 Discussion of Results

Cell growth profiles remained unaffected by phosphate concentrations (KH_2PO_4 and Na_2HPO_4) in medium for higher than $\frac{X}{30}$. For lower than 1/40-X phosphate concentration, the cells growth rate slowed down as evident from the slopes in the semi-log graph of cell dry weight vs. time (Figure 4.2.1a). The lower the phosphate concentration in the medium the slower the growth was. Exponential growth was observed until about 65 hours in all cases, followed by stationary phase up to 96 hours when the experiment was terminated.

The lipid fraction in dry cell mass has been presented in Figure 4.2.1b. Since phosphate is used in production of phospholipids present in cell membranes, it is not surprising that media with low phosphate concentrations had low lipid fractions. Significant lipid accumulation started after 45 hours of cultivation and it increased at a slow rate until cells reached stationary phase. As soon as cell mass reached to the stationary phase, lipid started to accumulate sharply and reached to maximum point in all cases. Lipid content declined from the peak point due to consumption of accumulated intracellular lipid.

The effect of phosphate content on cell behavior was plotted using the maximum concentrations of cell dry matter, lipid fractions in cells, and volumetric concentration of lipids in media against phosphate concentrations in Figure 4.2.2. Here results of additional experiments involving 1/50-X phosphates are also presented. Once again, we notice all of these cell-medium characteristics show a saturation type behavior where the concentrations were not affected anymore by the initial concentrations of phosphate.

a.

b.

c.

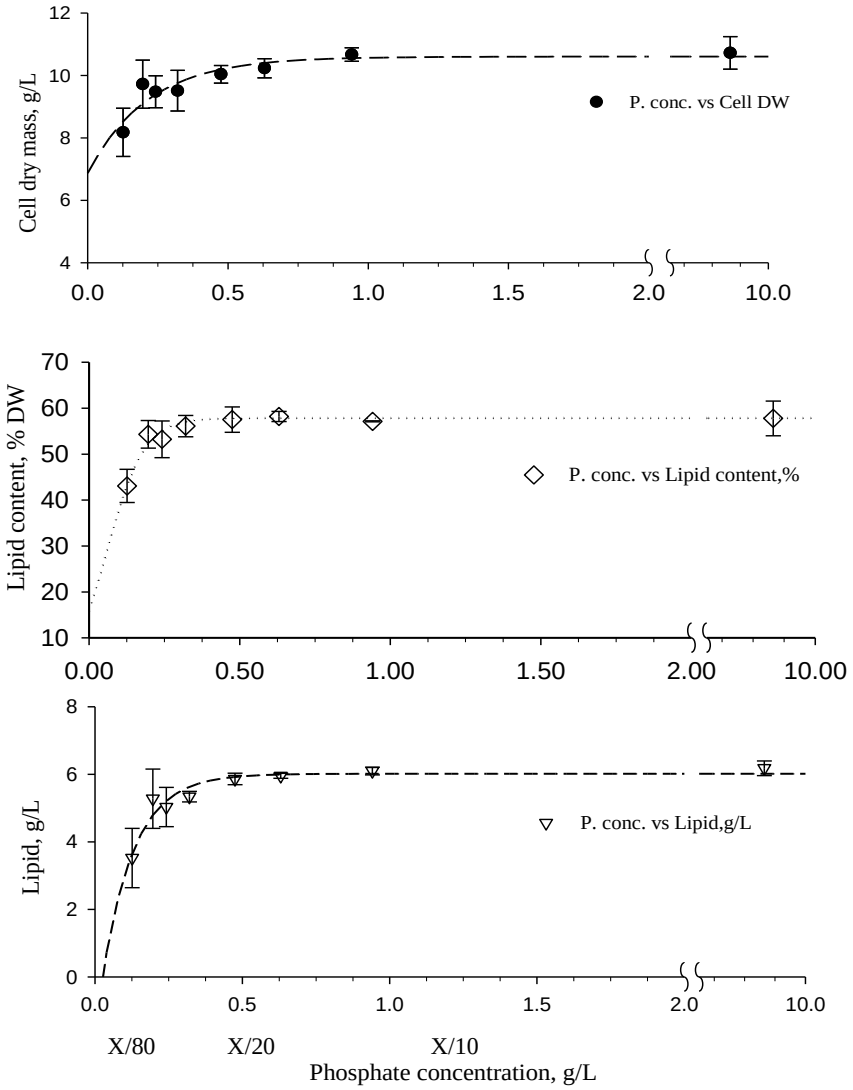


Figure 4.2.2: Maximum dry cell mass and lipid production with phosphate concentration

At the phosphate concentration was 0.48 g/L ($\frac{X}{21}$ g/L) which corresponded to 10.1 g/L of Dry cell biomass with 57.8% of dry mass as intracellular lipids or, alternatively, 5.86 g lipid per liter broth.

4.2.3 Fatty Acid Analysis

Lipid obtained from ASE extraction was processed as describe in section 3.4.6 and FAME was obtained from lipid samples according to the protocol described in section 3.3.4. The results are presented in Figure 4.2.3. Error bars were obtained at 95% confidence interval from six samples. The major fatty acids obtained from all samples were C16:0, C16:1, C18:1 and C18:2. The amount of C16:0, C16:1, C18:1, and C18:2 contributes around 95% of total lipids composition, and remaining was C18:0 (≈3-4%). There was no significant difference of fatty acid composition in reduced phosphate concentration (1/20-X) from 1-X phosphate.

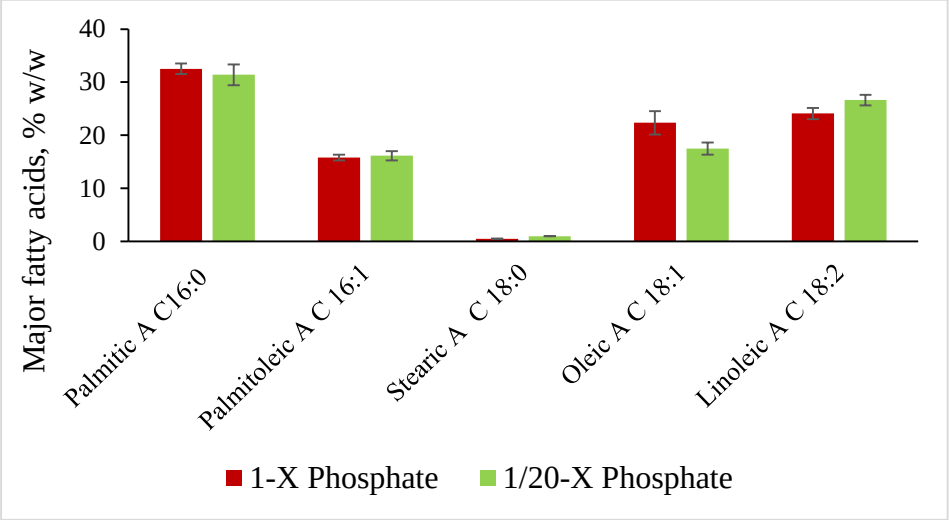


Figure 4.2.3: Fatty acid compositions of Lipomyces starkeyi grown on 1-X and 1/20-X phosphate medium

Unsaturated fatty acid was about 50-60%, which is favorable for biodiesel conversion. The obtained fatty acid composition is comparable with other oleaginous microorganisms and the current source of biodiesel presented in Table 4.2.1.

Table 4.2.1: Fatty acid compositions in microbial lipid and vegetable oil

		Palmitic Acid, % (C16:0)	Palmitoleic acid,% (C16:1)	Stearic acid, % (C18:0)	Oleic acid, % (C18:1)	Linoleic acid, % (C18:2)
Microbial sources	*Lipomyces starkeyi*	33-56	2-6	5-14	26-55	0.1-3
	Rhodortula toruloides	18-37	1	13-36	19-60	13-02
	Cryptococcus curvatus	17-25	-	12	55	8
	Yarrowia lipolytica	11	6	1	28	51
	Mortierella Isabellina	20-27	1-4	2-6	44-54	4.5-18
Vegetable Oil	Oil palm	32-59	-	1-8	27-52	5-14
	Sunflower	3-10	-	1-10	14-65	20-75
	Peanut	6-12.5	-	2.5-6	37-61	13-41
	Soybean oil	11	-	4	22	53
	Corn oil	12	-	0.9	25	61

4.3 Lipid Extraction Using Different Solvents

For these experiments, *Lipomyces starkeyi* was cultivated in a 4-L fermenter on starch in a fed-batch mode. The final cell dry mass concentration in this experiment after 200 hours of operation was 45 g/L and a centrifuge was used to get wet cells. The cells were freeze-dried at -80 ^{0}C.

Extraction of lipids was conducted from wet cells as well as freeze dried cells of *Lipomyces starkeyi*. The different solvent extraction protocols described in Chapter 3 was used with the wet cells as well as the freeze dried cells. Only the freeze-dried cells were subjected to an accelerated solvent extractor (ASE) with protocols described in Chapter 3. Several solvents (chloroform, methanol, isopropanol, hexane, and methyl tertiary butyl ether) were used either as individually or in a proportion which is described in materials and methods section. Results of these experiments are presented in Figure 4.3.1. In this figure, the amount of lipid extracted has been presented as a fraction of cell dry weight. The amount of lipid extracted with chloroform-methanol solvent system may be assigned as the total lipid fraction in the

cells. This was also the highest amount of lipids extracted from these cells of all the solvents. From this, it may be concluded that the cells produced in this fermentation had lipid content of 72% on dry-weight basis. It should be noted that use of this combination of polar (methanol) and non-polar (chloroform) solvents would result in extraction of phospholipids as well as neutral lipids (Halim et al. 2011). Consequently, 72% DW represents the total lipid content in the cells.

Use of wet cells for extraction of lipids resulted consistently in 40-50% less lipids compared to that from freeze-dried cells, irrespective of the solvent system used. For all the solvent systems, the amounts of lipids extracted by ASE and by the 5 different extraction methods discussed in section 3.4 (Materials and Methods) were almost identical (Figure 4.3.1), suggesting that the method used in this work is as efficient as the ASE method (which involves use of solvents at high pressures and high temperatures). On the other hand, the maximum amount of lipids extracted from wet cells was only 43.3% DW while using Methyl-tertiary-butyl-ethanol (MTBE) and methanol as solvent.

Hexane-Isopropanol (IPA) solvent system and Methyl-tertiary-butyl-ethanol (MTBE)-Methanol solvent system were also found to be quite efficient in extracting the lipids from dried cells. MTBE-Methanol, with 68.9% DW extracted lipids from freeze-dried cells, was almost as efficient as the chloroform-methanol system. With Hexane-IPA system, lipids extracted were 66% DW from Freeze-dried cells and 39% from the wet cells. Hexane and chloroform, on the other hand, were not as efficient as the other solvents. This was, however, expected given the fact that both of these solvents are non-polar. Hexane is the more non-polar of the two and it had the lowest amounts of lipids extracted from the cells. Chloroform

is slightly more polar (due to its dipole-moment) than hexane and it extracted slightly more lipids (perhaps some phospholipids).

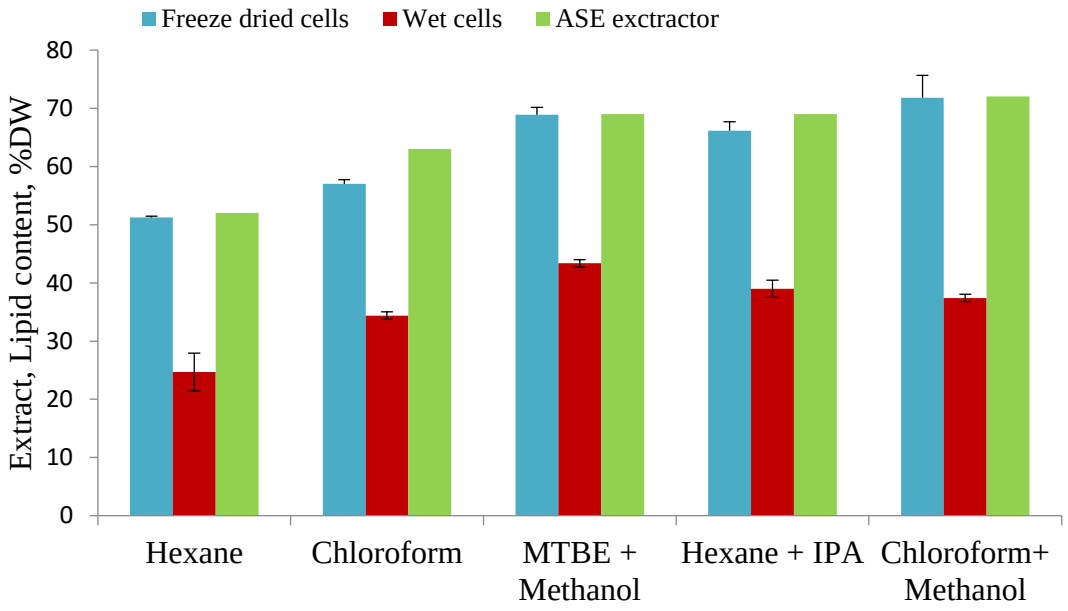


Figure 4.3.1: Comparison of lipid extraction methods using different solvents

Though chloroform-methanol was the best solvent system for lipid recovery, its classification as a probable human carcinogen precludes its widespread use (EPA, 2000). Hexane is the industry standard for extracting edible oil from dry matter mainly because of its low cost ($1.5/kg) and high volatility. Our results suggest that MTBE-Methanol and Hexane-IPA have lipid extraction efficiencies almost similar to chloroform-methanol system. MTBE offers ease of recovery, high oil solubility (Aremu et al., 2015; Johnson and Lusas, 1983), non-carcinogenic character, and low cost ($1/kg). As a result, MTBE and methanol could be a candidate as a potential solvent for lipid extraction.

CHAPTER 5: ECONOMIC EVALUATION

The goal of this work was to reduce the cost of production of microbial lipids by optimization of phosphate concentration, effective solvent selection, and evaluating uses of lignocellulosic hydrolysates as carbon sources. The following analysis shows the improvements that were achieved as a result of the experiments conducted in this work and their economic implications.

5.1 Cost of Fermentation Medium for Producing Gallon of Lipids

Bulk prices of different medium components were obtained from literature sources, including those from ICIS and/or Alibaba for the purpose of medium cost calculations. These are listed in Table 5.1.1.

Based on lipid production and the cost of elements in different media, costs per L medium and per gallon of lipid were calculated. Details of medium composition and their contributions per gallon of lipids formed are presented in Table 5.1.2 for the case of 1-X medium. For this medium, the medium constituents contributed $9.34 per gallon of lipid produced. Of this, the two major cost contributors were starch ($2.46 per gallon lipids) and phosphates ($6.7 per gallon lipids). In Table 5.1.3 are presented the lipids produced in media containing phosphate concentrations ranging from 1-X to 1/80-X along with the costs (total as well as those of starch and phosphates) per gal lipids produced. As the phosphate concentration was gradually reduced, the medium costs per gallon of lipid produced initially decreased from the values in 1-X medium due to excess phosphate present in it. But reductions beyond 1/20-X were counterproductive as it affected the metabolism in the cells. From the perspective of phosphates, medium composition corresponding to 1/20-X was optimal ($3.11 per gallon lipid produced). Of this cost, 11% were the costs of phosphates and

carbon source (starch) costs were 83%. Under these conditions, 15.8 mg phosphates were needed per kg equivalent glucose in the medium and phosphate related costs were reduced by a factor of 20.

The next cost item for the production of microbial lipids from *Lipomyces starkeyi* is that of carbohydrates. From the above discussion, it is clear the carbohydrates supplied as glucose or starch would contribute as much as 83% towards the cost of media per gallon of lipid produced. In the case of starch, the cost of starch per gallon of lipids was $2.58 (Table 5.1.3). The carbohydrate costs can be reduced by using lignocellulosic materials as sources of carbohydrates. According to a report from NREL (Humbird et al., 2011), the cost of carbohydrates from Lignocellulosic materials is $0.058 per kg equivalent glucose which is 1/3 that of commercial grade starch ($0.15/kg, Table 5.1.1). As per the previous research conducted in our laboratory with glucose and xylose, lipid yield of *Lipomyces starkeyi* on both of these sugars is identical, suggesting that lignocellulosic sugars can be effectively substituted for glucose. If that were so, the cost of carbohydrates would be reduced to $0.86 per gallon of lipids (under the same conversion efficiencies as found in our experiments), bringing the total medium costs down to $1.40 per gallon of lipid produced i.e. 15% of the costs using the 1-X media.

Table 5.1.1: Prices of medium components in bulk amount

Medium components		**Costs ($/kg)**	**Source**
Starch		0.15	Alibaba
$CaCl_2$		0.275	ICIS, Alibaba
$(NH_4)_2SO_4$		0.15	Alibaba
KH_2PO_4		1.2	Alibaba
Na_2HPO_4, 7 H_2O		1.1	Alibaba
$MgSO_4$, 7 H_2O		0.2	Alibaba
$FeSO_4$		0.3	Alibaba
$ZnSO_4.H_2O$		0.5	Alibaba
Vitamins	Inositol	5	Alibaba
	Ca Pantothenate	10	
	Biotin	50	
Trace elements	$CoCl_2$. $6H_2O$	70	Alibaba
	$MnSO_4$	0.5	
	$CuSO_4$	1.9	

Table 5.1.2: Cost of medium per gallon of lipids produced for 1-X media

Medium Components		Composition, g/L medium	Medium cost, $/gal lipid
Sweet Potato starch		27.3	2.46
$CaCl_2$		0.1	0.018
$(NH_4)_2SO_4$		0.5	0.063
KH_2PO_4		7	5.05
Na_2HPO_4. 7 H_2O		2.5	1.65
$MgSO_4$. 7 H_2O		1.5	0.09
$FeSO_4$		0.0082	0.0015
Vitamins		mg/L	
	Inositol	3	0.011
	Ca Pantothenate	0.6	
	Biotin	0.003	
Trace element			
	$CoCl_2$. $6H_2O$	9.41	0.08
	$MnSO_4$	0.007	
	$CuSO_4$	1.9	
Total cost of medium/gal lipid			9.34

Table 5.1.3: Cost of starch and phosphates per gallon lipid produced

Medium with variable phosphate (1 X ≡ 9.25 g Phosphates/L)	Lipid production, gal lipid/ m^3 medium	Total cost of medium ($/gal lipid)	Starch cost ($/ gal lipid)	Phosphate cost ($/gal lipid)	Cost of phosphates as % of total cost (%)
1-X	0.439	9.34	2.46	6.7	72
1/10-X	0.422	3.45	2.56	0.70	20
1/15-X	0.423	3.21	2.56	0.46	14
1/20-X	0.420	3.11	2.58	0.35	11
1/30-X	0.378	3.33	2.86	0.31	8
1/40-X	0.365	3.56	2.96	0.24	6
1/80-X	0.260	4.62	4.16	0.12	3

5.2 Cost of Solvent per Gallon of Lipid Extracted

Bulk prices of solvents were obtained from literature sources, including those from ICIS, and Alibaba for the purpose of cost calculations. Lipid recovery by solvent extraction was compared in terms of $/gallon of lipid extraction. The cost calculation considered only the cost of solvents after recovery that considers 1% to 2% loss during the process of recovery. The comparative cost has been presented in the following Table 5.2.1.

Table 5.2.1 Cost of solvents per gallon of lipid extracted

Solvents	Bulk price of Solvent, $/L	Lipid from freeze dried cells, g/g	Solvent required, L/ gal-Lipid	Cost of solvent, $/gal lipid (2% solvent loss)	Cost of solvent, $/gal lipid (1% solvent loss)
Hexane	0.66	0.52	14.45	2.71	1.35
Chloroform	0.97	0.63	11.93	3.30	1.65
Hexane + IPA	0.66 + 0.79	0.69	10.89	2.21	1.09
Chloroform + Methanol	0.97 + 0.28	0.72	10.89	2.21	1.10
MTBE + Methanol	0.70 + 0.28	0.69	10.44	1.88	0.94

**MTBE, Chloroform, Methanol, IPA price obtained from Alibaba, Hexane price from ICIS and Alibaba*

The volumes of solvents required per gallon of extracted lipids were scaled from the solvents used in ASE extraction of dry-cells. Since most of the solvents can be recovered from the extracted lipids for recycle, two scenarios were considered; in which solvent recovery was 98% and the other in which the solvent recovery was 99%. In all the cases, lowest solvent costs corresponded to those with high lipid recovery (i.e. MTBE+ Methanol, Hexane+IPA, Chloroform+Methanol). With 2% loss of solvents in the recovery process, solvents were still projected to be $1.88 per gallon of lipids (Table 5.2.1), that though 27% less than that when using hexane as solvent, are still considerable. As a result, it may be concluded that efficiency of solvent recovery process will play an important role in establishing the economics of microbial lipid production. These results also suggest that MTBE- Methanol may be a better solvent for lipid extraction, from the viewpoint of costs, i.e. $0.94 for a gallon of lipid.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

Conclusions

- The inhibitor tolerance of *Lipomyces starkeyi* is promising; it is able to grow in presence of most common inhibitors produced during acid hydrolysis of lignocellulosics. As shown in the results of the effect of inhibitors, the presence of higher concentration of inhibitors has effect on cell growth and lipid production.
- The inhibitory effect was not significant in all cases except furfural up to 500 mg/L of inhibitors in fermenting media. In case of furfural it was 400 mg/L.
- Most of the hydrolysates contain these compounds under this limit according to literatures (Table 2.6).
- Growth patterns of *Lipomyces starkeyi* suggested that seed cultures of the yeast should be prepared in media containing the inhibitory components and that the fermentation media be seeded with a heavy inoculum (significantly more than 2% v/v used in this study) in order to reduce the fermentation time.
- Studies involving concentration of phosphates in the medium suggested that 0.48 g/L phosphate in a medium containing 30 g/L concentration of glucose equivalents results in as same growth of cells and lipid production as higher phosphate levels.
- Extraction of lipids using wet cells consistently resulted in reduced efficiencies of extraction. Three solvents systems (MTBE-Methanol, Chloroform-methanol, and Hexane-isopropanol) showed higher extraction potential while using freeze-dried cells,

Recommendations

- The effect of inhibitors on cell growth and lipid production by *Lipomyces starkeyi* was investigated in this study only in presence of single inhibitor(s). These studies did not address the question if the effect of different inhibitors is simply additive or synergistic. Since several inhibitors are produced during the process of acid hydrolysis of lignocellulosic biomass, elucidation of their combined effect on cellular metabolism is critical in development of processes using lignocellulosic biomass.
- This study did not address the fate of inhibitors during the growth and lipid production by *Lipomyces starkeyi*. Quantitative analysis of inhibitors needs to be performed in a future study to identify the limits of pretreatment of acid-hydrolysates for high-density fed-batch production of lipids by this yeast.
- Even though this study established a ratio of 0.016 g phosphates per g glucose as optimal in a batch medium, chemostat studies should be performed to establish the C/P ratios in feed media for high density cultivations.
- Although use of wet cells in lipid extraction resulted in reduced extraction efficiencies, the economic impact of these reduced extraction efficiencies needs to be separately explored. Use of wet cells would leave significant lipids in cells, but also reduce the costs associated with drying of cells. At the same time, cell cake with significant lipids may turn out to fetch higher market price due to improved nutritional value of the cake. They may be used as fracking mud which has a higher economic value. Thus a holistic economic analysis of the downstream processing of microbial cake, including its impact on the extent of recovery of solvents, is recommended for future work.

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APPENDIX: RAW DATA

7.1 Phosphate optimization experiment:1

Experiment with control- 1X phosphate (1% seed), control- 1X phosphate (2% seed), 1/20-X phosphate (1% seed), 1/50-X phosphate (1% seed), and 1/50-X phosphate (2% seed).

Table 7.1 .1 Data for optical density and fluorescence intensity

1%Control

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.015	0.017	554	14.35	554	0.23	552	15.88	552	13.654	1
36	11	0.377	0.381	555	35.35	555	0.55	555	46.65	555	0.316	1
48	15	0.515	0.519	544	213.84	544	-0.60	543	195.21	543	-0.62	1
56	20	0.519	0.517	545	372.99	545	-0.58	547	267.36	541	-0.467	2
64	25	0.489	0.485	542	291.09	542	-0.44	544	319.56	545	-0.555	2
72	25	0.499	0.493	557	518.85	557	0.69	559	526.75	559	-0.144	2
80	25	0.559	0.552	545	870.19	545	0.20	545	870.19	545	-0.567	2
88	25	0.572	0.58	541	713.74	541	0.38	543	737.48	543	0.567	2
96	25	0.591	0.593	543	744.85	543	-0.63	542	691.85	542	0.345	2
104	25	0.595	0.599	571	477.34	571	-0.90	571	490.51	571	0.365	2

2 % Control

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.023	0.025	554	13.55	554	0.21	552	14.34	552	0.232	1
36	11	0.552	0.56	551	41.13	551	-0.06	556	26.59	556	0.365	1
48	15	0.567	0.562	546	226.10	546	0.25	545	215.21	545	0.258	1
56	20	0.483	0.487	546	379.97	546	0.33	554	403.84	554	0.025	2
64	25	0.469	0.474	543	381.27	543	0.26	545	425.43	545	0.365	2
72	25	0.484	0.485	564	460.60	564	0.32	560	504.92	560	0.345	2
80	25	0.544	0.55	541	620.58	541	0.87	543	651.63	543	0.964	2
88	25	0.524	0.521	545	898.34	545	0.02	544	668.14	544	0.896	2
96	25	0.571	0.574	546	986.58	546	-0.83	540	997.84	540	0.758	2
104	25	0.58	0.586	572	621.63	572	-1.67	568	628.72	568	0.635	2

Note: *ND: no. of dilution, OD: optical density, NR: Nile red, W.L: wave length, 1 & 2 denote samples*

1% ,X/50

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.016	0.15	554	11.43	554	0.45	552	15.88	552	0.122	1
36	11	0.196	0.193	560	15.53	560	1.24	538	19.70	538	0.085	1
48	15	0.318	0.313	548	87.64	548	0.40	551	87.10	551	0.048	1
56	20	0.347	0.35	545	224.73	545	0.96	547	214.89	547	0.036	2
64	25	0.369	0.365	546	483.54	546	0.15	546	448.44	546	0.058	2
72	25	0.381	0.384	545	657.17	545	0.37	545	666.38	545	0.014	2
80	25	0.426	0.429	542	608.97	542	0.70	543	632.08	543	0.025	2
88	25	0.463	0.468	544	821.69	544	0.70	544	611.18	544	0.365	2
96	25	0.498	0.499	545	735.87	545	0.03	544	758.58	544	0.986	2
104	25	0.505	0.511	550	619.62	550	0.10	549	638.13	549	0.574	2

2%,X/50

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.022	0.021	554	14.77	554	0.06	552	16.23	552	0.045	1
36	11	0.381	0.378	558	46.36	558	0.09	559	48.37	559	0.014	1
48	15	0.426	0.431	550	84.17	550	0.26	548	128.92	548	0.025	1
56	20	0.443	0.447	545	260.94	545	0.37	546	236.14	546	0.048	2
64	25	0.426	0.423	546	233.30	546	0.55	543	222.45	543	0.089	2
72	25	0.439	0.44	545	613.02	545	0.34	549	632.65	549	0.145	2
80	25	0.521	0.52	544	773.55	544	0.26	546	743.10	546	0.179	2
88	25	0.542	0.543	544	818.35	544	0.15	544	852.94	544	0.141	2
96	25	0.556	0.562	544	857.61	544	0.59	542	862.44	542	0.989	2
104	25	0.523	0.515	548	645.37	548	0.36	548	657.70	548	0.333	2

OD at 550 nm Fluorescence

1%,X/20

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.019	0.017	0.018	12.45	554	0.08	552	11.35	552	0.111	1
36	11	0.211	0.213	560	12.69	560	0.96	555	20.85	555	0.248	1
48	15	0.317	0.32	549	72.10	549	0.25	546	73.06	546	0.912	1
56	20	0.35	0.354	545	224.73	545	0.36	547	265.63	547	0.259	2
64	25	0.36	0.366	546	288.79	546	1.85	545	312.25	545	0.363	2
72	25	0.379	0.385	546	681.08	546	0.03	548	698.52	548	0.334	2
80	25	0.428	0.425	546	793.50	546	0.08	544	776.35	544	0.882	2
88	25	0.45	0.451	541	782.44	541	0.97	545	839.34	545	0.144	2
96	25	0.504	0.5	541	861.28	541	0.67	543	833.26	543	0.254	2
104	25	0.51	0.515	544	608.57	544	0.13	545	623.65	545	0.998	2

Note: *ND: no. of dilution, OD: optical density, NR: Nile red, W.L: wave length, 1 & 2 denote samples*

Table 7.1.2 Dry cell

mass measurement

	Flask	Volume filtered, mL	Filter paper, gm	Filter Paper+ dry cell, gm	wt. of dried cells, gm	Cell Mass conc., g/L
1% Control	A1	4	0.1234	0.17	0.0475	11.88
	A2	4	0.1209	0.16	0.0438	10.95
	A3	4	0.1271	0.18	0.0501	12.53
2 % Control	A1	4	0.122	0.17	0.0458	11.45
	A2	4	0.1238	0.17	0.0463	11.58
	A3	4	0.125	0.17	0.0472	11.80
1%, X/20	A1	4	0.1277	0.17	0.0422	10.55
	A2	4	0.1258	0.17	0.0403	10.08
	A3	4	0.1266	0.17	0.0454	11.35
1%, X/50	A1	4	0.1261	0.16	0.0364	9.10
	A2	4	0.1268	0.16	0.0363	9.08
	A3	4	0.1261	0.16	0.0321	8.03
2%, X/50	A1	4	0.1246	0.16	0.0373	9.33
	A2	4	0.1229	0.16	0.0369	9.23
	A3	4	0.1209	0.16	0.0429	10.73

Table 7.1.3 Lipid extraction measurement

	Freeze dried cells, gm	Wt. of Aluminum foil boat, gm	Aluminum. boat+lipid ,gm	wt. of Lipid, gm	gm lipid/gm DW
1%, control	1.012	5.146	5.5028	0.36	0.35
2%, control	1.018	5.7283	6.1394	0.41	0.40
1% , 1/20-X	1.0102	5.2316	5.506	0.27	0.27
1%, 1/50-X	1.0506	5.3246	5.5285	0.20	0.19
2%, 1/50-X	1.0452	5.572	5.811	0.24	0.23

Table 7.1.4 Calibration of optical density to dry weight

Samples were mixed from three flasks after harvesting at 104 hours

1%, Control

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	DW/Dil. Factor, Calculated dry wt, gm/L (from diluted sample)
	100	0.595	0.599	0.60	25.00	0.47
	80	0.505	0.506	0.51	25.00	0.38
	60	0.425	0.429	0.43	25.00	0.28
	40	0.314	0.312	0.31	25.00	0.19
	20	0.189	0.185	0.19	25.00	0.09
	0			0.00	0.00	0.00

2%, Control

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
	100	0.58	0.586	0.58	25.00	0.46
	80	0.499	0.50	0.50	25.00	0.37
	60	0.405	0.409	0.41	25.00	0.28
	40	0.315	0.309	0.31	25.00	0.19
	20	0.201	0.198	0.20	25.00	0.09
	0			0.00		0.00

1% , X/20

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
	100	0.51	0.515	0.51	25.00	0.43
	80	0.425	0.429	0.43	25.00	0.34
	60	0.352	0.356	0.35	25.00	0.26
	40	0.235	0.238	0.24	25.00	0.17
	20	0.126	0.13	0.13	25.00	0.09
	0	0.00	0	0.00		0.00

1%, X/50

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt., gm/L (from diluted sample)
	100	0.505	0.511	0.51	25.00	0.35
	80	0.425	0.423	0.42	25.00	0.28
	60	0.341	0.338	0.34	25.00	0.21
	40	0.234	0.231	0.23	25.00	0.14
	20	0.139	0.134	0.14	25.00	0.07
	0	0.00	0	0.00		0.00
2%, X/50						
	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt., gm/L (from diluted sample)
	100	0.523	0.515	0.52	25.00	0.39
	80	0.435	0.438	0.44	25.00	0.31
	60	0.354	0.351	0.35	25.00	0.23
	40	0.256	0.25	0.25	25.00	0.16
	20	0.123	0.125	0.12	25.00	0.08
	0			0.00		0.00

Table 7.1.5 Lipid calibration

Samples were mixed from three flasks after harvesting at 96 hours

1%, control

% dilution	With NR W.L	Intens.	Without NR W.L	Intens.	With NR W.L	Intrens.	W.L	ND	Avg. Fluor.	Lipid, gm/L
100%	571	377	571	0.794	571	390.506	571	3	383	3.84
80%	573	311	573	-0.643	568	305.911	568	3	309	3.07
60%	575	275	575	-0.868	569	262.225	569	3	269	2.30
40%	577	193	577	-0.916	579	205.601	579	3	200	1.54
20%	576	131	576	-0.861	577	116.722	577	3	125	0.77
0%									0	0.00

2%, control

% dilution	With NR W.L	Intens.	Without NR W.L	Intens.	With NR W.L	Intrens.	W.L	Avg. Fluor.	Lipid, gm/L
100%	572	422	572	0.576	568	428.723	568	425	2.41
80%	569	353	569	-0.443	568	338.435	568	346	1.93
60%	573	283	573	-0.568	574	288.822	574	287	1.45
40%	576	188	576	-0.516	574	175.046	574	182	0.97
20%	575	138	575	-0.861	581	124.551	581	132	0.48
0%								0	0.00

1% ,X/20

% dilution	With NR W.L	Intens.	Without NR W.L	Intens.	With NR W.L	Intrens.	W.L	Avg. Fluor.	Lipid, gm/L
100%	544	378	544	0.975	545	373.651	545	375	1.25
80%	544	320	544	-0.681	545	316.166	545	319	1.00
60%	547	253	547	-0.691	546	245.316	546	250	0.75
40%	548	174	548	-0.707	547	166.258	547	171	0.50
20%	550	102	550	-0.769	548	105.158	548	104	0.25
0%								0	0.00

1%, 1/50-X

% dilution	With NR W.L	Intens.	Without NR W.L	Intens.	With NR W.L	Intens.	Without NR W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	550	494	550	0.888	549	481.258	549	0.89	2	487	0.55
80%	546	404	546	-0.479	545	416.361	545	0.781	2	410	0.44
60%	548	327	548	-0.439	547	331.425	547	-0.799	2	330	0.33
40%	550	189	550	-0.794	549	174.304	549	-0.81	2	182	0.22
20%	551	97	551	-0.843	553	101.071	553	-0.879	2	100	0.11
0%										0	0.00

2%, 1/50-X

% dilution	With NR W.L	Intens.	Without NR W.L	Intens.	With NR W.L	Intens.	Without NR W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	548	542	548	0.896	548	512.23	548	1.808	2	526	0.55
80%	548	465	548	0.62	550	474.325	550	0.654	2	469	0.44
60%	547	388	547	-0.597	548	378.125	548	-0.681	2	384	0.33
40%	549	275	549	-0.607	547	264.265	547	-0.691	2	270	0.22
20%	549	121	549	-0.654	553	140.1234	553	-0.707	2	131	0.11
0%										0	0.00

Note: *ND: no. of dilution, NR: Nile red, W.L: wave length, Intens: Intensity, Fluor: Fluorescence*

7.2 Phosphate optimization experiment:2

Experiment with control- 1X phosphate (2% seed, 1/20-X), 1/20-X phosphate (1% seed), 1/50-X phosphate (1% seed), and 1/50-X phosphate (2% seed).

Table 7.2.1 Data for optical density and fluorescence intensity

Control

	OD at 550 nm			Fluorescence							
				Without NR1		With NR2		Without NR2		ND	
Hrs.	ND	OD1	OD2	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.022	0.021	14.77	554	0.06	552	16.23	552	0.045	1
48	15	0.499	0.513	396.1	544	8.1	544	381.0	544	6.2	1
60	25	0.423	0.429	703.9	543	9.1	544	660.7	544	11.2	1
66	25	0.475	0.447	756.6	545	10.4	544	738.6	544	14.9	1
72	25	0.496	0.501	791.3	544	13.8	543	749.1	543	15.2	1
80	30	0.429	0.441	669.3	545	14.7	542	716.2	542	8.6	2
86	30	0.552	0.548	867.8	546	6.8	545	890.7	545	7.6	2
94	32	0.472	0.473	594.9	545	7.1	545	599.2	545	7.9	2
96	33	0.409	0.399	363.8	544	3.5	542	342.6	542	2.4	3

X/10

	OD at 550 nm			Fluorescence							
				Without NR1		With NR2		Without NR2		ND	
Hrs.	ND	OD1	OD2	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.023	0.025	13.55	554	0.21	552	14.34	552	0.232	1
48	15	0.437	0.442	339.9	544	4.9	545	351.5	545	5.3	1
60	25	0.394	0.388	666.2	543	10.2	545	675.6	545	6.5	1
66	25	0.43	0.422	725.3	545	8.7	546	764.3	546	18.6	1
72	25	0.452	0.446	794.9	545	12.2	545	831.7	545	13.0	1
80	30	0.41	0.417	578.0	545	7.8	544	553.8	544	4.4	2
86	30	0.421	0.428	919.5	546	8.1	546	937.7	546	8.3	2
94	32	0.515	0.529	641.3	554	3.3	550	632.8	550	4.3	2
96	33	0.407	0.412	396.6	545	3.6	545	419.5	545	4.5	3

X/20

	OD at 550 nm			Fluorescence							
				Without NR1		With NR2		Without NR2		ND	
Hrs.	ND	OD1	OD2	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.015	0.017	14.35	554	0.23	552	15.88	552	13.654	1
48	15	0.386	0.393	316.6	545	5.5	544	345.7	544	3.3	1
60	25	0.352	0.359	745.1	547	5.1	547	769.0	547	5.9	1
66	25	0.388	0.382	696.7	547	6.2	546	729.2	547	5.5	1
72	25	0.404	0.414	788.2	543	10.8	548	791.7	548	8.8	1
80	30	0.411	0.407	686.4	546	10.5	545	720.1	545	11.9	2
86	30	0.477	0.482	922.9	545	7.9	543	906.1	543	10.3	2
94	32	0.483	0.478	687.9	547	5.9	543	698.1	543	5.4	2
96	33	0.372	0.365	451.1	546	3.6	544	436.5	544	4.1	3

X/40

	OD at 550 nm			Fluorescence							
				Without NR1		With NR2		Without NR2		ND	
Hrs.	ND	OD1	OD2	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.023	0.025	13.55	554	0.21	552	14.34	552	0.232	1
48	15	0.363	0.36	285.2	545	4.9	545	325.9	545	5.9	1
60	25	0.347	0.345	678.8	546	4.0	545	683.6	545	20.3	1
66	25	0.368	0.37	832.9	545	5.9	547	867.3	547	21.4	1
72	25	0.391	0.398	790.0	544	9.9	544	790.0	544	9.9	1
80	30	0.369	0.373	499.2	544	10.2	543	542.7	543	11.2	2
86	30	0.429	0.424	720.7	544	12.9	545	750.7	545	11.8	2
94	32	0.49	0.496	694.6	547	8.9	548	682.8	548	9.9	2
96	33	0.408	0.401	359.1	550	3.3	548	371.1	548	4.5	3

Note: *ND: no. of dilution, OD: optical density, NR: Nile red, W.L: wave length, 1 & 2 denote samples, Intens.: Intensity*

Table 7.2.2 Dry cell mass measurement

	Flask	Volume filtered, mL	Filter paper, gm	Filter Paper+ dry cell	Wt. of cells, gm	Cells conc. g/L	Avg. DW., g/L
Control	A1	4	0.1228	0.2	0.0427	10.7	
	A2	4	0.1245	0.2	0.0406	10.2	10.39±0.39
	A3	4	0.1274	0.2	0.0414	10.4	
1/10X	A1	4	0.1247	0.2	0.0368	9.2	
	A2	4	0.1241	0.2	0.0357	8.9	9.12±0.18
	A3	4	0.1219	0.2	0.037	9.3	
1/20X	A1	4	0.1246	0.2	0.0381	9.5	
	A2	4	0.1231	0.2	0.0413	10.3	9.89±0.34
	A3	4	0.1239	0.2	0.0393	9.8	
1/40X	A1	4	0.1246	0.2	0.0387	9.7	
	A2	4	0.1221	0.2	0.0408	10.2	9.86±0.37
	A3	4	0.1209	0.2	0.0395	9.9	

Table 7.2.3 Lipid extraction measurement

mixed samples from three flasks after harvesting at 96 hours

	Flask	wt. of tube, gm	tube+ lipid, gm	wt. of Lipid, gm	gm lipid/ gm biomass	Avg. gm lipid/gm biomass
Control	A1	13.621	13.9287	0.3	0.38	
	A2	13.582	13.9223	0.3	0.42	0.4
X/10	B1	13.762	14.0835	0.3	0.40	
	B2	13.790	14.1632	0.4	0.46	0.4
X/20	C1	13.663	13.9942	0.3	0.41	
	C2	13.695	14.0189	0.3	0.40	0.4
	D1	13.762	13.9932	0.2	0.33	
X/40	D2	13.559	13.7845	0.2	0.32	0.3

Table 7.2.4 Calibration of optical density to dry weight

Samples were mixed from three flasks after harvesting at 96 hours

Control

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	DW/Dil. Factor, Calculated dry wt, gm/L (from diluted sample)
	100	0.409	0.399	0.4	33.00	0.3
	80	0.330	0.332	0.3	33.00	0.3
	60	0.239	0.245	0.2	33.00	0.2
	40	0.181	0.188	0.2	33.00	0.1
	20	0.112	0.115	0.1	33.00	0.1
	0			0.0		0.0

X/10

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L
	100	0.407	0.412	0.4	33.00	0.3
	80	0.335	0.341	0.4	33.00	0.2
	60	0.259	0.254	0.3	33.00	0.2
	40	0.171	0.178	0.2	33.00	0.1
	20	0.097	0.098	0.1	33.00	0.1
	0			0.0	33.00	0.0

X/20

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L
	100	0.372	0.374	0.4	33.00	0.3
	80	0.273	0.276	0.3	33.00	0.2
	60	0.208	0.207	0.2	33.00	0.2
	40	0.164	0.168	0.2	33.00	0.1
	20	0.096	0.092	0.1	33.00	0.1
	0			0.0	33.00	0.0

X/40

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt., gm/L
	100	0.408	0.401	0.4	33.00	0.3
	80	0.306	0.302	0.3	33.00	0.2
	60	0.240	0.232	0.2	33.00	0.2
	40	0.150	0.161	0.2	33.00	0.1
	20	0.087	0.082	0.1	33.00	0.1
	0			0.0	33.00	0.0

Table 7.2.5 Lipid calibration

Samples were mixed from three flasks after harvesting at 96 hours

Control

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	545	226.87	545	-0.9	542	219.2	542	0.4	5	223.3	4.16
80%	575	168.97	565	-0.4	575	172.5	575	-0.8	5	171.4	3.32
60%	566	138.87	566	-0.6	563	136.9	563	-0.8	5	138.6	2.49
40%	570	98.45	570	-0.5	564	94.5	564	-0.8	5	97.2	1.66
20%	550	40.13	550	-0.9	560	42.6	560	-0.9	5	42.3	0.83
0%										0.0	0.00

X/10

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	572	232.25	572	-0.2	568	224.8	568	-0.6	5	228.9	3.92
80%	560	165.24	560	-0.7	558	159.1	558	-0.4	5	162.7	3.14
60%	563	142.35	563	-0.7	560	139.8	560	-0.8	5	141.8	2.35
40%	555	101.24	555	-0.7	558	95.9	558	-0.5	5	99.2	1.57
20%	565	65.21	565	-0.8	565	51.2	565	-0.8	5	59.0	0.78
0%										0.0	0.00

X/20

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	550	210.90	550	0.9	549	215.4	549	0.9	5	212.2	4.01
80%	546	169.08	546	-0.5	545	182.1	545	0.8	5	175.4	3.20
60%	548	132.04	548	-0.4	547	149.0	547	-0.8	5	141.1	2.40
40%	550	101.24	550	-0.8	549	92.5	549	-0.8	5	97.7	1.60
20%	551	40.21	551	-0.8	553	34.3	553	-0.9	5	38.1	0.80
0%										0.0	0.00

X/40

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	544	192.36	544	1.0	545	205.1	545	1.0	5	197.7	3.20
80%	548	145.03	548	0.6	550	153.3	550	0.7	5	148.5	2.56
60%	547	103.21	547	-0.6	548	112.3	548	-0.7	5	108.4	1.92
40%	549	85.37	549	-0.6	547	89.3	547	-0.7	5	88.0	1.28
20%	549	35.25	549	-0.7	553	52.4	553	-0.7	5	44.5	0.64
0%										0.0	0.00

7.3 Phosphate optimization experiment: 3

Experiment with control- 1X phosphate, 1/10-X phosphate, 1/20-X phosphate, and 1/40-X phosphate.

Table 7.3.1 Data for optical density and fluorescence intensity

Control

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.023	0.025	554	13.6	554	0.2	552	14.3	552	0.2	1
48	15	0.478	0.477	549	384.3	549	5.8	551	374.4	551	3.6	1
56	25	0.465	0.467	548	659.3	548	3.7	550	628.4	550	8.9	1
64	25	0.515	0.520	545	789.3	545	7.3	544	755.3	544	6.5	1
72	25	0.457	0.452	545	469.2	545	8.3	546	485.4	546	12.6	2
79	30	0.474	0.477	550	683.1	550	12.4	548	705.4	548	6.3	2
86	30	0.495	0.490	552	912.3	552	1.3	550	898.0	550	0.7	2
96	30	0.459	0.465	546	635.4	546	4.9	550	625.3	550	5.3	2

1/10X

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.015	0.017	554	14.3	554	0.2	552	15.9	552	13.7	1
48	15	0.461	0.459	548	326.1	548	3.5	545	341.3	545	3.9	1
56	25	0.446	0.444	545	536.3	545	8.8	550	522.0	550	5.2	1
64	25	0.497	0.5	545	689.4	545	7.5	552	656.4	552	6.4	1
72	25	0.431	0.435	548	425.3	548	6.5	544	412.3	544	7.4	2
79	30	0.464	0.468	545	554.0	545	3.5	546	553.8	546	0.0	2
86	30	0.478	0.485	545	859.6	545	5.8	548	898.8	548	6.0	2
96	30	0.449	0.458	550	540.4	550	2.2	550	581.1	550	3.2	2

1/20X

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.022	0.021	554	14.8	554	0.1	552	16.2	552	0.0	1
48	15	0.401	0.403	555	342.4	555	2.9	552	315.3	552	7.7	1
56	25	0.397	0.402	550	559.4	550	1.2	552	575.1	552	3.3	1
64	25	0.446	0.449	548	654.8	548	1.3	550	589.6	550	3.5	1
72	25	0.405	0.411	550	425.9	550	6.2	545	439.7	545	16.5	2
79	30	0.436	0.435	549	686.4	549	7.3	545	720.1	545	12.1	2
86	30	0.456	0.457	547	784.9	547	3.9	546	768.5	546	10.4	2
96	30	0.429	0.436	545	591.1	545	1.3	550	623.3	550	3.5	2

1/40X

	OD at 550 nm			Fluorescence								
				With NR1		Without NR1		With NR2		Without NR2		ND
Hrs.	ND	OD1	OD2	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.	
0	1	0.019	0.017	0.018	12.5	554	0.1	552	11.3	552	0.1	1
48	15	0.349	0.355	550	297.7	550	3.8	548	281.4	548	8.4	1
56	25	0.361	0.358	552	425.9	552	1.6	548	453.4	548	3.5	1
64	25	0.38	0.385	550	545.9	550	1.7	552	575.3	552	3.8	1
72	25	0.377	0.374	546	398.3	546	8.0	549	369.0	549	4.3	2
79	30	0.397	0.389	545	523.5	545	6.9	550	587.4	550	9.4	2
86	30	0.425	0.419	545	705.3	545	5.0	545	691.4	545	8.4	2
96	30	0.424	0.421	548	539.4	548	1.7	546	559.3	546	3.8	2

Note: *ND: no. of dilution, OD: optical density, NR: Nile red, W.L: wave length, 1 & 2 denote samples, Intens.: Intensity*

Table 7.3.2 Dry cell mass measurement

	Flask	Volume filtered, mL	Filter paper, gm	Filter Paper+ dry cell	Wt. of cells, gm	Cells conc. g/L	Avg DW., g/L
Control	A1	4	0.1233	0.1642	0.0409	10.225	10.06
	A2	4	0.1241	0.1637	0.0396	9.9	
	A3	4	0.1229	0.1632	0.0403	10.075	
1/10X	A1	4	0.1225	0.1623	0.0398	9.95	9.8
	A2	4	0.1229	0.1615	0.0386	9.65	
	A3	4	0.1231	0.1624	0.0393	9.825	
1/20X	A1	4	0.1256	0.1648	0.0392	9.8	9.55
	A2	4	0.1218	0.1590	0.0372	9.3	
	A3	4	0.1225	0.1607	0.0382	9.55	
1/40X	A1	4	0.1245	0.1607	0.0362	9.05	8.9
	A2	4	0.1224	0.1574	0.035	8.75	
	A3	4	0.1228	0.1585	0.0357	8.925	

Table 7.3.3 Lipid extraction measurement

	Sample	Wt. of tube, gm	tube+ lipid, gm	wt. of Lipid, gm	wt. of cells, gm	gm lipid/ gm biomass	Avg. gm lipid/gm biomass
Control	1	13.681	14.001	0.3196	0.8012	0.40	
	2	13.578	13.984	0.4063	0.8093	0.50	0.45
X/10	1	13.721	14.030	0.3088	0.8058	0.38	
	2	13.742	14.136	0.394	0.8085	0.49	0.44
X/20	1	13.575	13.929	0.3542	0.8009	0.44	
	2	13.710	14.032	0.3214	0.801	0.40	0.42
X/40	1	13.770	14.021	0.2507	0.7023	0.36	
	2	13.553	13.833	0.2802	0.7017	0.40	0.38

Table 7.3.4 Calibration of optical density to dry weight

Samples were mixed from three flasks after harvesting at 96 hours

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
	100	0.459	0.465	0.462	33.00	0.305
	80	0.350	0.348	0.349	33.00	0.244
Control	60	0.261	0.259	0.260	33.00	0.183
	40	0.181	0.188	0.185	33.00	0.122
	20	0.090	0.091	0.091	33.00	0.061
	0			0.000		0.000

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
	100	0.449	0.458	0.454	33.00	0.297
	80	0.337	0.341	0.339	33.00	0.238
1/10x	60	0.278	0.277	0.278	33.00	0.178
	40	0.190	0.191	0.191	33.00	0.119
	20	0.097	0.098	0.098	33.00	0.059
	0			0.00	33.00	0.000

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
	100	0.429	0.438	0.434	33.00	0.289
1/20x	80	0.303	0.311	0.307	33.00	0.232
	60	0.208	0.207	0.208	33.00	0.174
	40	0.164	0.158	0.161	33.00	0.116
	20	0.086	0.088	0.087	33.00	0.058
	0			0.000	33.00	0.000

	% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
	100	0.424	0.421	0.423	33.00	0.270
1/40x	80	0.306	0.302	0.304	33.00	0.216
	60	0.240	0.232	0.236	33.00	0.162
	40	0.150	0.161	0.156	33.00	0.108
	20	0.087	0.082	0.085	33.00	0.054
	0			0.000	33.00	0.000

Table 7.3.5 Lipid calibration

Samples were mixed from three flasks after harvesting at 96 hours

Control

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	549.0	245.5	549.0	0.5	544.0	210.5	544.0	0.9	5	227.3	4.53
80%	550.0	200.0	550.0	0.5	548.0	175.4	548.0	0.3	5	187.3	3.62
60%	544.0	160.2	544.0	0.6	548.0	151.4	548.0	0.8	5	155.1	2.72
40%	545.0	103.0	545.0	0.7	555.0	101.5	555.0	0.6	5	101.6	1.81
20%	544.0	42.7	544.0	0.7	550.0	38.7	550.0	0.6	5	40.0	0.91
0%										0.0	0.00

X/10

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	552.0	219.0	552.0	0.4	568.0	225.5	568.0	0.3	5	222.0	4.31
80%	550.0	170.8	550.0	0.9	558.0	175.5	558.0	0.9	5	172.3	3.45
60%	544.0	119.0	544.0	1.4	560.0	120.5	560.0	1.5	5	118.3	2.59
40%	545.0	75.5	545.0	1.9	558.0	74.5	558.0	2.0	5	73.0	1.72
20%	549.0	40.9	549.0	2.4	565.0	35.5	565.0	2.6	5	35.7	0.86
0%										0.0	0.00

X/20

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	550.0	221.3	550.0	0.5	549.0	235.9	549.0	0.2	5	228.2	4.01
80%	546.0	155.4	546.0	1.0	545.0	162.3	545.0	0.4	5	158.2	3.21
60%	548.0	131.2	548.0	0.4	547.0	149.2	547.0	0.7	5	139.7	2.41
40%	550.0	101.2	550.0	-0.1	549.0	89.4	549.0	0.6	5	95.0	1.60
20%	551.0	51.0	551.0	-0.6	553.0	55.4	553.0	0.5	5	53.3	0.80
0%										0.0	0.00

X/40

	With NR		Without NR		With NR		Without NR				
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intrens.	W.L	Intens.	ND	Avg. Fluor.	Lipid, gm/L
100%	544.0	205.2	544.0	0.4	545.0	201.2	545.0	0.8	5	202.6	3.29
80%	548.0	131.1	548.0	0.6	550.0	134.0	550.0	0.5	5	132.0	2.63
60%	547.0	110.2	547.0	0.9	548.0	105.3	548.0	0.3	5	107.2	1.98
40%	549.0	58.7	549.0	1.1	547.0	79.4	547.0	0.1	5	68.4	1.32
20%	549.0	35.3	549.0	1.4	553.0	43.9	553.0	0.2	5	38.8	0.66
0%										0.0	0.00

Note: *ND: no. of dilution, OD: optical density, NR: Nile red, W.L: wave length, 1 & 2 denote samples*

7.4 Phosphate optimization experiment:4

Experiment with control- 1X phosphate, 1/15-X, 1/20-X phosphate, 1/30-X phosphate.

Table 7.4.1 Data for optical density and fluorescence intensity

Control

		OD at 550 nm		Fluorescence							
Time, hrs	ND	OD1	OD2	Without NR1		With NR2		Without NR2		ND	
				Inten.	W.L	Inten.	W.L	Inten.	W.L	Inten.	
48	15	0.439	0.431	189.25	562	-1.761	570	197.1	570	-2.327	3
60	30	0.375	0.38	110.79	545	-1.364	544	102.5	544	-1.827	10
66	40	0.355	0.356	120.24	544	-1.120	545	115.6	545	-1.510	10
72	40	0.36	0.36	168.1	545	-0.086	546	144.3	546	-0.126	10
80	40	0.368	0.373	158.93	544	-0.035	545	140.4	545	-0.008	10
86	40	0.357	0.357	172.31	544	-0.012	544	196.9	544	-0.004	10
94	40	0.35	0.35	144.89	544	-0.010	544	150.5	544	-0.002	10
96	40	0.336	0.339	140.53	545	0.012	542	153.3	542	0.014	10

		OD at 550 nm		Fluorescence							
Time, hrs	ND	OD1	OD2	Without NR1		With NR2		Without NR2		ND	
				Inten.	W.L	Inten.	W.L	Inten.	W.L	Inten.	
48	15	0.301	0.305	78.68	544	-2.327	545	87.1	545	-1.761	3
60	30	0.318	0.315	88.17	543	-1.827	545	90.28	545	-1.364	10
66	40	0.305	0.301	105.2	545	-1.510	546	110.3	546	-1.120	10
72	40	0.312	0.314	160.15	545	-0.126	545	170.1	545	-0.086	10
80	40	0.354	0.359	163.2	545	-0.008	544	187.2	544	-0.035	10
86	40	0.339	0.346	169.25	546	-0.004	546	181.9	546	-0.012	10
94	40	0.342	0.349	147.81	554	-0.002	550	151.7	550	-0.010	10
96	40	0.339	0.346	129.4	545	0.014	545	117.2	545	0.012	10

1/20X

Time, hrs	ND	OD at 550 nm OD1	OD2	Fluorescence Without NR1 Inten.	W.L	With NR2 Inten.	W.L	Without NR2 Inten.	W.L	ND Inten.	
48	15	0.292	0.297	66.296	545	-1.761	544	59.11	544	-2.327	3
60	30	0.294	0.293	75.881	547	-1.364	547	81.45	547	-1.827	10
66	40	0.305	0.31	110.5	547	-1.120	546	119.5	547	-1.510	10
72	40	0.318	0.314	150.21	543	-0.086	548	155.3	548	-0.126	10
80	40	0.336	0.339	184.23	546	-0.035	545	192.7	545	-0.008	10
86	40	0.329	0.328	180.46	545	-0.012	543	196.9	543	-0.004	10
94	40	0.33	0.334	144.63	547	-0.010	543	138.1	543	-0.002	10
96	40	0.299	0.301	128.12	546	0.012	544	116.3	544	0.014	10

1/30X

Time, hrs	ND	OD at 550 nm OD1	OD2	Fluorescence Without NR1 Inten.	W.L	With NR2 Inten.	W.L	Without NR2 Inten.	W.L	ND Inten.	
48	15	0.294	0.289	81.87	545	-2.327	545	76.23	545	-1.761	3
60	30	0.297	0.303	84.84	546	-1.827	545	78.22	545	-1.364	10
66	40	0.251	0.248	105.23	545	-1.510	547	113.7	547	-1.120	10
72	40	0.288	0.291	140.1	544	-0.126	544	137	544	-0.086	10
80	40	0.325	0.324	167.69	544	-0.008	543	169.2	543	-0.035	10
86	40	0.331	0.332	191.28	544	-0.004	545	181.6	545	-0.012	10
94	40	0.329	0.327	141.1	547	-0.002	548	136.4	548	-0.010	10
96	40	0.321	0.325	128.11	550	0.014	548	138.6	548	0.012	10

Table 7.4.2 Dry cell mass measurement

	Flask	Volume filtered, mL	Filter paper, gm	Filter Paper+ dry cell, gm	Wt. of cells, gm	Dry cells conc. g/L
Control	A1	4	0.124	0.1639	0.0397	9.93
	A2	4	0.126	0.1648	0.0386	9.65
	A3	4	0.1233	0.1627	0.0394	9.85
1/15-X	A1	4	0.128	0.1672	0.0391	9.78
	A2	4	0.125	0.1645	0.0393	9.83
	A3	4	0.1241	0.1632	0.0391	9.78
1/20-X	A1	4	0.128	0.1663	0.0385	9.63
	A2	4	0.122	0.1669	0.0448	11.20
	A3	4	0.127	0.1681	0.0415	10.38
1/30-X	A1	4	0.126	0.1642	0.038	9.50
	A2	4	0.123	0.1622	0.0395	9.88
	A3	4	0.1239	0.1627	0.0388	9.70

Table 7.4.3 Lipid extraction measurement

	Flask	Wt. of tube, gm	tube+ lipid, gm	Wt. of Lipid, gm	Dry cell, gm	gm lipid/ gm biomass
Control	A1	13.6348	14.0528	0.418	0.811	0.52
	A2	13.5421	13.9421	0.4	0.805	0.50
1/15-X	B1	13.7542	14.1235	0.3693	0.8008	0.46
	B2	13.7421	14.0945	0.3524	0.8011	0.44
1/20-X	C1	13.7654	14.1653	0.3999	0.8014	0.50
	C2	13.7523	14.1325	0.3802	0.8017	0.47
1/30-X	D1	13.5463	13.9254	0.3791	0.8011	0.47
	D2	13.8563	14.2156	0.3593	0.8022	0.45

Table 7.4.4 Calibration of optical density to dry weight

Samples were mixed from three flasks after harvesting at 96 hours

Control,1X					DW/Dil factor
% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
100	0.34	0.339	0.338	40.00	0.246
80	0.27	0.279	0.277	40.00	0.192
60	0.21	0.21	0.209	40.00	0.144
40	0.14	0.144	0.142	40.00	0.096
20	0.08	0.08	0.078	40.00	0.048
0			0.000		0.000

1/15-X					
% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
100	0.30	0.303	0.300	40.00	0.245
80	0.24	0.239	0.239	40.00	0.196
60	0.20	0.191	0.193	40.00	0.147
40	0.13	0.126	0.129	40.00	0.098
20	0.07	0.066	0.066	40.00	0.049
0			0.00	40.00	0.000

1/20-X					
% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
100	0.30	0.301	0.300	40.00	0.243
80	0.24	0.244	0.244	40.00	0.194
60	0.20	0.194	0.197	40.00	0.146
40	0.13	0.127	0.127	40.00	0.097
20	0.07	0.067	0.066	40.00	0.049
0	0.00	0	0.000	40.00	0.000

1/30-X					
% dilution	OD measured at 550 nm		Avg. OD	dilution factor	Calculated dry wt, gm/L (from diluted sample)
100	0.29	0.286	0.288	40.00	0.242
80	0.22	0.229	0.226	40.00	0.194
60	0.18	0.232	0.205	40.00	0.145
40	0.12	0.12	0.122	40.00	0.097
20	0.09	0.082	0.085	40.00	0.048
0			0.000	40.00	0.000

Table 7.4.5 Lipid calibration

Control

	With NR		Without NR		With NR		Without NR		ND	Lipid,
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.		gm/L
100%	545	281.2	545	1.264	542	275.28	542	0.968	5	5.02
80%	575	205.3	565	0.958	575	210.84	575	0.366	5	4.01
60%	566	145.3	566	0.652	563	151.28	563	-0.236	5	3.01
40%	570	102.0	570	0.346	564	102.65	564	-0.838	5	2.01
20%	550	68.3	550	0.040	560	58.25	560	-1.440	5	1.00
0%										0.00

1/15-X

	With NR		Without NR		With NR		Without NR		ND	Lipid,
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.		gm/L
100%	572	260.354	572	0.651	568	251.029	568	0.236	5	4.41
80%	560	184.85	560	0.111	558	169.24	558	0.242	5	3.53
60%	563	152.05	563	-0.429	560	142.37	560	0.248	5	2.65
40%	555	108.05	555	-0.969	558	101.25	558	0.254	5	1.76
20%	565	49.31	565	-1.509	565	41.23	565	0.260	5	0.88
0%										0.00

1/20-X

	With NR		Without NR		With NR		Without NR		ND	Lipid,
% dilution	W.L	Intens.	W.L	Intens.	W.L	Intens.	W.L	Intens.		gm/L
100%	550	252.255	550	0.995	549	248.321	549	1.524	5	4.76
80%	546	182.36	546	0.654	545	169.24	545	0.369	5	3.81
60%	548	132.33	548	0.313	547	149.25	547	0.321	5	2.85
40%	550	125.37	550	-0.028	549	105.21	549	0.273	5	1.90
20%	551	54.46	551	-0.369	553	48.56	553	0.225	5	0.95
0%										0.00

1/30-X

% dilution	With NR W.L	With NR Intens.	Without NR W.L	Without NR Intens.	With NR W.L	With NR Intens.	Without NR W.L	Without NR Intens.	ND	Lipid, gm/L
100%	544	221.365	544	0.859	545	215.028	545	1.044	5	4.45
80%	548	189.24	548	0.577	550	178.32	550	0.654	5	3.56
60%	547	159.32	547	0.295	548	166.33	548	-0.681	5	2.67
40%	549	109.05	549	0.013	547	115.32	547	-0.691	5	1.78
20%	549	55.35	549	0.269	553	45.35	553	-0.707	5	0.89
0%										0.00

7.5 Effect of inhibitors on *Lipomyces starkeyi*

Experiment with Furfural, Vanillin, Syringaldehyde, Para hydroxy benzaldehyde, and 5-Hydroxy methyl furfural (HMF).

Table: 7.5.1 Data for optical density and fluorescence intensity

Furfural 0.05 g/L

	OD at 550 nm				With Nile Red Fluorescence Intensity					Without Nile Red Fluorescence Intensity			
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.13	0.13	0.23	0.23	571	15.6	15.5	20.5	24.3	3.4	4.0	3.7	4.9	1.0
24	0.96	0.92	0.86	0.86	571	55.3	68.3	74.3	57.3	3.4	4.0	3.7	4.9	1.0
48	5.50	5.56	7.58	7.50	574	168.0	132.6	163.0	168.0	4.6	5.5	8.0	6.6	2.0
56	7.96	7.92	9.80	9.84	574	343.0	252.1	302.0	343.0	5.0	6.0	8.7	7.2	2.0
64	9.6	9.6	12.8	12.7	573	410.0	325.3	370.0	410.0	3.0	2.7	2.7	4.4	2.0
72	10.5	10.5	12.8	12.8	569	356.0	356.3	394.0	356.0	7.4	6.5	6.6	10.7	2.0
80	11.5	11.5	13.0	13.0	570	411.0	436.5	393.0	411.0	9.4	8.2	8.3	13.6	2.0
88	12.0	11.9	13.1	13.1	572	530.0	485.2	495.0	530.0	9.4	7.6	8.5	11.7	2.0
96			12.9	12.9	568			407.0	468.0			7.5	8.4	2.0
104			12.6	12.6	571			446.0	470.0			7.6	10.3	2.0
114			12.0	11.9	579			442.0	433.0			9.3	12.3	2.0

Furfural 0.1 g/L

						With Nile Red Fluorescence Intensity					Without Nile Red Fluorescence Intensity			
	OD at 550 nm													
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.1	0.1	0.2	0.2	571	22.4	14.3	15.3	14.3	4.6	4.6	4.6	4.9	1
24	0.8	0.8	0.7	0.7	571	35.25	39.55	42.4	36.58	3.4	6.7	5.0	4.9	1
48	5.2	5.2	7.4	7.4	574	94.5	140.2	152.0	173.0	5.0	10.0	3.0	5.4	2
56	8.0	8.0	9.6	9.6	574	187.5	248.4	302.0	343.0	3.0	6.0	7.4	9.5	2
64	9.9	9.6	11.7	11.8	573	278.0	325.4	370.0	410.0	7.4	14.7	5.2	4.5	2
72	10.9	11.0	12.4	12.4	569	360.5	410.3	394.0	456.0	9.4	18.7	4.3	8.4	2
80	11.6	11.7	12.9	12.8	570	404.5	436.3	393.0	411.0	9.4	18.7	6.3	7.6	2
88	12.8	12.7	12.2	12.1	572	447.5	489.4	495.0	530.0	8.3	16.7	7.5	4.6	2
96			11.8	11.8	568			481.0	486.0			8.1	5.7	2
104			11.3	11.4	571			435.0	463.0			7.5	7.1	2
114			11.0	10.9	579			407.0	468.0			4.5	4.6	2

Furfural 0.2 g/L

						With Nile Red Fluorescence Intensity					Without Nile Red Fluorescence Intensity			
	OD at 550 nm													
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.14	0.14	0.22	0.22	571	14.3	24.4	14.5	15.5	7.5	5.4	8.3	7.5	1
24	0.46	0.5	0.56	0.55	571	22.4	28.4	31.3	21.3	4.6	4.6	3.7	4.9	1
48	3.66	3.7	6.6	6.62	574	156.1	178.5	137	138	3.4	6.7	3.24	0.86	2
56	6.18	6.04	9.24	9.2	574	284.9	274.9	291	343	5.0	10.0	7.58	7.50	2
64	8.6	8.7	11.7	11.8	573	308.9	328.1	341	339	3.0	6.0	9.80	9.84	2
72	9.2	9.1	11.6	11.6	569	378.3	397.4	365	307	7.4	14.7	5.6	4.6	2
80	10.9	11.0	12.2	12.2	570	418.8	442.4	403	450	9.4	18.7	4.5	8.3	2
88	10.7	10.8	12.5	12.5	572	375.6	398.1	420	452	9.4	18.7	8.5	7.3	2
96			11.4	11.4	568			398	431			8.3	6.5	2
104			11.3	11.4	571			399	403			6.2	8.6	2
114			11.0	11.0	579			372	355			7.5	7.5	2

Furfural 0.4 g/L

	OD at 550 nm					With Nile Red Fluorescence Intensity				Without Nile Red Fluorescence Intensity				
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.2	0.21	0.2	0.2	571	7.0	8.0	10.0	4.0	5.6	6.8	6.1	8.4	1
24	0.35	0.35	0.33	0.33	571	32.25	28.25	47.5	35.3	4.5	5.5	4.9	6.8	1
48	4.44	4.38	4.08	4.1	574	78.0	82.0	67.0	71.0	3.4	4.1	3.7	5.0	2
56	7.2	7.12	6.96	7.04	574	212.0	210.0	176.0	185.0	5.0	6.1	5.4	7.5	2
64	9.4	9.44	9.44	9.36	573	340.0	305.0	328.0	308.0	3.0	3.7	3.3	4.5	2
72	10.7	10.7	10.0	10.2	569	315.0	384.0	322.0	323.0	7.4	9.0	8.0	11.0	2
80	11.5	11.6	11.2	11.1	570	402.0	317.0	405.0	378.0	9.4	11.4	10.2	14.0	2
88	11.8	11.8	11.3	11.4	572	432.0	402.0	448.0	411.0	9.4	11.4	10.2	14.0	2
96	11.2	11.2	11.4	11.4	568	399.0	440.0	450.0	459.0	8.3	10.2	9.1	12.5	2
104	11.4	11.3	11.2	11.2	571	389.0	401.0	449.0	439.0	8.4	10.2	9.1	12.5	2
114	11.2	11.3	11.1	11.0	579	323.3	365.5	380.4	383.4	8.4	10.2	9.1	12.5	2

Vanillin 0.25 g/L

	OD at 550 nm					With Nile Red Fluorescence Intensity				Without Nile Red Fluorescence Intensity				
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.24	0.24	0.24	0.24	571	12.00	15.00	8.00	9.00	4.56	4.1	4.9	6.4	1
24	0.65	0.65	0.64	0.64	571	18	14	22	14	8.25	7.4	8.9	11.6	1
48	5.86	5.82	5.76	5.74	574	120	128	116	118	3.37	3.0	3.6	4.7	2
56	9.0	9.0	9.2	9.2	574	264	281	247	259	4.99	4.5	5.4	7.0	2
64	11.4	11.4	10.6	10.6	573	376	352	306	298	3.02	2.7	3.3	4.2	2
72	11.6	11.5	11.4	11.3	569	321	368	261	305	7.37	6.6	8.0	10.3	2
80	11.9	12.0	11.7	11.5	570	356	446	378	349	9.37	8.4	10.1	13.1	2
88	11.2	11.3	12.4	12.5	572	459	466	366	403	9.37	8.4	10.1	13.1	2
96	11.1	11.1	11.9	12.0	568	440	465	504	488	8.34	7.5	9.0	11.7	2
104	11.4	11.5	11.4	11.4	571	444	466	478	436	8.36	7.5	9.0	11.7	2
114	11.1	11.1	10.9	11.0	579	437	418	426	408	5.38	4.8	5.8	7.5	2

Vanillin 0.5 g/L

						With Nile Red Fluorescence Intensity				Without Nile Red Fluorescence Intensity				
	OD at 550 nm													
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.1	0.1	0.2	0.2	571	10.3	12.5	8.00	9.00	4.6	3.2	4.2	5.1	1
24	0.1	0.1	0.5	0.5	571	15.3	18.5	15.5	18.5	5.5	3.9	5.1	6.1	1
48	0.3	0.3	1.8	1.8	574	24.5	45.3	35.5	41.2	3.4	2.4	3.1	3.7	2
56	0.5	0.5	4.3	4.4	574	111.5	170.6	50	51	5.0	3.5	4.6	5.5	2
64	1.0	1.0	7.8	7.8	573	155.5	205.3	200	270	3.0	2.1	2.8	3.4	2
72	1.7	1.7	10.1	10.2	569	256.3	298.4	220	234	7.4	5.2	6.8	8.2	2
80	4.0	4.0	10.2	10.3	570	351.0	328.3	333	324	9.4	6.6	8.6	10.4	2
88	6.7	6.5	10.7	10.7	572	378.9	389.3	485	391	9.4	6.6	8.6	10.4	2
96			10.9	10.9	568			442	421	8.3	5.9	7.7	9.3	2
104			11.1	11.0	571			475	458	8.4	5.9	7.7	9.3	2
114			10.9	10.9	579			456	421	6.9	4.9	6.4	7.7	2

Syringaldehyde 0.5g/L

						With Nile Red Fluorescence Intensity				Without Nile Red Fluorescence Intensity				
	OD at 550 nm													
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.1	0.1	0.29	0.29	571	12.4	14.6	7.0	9.0	4.56	12.6	8.9	11.6	1
24	0.4	0.4	0.76	0.76	571	35.3	25.5	18.3	21.3	8.25	9.8	7.0	9.0	1
72	7.4	7.4	10.8	10.9	569	328.0	356.9	315.0	312.0	7.37	5.3	3.8	4.9	2
80	8.6	8.6	11.1	11.1	570	362.0	374.4	437.0	477.0	9.37	8.4	10.1	5.3	2
88	10.6	10.7	11.7	11.8	572	310.2	345.4	447.0	404.0	9.37	8.4	10.1	9.2	2
96			11.1	11.1	568			338.0	330.0	8.34	7.5	9.0	6.2	2
104			10.8	10.7	571			308.0	328.0	8.36	7.5	9.0	7.2	2
114			10.4	10.5	579			313.0	320.0	4.29	3.9	4.6	5.7	2

Syringaldehyde 1g/L

						With Nile Red					Without Nile Red			
		OD at 550 nm				Fluorescence Intensity					Fluorescence Intensity			
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	NR1	NR2	NR3	NR4	ND
0	0.1	0.1	0.28	0.29	571	3.0	4.0	8.0	9.0	5.5	4.6	5.8	5.4	1
24	0.2	0.2	0.43	0.43	571	38.0	29.4	15.5	18.5	4.56	4.5	4.1	2.9	1
48	1.9	1.8	2.64	2.6	574	51.5	75.4	41.0	44.0	3.37	8.4	7.6	5.4	2
56	4.6	4.8	5.24	5.28	574	175.0	198.4	113.0	110.0	4.99	7.6	6.8	4.8	2
64	5.3	5.6	8.56	8.48	573	230.0	208.4	247.0	241.0	3.02	4.6	4.1	2.9	2
72	6.7	6.7	8.99	8.85	569	274.0	298.4	239.0	232.0	7.37	5.7	5.1	3.6	2
80	7.4	7.4	9.59	9.75	570	358.2	365.6	333.0	376.0	9.37	6.7	6.0	4.3	2
88	7.9	7.8	9.98	10	572	348.5	385.6	399.0	482.0	9.37	7.7	6.9	4.9	2
96			10.1	10.1	568			414.0	399.0	8.34	8.7	7.8	5.6	2
104			10.3	10.4	571			384.0	372.0	8.36	9.7	8.7	6.2	2
114			10.1	10.2	579			404.0	372.0	7.29	10.7	9.6	6.8	2

PHB 0.25g/L

							With Nile Red						Without Nile Red						
	OD at 550 nm						Fluorescence Intensity												
Hrs.	OD1	OD2	OD3	OD4	OD5	OD6	W.L	NR1	NR2	NR3	NR4	NR5	NR6	NR1	NR2	NR3	NR4	NR5	NR6	ND
0	0.11	0.11	0.12	0.11	0.09	0.09	571	4.0	2.0	5.0	4.0			8.25	7	4.56	4.1	4.9	6.4	1
24	0.03	0.04	0.03	0.03	0.03	0.04	571	147.6	144.8	162.4	166.6	130	132	3.37	3	8.3	7.4	8.9	11.6	1
48	0.22	0.22	0.20	0.20	0.22	0.22	574	252.4	225.5	277.7	259.4	222	205	4.99	4	3.37	3.0	3.6	4.7	3
60	0.38	0.38	0.37	0.36	0.36	0.35	574	380.4	406.5	418.4	467.5	335	370	3.02	3	4.99	4.5	5.4	7.0	3
78	0.30	0.30	0.30	0.30	0.29	0.29	569	436.8	449.5	480.4	516.9	384	409	9.37	8	10.1	3.7	4.9	3.4	3
88	0.31	0.30	0.29	0.29	0.29	0.29	570	479.3	481.3	527.3	553.5	422	438	9.37	8	10.1	8.0	6.6	4.6	3
96	0.29	0.29	0.29	0.29	0.29	0.29	572	453.2	437.5	498.6	503.1	399	398	8.34	8	9.0	8.7	7.2	5.1	3
106	0.3	0.3	0.31	0.31	0.3	0.3	568	389.3	371.9	428.3	427.6	343	338	8.36	8	9.0	2.7	4.4	3.1	3
118	0.3	0.3	0.31	0.3	0.3	0.3	571	333.7	345.4	367	397.2	294	314	5.38	5	5.8	6.6	10.7	7.5	3
128	0.3	0.3	0.3	0.3	0.3	0.3	579	298.4	247.4	328.2	284.5	263	225	7.5	8	5.9	8.3	13.6	9.5	3
140	0.3	0.3	0.3	0.3	0.31	0.31	578	262.9	217.6	289.1	250.2	231	198	7.6	10	7.2	8.5	11.7	8.2	3

PHB 0.5 g/L

					With Nile Red					Without Nile Red					
	OD at 550 nm				Fluorescence Intensity										
Hrs.	OD1	OD2	OD3	OD4	W.L	NR1	NR2	NR3	NR4	W.L	NR1	NR2	NR3	NR4	ND
0	0.11	0.11	0.12	0.12	571	4.0	2.0	5.0	4.0	571	11.7	8	9.1	10.3	1
24	0.35	0.35	0.34	0.34	571	27.1	27.6	30.05	31.42	571	8.4	6	6.5	7.36	1
60	0.17	0.18	0.18	0.18	574	86.4	107.2	95.9	122.2	574	12.3	9	9.6	10.8	3
70	0.32	0.32	0.33	0.33	573	108.5	107.0	120.5	122	573	3.7	3	2.9	3.22	3
78	0.43	0.43	0.44	0.44	569	481.5	427.9	534.4	487.9	569	3.7	3	2.9	3.22	3
88	0.29	0.29	0.29	0.28	570	499.7	461.4	554.6	526	570	5.0	3	3.9	4.37	3
96	0.28	0.28	0.28	0.27	572	475.4	452.3	527.7	515.6	572	5.4	4	4.2	4.77	3
106	0.3	0.3	0.3	0.3	568	419.3	399.6	465.4	455.5	568	3.3	2	2.6	2.89	3
118	0.3	0.3	0.3	0.3	571	354.4	343.0	393.3	391	571	8.0	6	6.2	7.05	3
128	0.29	0.29	0.3	0.3	579	312.4	326.4	346.7	372.1	579	10.2	7	7.9	8.96	3
140	0.31	0.31	0.32	0.3	578	278.4	218.2	309	248.8	578	8.78	6	6.8	7.73	3

HMF 0.25 g/L

									With Nile Red Fluorescence Intensity						Without Nile Red Fluorescence Intensity				
	OD at 550 nm																		
Hrs.	OD1	OD2	OD3	OD4	OD5	OD6	ND	W.L	NR1	NR2	NR3	NR4	NR5	NR6	NR1	NR4	NR5	NR6	ND
0	0.12	0.12	0.13	0.13	0.11	0.11	1	571	12.0	9.0	11.0	8.0	15	18.0	3.7	4.4	5.3	3.4	1
24	0.04	0.04	0.04	0.04	0.04	0.04	20	571	21.0	24.0	28.0	38.0	45	41.0	4.8	5.2	6.3	4.6	1
48	0.23	0.23	0.24	0.24	0.25	0.25	20	574	208.4	200.4	192.2	215.5	205	195.4	3.6	5.6	6.8	5.1	3
60	0.37	0.38	0.4	0.41	0.4	0.39	20	574	166.9	159.1	175.6	185.2	175	154.2	3.9	3.4	4.1	3.1	3
70	0.48	0.48	0.48	0.48	0.49	0.49	20	573	286.8	266.9	285.4	292.4	264	278.3	4.3	3.7	4.5	7.5	3
78	0.52	0.52	0.53	0.54	0.53	0.53	20	569	331.2	303.6	311.3	317.3	315	333.2	3.6	6.6	7.9	9.5	3
88	0.31	0.3	0.3	0.3	0.3	0.3	40	570	457.5	459.7	441.3	457.3	437	467.4	4.4	3.1	3.8	8.2	3
96	0.3	0.3	0.3	0.3	0.3	0.3	40	572	495.0	483.8	475.2	471.3	496	485.5	5.4	5.8	7.0	5.9	3
106	0.3	0.3	0.29	0.28	0.3	0.3	40	568	453.2	437.5	444.4	418.3	425	452.5	7.3	5.3	6.3	7.2	3
118	0.29	0.29	0.29	0.3	0.3	0.3	40	571	389.3	371.9	365.3	379.4	370	389.3	9.3	3.2	3.8	8.6	3
128	0.28	0.29	0.29	0.29	0.29	0.3	40	579	225.2	240.5	215.4	235.4	229	238.5	4.4	4.0	4.2	2.6	3
140	0.29	0.29	0.3	0.3	0.3	0.3	40	578	210.3	204.4	191.3	223.5	211	208.6	8.3	4.6	5.2	2.6	3

HMF 0.5 g/L

									With Nile Red Fluorescence Intensity						Without Nile Red Fluorescence Intensity				
	OD at 550 nm																		
Hrs.	OD1	OD2	OD3	OD4	OD5	OD6	ND	W.L	NR1	NR2	NR3	NR4	NR5	NR6	NR1	NR4	NR5	NR6	ND
0	0.13	0.12	0.12	0.12	0.12	0.12	1	571	4.0	2.0	5.0	4.0	571	3.4	8.4	7.4	7.7	6.41	1
24	0.57	0.57	0.55	0.54	0.56	0.56	20	574	70.7	83.5	84.8	63.6	92.6	78.1	10.3	9.0	9.43	6.48	1
60	0.32	0.32	0.34	0.34	0.34	0.34	20	572	145.7	150.6	174.9	131.2	167	160.9	3.66	3.22	3.37	2.67	3
78	0.49	0.49	0.47	0.48	0.49	0.49	20	568	394.3	298.3	473.1	354.8	331	435.3	4.96	4.37	4.57	5.77	3
88	0.3	0.29	0.3	0.3	0.3	0.3	40	571	441.6	460.9	529.9	397.4	512	487.5	5.43	4.77	4.99	6.31	3
96	0.29	0.29	0.3	0.3	0.3	0.3	40	570	433.3	418.3	520.0	390.0	464	478.4	3.3	2.9	3.02	1.94	3
106	0.31	0.31	0.3	0.3	0.3	0.3	40	573	374.0	359.4	448.8	336.6	399	412.9	8.0	7.0	7.37	4.74	3
118	0.3	0.3	0.31	0.31	0.31	0.31	40	571	225.2	240.5	270.3	202.7	267	248.6	10.2	9.0	9.37	6.02	3
128	0.3	0.3	0.3	0.29	0.29	0.3	40	577	205.5	198.4	246.6	185.0	220	226.9	8.8	7.7	8.08	6.13	3
140	0.29	0.29	0.3	0.29	0.3		40	577	178.3	191.3	213.9	160.5	212	196.8	6.27	5.52	5.77	5.45	3

HMF 1 g/L

	OD at 550 nm								With Nile Red Fluorescence Intensity						Without Nile Red Fluorescence Intensity					
Hrs.	OD1	OD2	OD3	OD4	OD5	OD6	ND	W.L	NR1	NR2	NR3	NR4	NR5	NR6	NR1	NR2	NR4	NR5	NR6	ND
0	0.12	0.12	0.11	0.1	0.11	0.11	1	571	2.0	5.0	4.0	571	3.4	3.7	1.52	2	2.6	1.8	2.0	1
24	0.42	0.42	0.42	0.41	0.4	0.39	20	571	16.2	18.8	18.7	17.1	17.9	15.3	3.69	4	6.3	4.38	4.9	1
48	0.04	0.04	0.03	0.03	0.04	0.04	20	574	65.4	66.4	75.2	68.7	63.1	61.5	4.7	5	8.0	5.57	6.2	3
60	0.1	0.1	0.1	0.09	0.1	0.09	20	574	27.7	24.2	31.8	29.1	22.9	26.0	4.78	5	6.9	4.81	5.4	3
70	0.22	0.22	0.23	0.23	0.22	0.22	20	573	65.5	70.0	75.3	68.8	66.5	61.6	4.25	5	4.91	3.43	3.83	3
78	0.29	0.29	0.28	0.28	0.29	0.29	20	569	295.0	277.9	339.2	309.7	264	277.3	4.3	5	6.01	4.21	4.69	3
88	0.22	0.22	0.23	0.23	0.24	0.24	40	570	394.8	374.0	454.1	414.6	355	371.2	5.22	6	7.23	5.06	5.64	3
96	0.23	0.23	0.21	0.21	0.23	0.23	40	572	429.9	422.0	494.4	451.4	401	404.1	4.92	6	3.57	2.5	2.78	3
106	0.27	0.27	0.28	0.28	0.27	0.27	40	568	399.3	408.3	459.2	419.3	388	375.4	2.01	2	6.5	4.52	5.0	3
118	0.29	0.29	0.3	0.3	0.31	0.31	40	571	271.3	291.3	312.0	284.9	277	255.0	2.98	3	2.6	1.84	2.1	3
128	0.29	0.29	0.3	0.3	0.31	0.31	40	579	240.4	235.0	276.4	252.4	223	225.9	1.8	2	3.9	2.73	3.0	3
140	0.29	0.29	0.28	0.28	0.3	0.3	40	579	191.7	176.3	220.5	201.3	167	180.2	4.39	5	6.2	4.35	4.9	3

HMF 1.25 g/L

	OD at 550 nm								With Nile Red Fluorescence Intensity						Without Nile Red Fluorescence Intensity					
Hrs.	OD1	OD2	OD3	OD4	OD5	OD6	ND	W.L	NR1	NR2	NR3	NR4	NR5	NR6	NR1	NR3	NR4	NR5	NR6	ND
0	0.11	0.12	0.12	0.12	0.11	0.11	1	571	12.0	9.0	7.0	8	3.4	3.7	4.3	3.7	2.6	2.9	2.03	1
24	0.37	0.37	0.36	0.37	0.34	0.35	20	571	34.6	29.3	39.4	32.2	28.0	26.1	5.47	4.7	3.3	3.7	2.59	1
60	0.5	0.5	0.5	0.51	0.49	0.48	20	574	36.6	39.7	41.7	43.6	29.7	35.3	3.37	2.9	2.0	2.3	1.59	3
70	0.6	0.6	0.6	0.6	0.56	0.55	20	573	59.3	48.0	67.6	52.8	48.0	42.7	4.13	3.6	2.5	2.8	1.95	3
88	0.11	0.12	0.1	0.1	0.11	0.11	40	570	90.2	120.1	102.8	132.1	73.1	106.9	2.45	2.1	1.5	1.7	1.16	3
96	0.16	0.16	0.12	0.12	0.14	0.14	40	572	133.4	148.9	152.0	163.8	108	132.5	4.43	3.8	2.7	3.0	2.1	3
106	0.25	0.26	0.26	0.26	0.25	0.25	40	568	158.3	168.7	180.4	185.5	128	150.1	1.81	1.6	1.1	1.2	0.85	3
118	0.2	0.2	0.21	0.21	0.2	0.2	40	571	175.4	188.4	199.9	207.2	142	167.6	2.68	2.3	1.6	1.8	1.27	3
128	0.23	0.23	0.24	0.24	0.24	0.24	40	579	189.5	199.0	216.0	218.9	153	177.1	4.27	3.7	2.6	2.9	2.02	3
140	0.25	0.25	0.25	0.25	0.26	0.26	40	579	197.5	201.5	225.1	221.7	160	179.4	5.43	4.7	3.29	3.67	2.57	3

Control

									With Nile Red Fluorescence Intensity					Without Nile Red Fluorescence Intensity						
	OD at 550 nm																			
Hrs.	OD1	OD2	OD3	OD4	OD5	OD6	ND	W.L	NR1	NR2	NR3	NR4	NR5	NR6	NR1	NR3	NR4	NR5	NR6	ND
0	0.1	0.1	0.07	0.07	0.09	0.09	1	573	8	9	8.5	7.8	6	4.5	4.9	3.8	4.3	4.5	2.67	1
24	0.05	0.05	0.05	0.04	0.06	0.06	20	572	70.2	82.5	78.6	77.2	70	78.4	4.9	3.8	4.3	4.5	2.67	1
48	0.31	0.31	0.32	0.32	0.32	0.31	20	573	155.0	164.5	173.6	170.5	140	156.3	6.6	5.2	5.8	6.1	5.77	3
60	0.46	0.47	0.46	0.46			20	574	220.1	299.9	246.5	242.1	255	284.9	7.2	5.6	6.4	6.7	6.31	3
70	0.51	0.51	0.52	0.51	0.52	0.52	20	573	443.0	396.8	496.1	487.3	337	377.0	4.4	3.4	3.9	4.0	1.94	3
78	0.31	0.31	0.31	0.31	0.31	0.32	20	570	468.7	451.2	524.9	515.6	384	428.6	10.7	8.3	9.4	9.8	4.74	3
88	0.31	0.31	0.31	0.31	0.31	0.31	40	570	486.8	513.3	545.2	535.5	436	487.6	13.6	10.6	11.9	12.5	6.02	3
96	0.3	0.3	0.3	0.29	0.3	0.3	40	572	436.3	411.4	488.7	480.0	350	390.8	11.7	9.1	10.3	10.8	6.13	3
106	0.31	0.31	0.31	0.31	0.3	0.31	40	576	375.7	355.2	420.8	413.3	302	337.5	8.4	6.5	7.4	7.7	5.45	3
118	0.3	0.31	0.3	0.3	0.3	0.3	40	572	245.8	229.4	275.3	270.4	195	217.9	10.3	8.0	9.0	9.4	5.51	3
128	0.3	0.3	0.3	0.3	0.3	0.31	40	583	202.4	219.3	226.6	222.6	186	208.4	12.3	9.6	10.8	11.3	6.69	3
140	0.31	0.3	0.29	0.3	0.31	0.3	40	583	145.1	178.2	162.5	159.6	151	169.3	3.66	2.9	3.22	3.37	2.27	3

Control

						With Nile Red Fluorescence Intensity					Without Nile Red Fluorescence Intensity			
	OD at 550 nm													
Hrs.	OD 1	OD 2	OD 3	OD 4	W. L	NR 1	NR 2	NR 3	NR 4	W.L	NR1	NR2	NR3	NR4
0	0.1	0.1	0.3	0.3	571	12.4	8.3	8.2	4.3	571	3.4	4.0	3.7	4.9
24	1.2	1.2	1.0	1.0	571	48	35	38.3	48.6	571	3.4	4.0	3.7	4.9
48	6.8	6.8	7.7	7.7	574	128	116	107	110	574	9.4	6.5	10.3	7.2
56	9.3	9.3	10.6	10.6	574	214	284	327	347	574	11.3	7.9	12.3	8.6
64	10.3	10.5	12.2	12.4	573	308	355	389	371	573	3.4	2.7	3.7	2.6
72	11.4	11.4	13.2	13.2	569	390	374	355	389	569	3.4	2.7	3.7	2.6
80	13.4	13.4	13.6	13.6	570	408	423	449	483	570	4.6	5.8	5.0	3.5
88	13.0	13.0	13.9	13.9	572	493	487	492	510	572	5.0	6.3	5.4	3.8
96	13.0	13.0	13.8	13.8	568			398	432	568	3.0	4.5	4.9	5.8
104	13.0	13.0	13.6	13.5	571			418	417	571	3.5	4.5	7.5	5.6
114	13.0	13.0	12.9	12.9	579			426	435	579	5.2	4.8	5.4	6.2

Table 7.5.2 Dry cell mass measurement

		Wt. of filterpaper, gm	Filter paper+ Dry cell, gm	Dry cell,gm	Dw,g/L
Control	A	0.1263	0.1642	0.0379	9.4
	B	0.1246	0.1618	0.0372	9.3
	C	0.1239	0.1607	0.0368	9.2
Furfural 0.05 g/L	A	0.1231	0.1565	0.0334	8.3
	B	0.1217	0.1543	0.0326	8.15
	C	0.1233	0.1557	0.0324	8.1
Furfural 0.1 g/L	A	0.1234	0.1585	0.0351	8.7
	B	0.1219	0.1578	0.0359	8.95
	C	0.1224	0.1571	0.0347	8.65
Furfural 0.2 g/L	A	0.1236	0.1525	0.0289	7.25
	B	0.1238	0.1515	0.0277	6.95
	C	0.1227	0.1509	0.0282	7.05
Vanillin 0.5 g/L	A	0.122	0.1494	0.0274	6.85
	B	0.1233	0.1513	0.028	7
	C	0.1235	0.1511	0.0276	6.9
Syringaldehyde 0.5 g/L	A	0.1248	0.1574	0.0326	8.15
	B	0.127	0.162	0.035	8.75
	C	0.1241	0.1577	0.0336	8.4
Syringaldehyde 1 g/L	A	0.1237	0.151	0.0273	6.85
	B	0.1221	0.143	0.0209	5.25
	C	0.1223	0.1461	0.0238	5.95

Note: Broth filtered-4 mL

Dry cell mass measurement

	Flask	Wt. of filter paper, gm	Filter paper+ Dry cell, gm	Dry cell, gm	Dry cell wt. g/L
Control	A	0.1236	0.158	0.0344	8.60
	B	0.1238	0.1579	0.0341	8.53
	C	0.1235	0.1578	0.0343	8.58
Furfural 0.05 g/L	A	0.122	0.1561	0.0341	8.53
	B	0.1233	0.1551	0.0318	7.95
	C	0.1241	0.157	0.0329	8.23
Furfural 0.1 g/L	A	0.1248	0.1582	0.0334	8.35
	B	0.127	0.1568	0.0298	7.45
	C	0.1237	0.1551	0.0314	7.85
Furfural 0.2 g/L	A	0.1237	0.1552	0.0315	7.88
	B	0.1246	0.1549	0.0303	7.58
	C	0.1227	0.1535	0.0308	7.70
Furfural 0.4 g/L	A	0.1231	0.1531	0.03	7.50
	B	0.1217	0.1528	0.0311	7.78
	C	0.1223	0.1528	0.0305	7.63
Vanillin 0.25 g/L	A	0.1234	0.1536	0.0302	7.55
	B	0.1219	0.1542	0.0323	8.08
	C	0.1231	0.1543	0.0312	7.80
Vanillin 0.5 g/L	A	0.1237	0.1525	0.0288	7.20
	B	0.1225	0.1548	0.0323	8.08
	C	0.1219	0.1521	0.0302	7.55
Syringaldehyde 0.5 g/L	A	0.1231	0.1504	0.0273	6.83
	B	0.1218	0.1512	0.0294	7.35
	C	0.1235	0.1517	0.0282	7.05
Syringaldehyde 1 g/L	A	0.1241	0.1481	0.024	6.00
	B	0.1252	0.1528	0.0276	6.90
	C	0.1214	0.1473	0.0259	6.48

Table 7.5.3 Lipid extraction measurement

	Cell dry wt., gm	Wt. of tube, gm	Wt. of tube +lipid, gm	wt. of lipid,gm	gm lipid / gm DW
Furfural 0.05 g/L	0.508	13.9299	14.17	0.24	0.47
	0.5089	13.8865	14.08	0.19	0.38
Furfural 0.1 g/L	0.213	14.2997	14.39	0.09	0.40
	0.5121	13.9842	14.18	0.20	0.39
Furfural 0.1 g/L	0.516	13.7922	13.98	0.19	0.37
	0.5056	13.9254	14.09	0.16	0.32
Furfural 0.2 g/L	0.512	13.8744	14.05	0.18	0.35
	0.5065	13.8952	14.09	0.19	0.38
Vanillin 0.25 g/L	0.513	13.7454	13.93	0.18	0.35
	0.5107	14.1245	14.31	0.19	0.37
Vanillin 0.5 g/L	0.511	13.825	13.92	0.10	0.19
	0.5099	13.9524	14.08	0.13	0.26
Syringaldehyde 0.5 g/L	0.514	14.1251	14.31	0.19	0.36
	0.5045	14.0854	14.27	0.18	0.36
Syringaldehyde 1 g/L	0.511	13.9133	14.05	0.14	0.28
	0.5045	14.0231	14.19	0.17	0.33
Control	0.510	14.3846	14.54	0.16	0.31
	0.5099	13.9852	14.29	0.30	0.60

Lipid extraction measurement

	Cell dry wt., gm	Wt. of tube, gm	Wt. of tube +lipid, gm	wt. of lipid,gm	gm lipid / gm DW
HMF 0.25 g/L	0.7098	25.4115	25.7134	0.30	0.43
	0.7079	24.9577	25.2949	0.34	0.48
HMF 0.5 g/L	0.7021	24.8993	25.2105	0.31	0.44
	0.7091	25.2122	25.5502	0.34	0.48
HMF 1 g/L	0.7045	25.411	25.7392	0.33	0.47
	0.7081	25.1268	25.4202	0.29	0.41
HMF 1.25 g/L	0.7017	25.4163	25.7326	0.32	0.45
	0.7092	25.9527	26.2684	0.32	0.45
PHB 0.25 g/L	0.7055	25.9088	26.2256	0.32	0.45
	0.7103	25.6385	25.9507	0.31	0.44
PHB 0.5 g/L	0.501	25.6904	25.9111	0.22	0.44
	0.5825	25.0214	25.2517	0.23	0.40
Control	0.7031	25.3705	25.7113	0.34	0.48
	0.7127	25.5625	25.9085	0.35	0.49

Table 7.5.4 Calibration of dry weight

		OD1	OD2	Avg. OD	ND	OD	dry wt, gm/L
HMF 0.25 g/L	100%	0.398	0.397	0.3975	30	11.9	9.0
	80%	0.291	0.29	0.2905	30	8.7	7.2
	60%	0.234	0.235	0.2345	30	7.0	5.4
	40%	0.144	0.146	0.145	30	4.4	3.6
	20%	0.082	0.079	0.0805	30	2.4	1.8
	0%			0		0.0	0.0

		OD1	OD2	Avg	ND	OD	dry wt, gm/L
HMF 0.5 g/L	100%	0.403	0.405	0.404	30	12.1	8.8
	80%	0.299	0.297	0.298	30	8.9	7.0
	60%	0.244	0.245	0.2445	30	7.3	5.3
	40%	0.148	0.149	0.1485	30	4.5	3.5
	20%	0.085	0.084	0.0845	30	2.5	1.8
	0%			0		0.0	0.0

		OD1	OD2	Avg	ND	OD	dry wt, gm/L
HMF 1 g/L	100%	0.381	0.397	0.383	30	11.5	8.5
	80%	0.285	0.286	0.2855	30	8.6	6.8
	60%	0.229	0.232	0.2305	30	6.9	5.1
	40%	0.161	0.163	0.162	30	4.9	3.4
	20%	0.074	0.075	0.0745	30	2.2	1.7
	0%			0		0.0	0.0

		OD1	OD2	Avg	ND	OD	dry wt, gm/L
HMF 1.25 g/L	100%	0.384	0.382	0.383	30	11.5	8.1
	80%	0.296	0.297	0.2965	30	8.9	6.5
	60%	0.228	0.225	0.2265	30	6.8	4.8
	40%	0.139	0.142	0.1405	30	4.2	3.2
	20%	0.066	0.067	0.0665	30	2.0	1.6
	0%			0		0.0	0.0

		OD1	OD2	Avg.OD	ND	OD	dry wt, gm/L
PHB 0.25 g/L	100%	0.399	0.399	0.399	30	12.0	8.8
	80%	0.302	0.303	0.3025	30	9.1	7.0
	60%	0.204	0.206	0.205	30	6.2	5.3
	40%	0.117	0.118	0.1175	30	3.5	3.5
	20%	0.056	0.057	0.0565	30	1.7	1.8
	0%			0		0.0	0.0

		OD1	OD2	Avg.OD	ND	OD	dry wt, gm/L
PHB 0.5 g/L	100%	0.403	0.401	0.402	30	12.1	8.6
	80%	0.31	0.309	0.3095	30	9.3	6.9
	60%	0.216	0.217	0.2165	30	6.5	5.2
	40%	0.118	0.119	0.1185	30	3.6	3.4
	20%	0.074	0.075	0.0745	30	2.2	1.7
	0%			0		0.0	0.0

		OD1	OD2	Avg.OD	ND	OD	dry wt, gm/L
Control	100%	0.412	0.415	0.4135	30	12.4	9.1
	80%	0.333	0.323	0.328	30	9.8	7.3
	60%	0.218	0.219	0.2185	30	6.6	5.5
	40%	0.145	0.144	0.1445	30	4.3	3.6
	20%	0.065	0.066	0.0655	30	2.0	1.8
	0%			0		0.0	0.0

Note: *OD: Optical Density, ND: Number of dilution, 1 & 2 denote samples*

Table 7.5.5 Calibration of lipid

	With NR1			Without NR1		With NR1			Without NR1		
	%	W.L	Intens.	Flu	ND	W.L	Intens.	Intens.	ND	Avg. Intens.	lipid g/L
HMF 0.25 g/L											
	100	579	197.2	8.254	5	594	163.3	7.4	6	172.4	4.07
	80	578	166.6	7.256	5	582	154.2	6.4	6	153.6	3.25
	60	579	120.3	4.545	5	582	122.0	5.4	5	116.2	2.44
	40	579	81.1	6.256	5	582	78.9	9.0	5	72.4	1.63
	20	582	37.7	3.232	5	576	30.0	2.9	5	30.8	0.81
	0				5				5	0.0	0.00
HMF 0.5 g/L											
	100	577	178.3	9.256	5	586	191.3	7.3	5	176.5	4.04
	80	583	136.5	8.365	5	582	136.0	7.4	5	128.4	3.23
	60	584	95.4	11.229	5	585	93.7	10.0	5	83.9	2.43
	40	573	72.7	9.501	5	584	61.8	8.7	5	58.1	1.62
	20	583	28.0	4.871	5	583	16.7	4.3	5	17.8	0.81
	0									0.0	0.00
HMF 1 g/L											
	100	581	191.7	4.183	5	582	176.3	4.3	5	179.8	3.75
	80	581	156.8	2.563	5	582	129.8	2.5	5	140.7	3.00
	60	574	118.5	4.432	5	580	68.6	3.9	5	89.4	2.25
	40	582	90.5	2.712	5	584	61.9	2.7	5	73.5	1.50
	20	579	40.0	5.068	5	578	21.4	5.3	5	25.5	0.75
	0									0.0	0.00
HMF 1.25 g/L											
	100	580	197.5	30.911	5	575	201.5	31.6	5	168.3	3.62
	80	580	151.4	14.132	5	584	121.5	13.1	5	122.8	2.89
	60	581	101.4	15.656	5	586	98.0	12.9	5	85.5	2.17
	40	577	29.3	4.385	5	590	78.2	3.2	5	50.0	1.45
	20	580	28.5	3.237	5	581	28.7	3.3	5	25.3	0.72
	0									0.0	0.00
PHB 0.25 g/L											
	100	582	262.9	4.691	5	578	217.6	5.4	5	235.2	3.91
	80	576	213.7	7.191	5	585	161.2	6.0	5	180.8	3.13
	60	582	169.3	3.007	5	583	111.2	3.0	5	137.3	2.35
	40	579	103.5	2.589	5	575	70.8	2.5	5	84.6	1.56

PHB 0.5 g/L										
100	577	278.4	7.662	5	584	218.2	6.9	5	241.0	3.59
80	579	157.7	6.767	5	580	181.4	6.9	5	162.7	2.88
60	579	93.4	6.852	5	575	120.3	4.2	5	101.3	2.16
40	575	51.3	7.403	5	584	84.6	7.4	5	60.6	1.44
20	579	32.1	2.589	5	575	30.7	2.5	5	28.8	0.72
0									0.0	0.00
Control										
100	583	202.4	7.801	5	581	198.2	8.1	5	192.3	4.41
80	583	124.7	4.253	5	584	139.9	5.5	5	127.5	3.53
60	585	103.3	4.122	5	581	102.2	4.9	5	98.2	2.65
40	579	84.7	3.722	5	580	54.3	3.9	5	65.7	1.77
20	585	27.7	5.923	5	577	21.7	5.9	5	18.8	0.88
0		0.0				0.0			0.0	0.00

Note: *ND: no. of dilution, OD: optical density, NR: Nile red, W.L: wave length, 1 & 2 denote samples, Intens. Intensity*

Table 7.5.6 Glucose measurement

Hrs.	Control g/L		Furfural 0.05 g/L		Furfural 0.1 g/L		Furfural 0.2 g/L		Furfural 0.4 g/L		Vanillin 0.25 g/L		Vanillin 0.5 g/L	
0	29.7	28.9	30.6	31.3	31.2	30.2	29.3	31.9	29.5	32.2	30.2	28.7	29.6	31.3
	31.6		31.4		31.3		31.0						30.8	
24	28.4	27.4	29.3	31.2	30.2	29.3	29.1	30.3	29.3	31.3	29.3	28.6	28.9	30.7
	30.1		30.1		30.3		29.3						30.2	
48	12.1	13.8	16.9	12.2	11.1	11.6	11.3	13.4	19.1	20.4	17.4	17.8	23.8	24.7
64	5.1	4.8	8.6	7.9	7.5	7.6	8.6	8.9	13.2	13.9	11.1	10.5	19.5	20.1
	8.9		10.6		12.0		17.6						24.8	
72	2.1	2.5	3.2	2.9	3.5	2.3	2.3	3.3	3.8	1.8	1.5	2.4	9.5	9.2
													14.2	
80	0	0	0	0	0	0	0	0	0	0	0	0	4.1	3.8
	0		1.4		2.6		2.6						10.2	
104	0	0	0	0	0	0	0	0	0	0	0	0	2	0

Hrs.	Syringaldehyde 0.5 g/L	Syringaldehyde 1 g/L	
0	30.5	31.2	29.2
	31.4		31.9
24	29.5	30.9	29.2
	30.1		29.6
48	17.5	16.6	24.9
64	8.2	8.9	16.2
	13.8		22.9
72	2.8	1.7	5.4
			8.7
80	2.5	3.1	2.2
	5.9		2.5
104	0	0	0

Glucose measurement

	HMF 0.25 g/L			HMF 0.5 g/L			HMF 1 g/L			HMF 1.25 g/L		
Hr	A	B	C	A	B	C	A	B	C	A	B	C
0	29.12	31.24	32.33	31.23	28.36	29.35	33.17	32.25	31.45	32.25	31.25	32.25
24	25.55	28.35	24.52	28.23	27.35	27.55	32.21	29.58	30.1	31.25	31.5	31.25
48	20.95	17.23	19.52	25.82	27.94	24.91	31.86	29.85	28.18	29.52	30.6	29.68
70	5.82	2.65	2.03	3.89	4.14	3.62	16.41	15.04	18.96	22.38	24.56	23.25
78	3.95	2.43	1.44	3.26	3.33	3.18	13.84	12.74	14.46	15.54	18.21	16.54
106	0	0	0	0	0	0	5.35	5.96	7.55	7.95	8.21	7.25
128	0	0	0	0	0	0	0	0	0	0	0	0

	PHB 0.25 g/L			PHB 0.5 g/L		Control		
Hr	A	B	C	A	B	A	B	C
0	32.25	29.35	31.25	32.32	31.25	31.84	30.48	31.25
24	31.25	29.65	31.12	31.25	31.52	29.25	28.36	27.22
48	19.21	17.25	14.53	17.56	16.85	12.56	10.69	11.1
70	6.35	8.85	7.63	14.48	12.25	5.56	4.53	4.49
78	4.36	4.36	3.61	9.35	8.25	2.53	2.12	3.51
106	0	0	0	5.35	4.28	0	0	0
128	0	0	0	0	0	0	0	0

Note: *A, B, C denote flask*

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Major: Chemical Engineering
Title of Thesis: Economical Lipid Production by *Lipomyces starkeyi*
Thesis Director: Dr. Ramalingam Subramaniam
Pages in Thesis: 167; Words in Abstract: 280

ABSTRACT

Microbial lipids can be produced by the oleaginous yeast *Lipomyces starkeyi* from a wide range of carbohydrates, including hexose and pentose sugars. The main barrier for commercial production of microbial lipids is their production costs. The major cost contributing factors in production of microbial lipids are the medium components, processing, and lipid recovery from cells. Medium cost comprises of carbon substrate and other essential nutrients, mainly phosphate. The carbohydrate costs can be reduced by using lignocellulosic materials as feedstocks. But the process of extraction of sugars from lignocellulosic materials produces toxic byproducts. The effect of byproducts, such as furfural, 5-hydroxy methyl furfural (HMF), vanillin, para hydroxy benzaldehyde (PHB), and syringaldehyde, were examined for cell growth and lipid production by *Lipomyces starkeyi.* The inhibitory effects of these byproducts were not significant in all cases up to 500 mg/L of inhibitors in fermenting media. Under the phosphate optimization, phosphate concentration corresponding to 1/20-X was found optimal and the medium cost can be reduced from $9.34 per gallon lipid to $3.11 per gallon lipid. For extraction of lipid, methyl tertiary butyl ether-methanol solvent system showed better potential than other solvents studied in lipid extraction from the economical view point.

BIOGRAPHICAL SKETCH

Sharif Rahman completed his Bachelor of Science in Chemical Engineering from Bangladesh University of Engineering and Technology in 2005. Later, he worked in chemical process industry for five years in Bangladesh before entering in graduate study in Chemical Engineering at UL Lafayette in 2011. He received his Masters of Science in Chemical Engineering in spring 2016. He was born on December 7, 1979 in Dhaka, Bangladesh. He is the youngest son of Md. Motiur Rahman and Sahara Banu.