DEVELOPING NOVEL BIOLOGICAL PROCESSES TO CONVERT LIGNOCELLULOSE INTO LIPID BASED BIOFUEL

By

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ABSTRACT

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Microbial lipids for biofuel production have recently attracted increasing attention because of their advantages over plant oils such as fast growth, year-round production, less demand on space, and easy scale-up. Particularly, microbes are able to utilize a variety of carbon sources (glucose, xylose, glycerol, starch, and lignocellulose) for the production of cell mass and lipids, which greatly improves the flexibility of microbial lipid based biofuel production. Among these carbon sources, lignocellulose, the most abundant and renewable source in nature, is considered as an ideal raw material for microbial lipid production. Some oleaginous microbes (those can accumulate more than 20% of intracellular lipid), such as *Mortierella isabellina*, *Rhodotorula glutinis,* and *Candida curvata* etc., are able to uptake both glucose and xylose from lignocellulose and accumulate a relatively large amount of lipid in the cell. Despite their promise, the full potential of lignocellulose for microbial lipid fermentation has yet to be unlocked because of the challenges associated with its decomposition into fermentable sugars and low fermentation efficiency due to inhibitors generated from thermal/chemical pretreatment. In addition, liquid-solid separation, water usage, and low xylan utilization are other major issues impeding commercial microbial lignocellulose based lipid production.

In response to addressing the aforementioned issues, the novel biological process of combined hydrolysis, which based on our co-hydrolysis process, was developed in the current study to efficiently release mono-sugars and acetate from lignocelluloses,

and satisfy the need of fungal fermentation to accumulate lipids. Besides that, little is known about the effect of inhibitors from lignocellulose hydrolysis on oleaginous fungal cell growth and lipid production, so their individual and synergistic effects were studied as well. ^{13}C isotope traced experiment was carried on to illustrate the carbon utilization regarding cell growth and lipid accumulation in the presence of inhibitors such as furfural and HMF. This study would play an important role in terms of microbial lipid fermentation from lignocellulose using oleaginous fungal fermentation platform.

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KEY TO ABBREVIATIONS AND SYMBOLS

Abbreviations:

M.isabellina: Mortierella isabellina

C/N ratio: Carbon/nitrogen molar ratio

$v \cdot v^{-1}$: Volume/volume

FPU: Fiber paper unit

HPLC: High performance liquid chromatography

LC-MS: Liquid chromatography-mass spectrometry

GC: Gas chromatography

HMF: Hydroxymethylfurfural

FID: Flame ionization detector

PUFA: Poly unsaturated fatty acid

C14:0: Myristic acid

C16:0: Palmitic acid

C16:1: Palmitoleic acid

C18:0: Stearic acid

C18:1: Oleic acid

C18:2: Linoleic acid

C18:3: Linolenic acid

DHA; docosahexaenoic acid

ARA: Arachidonic acid

EPA: Eicosapentaenoic acid

GLA: γ-linolenic acid

FAME: Fatty acid methyl ester

TCA: Tricarboxylic acid cycle/citric acid cycle

Asp: Aspartate

Phe: Phenylalanine

ATP: Adenosine triphosphate

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

Symbols

X/S: Cell mass per consumed substrate

L/S: Lipid per consumed substrate

L/X: Lipid per cell mass

CHAPTER 1. INTRODUCTION

1.1 Introduction

Biodiesel has been widely accepted as an alternative to petroleum derived diesel fuels. It is non-toxic, biodegradable, has a favorable emission profile, and can be produced from a variety of resources such as soybean, palm, sunflower, jatropha, waste oils and animal fats through simple transesterification reaction (4, 18). However, the rising concerns regarding the usage of food resources for biofuel production, along with the increasing energy demand has forced the biodiesel industry to search for alternative oil (lipid) sources. Much focus has shifted to non-edible oil sources such as microbial lipids (oils), which are usually produced by oleaginous microorganisms (with intracellular lipid content over 20%).

1.2 Microbial lipids

Microbial lipids have been widely studied for nutraceutical production in the past decades. They play a critical role in maintaining human health from medical perspective (12, 134). Microbial lipids are the sources of important dietary supplements of polyunsaturated fatty acids (PUFA) such as γ-linolenic acid (GLA), which has selective anticancer characteristics (85); while docosahexaenoic acid (DHA), arachidonic acid (ARA) and eicosapentaenoic acid (EPA) are essential fatty acids in infant brain development, eye function, hormones synthesis (130). Numerous studies have been conducted regarding the microbial production of these PUFA, such as *Cunninghamella echinulata* (43), *Mortierella isabellina* (114), *Mortierella ramanniana* (38)*, Mortierella aplina (79),* and *Mucor rouxii* (102) for GLA accumulation; *Schizochytrium limacinum* (25), *Crypthecodimium cohnii* (25, 31, 32, 50, 123, 157), *Schizochytrium G13/2s* (50) for DHA production; *Sirodotia kylin* (14); *Mortierella alpina* (115, 182), *Parietochloris incise*

(16) for ARA fermentation; (14, 16, 115), *Candida guilliermondii* (56, 57), and *Achly a sp*. Ma-2801 (3) for EPA production.

With the increasing demand on biodiesel, microbial lipids recently attract much attention as alternative feedstock to replace vegetable oils and animal fats for biodiesel production. It has been reported that extensive usage of vegetable oils and animal fats has significantly influenced the food supply and price (137). Compared to other plant oils, microbial lipids have many advantages, such as shorter life cycle, reduced labor requirement, less affection by venue, minimum season and climate influences, and easier to scale up (94). Microbial lipids, whose fatty acid composition is similar to that of vegetable oils, primaryily composed of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), could be used as an excellent alternative feedstock for biodiesel production. The oleaginous microorganisms are found in the microalgal, bacteria, yeast and fungal groups, but not all of them are available for biodiesel production (105).

Microalgae is considered as cell factories for biofuel production, which are driven by sunlight as energy source and CO_2 as carbon source. They can produce methane, bioethanol, biodiesel (9, 51) and biohydrogen (46, 82, 104). The stored lipid in microalgae is mainly triglyceride (>80%), with a fatty acid composition rich in C16 and C18 as well as unsaturated fatty acids such as C16:1, C18:1. The average lipid content in algae varies from 1% to 70% (136). The representative lipid producing microalgae are *Nannochloropsis oculata* (26), *Neochloris oleoabundans* (94), *Cladophora fracta* (36), *Chlorella protothecoides* (36, 161, 162), and *Chlorella vulgaris* (94). Due to their high lipid content, microalgae are one of the promising alternatives for biodiesel production. Compared with energy crops, they have higher cell mass accumulation and faster growth (105). However, compared to other oleaginous microbes, the major drawbacks of the

microalgal lipid production are: they need a larger amount of water to culture, and the cell density is usually lower. So these issues are the key barriers to limit large-scale microalgal lipid production (94).

Bacteria are another group of microbes for lipid accumulation. They have relatively lower lipid content compared with microalgae, The average lipid content in bacteria cell mass is usually around 20-40% (105) with some exceptional cases of up to 80%. For instance, the actinomycete (a heterogeneous group of gram-positive bacteria) are able to accumulate up to 70% cellular lipid from carbon source such as glucose under growth stressed conditions (5). *Arthrobacter* AK19 was able to accumulate around 78% intracellular lipid using glucose as carbon source with a culture time of 13 days (122). *Gordonia* sp.DG could accumulate around 71% lipid in its cell with 4 days culture using valerate as substrate (55). While for *R.opacus* PD630, it can utilize various substrates as gluconate, olive oil, carob waste and molasses to accumulate more than 50% lipid inside the cell (5, 55, 151). However, even though bacteria usually accumulate lipid in a short time and are relatively easy to culture (67), their lipid in general is not suitable for biodiesel production. Some research has focused on genetic modification of bacteria to accumulate fatty acids for biodiesel production. It was reported that an engineered *E.coli* could produce free fatty acids that are suitable for transesterification of biodiesel production (99).

Besides algae and bacteria, yeast and fungi are widely recognized as favorable oleaginous microbes (1, 121). Oleaginous yeast are highly productive with remarkable cell density and lipid yield (94). There are many yeast strains capable of lipid accumulation on various carbon sources, such as glucose, starch, stearin, glycerol, and cheese whey. *Rhodotorula glutinis* can accumulate intracellular lipid up to 70% in the cell mass, with a triacylglycerol concentration of 67 g/L in the fermentation broth (19). *Lipomyces starkeyi* ,

Rhodosporidium toruloides and *Trichosporon fermentans* are able to accumulate 56%, 67.5% and 62% intracellular lipid using glucose as carbon source (7) (95, 180). *Rhodotorula mucilaginosa* had the ability of converting cassava starch into lipid with a cellular content of 53% in 5 days (93). *Yarrowia lipolytica*, which utilized stearin as substrate, accumulated 52% cellular lipid with a culture time of 4 days, while it was 43% when using glycerol as substrate for 0.5-1 day culture (110, 111). *Cryptococcus curvatus* accumulated 46% and 53% intracellular lipid when utilizing whey and glucose as carbon source, respectively (60, 103). Compared with yeast, fungal lipid accumulation usually needs a bit longer culture time. *Mortierella ramanniana* MM15-1 accumulated 68% intracellular lipid using glucose as substrate in 9 days culture (62). *Mortierella isabellina* ATHUM2935 was able to accumulate 64%, 53% and 50% intracellular lipid using xylose, raw glycerol and glucose as substrate, respectively, while the lipid concentration was 6.1, 3.3, and 18.1 g/L with 15, 11, and 10.4 days culture (44, 114). Another oleaginous fungus *Cunninghamela echinulata* ATHUM 4411 accumulated 53.6% and 26% lipid with xylose and crude glycerol as carbon sources, respectively (44). Basically, oleaginous yeast and fungi accumulate triacylglycerol rich in polyunsaturated fatty acids with carbon chain length of 16 and 18 fatty acids (84). For instance, the oleaginous fungus *Mortierella isabellina* ATHUM 2935 accumulated 44-56 % oleic acid, 10-16% linoleic acid (44); the oleaginous yeast *Rhodosporidium toruloides* accumulated 47% oleic acid and 13% linoleic acid (95). Based on the aforementioned information, oleaginous yeasts and fungi are both promising candidates of oil sources for biodiesel production.

When it comes to biodiesel production using microbial lipid as its feedstock, one of the major barriers is the cost of microbial biodiesel production including both lipid fermentation and transesterification, particularly the former one. The fermentation substrates are the key component that influence the lipid fermentation cost. Under ideal

circumstances, they should be low enough to make the biodiesel production competitive compared with other sources of diesel. In order to reduce the cost of microbial lipid fermentation, considerable effort has been devoted to use the low cost substrates, such as molasses (21, 180), glycerol (25, 42, 110, 112, 117), whey (2, 30, 146), monosodium glutamate wastewater (164-166), sewage sludge (7), olive oil mill wastewater (168), sweet sorghum extract (39), tomato waste hydrolysate (45), N-acetylglucosamine hydrolysate (156, 173), and starch hydrolysate (181). Though the above-mentioned low cost substrates could be used to decrease the cost of microbial lipid production, the major barrier that might prevent its commercial application is the transportation and continuous supply, which would lead to unfavorable microbial lipid production (67). An alternative would be to use lignocellulose, the most widely available renewable materials on earth as feedstock for microbial lipid production, hence to address the above mentioned issues effectively.

1.3 Lignocellulose

Lignocellulose has been considered as a promising candidate of material for biofuel and biochemical production. It is the most abundant natural polymer in the biosphere, and interest in using it as a feedstock for the production of advanced biofuels has gained momentum in recent years as shown by various government directives. The Renewable Fuels Standard 2 provision in the United States Energy Independence and Security Act of 2007 mandates the production of 36 billion gallons of biofuels by 2022, with 16 billion gallons coming from lignocellulosic sources while capping conventional biofuels (i.e. corn starch-based ethanol) at 15 billion gallons (28). Support for lignocellulosic biofuels is not only owed to its large supply, but also its ability to mitigate greenhouse gas emissions, avoid competition with food resources, stimulate rural economies, and provide a stable and secure source of energy production (28). Many sources of lignocellulosic biomass

exist, including municipal solid wastes, pulp and paper wastes, forest and agricultural residues, dedicated woody and herbaceous perennial energy crops.

Lignocellulose is mainly composed of cellulose, hemicellulose and lignin. The composition of lignocellulose depends on plant species, age and growth conditions. Distribution of cellulose, hemicelluloses and lignin varies significantly between different plants (81). Its bioconversion generally includes pretreatment, enzymatic hydrolysis and microbial fermentation (127). A variety of lignocellulose pretreatment technologies are available today, which encompass a wide range of physical, chemical, and biological processes. Among which, chemical pretreatments are considered to be the most promising for future biorefineries, mainly including acid, alkali, ionic liquid, and oxidative pretreatments (29, 48). The overall goal of pretreatment is to increase the enzymatic convertibility of cellulose and hemicellulose by alteration or removal of hemicellulose and/or lignin, increase surface area and decrease the crystallinity of cellulose (106, 158). After pretreatment, cellulolytic enzyme cocktail including exo-1,4-β-D-glucanase, endo-1,4-β-D-glucanase, and 1,4-β-D-glucosidase is added, these enzymes work synergistically to hydrolyze cellulose (the largest part of lignocellulose) to glucose (145), which is the preferred carbon source for many microorganisms. Hemicellulose is the second most common polysaccharide in lignocellulose, while xylan is the most abundant part in hemicellulose. Depend on the pretreatment methods, xylan is either partially removed or degraded. For the remained xylan, xylanolytic enzyme mixture including endo-xylanase, exo-xylanase, β –xylosidase and α-L-arabinofuranosidase is required in order to effectively hydrolyze and release monomer sugar mainly xylose from hemicellulose (81, 129). After pretreatment and enzymatic hydrolysis, glucose and xylose are the two most abundant mono-sugars released from lignocellulosic biomass. Thus, depending on the desired fuels, the specific biofuel-producing microbe can be applied to convert these

sugars into targeted products. For instance, ethanol production is usually carried out by yeast such as *Saccharomyces cerevisiae*.

1.4 Microbial lipid from lignocellulose

Development of microbial biodiesel production from lignocellulose is at its early stage compared to lignocellulosic ethanol production. There are usually three steps involved in microbial lipid fermentation: fermentable sugars release from lignocellulose, lipids accumulation from these sugars by oleaginous microbes, and then transesterification of lipids to biodiesel (177). The pretreatment and enzymatic hydrolysis of lignocellulose has been studied for several decades and are mature technologies, transesterification of lipid into biodiesel using either chemical or biological methods has also been researched for a long time (67). Thus, the focus of this study was on the microbial lipid fermentation from lignocellulose.

Current research and development on lipid accumulation from lignocellulose mainly focuses on oleaginous yeasts as the biocatalysts (Table 1). While regarding oleaginous yeast lipid fermentation from lignocellulose, the major substrates were detoxified lignocellulosic hydrolysates, since yeast are sensitive to the inhibitory effect caused by those inhibitors (weak acids, furan derivatives, phenolic compounds) generated during pretreatment of lignocellulose. *Trichosporon cutaneum, Rhodotorula garminis,* and *Trichosporon coremiiforme* were the strains that have been applied to converted corn stover hydrolysate into lipids (49, 65, 68, 75, 97). *Trichosporon fermentans* and *Yarrowia lipolytica* was used to produce lipids from pretreated rice straw, rice bran and sugarcane bagasse (69, 74, 142, 143). *Trichosporon dermatis* was adopted to ferment organic solvent pretreated corncob for lipid accumulation (66). There are only a few studies on lipid accumulation on non-detoxified lignocellulosic hydrolysates.

Table 1 Yeast lipid fermentation from lignocellulose

Strains	Feedstocks	Lipid yield (g/L)	Lipid content (%,w/w)	Lipid productivity (g/L/h)	References
Cryptococcus curvatus	Wheat straw	5.8	33.5	0.04	(169)
Rhodotorula glutinis	Wheat straw	3.5	25	0.028	(169)
Lipomyces starkeyi	Wheat straw	4.6	31.2	0.032	(169)
Rhodosporidium toruloides	Wheat straw	2.4	24.6	0.017	(169)
Trichosporon dermatis CH007	Corncob	9.8	40.1	0.058	(66)
Trichosporon coremiiforme	Corncob	7.7	37.8	0.04	(68)
Trichosporon fermentans	Sugarcane bagasse	15.8	N.A.	0.073	(69)
Yarrowia lipolytica Po1g	Sugarcane bagasse	6.7	58.5	0.07	(143)
Trichosporon.cutaneum	Corn stover	3.2	N.A.	0.034	(97)
Trichosporon.cutaneum	Corn stover	7.6	39.2	0.079	(65)
Trichosporon.cutaneum cx1	Corn stover	3.1	23.5	0.052	(75)
Rhodotorula garminis	Corn stover	14.1	34	0.21	(49)
Cryptococcus curvatus	Corn stover	6.0	N.A.	0.125	(53)
Rhodosporidium Toruloides Y4	Corn stover	5.5	36.4	0.034	(160)
Trichosporon fermentans CICC 1368	Rice straw	11.5	40.1	0.06	(74)
Yarrowia lipolytica Po1g	Rice bran	5.2	48	N.A.	(142)

Yu et al. reported that *Cryptoccus curvatus, Rhidotorula glutinis, Rhidosporidium toruloides* and *Lipomyces starkeyi* were able to accumulate lipid from non-detoxified wheat straw (169). The inhibitory effect from the compounds of lignin and carbohydrate degradation was a major hurdle regarding oleaginous yeast lipid fermentation. The type and extent of these inhibitors depend on the cell wall composition of the biomass and pretreatment methods (87, 88). In general, there are mainly three types of biological inhibitors: weak acids (acetic acid, formic acid, levulinic acid), furan derivatives (furfural

and hydroxymethylfurfural), and phenolic compounds (coumaric acid, ferulic acid, 4-hydroxybenzaldehyde, syringaldehyde, 4-hydroxybenzoic acid, vanillin, catechol) (108). Acetic acid is generated during hemicellulose degradation under high temperature and pressure condition, furfural and hydroxymethylfurfural (HMF) were degraded from xylose and hexose; formic acid and levulinic acids are generated when furfural and hydroxymethylfurfural are broken down (144). Phenolic compounds are formed as a result of partial lignin degradation (11) as well as carbohydrate degradation (139). For instance, vanillin is generated by degradation of one of the lignin units guaiacylpropane (80), syringaldehyde is formed because of syringylpropane unit degradation in lignin (80).

Acids are categorized into strong or weak acids, depending on their dissociation constant, K_a, the negative logarithm of which is defined as pK_a. The concentration of undissociated acid is a function of pH and pK_a, and increases with decreasing pH (108). Undissociated weak acids are liposoluble; they can diffuse across the plasma membrane and enter the cell cytosol (8, 147, 148, 153). In the cytosol, the acids dissociate due to the neutral intracellular pH in the cytosol, leading to a cytosolic pH drop (109). The decrease of the cytosolic pH triggers the plasma membrane ATPase that pumps protons out of the cell at the expense of ATP hydrolysis, so additional ATP must be generated to maintain the neutral intracellular pH in the cytosol, which causes the inhibition of cell growth (128). Since the concentration of undissociated acids in lignocellulosic hydrolysates is very dependent on pH, pH is a crucial variable during lipid fermentation. Furan derivatives furfural and hydroxymethylfurfural influence the glycolysis and Krebs cycle fluxes, thus adversely influencing energy metabolism (63, 108, 171), leading to a longer lag phase for microbial cell growth (107). The phenolic compound integrates with cell membrane and cause the loss of membrane integrity, affect their ability as selective hurdles and enzyme matrices (61). Besides the individual inhibitory effect on microorganisms, the interactive

effects of these inhibitors were also noticed and commonly found to be synergistic (23, 64, 176).

Due to the inhibitory effect of aforementioned inhibitors on yeasts, the lipid yield and lipid productivity of yeast fermentation on lignocellulosic hydrolysate was usually lower compared with the synthetic media (23, 74, 160, 169). Besides that, most oleaginous yeast cannot utilize xylose for lipid production, while xylose is the second most abundant monomer sugar in lignocellulosic enzymatic hydrolysate, so it is very valuable from techno-economic standpoint to convert this fraction into lipids. Discovering and developing robust oleaginous microorganisms that can efficiently convert fermentable sugars (glucose and xylose) from lignocellulose and bear inhibitory effect from various inhibitors would be essential to realizing a lignocellulose based biodiesel production.

Oleaginous fungi seem to be a promising candidate in terms of addressing these issues for microbial lignocellulosic lipid accumulation (Table 2). The advantages of oleaginous fungi were their unique ability of converting both glucose and xylose into lipid; their physiological properties also determine that they are generally robust and had better tolerance to the harsh environment compared with oleaginous yeast (58). One of the widely studied fungal strains for microbial lipid fermentation is *Mortierella isabellina*, the lignocellulosic biomass used including sweet sorghum (41), rice hull (40), soybean hull (175), as well as dilute acid pretreated wheat straw hydrolysate (172). Other strains such as *Mucor circinelloides* was applied on avicel enzymatic hydrolysate to produce lipid (154). However, they usually grow slower compared with yeast. Therefore, a fast growing oleaginous fungus must be discovered in order to efficiently utilize both glucose and xylose from lignocellulosic hydrolysates.

Table 2 Lipid fermentation from lignocellulose by fungi

Strains	Feedstocks	Lipid yield ($g \cdot L^{-1}$)	Lipid content (%)	Lipid productivity ($g \cdot L^{-1} h^{-1}$)	References
A.terreus	Wheat straw	1.52	20	N.A.	(179)
M. isabellina	Wheat straw	2.63	39.4	N.A.	(179)
M. vinacea	Wheat straw	2.46	32.7	N.A.	(179)
T.lanuginosus	Wheat straw	0.78	20.5	N.A.	(179)
R.oryzae	Wheat straw	0.84	16.1	N.A.	(179)
C.elegans	Wheat straw	0.80	17.0	N.A.	(179)
M. isabellina ATHUM2935	Rice hull	3.6	64.3	0.0096	(40)
M. isabellina ATHUM2935	Sweet sorghum	9.2	43	0.038	(39)
M.isabellina NRRL 1757	Wheat straw	4.36	36.3	0.03	(172)
M. isabellina ATHUM2935	Sweet sorghum	110(mg/gds)[a]	25	0.055	(41)
M. isabellina IFO 7884	Soybean hull	47.9(mg/gds)	N.A.	N.A.	(175)
A.oryzae A-4	Wheat straw + bran	62.9(mg/gds)	N.A.	0.44(mg/g/h)	(96)
Microsphaeropsis sp. *Colletotrichum* sp.DM06	Wheat straw + bran	80(mg/gds)	10.2	0.33(mg/g/h)	(116)
	Rice straw	68.2(mg/gds))	N.A.	0.28(mg/g/h)	(37)
Alternaria sp.DM09	Rice straw	60.3(mg/gds)	N.A.	0.25(mg/g/h)	(37)
Mucor circinelloides	Avicel	0.7	3.32	N.A.	(154)

N.A. Not available
a: gds means gram dry substrate

1.5 Problems with lignocellulose bioprocessing

Regarding traditional lignocellulose bioprocessing, after dilute acid pretreatment, one of the most widely used pretreatment method, the solids are separated from the liquid stream, washed to neutralize and detoxify the remaining solids, then subjected to enzymatic hydrolysis in order to extract glucose (34). While the discarded liquid stream contains the second most abundant monomer sugar from lignocellulose, xylose, so it is critical to recover and convert this fraction into targeted product. The traditional

lignocellulose processes require large amount of water to detoxify and neutralize the remaining solid fiber, separation of the solid and liquid streams is time consuming, and contamination might be introduced during these operations (138). Therefore, development of novel biological process, which could utilize the most of carbohydrates from lignocellulose, eliminate liquid-solid separation and detoxification of the pretreated solids, and directly carry out enzymatic hydrolysis after pretreatment, would make a significant contribution to the advanced biofuels production from lignocellulose.

1.6 Objectives

The goal of this project is to develop novel biological processes to convert lignocellulose into lipid based biofuel. This process will integrate chemical pretreatment, enzymatic hydrolysis and oleaginous fungal lipid accumulation to achieve an efficient lignocellulose conversion for lipid production. The specific objectives of this research mainly include the following:

1. Screen for a microbe that could efficiently utilize glucose and xylose from lignocellulose for microbial lipid fermentation.
2. Develop a combined chemical pretreatment and enzymatic hydrolysis method for efficient lignocellulose bioprocessing, which is able to integrate well with microbial lipid fermentation.
3. Identify key inhibitory compounds derived from the pretreatment of lignocellulosic biomass corn stover and investigate their effects on microbial growth and lipid production with the help of ^{13}C trace study.

CHAPTER 2. EVALUATION OF LIPID ACCUMULATION FROM LIGNOCELLULOSIC SUGARS BY MORTIERELLA ISABELLINA FOR BIODIESEL PRODUCTION

Zhenhua Ruan, Michael Zanotti, Xiaoqing Wang, Chad Ducey, Yan Liu

2.1 Abstract

The filamentous fungus *Mortierella isabellina* ATCC42613 was used to assess the conversion of different carbon sources (glucose, xylose, mixed glucose/xylose, acid and alkali treated corn stover hydrolysate) in submerged media to lipid. Glucose and xylose cultures composed of varying initial sugar concentrations (28.1-91.7 $g \cdot L^{-1}$, and 26.6-90.9 $g \cdot L^{-1}$ respectively) showed a positive correlation to lipid accumulation, with significant quantities occurring at the upper limit of the substrate range (10.2, and 8.8 $g \bullet L^{-1}$ lipid respectively). While lipid concentrations increased with each incremental glucose and xylose level, the lipid yield (0.41-0.44, and 0.39-0.43 $g \bullet g^{-1}$ cell mass respectively), and intracellular fatty acid composition remained relatively constant. Additionally, sulfuric acid hydrolysate, without detoxification, exhibited greater cell mass, and equivalent lipid production compared to synthetic medium with similar initial glucose and xylose concentrations. These results elucidate the potential of utilizing filamentous fungal fermentation to accumulate lipids from lignocellulosic biomass for biodiesel production.

Keywords: *Mortierella isabellina*; diluted acid and alkaline pretreatments; enzymatic hydrolysis; hydrolysate of corn stover; filamentous fungal lipid fermentation.

2.2 Introduction

Biodiesel is viewed as an attractive alternative to diesel fuel due to its positive environmental characteristics. It is non-toxic, biodegradable, has a favorable emissions profile, and is produced from a variety of renewable resources including: soybean, palm, sunflower, rapeseed, jatropha, and waste oils (4, 18). However, growing concerns over the use of food crops for fuel production, as well as the rising global energy demand has put pressure on the biodiesel industry to find alternative sources of oil. Attention has shifted to non-edible oil sources such as those produced from oleaginous microorganisms (those organisms with lipid content in excess of 20%). Microbial lipids are viewed as a possible alternative for industrial production because their fatty acid composition is similar to that of vegetable oils, as well as the fact that they are rich in polyunsaturated fatty acids, such as γ-linolenic acid, which are often used in dietary supplements and for infant nutrition (74). The major fatty acids present in the lipids produced by oleaginous microorganisms are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3); all of which can be converted to biodiesel through a transesterfication reaction.

However, the limiting factor for microbial lipid production is often the organism's low productivity coupled with the high expense of its glucose substrate (96). In order to overcome cost barriers, a cheaper source of carbon is desired. Lignocellulosic biomass such as corn stover is of great interest due to its abundance and potential to serve as an inexpensive feedstock of mono-sugars for lipid production. Both glucose and xylose can be extracted from such sources, with glucose being the more abundant and easier metabolized of the two. However, large amounts of xylose are also produced during lignocellulosic biomass pretreatment (91). Organisms that can utilize both C-5 and C-6

sugars for fermentation are thus highly desired in order to increase the efficiency of lipid production from lignocellulosic materials. The wide availability of lignocellulosic material coupled with their cheap cost and ease of xylose extraction makes them potentially attractive substrates for microbial lipid accumulation.

In general, lipids are accumulated in oleaginous organisms due to the presence of the ATP-dependent citrate lyase. Under nutrient limited conditions, especially nitrogen limitation with an abundant carbon source (high carbon: nitrogen ratio), AMP deaminase is up-regulated eventually leading to the presence of citrate in the cell cytosol. Citrate is cleaved by the ATP-dependent citrate lyase producing acetyl-CoA, which is used in fatty acid biosynthesis. It is by this pathway that oleaginous microorganisms shift their carbon sources away from growth and toward lipid production (119). Though glucose is the more easily assimilated of the two major lignocullosic carbon sources, studies have investigated oleaginous fungal lipid production using xylose as the carbon source as well as both glucose and xylose simultaneously. Xylose (80 $g \cdot L^{-1}$, $C \cdot N^{-1}$ ratio of 285) was used as the sole carbon source to cultivate the oleaginous molds *M.isabellina* ATHUM 2935 and *Cunninghamella echinulata* ATHUM 4411 resulting in lipid concentrations of 6.1 $g \cdot L^{-1}$ and 6.7 $g \cdot L^{-1}$ respectively at 360 h, however, more than 25 $g \cdot L^{-1}$ of unconsumed xylose remained in the media (44). Dey et al., (2011) have reported lipid accumulations from xylose of 2.2 $g \cdot L^{-1}$ for the oleaginous mold *Colletotrichum sp*.DM06, and 4.3 $g \cdot L^{-1}$ for *Alternaria sp*.DM09 under nitrogen stressed conditions respectively (37). The oleaginous yeast *Trichosporon cutaneum* AS 2.571 assimilated glucose and xylose simultaneously, and accumulated intracellular lipid up to 59 wt% with a lipid coefficient up to 0.17 g/g sugar, upon cultivation on a 2:1 glucose/xylose mixture (65).

Additionally, a variety of research has been conducted on oleaginous mold lipid accumulation from pretreated lignocellulosic biomass enzymatic hydrolysate. When using

a mixture of wheat straw and bran as feedstock with solid culture, 80 milligram lipid per gram of dry solid cell mass ($mg \cdot gds^{-1}$) was accumulated by the oleaginous mold *Microsphaeropsis sp* (116). For semi-solid culture, 11 g oil /100 g dry sweet sorghum was production (41). Researchers using rice straw and wheat bran under solid culture produced 68.2 $mg \cdot gds^{-1}$and 60.32 $mg \cdot gds^{-1}$ lipid using *Colletotrichum sp* (DM06) and *Alternaria sp*. (DM09) (37). The mutant strain *Mortierella. alpina* (MAI502-8) accumulated 27.4 and 10.05 $g \cdot L^{-1}$ cell mass and lipid respectively from a mixture of glucose and xylose media (5:3 wt/wt) over 11 days of total culture time (152). Under varying $C \cdot N^{-1}$ ratios (35, 44 and 57), lipid accumulated in *Mortierella isabellia* ATHUM 2935 was 36%, 51.2% and 64.3%, respectively using rice hull hydrolysate (40). However, limited studies exist which examine filamentous fungal lipid accumulation in submerged cultures using corn stover enzymatic hydrolysate after sulfuric acid or sodium hydroxide pretreatments. In this study, experiments were carried out in order to investigate the effects of different carbon sources on lipid accumulation using the oleaginous filamentous fungus *M.isabellina* under nitrogen stressed conditions. The carbon sources include: glucose, xylose, acid and alkaline pretreated corn stover coupled with enzymatic hydrolysis, as well as a glucose/xylose mixture acting as artificial hydrolysate.

2.3 Materials and methods

2.3.1 Microorganisms and Culture conditions

Mortierella isabellina ATCC 42613 was obtained from the American Type Culture Collection (Manassas, VA). This strain was first cultured on potato dextrose agar (Sigma, USA) to produce spores at 30ºC. After 14 days cultivation, the spores were washed from the agar with sterile distilled water as a spore suspension and maintained at 4℃. Seed cultures were grown with 24 $g \cdot L^{-1}$ potato dextrose broth (Sigma) with 8 $g \cdot L^{-1}$ yeast extract

at 25℃ and 190 rpm for 2days in a rotary shaker (Thermal Scientific) with a spore concentration of 1-2×10^7 spore·mL^{-1} calculated by a hemocytometer . The submerged batch culture salt medium was: KH_2PO_4 (1 g·L^{-1}) (Mallinckrodt Bakker); $MgCl_2 \cdot 6H_2O$ (0.5 g·L^{-1}) (Mallinckrodt Bakker); $ZnSO_4 \cdot 7H_2O$(0.0014 g·L^{-1}) (Sigma) ; $MnSO_4 \cdot H_2O$ (0.0016 g·L^{-1}) (Sigma); $CoCl_2 \cdot 6H_2O$(0.0036 g·L^{-1}) (Sigma); $FeSO_4 \cdot 7H_2O$ (0.00275 g·L^{-1}) (Sigma); 2.74 g·L^{-1} yeast extract (DOT Scientific Inc.) was used as the sole nitrogen source. The carbon sources used were glucose, xylose, artificial corn stover hydrolysate (composed of a mixture of glucose and xylose), and enzymatic hydrolysate of corn stover after sulfuric acid and sodium hydroxide pretreatments. The pH of the medium was adjusted to 6.0±0.1 before autoclaving. Cultures were carried out at various initial sugar concentrations. For batch culture, 250mL Erlenmeyer flasks were filled with 50 mL of growth medium and sterilized at 121℃ for 15min. The growth medium was inoculated with a 10 % (v·v^{-1}) seed culture and cultivated at 25±1℃ on a rotary shaker (Thermal Scientific) with an agitation speed of 180rpm.

2.3.2 Enzymatic hydrolysates of corn stover after acid and alkaline pretreatments for filamentous fungal lipid production

Corn stover was obtained from the Michigan State University Crop and Soil Science Teaching and Research Field Facility, and samples were air-dried and ground on site using a mill (Willey Mill, Standard Model No. 3, Arthur H. Thomas, Philadelphia, PA) with 2 mm size opening. Two different pretreatment methods were applied to the ground corn stover (Yue et al., 2010): dilute acid pretreatment (1% w·w^{-1} H_2SO_4, 121°C, 2h, 10% corn stover dry solid (ds)), and dilute alkali pretreatment (1% w·w^{-1} NaOH, 121°C, 2h, 10% corn stover dry solid (ds)). After pretreatment, and without any detoxification, both samples were filtered with 4 layers of cheese cloth, and the hydrolysate liquids were

neutralized using either $CaCO_3$ or H_2SO_4 to a pH of 5±0.1. The solid fiber and liquid samples were stored separately at 4°C for later use.

For either sodium hydroxide treated corn stover (10.08 g dry matter) or sulfuric acid treatment (10.0g dry matter), the corresponding liquid was centrifuged and added back to its respective residual fiber. The enzyme Accellerase 1000® was loaded at 26FPU·g^{-1} dry fiber, and 0.05 M citrate buffer (pH 4.8) was added to create a 200 g solution (5% of dry matter in the solution) respectively. The solution was placed in a rotary shaker at 150 rpm under 50°C for 72h. After enzymatic hydrolysis, the hydrolysate was separated from the remaining fiber via centrifugation (3700 rpm × 20min) for further use in filamentous fungal lipid cultivation.

2.3.3 Analytical methods

Mycelia were collected by filtration and washed twice with distilled water. Cell mass was determined by drying under 105±1°C overnight to get a constant weight. Glucose, xylose, cellobiose, arabinose, galactose, mannose, acetic acid, formic acid, furfural and Hydroxymethylfurfural (HMF) were detected by an HPLC (Agilent 1100) equipped with a Bio-rad Aminex HPX-87H or 87P analytical column and a refractive index detector. The mobile phase was 0.005 M sulfuric acid (flow rate of 0.6 mL·min^{-1}), and Millipore water (flow rate of 0.6mL·min^{-1}) in the HPX-87H and HPX-87P columns respectively. Column temperatures were set to 65 and 60°C respectively. High purity glucose, xylose, cellobiose, arabinose, galactose, mannose, acetic acid, formic acid, furfural and HMF from sigma were used as the standards. Dried mycelia were ground in a mortar and used for lipid extraction following the method of Bligh and Dyer (17). Total lipid was determined gravimetrically. The extracted lipid was subjected to methanolysis to form fatty acid methyl esters (78), which were analyzed by a gas chromatographer (Agilent) equipped

with an Agilent J&W DB-23 capillary column (30m × 0.25mm). Helium was used as the carrier gas. The column temperature was programmed to 50 ℃ for 2min, then raised to 180 ℃ at an increasing rate of 10℃·min^{-1}, held at 180 ℃ for 5min, and then raised to 240 ℃ at an increasing rate of 5℃·min^{-1} and kept there for 1min. The injector (with injection volume of 1μL) and FID detector temperatures were maintained at 240 ℃ and 280 ℃, respectively. Methyl esters of myristic, palmitic, palmitoleic, stearic, oleic, linoleic, γ-linolenic acids, were used as standards for fatty acid identification and quantification. Protein and carbohydrate content of the filamentous fungal mycelia were analyzed according to the method of Bidochka with a slight modification (15). For protein determination, 2.0 mL of 0.5 mol·L^{-1} NaOH was added to the 0.1 g mycelial samples in 20mL glass vials, capped with aluminum slide caps, and placed in a boiling water bath for 15 min. The tubes were cooled to 25 ℃, and 0.50 mL of 1.0 mol·L^{-1} HCl and 0.50 mL of 0.1 mol·L^{-1} Tris-HCl buffer (pH 7.5) were sequentially added. The protein concentration was estimated according to the BCA kit analysis method. For carbohydrate analysis, samples of mycelia were placed in test tubes and cooled on ice. A 5mL sample of anthrone reagent (5 mL of absolute ethanol, 200 mg of anthrone, 95 mL of 75% w·w^{-1} H_2SO_4) was added to the 0.1 g dry mycelial samples in 20mL glass vials, boiled for 10 min, and then returned to an ice bath. After cooling the samples, the solution was measured at an absorption spectrum of 625 nm; glucose was used as the standard.

2.4 Results and discussion

2.4.1 Effect of glucose and xylose concentration on cell mass production and lipid accumulation

M.isabellina ATCC42613 was cultured in nitrogen stressed medium at various initial glucose and xylose concentrations in order to examine changes in lipid accumulation in relation to cell mass production. Under fixed nitrogen conditions (2.74 $g \cdot L^{-1}$ yeast extract), incremental increases in glucose and xylose concentrations resulted in gradual increases in cell mass production up to appreciable levels of 22.9 $g \cdot L^{-1}$ and 21.6 $g \cdot L^{-1}$ respectively without optimization of fermentation conditions. Lower initial sugar concentrations showed higher cell mass and lipid productivities (Table 3). The highest lipid (10.2 $g \cdot L^{-1}$) and cell mass concentration (22.9 $g \cdot L^{-1}$) for glucose both occurred at an initial $C \cdot N^{-1}$ ratio of 309.2. However, the highest lipid content (44.8%) was seen at a $C \cdot N^{-1}$ ratio of 275.7 (Table 1). On xylose medium, the mold presented its highest lipid content (42.8%) under an initial $C \cdot N^{-1}$ ratio of 110.3, while the highest cell mass (21.6 $g \cdot L^{-1}$) and lipid (8.8 $g \cdot L^{-1}$) produced were observed under an initial $C \cdot N^{-1}$ ratio of 306.9. Interestingly, it was observed that the percentage of lipid accumulated in cell mass kept quite constant ($Y_{L/X}$ 0.41-0.44 $g \cdot g^{-1}$ for glucose and $Y_{L/X}$ 0.39-0.44 $g \cdot g^{-1}$ for xylose respectively) during the entire range of initial glucose and xylose concentrations investigated. Similar results were obtained for *M. isabellina* ATHUM 2935 for lipid accumulation on high concentration of glucose media under nitrogen limited conditions, where lipid contents varied from 50% to 55% upon changing the $C \cdot N^{-1}$ molar ratios from 150:1 to 340:1 (114).

Table 3 Cell mass and lipid accumulation of *M.isabellina* in medium with various initial glucose or xylose concentrations

Initial concentration ($g \cdot L^{-1}$)	Initial C/N* ($mol \cdot mol^{-1}$)	Cell mass (X, $g \cdot L^{-1}$)	Lipid (L, $g \cdot L^{-1}$)	$Yield_{L/X}$ ($g \cdot g^{-1}$)	Cell mass Productivity ($g \cdot L^{-1} \cdot h^{-1}$)	Lipid Productivity ($g \cdot L^{-1} \cdot h^{-1}$)
Glucose						
28.1	70.3	11.4±0.1	5.0±0.3	0.44±0.022	0.20	0.088
35.4	88.1	13.3±0.5	5.5±0.0	0.41±0.001	0.14	0.059
46.0	137.7	14.7±0.6	5.9±0.6	0.40±0.033	0.088	0.035
73.7	241.6	20.7±0.6	8.9±0.5	0.43±0.036	0.051	0.022
82.5	275.7	22.3±0.6	10.0±1.8	0.45±0.04	0.055	0.025
91.7	309.2	22.9±0.6	10.2±0.2	0.44±0.013	0.051	0.023
Xylose						
26.6	66.8	9.8±0.2	3.8±1.0	0.39±0.1	0.14	0.053
39.1	110.3	11.4±0.3	5.0±0.1	0.43±0.002	0.12	0.048
45.7	136.9	14.2±1.0	5.7±0.6	0.40±0.023	0.084	0.032
66.3	212.5	17.5±2.5	6.5±0.6	0.37±0.052	0.061	0.021
79.7	266.4	18.9±0.6	7.9±0.5	0.42±0.034	0.046	0.016
90.9	306.9	21.6±1.1	8.8±0.4	0.41±0.024	0.048	0.018

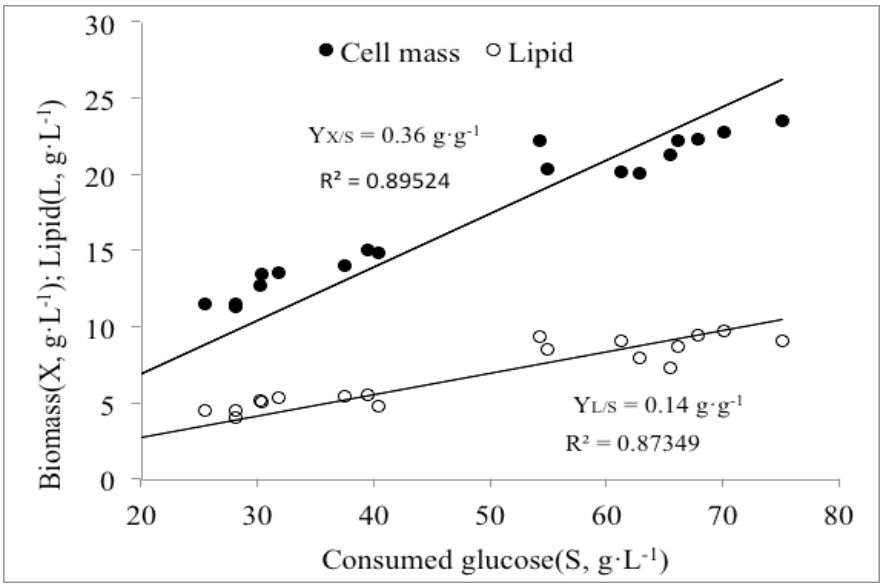
Data points are the means of three replicate determinations with standard deviations.
*:For initial $C \cdot N^{-1}$ ratio in the medium, it was assumed that yeast extract contained 12% $w \cdot w^{-1}$ of carbon source and 7% $w \cdot w^{-1}$ of nitrogen source.

The final pH of the broth ranged from 5.2 to 5.8, showing a slight decrease from an initial pH of 6 in all growth conditions due to the production of organic acids (e.g. oxalic acid) in low concentrations.

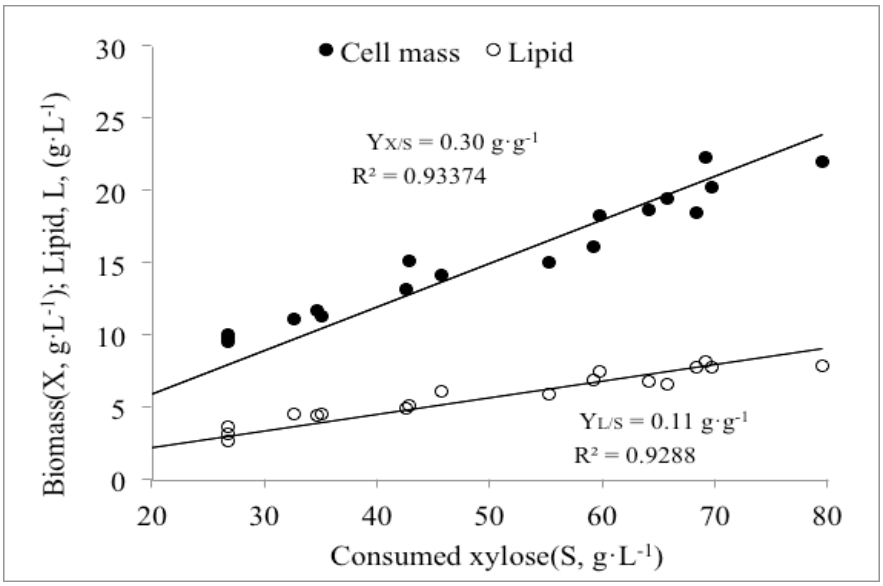
2.4.2 Effect of consumed C/N ratio on mycelia growth and lipid production

The sugar was rapidly consumed under low initial sugar concentrations (e.g. 28.1g·L^{-1} glucose or 26.6 g·L^{-1} xylose) with a consumption rate of 0.41g·$L^{-1}h^{-1}$ and 0.39g·$L^{-1}h^{-1}$ respectively. However, the consumption rates for high initial glucose and xylose concentrations decreased, especially during the later growth stages, after long fermentation times (e.g. 408h), with 21.5 g·L^{-1} glucose and 16.5 g·L^{-1} xylose remaining unutilized at 82.5 g·L^{-1} and 79.7 g·L^{-1} initial glucose and xylose media respectively. Similar phenomena was also observed by other researchers (20, 44, 52).

The filamentous fungus presented constant and impressive cell mass and lipid yields of $Y_{X/S}$ and $Y_{L/S}$ at 0.36g·g^{-1} and 0.14 g·g^{-1} on glucose medium regardless of the initial glucose concentrations (Figure 1a). Similar trends were observed on xylose medium; total cell mass and lipid yields were 0.30 g·g^{-1} and 0.11 g·g^{-1} respectively (Figure 1b). However, other oleaginous Zygomycetes cultivated on glucose medium, presented significantly lower lipid yields (0.03-0.14 g·g^{-1}) compared with the strain under study (52, 83). When cultured on xylose medium with a C·N^{-1} ratio of 285, the overall $Y_{L/S}$ of the mold *M.isabellina* ATHUM 2935 was 11.3%; similar to our results (44). For glucose metabolism, the maximum theoretical lipid to glucose yield is 31%. This study achieved a 14% conversion yield of lipid from glucose.



(a)



(b)

Figure 1 Cell mass and stored lipid produced by *M.isabellina* as function of consumed glucose or xylose in various initial sugar concentrations

Documented conversion yields above 20% are rare, indicating there may be some limiting factor associated with this threshold for lipid accumulation from glucose (44). Different yields in glucose (14%) and xylose (11%) cultures were observed in this study, indicating the possibility that xylose was metabolized through the pentose phosphate pathway, which

is less efficient than glucose metabolism for lipid production in this filamentous fungus. Similar results were also observed by other researchers (44).

2.4.3 Intracellular lipid fatty acid profiles

To evaluate the potential of using the filamentous fungal lipid as biodiesel substrate, its fatty acid profile was determined (Table 4). FAME analysis indicated that the fatty acid compositions of the lipid from glucose and xylose grown cells were quite similar. In all cultures, it was observed that oleic acid (C18:1) was the dominant intracellular fatty acid. Palmitic acid (C16:0) was found to be the second most abundant fatty acid, whereas linolenic acid (C18:2) was present to a lesser degree. Traces of palmitoleic (C16:1), stearic (C18:0) and γ- linolenic acids (C18:3) were also found in the reserved lipid as well. The entire fatty acid profile present in the filamentous fungal lipid has potential utility for biodiesel production as has been documented for vegetable oils and fungi (54, 114, 149).

Table 4 Effect of initial sugar concentrations on fatty acid composition of *M.isabellina* stored lipid

Initial sugar ($g \cdot L^{-1}$)	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 6,9,12}$C18:3	Others*
Glucose							
28.1	24.6±0.33	2.49±0.01	3.79±0.08	54.5±0.28	10.6±0.23	3.08±0.02	0.97±0.02
35.4	22.5±0.44	2.62±0.05	3.24±0.02	55.5±0.61	11.9±0.13	3.09±0.05	1.13±0.02
46	23.0±0.09	1.99±0.06	4.48±0.05	55.6±0.60	11.1±0.17	2.78±0.05	1.07±0.02
73.7	27.3±0.36	2.53±0.04	3.36±0.01	54.0±0.67	7.91±0.08	2.25±0.02	2.61±0.02
82.5	20.6±0.14	2.51±0.05	2.53±0.02	59.5±0.77	12.9±0.10	0.36±0.01	1.65±0.02
91.7	20.0±0.07	2.32±0.04	1.74±0.03	58.4±0.70	12.5±0.12	3.21±0.041	1.23±0.02
Xylose							
26.6	23.7±0.29	2.61±0.01	4.32±0.07	53.9±0.42	11.4±0.15	2.81±0.07	1.29±0.05
39.1	24.6±0.33	2.85±0.07	3.59±0.06	50.9±0.57	13.0±0.25	3.41±0.02	1.65±0.03
45.7	29.6±0.16	2.83±0.04	3.60±0.05	54.5±0.75	7.10±0.08	1.65±0.01	0.72±0.02
66.3	21.5±0.28	3.29±0.04	2.29±0.04	54.6±0.56	13.7±0.07	3.43±0.06	1.18±0.02
79.7	24.2±0.23	3.51±0.07	2.23±0.01	54.1±0.65	11.9±0.05	3.47±0.03	0.56±0.08
90.9	25.6±0.15	3.59±0.08	2.44±0.26	52.7±0.67	10.8±0.08	2.87±0.05	2.07±0.04

Data points are the means of three replicate determinations with standard deviations.
*others are C14:0, C20:0, C20:1.

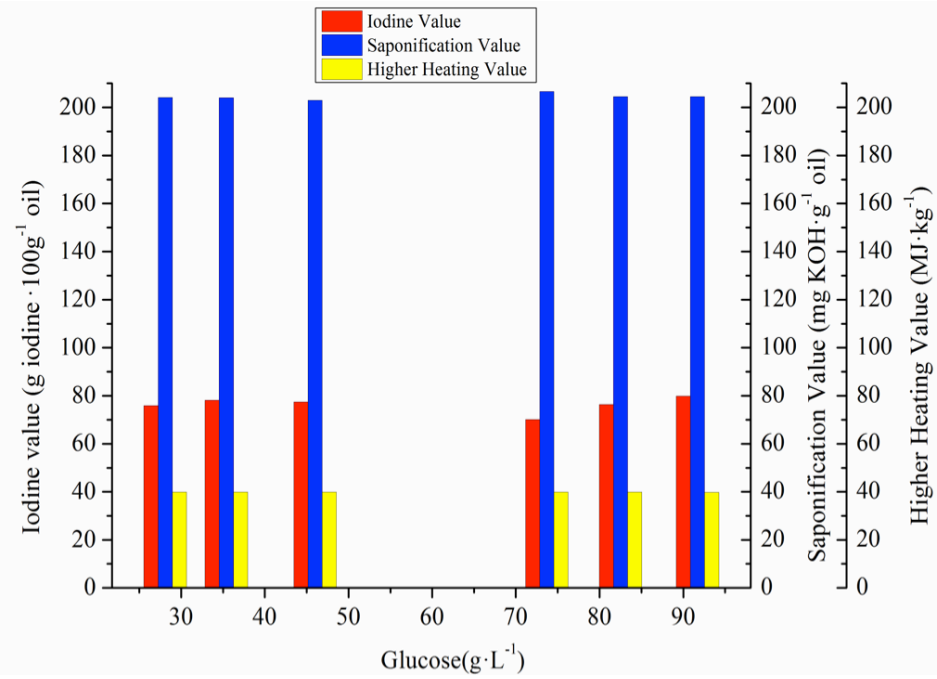
Table 4 (cont'd)

Slight changes to the fatty acid composition in the stored lipid were observed regardless of the cultural conditions such as fermentation time or the initial glucose or xylose concentrations (Table 4), consistent with other reports (114). Oleic acid (C18:1) concentrations in glucose and xylose mediums ranged from 54.6% to 60.2% and 50.9% to 54.6% respectively, differing from other Zygomycetes cultivated on glucose medium, where the fatty acid composition of intracellular lipid varied significantly depending on the fermentation time (83).

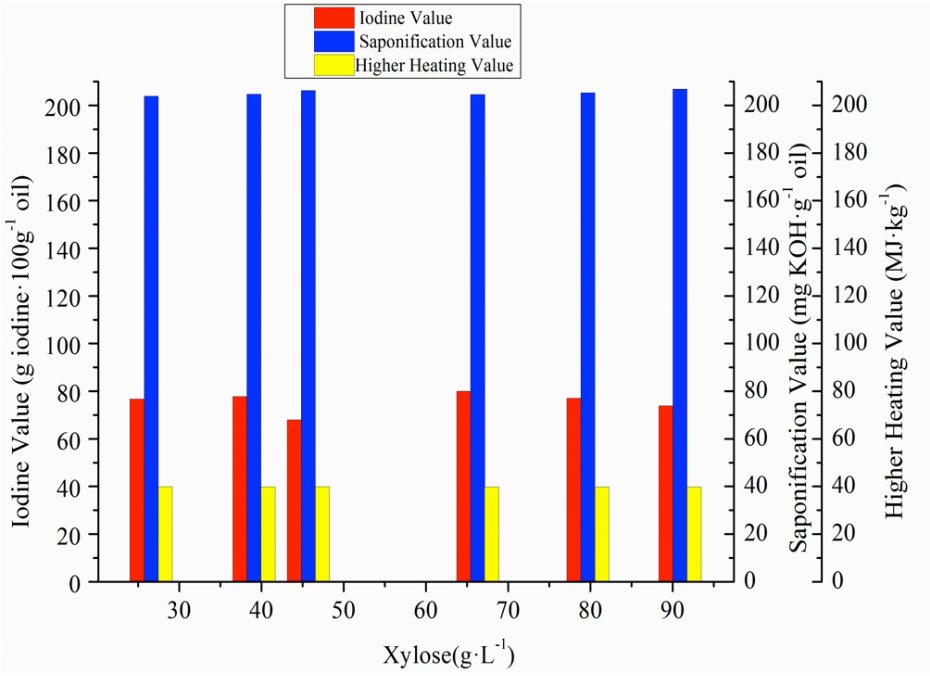
2.4.4 Prediction of biodiesel properties of the filamentous fungal lipids

The iodine value, saponification value and higher heating values of the fatty acid methyl esters are important characteristics in determining the quality of the produced biodiesel. The iodine value is the amount of iodine (in grams) necessary to saturate 100 g of oil sample. Saponification values are milli-grams of KOH necessary to saponify 1 g of oil sample (35). Higher heating value is the amount of heat produced by the complete combustion of one gram of fuel including the latent heat of vaporization of water in the combustion product (118). These biodiesel values can be predicted from a given lipid source by using its fatty acid composition (C16:0, C18:0, C18:1, C18:2 and C18:3) as inputs into the predictive model (54).

Some variations in the filamentous fungal lipid iodine value were observed, ranging from 71.33 to 80.66 g iodine/100g oil and 68.24 g to 80.12 g iodine/100g oil for glucose and xylose cultures respectively (Figure 2).



(a)

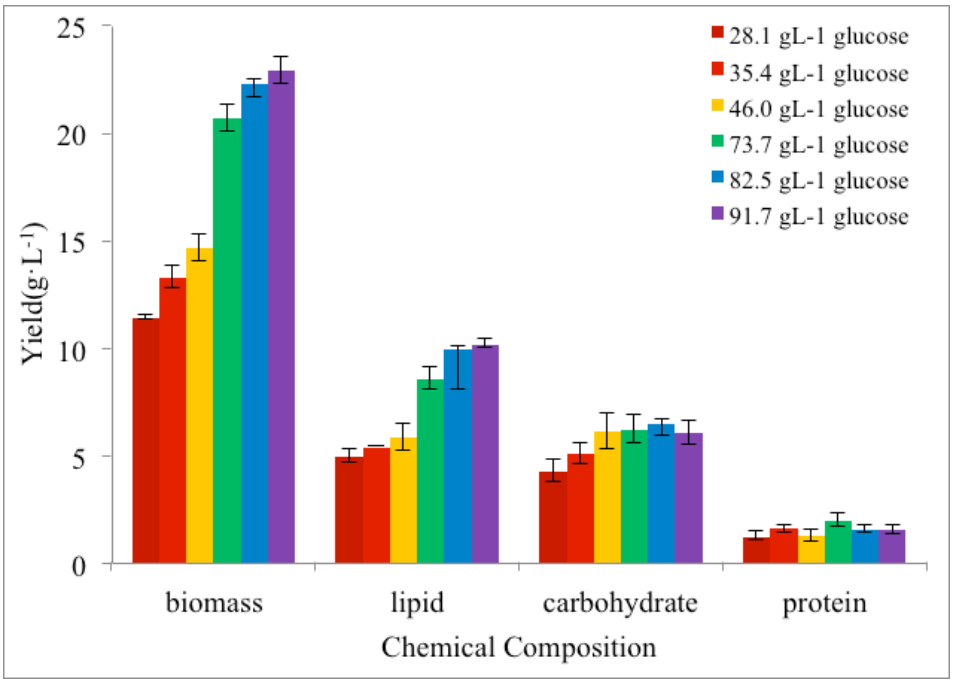


(b)

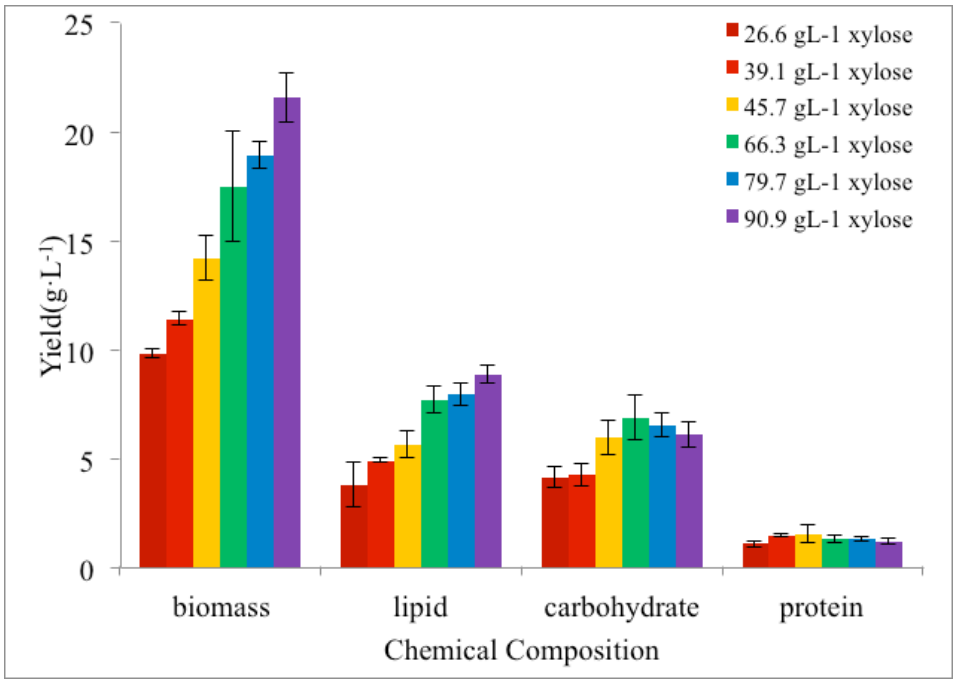
Figure 2 Chemical properities of *M.isabellina* lipid from glucose (a) and xylose (b) for biodiesel production

2.4.5 Effect of glucose and xylose concentration on cell mass composition

Although extensive work has been performed on the accumulation of fungal lipids for fuel purposes, there seems to be limited information regarding the potential for byproduct extraction alongside biodiesel production. Solid byproducts would result from oil processing for biodiesel production. If not contaminated with toxic solvents such as hexane, they could be valuable sources of animal feed as they are rich in protein content (86). Thus it is necessary to investigate the cell mass composition for a better understanding and utilization of these fungal biodiesel byproducts. Figures 3(a) and 3(b) show the mycelia lipid, protein, and carbohydrate content of *M.isabellina* grown on nitrogen stressed conditions with various initial concentrations of glucose or xylose as carbon source.



(a)



(b)

Figure 3 Effect of various initial glucose (a) or xylose (b) concentration on *M.isabellina* dry cell mass composition.

Along with consistency in its lipid production (41-44 % w·w^{-1} of the cell mass as intracellular lipid), the filamentous fungus also produced relatively constant carbohydrate

Figure 3 (cont'd)

(4.28-7.75g·L^{-1}) and protein (1.4-2.2 g·L^{-1}) amounts when cultured on glucose medium under experimental conditions. Similar results were seen with various initial xylose concentrations, as 4.15-6.96 g·L^{-1}carbohydrate and 1.17-1.78 g·L^{-1} protein were accumulated in the filamentous fungal cell mass respectively.

2.4.6 Evaluation of lignocellulosic hydrolysates on lipid productio

Lignocellulosic material (corn stover) was evaluated for its potential use in lipid production by cultivating *M. isabellina* on its hydrolysate pretreated under both dilute acid and alkaline conditions. As a comparison, artificial media containing no inhibitors was also prepared with glucose and xylose concentrations mimicking those found in both the acid and alkaline pretreated enzymatic hydrolysate. 2.74 g·L^{-1} of yeast extract was added to all media for a C·N^{-1} ratio of approximately 73±4 (mol·mol^{-1}) (Table 5).

Table 5 Cell mass and lipid accumulation of *M.isabellina* using diluted acid and alkaline treated corn stover enzymatic hydrolysate and synthetic medium

Carbon Source	C·N^{-1}* (mol·mol^{-1})	Glucose (g·L^{-1})	Xylose (g·L^{-1})	Cell mass (X, g·L^{-1})	%Yield $_{L/X}$ (g·g^{-1})	Lipid (L, g·L^{-1})	Lipid Productivity (g·L^{-1}·h^{-1})
Synthetic Acid Hydrolysate	77.4	16.85±0.13	14.14±0.42	12.59±0.15	38.36±0.23	4.82±0.01	0.067
Acid Hydrolysate	77.4	17.0±0.1	14.0±0.1	14.08±0.18	34.47±2.13	4.78±0.3	0.050
Synthetic Alkaline Hydrolysate	75.7	24.0±0.2	6.3±0.5	12.24±0.05	30.16±1.51	3.28±0.18	0.050
Alkaline Hydrolysate	70.7	21.9±0.3	6.4±0.1	10.90±1.06	29.47±4.26	2.48±1.13	0.027

Data points are the means of three replicate determinations with standard deviations.

Table 5 (cont'd)

The acid hydrolysate had a lower glucose concentration (17.0g·L^{-1}), and higher xylose concentration (14.0 g·L^{-1}) than alkaline hydrolysate (24.0 g·L^{-1} glucose, and 6.4 g·L^{-1} xylose respectively). The artificial and authentic corn stover hydrolysate sugar concentrations and fermentation results regarding cell mass, lipid, and lipid productivity of *M. isabellina* cultivation on these four substrates are shown in table 5. Degradation products from chemical pre-treatment of biomass usually include the following classes of inhibitors: carboxylic acids, furans, phenols and inorganic salts, with phenols showing the greatest negative effect on fermentation. Low molecular weight (MW) organic salts are able to penetrate cell membranes, whereas fermentation inhibitors with high MW influence the expression and activity of sugar and ion transporters in the cell membrane. The toxic effects of inhibitors such as acetic acid, furfural, and HMF produced during pretreatment of lignocellulosic biomass on microbial cell growth and metabolism of ethanologenic bacteria and yeasts is well studied (88). Several reports also detail the negative influence inhibitors have on lipid accumulation by oleaginous yeasts (23, 64, 74, 75). However, information regarding the effect these inhibitors have on oleaginous filamentous fungal lipid accumulation is quite limited. The following potential inhibitors were present in the acid treated enzymatic hydrolysate: furfural (0.073±0.024 g·L^{-1}), hydroxymethylfurfural (0.032±0.0052g·L^{-1}), and acetic acid (2.71±0.04 g·L^{-1}). Even in the presence of these inhibitors and without any detoxification, the maximum filamentous fungal cell mass concentration was obtained with the acid pretreated enzymatic hydrolysate (14.08 g·L^{-1}), higher even than its artificial equivalent (12.59 g·L^{-1}). This may be due to the utilization of acetic acid (23) and small amounts of minor sugars (cellobiose, galactose and arabinose) present in the hydrolysate medium (24, 64). The filamentous fungus consumed acetic acid and glucose simultaneously, with acetic acid depletion

Table 5 (cont'd)

occurring at 40 h of the total 90 h of cultivation time. This indicates the possibility that acetic acid not only participates in cell mass growth, but may also contribute to the accumulation of microbial lipid for this particular mold (23, 47). Despite a slightly decreased glucose consumption rate (data not shown), lipid production in the acid hydrolysate was equivalent to that found in the artificial hydrolysate (4.78 $g \bullet L^{-1}$ versus 4.82 $g \bullet L^{-1}$ respectively), which was also the maximum concentration found in all media. The slight decrease in lipid yield, cell mass productivity and lipid productivity may be due to the deleterious effects from the other inhibitors produced during acid pretreatment.

Furfural and HMF were not detected in the enzymatic hydrolysate of sodium hydroxide pretreated corn stover. Acetic acid and formic acid were present with concentrations of 3.47±0.05 $g \cdot L^{-1}$ and 1.36±0.05 $g \cdot L^{-1}$ respectively. Acetic acid and formic acid were also consumed simultaneously with the consumption of glucose, with depletion occurring within 60 h and 43 h respectively of the total 114 h cultivation time. The cell growth and glucose consumption rates in the alkaline hydrolysate were much lower compared to its artificial media (data not shown). Lower cell mass, lipid yield, lipid content and lipid productivity were also observed in the alkaline hydrolysate (data in table 3), which may be due to the inhibitory effects of phenols and carboxylic acids present in the media (88). Further investigation on the detailed impact of inhibitors such as acetic acid, formic acid, phenolic compounds on lipid accumulation of this filamentous fungus needs to be conducted.

The greater growth and lipid production in the acid treated hydrolysate compared to the alkaline treated hydrolysate may be attributed to the greater concentration of inhibitors and the presence of sodium salts found in the later treatment. The composition of the carbon sources in the media may have also mitigated some of the adverse effects of the

inhibitors resulting in greater growth and lipid production in the acid hydrolysate media. The effects of these inhibitors on growth and lipid production may vary not only with their concentrations in the media, but the composition of the media itself since glucose and xylose utilize different metabolic pathways which the inhibitors would alter to varying degrees (174). These results show that carbon sourced from lignocellulosic biomass can be used to cultivate M. isabellina and, without detoxification, produce equivalent fermentation products as cell mass and lipid compared to growth on synthetic media (glucose and xylose mixture). This leads to the prospects of using an already cheap and abundant carbon source for filamentous fungal lipid production, which is made even more attractive since costs associated with detoxification of inhibition products could possibly be minimized.

A number of researchers reported growing oleaginous molds on glucose, xylose and lignocellulosic biomass for lipid accumulation (Table 6). The current study indicates that *M.isabellina* ATCC42613 is one such microorganism of interest as it exhibited noticeable production of stored lipid on higher glucose or xylose medium.

Table 6 Lipid production of oleaginous molds in different fermentation conditions

Strain	Carbon source	Lipid ($g.L^{-1}$)	Lipid content	Lipid productivity ($g \cdot L^{-1} \cdot h^{-1}$)	Reference
Mortierella isabellina ATHUM2935	Glucose	18.1	50.	0.072	(114)
	Commercial	9.9	74	0.042	(21)
	Commercial	7.4	61.	0.018	(21)
	Glucose	12	44.	0.033	(44)
	Xylose	6.1	64.	0.017	(44)
	Raw glycerol	3.3	53.	0.0092	(44)
	Rice hull	2.3	64.	0.0096	(40)
Cunninghamella echinulata ATHUM 4411	Glucose+TWH	7.8	25	0.046	(22)
	Xylose	6.7	53.	0.0186	(44)
	Raw glycerol	2.0	53.	0.0056	(44)
	Commercial	3.9	30	0.0126	(21)
	Commercial	3.6	21	0.0089	(21)
Cunninghamella echinulata	Glucose	8.03	26.	0.024	(22)
Colletotrichum	Xylose	2.2	29.	0.0092	(37)
*Alternaria sp.*DM09	Xylose	4.3	41.	0.018	(37)
Epicoccum	molasses	26.8	80	0.079	(90)
A.oryzae A-4	Wheat straw + bran	62.9(mg·gds)	N.	$0.44(mg \cdot g^{-1} \cdot h^{-1})$	(96)
Microsphaeropsis sp.	Wheat straw + bran	80(mg·gds)	10.	$0.33(mg \cdot g^{-1} \cdot h^{-1})$	(116)
Colletotrichum	Rice straw +wheat	68.2(mg·gds)	N.	$0.28(mg \cdot g^{-1} \cdot h^{-1})$	(37)
*Alternaria sp.*DM09	Rice straw +wheat	60.3(mg·gds)	N.	$0.25(mg \cdot g^{-1} \cdot h^{-1})$	(37)
Mortierella isabellina	Sweet sorghum	0.11(g·gds)	25	$0.055(g \cdot g^{-1} \cdot h^{-1})$	(41)
Mortierella isabellina ATCC42613	Glucose	10.2	44.	0.023	This
	Xylose	8.8	40.	0.018	This
	Acid CSH[a]	25.2(mg/gds)	34.	0.05	This
	Alkaline CSH[b]	16.1(mg/gds)	29.	0.027	This

a: 1% ($w \cdot w^{-1}$) sulfuric acid treated enzymatic corn stover hydrolysate.
b: 1% ($w \cdot w^{-1}$) sodium hydroxide treated enzymatic corn stover hydrolysate.
N.A.: Not available.

2.5 Conclusions

The filamentous fungus *M. isabellina* ATCC 42613, when cultivated on various carbon sources under stressed nitrogen conditions, is not only able to generate significant amounts of mycelia and lipid, but can also stably convert glucose and/or xylose to cellular lipid at a constant conversion yield while maintaining a stable intracellular fatty acid profile with appreciable chemical properties for biodiesel application. Lipid and cell mass conversion yields of enzymatic hydrolysates of sulfuric acid pretreated corn stover without washing and detoxification show comparable results to artificial hydrolysate without any inhibitors, indicating the promising future of utilizing lignocellulosic biomass for lipid production from filamentous fungal cultivation.

CHAPTER 3. OLEAGINOUS FUNGAL LIPID FERMENTATION ON COMBINED ACID-AND ALKALI-PRETREATED CORN STOVER HYDROLYSATE FOR ADVANCED BIOFUEL PRODUCTION

Zhenhua Ruan, Michael Zanotti, Steven Archer, Wei Liao, Yan Liu

3.1 Abstract

A combined hydrolysis process, which first mixed dilute acid- and alkali-pretreated corn stover at a 1:1 (w/w) ratio, directly followed by enzymatic saccharification without pH adjustment, has been developed in this study in order to minimize the need of neutralization, detoxification, and washing during the process of lignocellulosic biofuel production. The oleaginous fungus *M. isabellina* was selected and applied to the combined hydrolysate as well as a synthetic medium to compare fungal lipid accumulation and biodiesel production in both shake flask and 7.5L fermentor. Fungal cultivation on combined hydrolysate exhibited comparable cell mass and lipid yields with those from synthetic medium, indicating that the integration of combined hydrolysis with oleaginous fungal lipid fermentation has great potential to improve performance of advanced lignocellulosic biofuel production.

Keywords: biodiesel, combined hydrolysis, lignocellulosic biomass, oleaginous fungus, lipid accumulation

3.2 Introduction

Lignocellulosic biomass is one of the most abundant and renewable sources in nature. Many studies have explored the possibility of using it as a feedstock for advanced biofuels, particularly bio-ethanol production (101). However, investigations of utilizing lignocellulose for microbial lipid production are still relatively limited. The ability to convert fermentable sugars from lignocellulosic material to lipid in a cost-effective manner is a key technological challenge to fully unlocking the commercial potential for such a process (100). Three major steps identified in the production of microbial oil from lignocellulosic biomass include: hydrolyzing the lignocellulose into fermentable sugars; metabolizing those sugars by oleaginous microorganisms into microbial lipids; and finally generating biodiesel from the microbial lipids (178). Unfortunately, fermentation of lignocellulosic hydrolysate is often preceded by a washing and detoxification steps that require a large amount of water and chemicals (155). In order to develop a technically and economically feasible process for lignocellulosic biofuel production, feedstock pretreatment and enzymatic hydrolysis need to be optimized according to physiological characteristics of the target microorganism.

Lignocellulose is a naturally recalcitrant material consisting of a heterogeneous matrix of three macromolecules: cellulose, hemicellulose and lignin. Physico-chemical pretreatment disrupts the structure of lignocellulosic biomass, removes substrate-specific barriers to enzymatic hydrolysis, and thus improves its digestibility. While pretreatment is a crucial step in the biological conversion of lignocellulose to biofuels, it has likewise been identified as the second most expensive unit in the production of lignocellulosic ethanol preceded by feedstock cost (106). Several thermochemical pretreatment methods have been employed to overcome the recalcitrance nature of lignocellulose, including

dilute acid, ammonia fiber expansion, hot water, dilute alkali and organo-solvent methods (6). Among these pretreatment methods, dilute sulfuric acid pretreatment has been widely studied due to its efficacy and relatively low cost. It disrupts covalent bonds, hydrogen bonds and Van der Waals forces that link cellulose to lignin and hemicellulose, which consequently enhance cellulose conversion (92). Sodium hydroxide treatment is another widely used pretreatment method, and has been successfully implemented on a variety of lignocellulosic feedstocks (163). The sodium hydroxide pretreatment cleaves the ester bond linking lignin and xylan as well as the glycosidic bond of the polysaccharides, which reduces the degree of polymerization and crystallinity, causes fiber swelling, extracts lignin, and ultimately improves the enzymes access to the polysaccharide chains (140).

The biomass pH after either sulfuric acid pretreatment (generally lower than 2) or sodium hydroxide pretreatment (usually over 10) needs to be adjusted prior to enzymatic hydrolysis. This requires significant chemical inputs for the pH adjustment, as well as substantial water for solid washing following the solid-liquid separation, in which the toxin-containing liquid is discarded, and buffer is later added for the enzymatic hydrolysis. In order to cope with this issue, a new combined hydrolysis process was developed in the current study, which mixed the dilute acid and dilute alkali pretreated corn stover slurries at a 1:1 (w/w) ratio (pH value of 4.2-5.2), removed solid washing and buffer adding, and directly applied enzymes on the neutralized slurry to generate combined hydrolysate.

Combined hydrolysis has its own challenges, such as requiring microorganisms that not only withstand a variety of toxins from pretreatment, but also hold the ability to efficiently ferment both hexose (glucose) and pentose (xylose) in the hydrolysate. There are only a few studies that address both issues. The extreme thermophilic bacterium *Thermotoga elfii* metabolized glucose and xylose in miscanthus hydrolysate for hydrogen production (33), and the yeast *Scheffersomyces stipites* CBS6054 was capable to convert

glucose and xylose in giant reed hemicellulose hydrolysate to ethanol (132). Gong et. al., reported *Cryptococcus curvatus* was able to simultaneously consume glucose and xylose from ionic liquid pretreated corn stover (53). The yeast strain *Trichosporon cutaneum* AS 2.571 assimilated glucose and xylose simultaneously during corn stover hydrolysate lipid fermentation (76). Our previous studies found that the oleaginous mold *Mortierella isabellina* ATCC 42613 growing on undetoxified corn stover hydrolysate could accumulate lipid comparable to those from synthetic medium (125, 126).

Thus, the objectives of the current study are to investigate the performance of combined hydrolysis, and evaluate fungal lipid accumulation on the combined hydrolysate. The integrated process formulates and demonstrates a technically and economically feasible strategy to generate advanced lignocellulosic biofuel.

3.3 Materials and Methods

3.3.1 Materials

Corn stover was obtained from Michigan State University Crop and Soil Science Teaching and Research Field Facility. It was air-dried and ground using a mill (Willey Mill, Standard Model No. 3, Arthur H. Thomas, Philadelphia, PA) with a 2 mm size screen; then the ground biomass was sieved to make a particle size distribution less than 30 mesh (< 1.6 mm) but greater than 80 mesh (> 1 mm). The ground corn stover sample was analyzed for cellulose, xylan, and lignin content according to the National Renewable Energy Laboratory's (NREL) analytical procedure for determination of structural carbohydrates and lignin (135). Results showed a composition of 36.3% cellulose, 22.0% xylan, and 18.6% lignin on a dry weight basis. The moisture content was approximately 7% (total weight basis). The milled corn stover was kept at 4 °C for long-term storage.

3.3.2 Combined hydrolysis

Each sample was treated in a screw cap 125 mL serum bottle and placed in an autoclave (Brinkmann 2540M, Tuttnauer, Hauppauge, NY). Dilute acid and alkali pretreatments were carried out at sulfuric acid and sodium hydroxide concentrations of 2% ($w \cdot w^{-1}$), respectively. A retention time of 1 h and reaction temperature of 130 ˚C were applied on both pretreatments, which were the optimal conditions for individual pretreatment concluded from previous studies (126, 141). Biomass concentration for pretreatment was set at 10% dry matter. After pretreatment, the slurries of dilute acid and alkali treated corn stover were first mixed thoroughly at a 1:1 (w/w) ratio and then directly subjected to enzymatic hydrolysis without pH adjustment.

The combined slurry was hydrolyzed using commercial enzyme mixtures. The mixtures consisted of cellulase (Accellerase 1500®, protein content 85.15 mg mL^{-1}, lot number 3016295230; Genencor, Palo Alto, CA) and xylanase (Accellrase XY, protein content 43.63 mg mL^{-1}, lot number 4900667792; Genencor, Palo Alto, CA). Three different enzyme loadings per gram of the initial dry fiber were applied on the combined corn stover slurry: enzyme loading (a) of 21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY, enzyme loading (b) of 10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY, and enzyme loading (c) of 5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY. The enzymatic reaction was carried out for 70 h at 50 °C and 150 rpm agitation. The hydrolysate was centrifuged at 7025 × g for 5 min to separate the liquid solution from the residual solids. The clear liquid solution was called combined hydrolysate, and stored in a 4 °C refrigerator for further use.

The sugar yield was used to evaluate the performance of combined hydrolysis. It was determined by the ratio of the measured amount of sugars (glucose and xylose) in

combined hydrolysate to the dry weight of initial biomass and also calculated as the percentage of theoretical glucose and xylose yields from initial cellulose and xylan in corn stover.

3.3.3 Microorganism and culture conditions

Seed and fermentation media for *M. isabellina* culture were previously reported (125, 126). Yeast extract (DOT Scientific Inc., Burton, MI) was used as the sole nitrogen source. The carbon sources were the combined corn stover hydrolysate and the control of synthetic medium (composed of a mixture of glucose, xylose and acetate, with concentrations similar to those found in the combined hydrolystate). The nitrogen source in the culture media was 3.6 $g \cdot L^{-1}$ yeast extract. The mineral salts in the culture media were 1 $g \cdot L^{-1}$ KH_2PO_4, 0.5 $g \cdot L^{-1}$ $MgCl_2 \cdot 6H_2O$, 0.0014 $g \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.0016 $g \cdot L^{-1}$ $MnSO_4 \cdot H_2O$, 0.0036 $g \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$, and 0.00275 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$. The pH of the fermentation media was adjusted to 6.0±0.1 before autoclaving. 250 mL Erlenmeyer flasks filled with 50 mL of media were sterilized at 121°C for 15 minutes. The medium was inoculated with a 10% ($v \cdot v^{-1}$) seed and cultivated at 25±1 °C on a rotary shaker (Thermal Scientific, Odessa, Texas) with an agitation speed of 180 rpm. The fungal lipid culture using combined hydrolysate medium was also scaled up and carried out at a 7.5L fermentor (New Brunswick Bioflo 115® bioreactor, Eppendorf, Inc. CT). Compressed air was used and the dissolved oxygen was maintained between 1.2-2.4 mg/L with 1 vvm (volume air per volume medium per minute) aeration rate and 200 rpm agitation. Fermentation pH was kept at around 6.0 and temperature was controlled at 25 °C during the culture. Substrate concentrations (glucose, xylose and acetate) were monitored to determine the completion of fermentation for both flask and fermentor cultures.

3.3.4 Biodiesel production

The lipids in fungal biomass were extracted using a mixed solvent of hexane and isopropanol at a hexane:isopropanol ratio of 3:2 (v/v) (59). The extracted lipids were directly converted into fatty acid methyl esters (FAMEs) using a transesterification approach (78). The resulted FAMEs were analyzed by GC-MS and biodiesel yield was calculated based on the FAMEs.

3.3.5 Analytical methods

Glucose, xylose, acetic acid, furfural and hydroxymethylfurfural (HMF) in the combined hydrolysate as well as fermentation broth were determined by High Performance Liquid Chromatography (HPLC) (Shimadzu prominence), which equipped with a Bio-rad Aminex HPX-87H analytical column and a refractive index detector. The mobile phase was 0.005 $mol \cdot L^{-1}$ sulfuric acid at a flow rate of 0.6 $mL \cdot min^{-1}$. The oven temperature was set at 65°C. HPLC grade standards including glucose (Catalog Number: 49158), xylose (Catalog Number: 95729), sodium acetate (Catalog Number: S8750), HMF (Catalog Number: 53407), furfural (Catalog Number: 185914) were purchased from Sigma-Aldrich, St. Louis, MO.

Mycelia were collected by filtration and washed twice with distilled water. Cell mass was determined by drying at 75 ± 1 °C to obtain a consistent weight. Dried mycelia were ground in a mortar and used for lipid extraction. Total lipid was determined gravimetrically (125).

FAME analysis was performed using an Agilent 6890N gas chromatograph equipped with an Agilent DB-23 column (30 m, 250 μm, 0.25 μm) and a CTC PAL auto-sampler. The GC was connected to an Agilent 5975 single quadrupole mass spectrometer equipped

with a 70 eV electron ionization source. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The inlet was operated in the splitless mode at a temperature of 250°C. An injection volume of 1 μL was used. The column temperature was programmed as follows: holding at 50°C for 2 minutes, increasing to 180 °C at 10 °C/minute and holding at 180°C for 5 minutes, and further increasing to 240 °C at 5 ^{o}C/minute and holding at 240°C for 1 minute. A mixture of C8-C24 FAMEs (Catalog No: 18918-1 AMP, Sigma -Aldrich, St. Louis, MO) was used as the external standards. The mixture included methyl arachidate, methyl behenate, methyl decanoate, methyl cis-13-docosenoate, methyl dodecanoate, methyl linoleate, methyl linolenate, methyl myristate, methyl octanoate, methyl oleate, methyl palmitate, methyl palmitoleate and methyl stearate, methyl tetracosanoate, Nonadecanoic acid methyl ester (C19:0) (Catalog No: 646-30-0, Sigma-Aldrich, St. Louis, MO) was used as the internal standard (10 μg/mL).

3.3.6 Statistical analysis

A general linear model using R software (R Version 2.15.0, Vienna, Austria) was applied to perform an analysis of variance (ANOVA) and multiple comparisons on the experimental data.

3.4 Results and discussion

3.4.1 Combined hydrolysis of corn stover for fermentable sugars production

The combined hydrolysis of corn stover was performed by applying enzymatic hydrolysis on mixed dilute acid and alkali-pretreated corn stover slurry. Three different enzyme loadings as indicated in Section 2.2 were applied. Fig. 4 showed the time course of glucose and xylose concentrations as a function of various enzyme loadings for combined hydrolysis. Glucose concentration indicated the typical batch hydrolysis pattern,

with a rapid glucose release at the beginning of the process, followed by a plateau with litter further increase. Enzyme loading (a) on the combined slurry released 28 g L^{-1} glucose at 17 h, which was 92% of that released at 70 h of enzymatic hydrolysis; while in the same reaction duration, 24 g·L^{-1} and 14 g·L^{-1} glucose were produced with enzyme loadings (b) and (c), respectively, which corresponded to 80% and 62% of those generated at 70 h. The pattern of xylan hydrolysis was different from cellulose hydrolysis. Xylose concentrations were gradually increased with the progression of reaction time (Fig. 4). Enzyme loading (a) released 84 % (16 g·L^{-1}) of the final xylose generated at 70h enzymatic hydrolysis; and enzyme loadings (b) and (c) delivered 80% and 77% of that produced at 70 h with corresponding concentration of 13 and 10.5 g·L^{-1}.

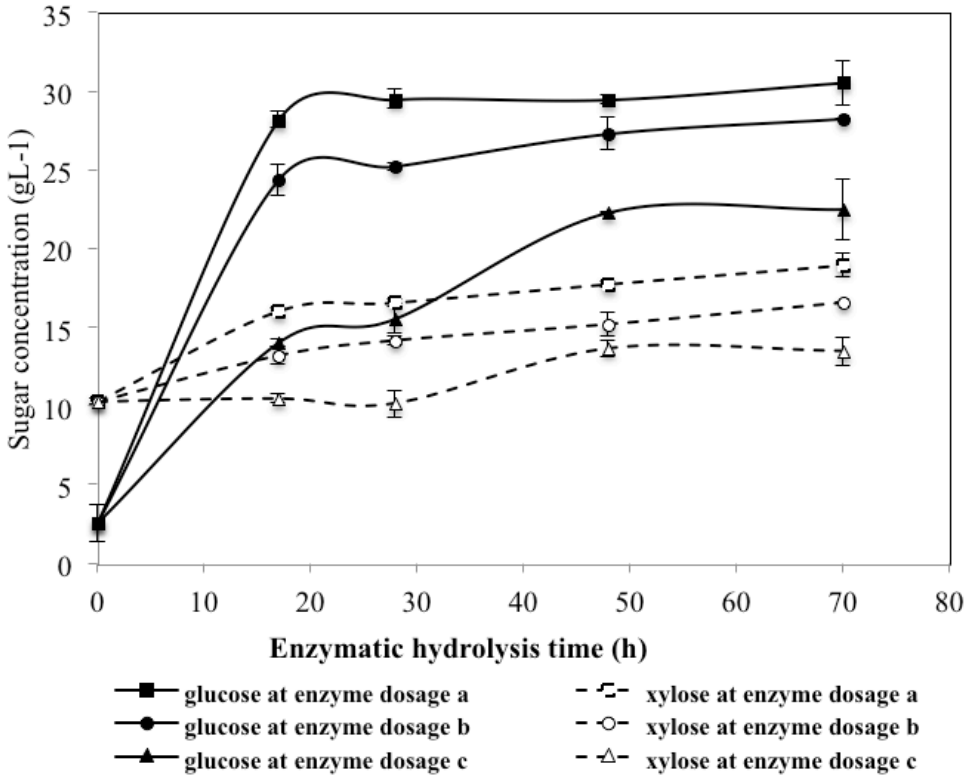


Figure 4 Changes in monomeric glucose (Glc) and xylose (xyl) concentration during enzymatic hydrolysis of combined slurry

Table 7 and Fig. 5 presented the combined sugar (glucose+xylose) yields and inhibitor concentrations from combined hydrolysis. The sugar yield at enzyme loading (a) reached 0.45 $g \cdot g^{-1}$ dry initial biomass, which was 74% of the theoretical yield. While enzyme loadings (b) and (c) had the sugar yields of 0.41 $g \cdot g^{-1}$ (67% of the theoretical yield) and 0.32 $g \cdot g^{-1}$ (54% of the theoretical yield) dry initial biomass, respectively. Statistical analysis elucidated that there was no significant ($p>0.05$) difference on combined sugar yield between enzyme loadings (a) and (b), while the combined sugar yield from enzyme loading (c) was significantly ($p<0.05$) lower than the other two enzyme loadings.

Therefore, considering both enzyme loading and hydrolysis performance, enzyme loading (b) (50% of the enzyme loading (a)) was chosen to prepare the combined hydrolysate for the following fermentation experiments.

Table 7 Fermentable sugars yield and inhibitory compound concentrations at the end of combined hydrolysis (70h)

Enzyme loadings	Sugars		Inhibitory compounds		
	Glu+Xyl ($g \cdot g^{-1}$ dry biomass)	Glu+Xyl (% of theoretical yield)	Acetate ($g \cdot L^{-1}$)	HMF ($mg \cdot L^{-1}$)	Furfural ($g \cdot L^{-1}$)
21.2 mg Accellerase 1500 + 1.08 mg Accellerase XY	0.446	74.2	4.48	11.9	0.147
10.6 mg Accellerase 1500 + 0.54 mg Accellerase XY	0.404	67.2	4.62	11.8	0.142
5.4 mg Accellerase 1500 + 0.27 mg Accellerase XY	0.324	54.0	4.38	12.5	0.160

(1) NREL's analysis of structural carbohydrate and lignin: cellulose: 36.3 %(w/w); xylan: 22.0 %(w/w); lignin: 18.6 %(w/w)
(2) Dilute acid and alkali pretreatment was carried out at 10% (w/w) dry solids.
(3) Data represent the average of two replicates.

In Fig.5, the glucose and xylose combined theoretical yield was calculated as the percent of theoretical combined glucose and xylose yield from corn stover; while glucose and xylose combined theoretical yield of biomass was determined by the ratio of the measured amount of sugars (glucose and xylose) in combined hydrolysates to the dry weight of initial corn stover. The enzyme loading per gram dry mass for each treatment was at three levels, loading (a: 21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY), loading (b: 10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY), loading (c: 5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY).

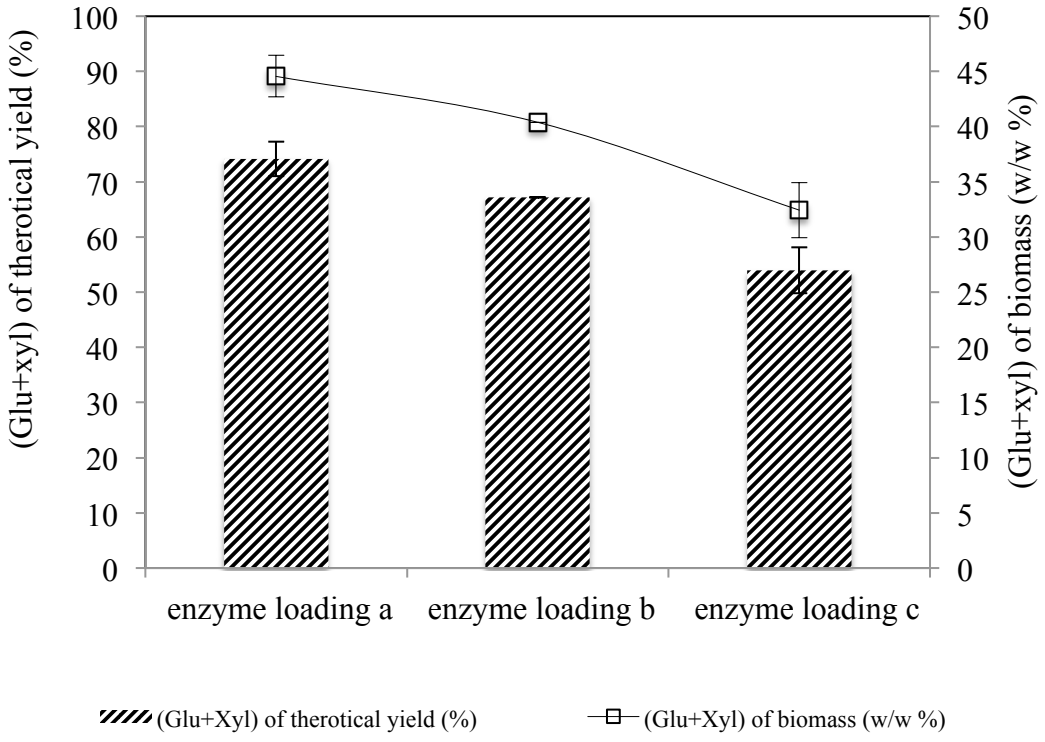


Figure 5 Effect of enzyme loadings on combined glucose and xylose yield

3.4.2 Microbial lipid fermentation from combined hydrolysate

3.4.2.1 Fungal lipid production of shake flask culture

Microbial lipid fermentations using *M.isabellina* ATCC 42613 on the combined hydrolysate and synthetic medium were carried out to elucidate the effects of combined hydrolysis on fungal lipid accumulation. The carbon sources in the combined hydrolysate media for the shake flask culture were 22.2 g L^{-1} glucose, 12 g L^{-1} xylose, and 3.7 g L^{-1} acetate. While the synthetic medium included 17.9 g L^{-1} glucose, 12.5 g L^{-1} xylose, and 3.9 g L^{-1} acetate. Changes of cell mass, lipid content, glucose, xylose and acetate during the fermentation were presented in Fig. 6.

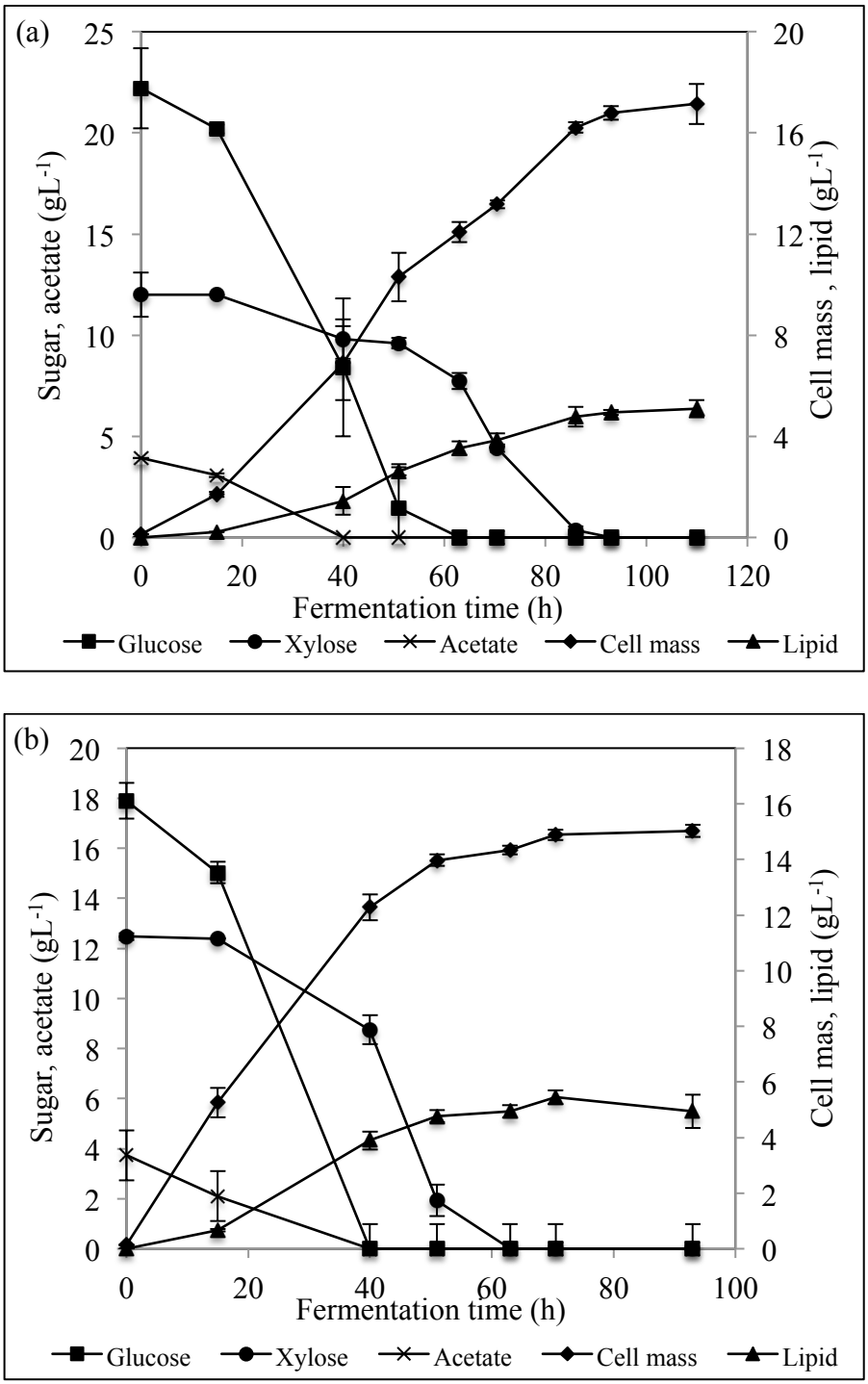


Figure 6 *M.isabellina* fermentation of lipid fermentation on combined hydrolysate (a) and synthetic medium (b)

Figure 6 (cont'd)

The trends of sugar and acetate consumption, cell mass and lipid accumulation were similar for fermentation on both combined hydrolysate and synthetic medium. However, fungal growth on the combined hydrolysate was delayed by approximately 20 h compared to the fermentation on synthetic medium. The concentrations of cell mass and lipid from the fermentation on synthetic medium were quickly increased to 5.26 and 0.67 $g \cdot L^{-1}$, respectively in the first 15 h of the culture, and lipid content in the cell mass was about 13% (w/w). While, only slight increase in cell mass and small consumption of glucose were observed for the culture on the combined hydrolysate, which the cell mass and lipid concentration were 1.7 and 0.22 $g \cdot L^{-1}$ at 15 h. With the extension of the culture, the fermentation on synthetic medium continuously accumulated cell mass and lipid. The cell mass, lipid concentration and lipid content increased to 12.28 $g \cdot L^{-1}$, 3.89 $g \cdot L^{-1}$, and 35.16% ($w \cdot w^{-1}$) at 40 h. The fermentation was then slowed down after glucose, xylose and acetate were completely utilized. The maximum cell mass and lipid concentration of 14.9 and 5.4 $g \cdot L^{-1}$ were achieved at 70 h with a lipid content of 36.6 % ($w \cdot w^{-1}$). Meanwhile, the fermentation on combined hydrolysate started taking off after 15 h of the culture. It took 86 h to consume all of the glucose, xylose, and acetate. The maximum cell mass and lipid concentrations of 16.8 and 5.1 $g \cdot L^{-1}$ were reached at 93 h with a lipid content of 30 % ($w \cdot w^{-1}$). Considering lipid yield with respect to sugars and acetate in the media, the fermentation on the combined hydrolysate had 0.14 $g \cdot g^{-1}$, which is slightly lower than 0.16 $g \cdot g^{-1}$ of the fermentation from the synthetic medium (Table 3). The prolonged lag phase and lower lipid yield may be caused by various inhibitory compounds in the combined hydrolysate such as furfural, HMF, acetate (Table 4) (125, 126). Similar inhibitory effects of such compounds were observed by other researchers (23, 64, 74).

Despite a slightly lower yield and longer lag phase, *M. isabellina* lipid accumulation

on the combined corn stover hydrolysate demonstrated superior lipid productivity compared to those on other lignocellulosic hydrolysates (Table 8). In addition, using both acid and base to effectively pretreat the feedstock, instead of just using either of them to neutralize the pretreated slurry, also significantly enhances the efficiency of chemical use during the refining process (50% reduction of chemical loading per unit feedstock), and alleviates the pressure on the desalination of the fermentation effluent treatment. Thus, combined hydrolysis has potential to be integrated with oleaginous fungal lipid accumulation for advanced lipid-based fuel production.

Table 8 Lipid production of *M.isabellina* from various feedstocks

Strain	Feedstocks	Lipid Yield ($g \cdot L^{-1}$)	Lipid content (%)	Lipid productivity ($g \cdot L^{-1} \cdot h^{-1}$)	References
M. isabellina ATHUM2935	Starch	3.7	35.6	0.021	(113)
M. isabellina ATHUM2935	Raw glycerol	4.4	51.0	0.0105	(112)
M. isabellina ATHUM2935	Cheese whey	4.0	17.3	0.0234	(146)
M. isabellina ATHUM2935	Raw glycerol	3.3	53.2	0.0092	(44)
M. isabellina ATHUM2935	Rice hull	3.6	64.3	0.0096	(40)
M. isabellina ATHUM2935	Sweet sorghum	9.2	43	0.038	(39)
M.isabellina NRRL 1757	Wheat straw	4.36	36.3	0.03	(172)
M. isabellina ATHUM2935	Sweet sorghum	110(mg/gds)[f]	25	0.055	(41)
M. isabellina IFO 7884	Soybean hull	47.9(mg/gds)	N.A.	N.A.	(175)
M. isabellina ATCC42613	Corn stover[a]	25.2(mg/gds)	34.4	0.05	(125)
	Corn stover[b]	16.1(mg/gds)	29.5	0.027	(125)
	Corn stover[c]	62.8(mg/gds)	24.8	0.036	(126)
	Switchgrass	72.2(mg/gds)	35.6	0.0375	(126)
	Miscanthus	49.9(mg/gds)	32.2	0.032	(126)
	Giant reed	42.1(mg/gds)	21.2	0.022	(126)
	Corn stover[d]	60(mg/gds)	30.0	0.055	Current study

N.A.: Not available
a: 1% (w w^{-1}) Sulfuric acid treated corn stover enzymatic hydrolysate.
b: 1% (w w^{-1}) Sodium hydroxide treated corn stover enzymatic hydrolysate.
c: 2% (w w^{-1}) Sulfuric acid treated corn stover co-hydrolysate
d: 2% (w w^{-1}) Sulfuric acid and 2% (w w^{-1}) sodium hydroxide treated corn stover combined hydrolysate
f: gds means gram dry substrate

3.4.2.2 Fungal lipid production using 7.5 L bioreactor

Fungal lipid accumulation was also carried out in a 7.5 L bioreactor using the combined hydrolysate medium including carbon sources of 28.6 g L^{-1} glucose, 16.1 g L^{-1} xylose, and 3.4 g L^{-1} acetate. The fungus experienced a lag phase around 2d, and then had a similar growth pattern with the flask culture on the combined hydrolysate. Most of the

substrates were consumed after 184 h culture. The culture generated 18.7 g L^{-1} cell mass and 6.9 g L^{-1} cellular lipid (Table 9). While the lipid productivity and lipid yield with respect to the total amount of carbon sources (glucose, xylose, and acetate) were 0.90 $g \cdot L^{-1} \cdot d^{-1}$ and 0.15 $g \cdot g^{-1}$, respectively (Table 9). The results showed that cell mass and lipid production from the 7.5 L fermentation were similar with those from the flask culture, which, to certain extent, indicated that lipid production using *M. isabellina* could be effectively scaled up.

Table 9 Microbial lipid accumulation of *M.isabellina* on combined corn stover hydrolysate and synthetic hydrolysate

Culture methods	Carbon Sources	$C \cdot N^{-1}$ (mol· mol^{-1})	Glucose ($g \cdot L^{-1}$)	Xylose ($g \cdot L^{-1}$)	Acetate ($g \cdot L^{-1}$)	$Lipid_{max}$ (L, $g \cdot L^{-1}$)	% Yield $max_{L/X}$ ($g \cdot g^{-1}$)	% Yield $max_{L/S}$ ($g \cdot g^{-1}$)	Lipid productivity ($g \cdot L^{-1} \cdot d^{-1}$)
Flask	Synthetic hydrolysate	58.2	17.9	12.5	3.7	5.4	36.6	16.0	1.8
	Combined hydrolysate	65.3	22.2	12.0	3.9	5.1	29.7	13.7	1.3
Fermentor	Combined hydrolysate	91.0	28.6	16.1	3.4	6.9	37.0	14.7	0.90

Data points are the means of two replicate determinations

3.4.3 Lipid and biodiesel production using the novel combined hydrolysis

According to our previous study of oleaginous fungal lipid accumulation on pure glucose, xylose and corn stover co-hydrolysate (125, 126), 170 kg and 140 kg of lipid could be produced per ton of glucose and xylose respectively, and 62 kg of lipid per ton dry corn stover could be obtained from *M. isabellina* fermentation. It has been reported that theoretical biodiesel yield per ton of glucose and xylose would be 88 and 40 gallons, respectively (179). The theoretical biodiesel yield using *M. isabellina* for microbial lipid

production is 57 gallons per ton of corn stover. Based on the lipid yield of *M. isabellina* in this study using 7.5 L bioreactor culture, 60 kg lipid per ton dry corn stover could be produced by *M. isabellina* fermentation on the combined hydrolysate (Table 9), 3.6 gallon biodiesel could be produced from this 60 kg fungal lipid by direct methanolysis using the Indarti transesterification method (78). Even though lipid and biodiesel yields from the current study are lower than the theoretical yields, *M. isabellina* fermentation on combined hydrolysate has demonstrated better performance than other oleaginous microbes such as *Trihisporon cutaneum* and *Rhodotorula graminis* (49, 97). *Trihisporon cutaneum* was cultured on detoxified corn stover hydrolysate, while *Rhodotorula graminis* fermented undetoxified corn stover hydrolysate. Both fermentations produced approximately 46 kg lipid. It is believed that both lipid and biodiesel yields from *M. isabellina* could be further improved with optimization of combined hydrolysis and fermentation process as well as microbial lipid transesterification. The study is being fulfilled in authors' research group.

3.5 Conclusion

This study demonstrated that combined hydrolysis process was a technically feasible and promising strategy that maximized lignocellulosic biomass conversion. It minimized the need for detoxification, solid washing, and buffer addition for enzymatic hydrolysis, resulting in significantly reduced water and chemical usages. *M. isabellina* ATCC 42613 exhibited unique characteristics of utilizing glucose, xylose and acetic acid in the combined hydrolysate to accumulate fungal lipids, as well as tolerating a variety of inhibitors from feedstock pretreatment. The results indicated that the integration of *M. isabellina* lipid fermentation with novel combined hydrolysis would have great potential to advance lignocellulosic biofuel production.

CHAPTER 4 MORTIERELLA ISABELLINA GROWTH TOLERANCE AND CARBON UTILIZATION UNDER THE EFFECT OF INHIBITORY COMPOUNDS IN LIGNOCELLULOSIC HYDROLYSATES

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4.1 Abstract

Oleaginous fungus *Mortierella isabellina* showed excellent lipid conversion on non-detoxified lignocellulosic. This study investigated the effects of inhibitory compounds (furfural, hydroxymethylfurfrual, ferulic and coumaric acids) in lignocellulosic hydrolysate on *M.isabellina* cell growth and lipid production. For individual inhibitor, *M.isabellina* could tolerate furfural (~1g/L), hydroxymethylfurfrual (~2.5 g/L), ferulic (~0.5 g/L) and coumaric acid (~0.5 g/L) with normal growth rates. Synergistic effect of these inhibitors (2 g/L furfural, 0.4 g/L hydroxymethylfurfrual, 0.02g/L ferulic and 0.02g/L coumaric acid) moderately reduces total fungal growth (by 28%) and lipid titer (by 37%), while the presence of these inhibitors has minor impact on cell lipid contents and lipid profiles. ^{13}C-carbon tracing has revealed that *M. isabellina* can simultaneously utilize glucose and acetate in the presence of inhibitors. Acetate is mainly assimilated for synthesis of lipid and TCA cycle amino acids. Xylose utilization is strongly suppressed by the presence of glucose, and only contributes to fungal culture in the late growth phase. In addition, yeast extract not only plays main role as the carbon and nitrogen sources for biomass growth, it also contributes to lipid synthesis (~9% during the growth phase).

Key words: TCA cycle, Xylose, oleaginous fungus, lipid accumulation, ^{13}C isotope

4.2 Introduction

Microbial lipid production from lignocellulosic materials generally undergoes three main steps: pretreatment, enzymatic hydrolysis for mono-sugars release, and microbial lipid fermentation. During pretreatment, various inhibitors are generated, mainly including weak acids (acetic acid, formic acid, and levulinic acid), furan derivatives (furfural and hydroxymethylfurfural (HMF)), and phenolic compounds (such as vanillin and syringaldehyde). Most of these inhibitors are toxic to microbes, hindering their cell growth and lipid accumulation. Many studies have been conducted to delineate the effect of lignocellulosic inhibitors on oleaginous yeast lipid fermentation (23, 64, 70, 71, 73, 170, 174, 176). It was reported that aldehydes were more toxic to yeasts than other inhibitors (70, 72, 73). Among different aldehyde compounds, HMF is less toxic than aromatic aldehydes and furfural. In addition, these studies also concluded that the inhibitory effects impacted cell growth more than lipid accumulation.

Economical lipid production requires a robust microbial host that shows both high lipid productivity and strong tolerance to common inhibitors from lignocellulosic hydrolysates. Our previous studies indicated that oleaginous fungus *M. isabellina* had a comparable growth and lipid accumulation performance on lignocellulosic hydrolysates with the culture on synthetic sugar medium (124-126). It was also elucidated that, unlike other yeasts, *M. isabellina* has the capability to effectively utilize acetate up to 7.2 g/L as a sole carbon source for lipid production (98). In order to better understand *M. isabellina* growth and lipid accumulation on lignocellulosic hydrolysates, this study was to delineate the effect of representative lignocellulosic inhibitors (furfural, HMF, coumaric acid, ferulic acid) on *M.isabellina* carbon utilization.

4.3 Materials and Methods

4.3.1 Microorganism and culture conditions

Mortierella isabellina ATCC 42613 was obtained from the American Type Culture Collection (Manassas, VA). The spore and seed culture was previously reported (125). Synthetic lignocellulosic hydrolysate as the fermentation medium contained: glucose 20 g/L, xylose 15 g/L, acetate 4 g/L, KH_2PO_4 1 g/L, $MgCl_2 \cdot 6H_2O$ 0.5 g/L, $ZnSO_4 \cdot 7H_2O$ 0.0014 g/L, $MnSO_4 \cdot H_2O$ 0.0016 g/L, $CoCl_2 \cdot 6H_2O$ 0.0036 g/L, and $FeSO_4 \cdot 7H_2O$ 0.00275 g/L, and yeast extract 2.4 g/L. The pH of the medium was adjusted to pH 6.0. The fermentation medium was sterilized at 121°C for 15 min. 10% v/v seed culture (5 mL) was inoculated in a 250 mL conical flask containing 45 mL of the fermentation medium, and the cultivation was carried out on a rotary shaker at 25°C with an agitation speed of 180 rpm.

4.3.2 Effects of inhibitors on cell growth and lipid fermentation

Furfural, HMF, coumaric acid, and ferulic acid were selected as the representative lignocellulosic inhibitors. Their concentration ranges were: furfural (0-3.84 g/L), HMF (0-5.12 g/L), coumaric acid (0-0.5 g/L), and ferulic acid (0-0.5 g/L). All inhibitor stock solutions were sterilized using 0.2 μm-sterilized membranes. The effect of mixed inhibitors was carried out using three different inhibitor compositions (Inhibitor combination-1, 2, 3). Inhibitor combination-1 included 0.5 g/L of furfural, 0.1 g/L of HMF, 0.025 g/L of coumaric acid and 0.025 g/L of ferulic acid; Inhibitor combination-2 contained 1g/L of furfural, 0.2g/L of HMF, 0.05g/L of coumaric acid, 0.05g/L of ferulic acid; and Inhibitor combination-3 was with 2g/L of furfural, 0.4g/L of HMF, 0.02g/L of coumaric acid and 0.02g/L of ferulic acid. Kinetics of *M. isabellina* was also studied on the selected inhibition combination. Sugar and acetic acid consumption, and cell mass and

lipid accumulation were monitored during the course of fermentation. Fermentation on synthetic medium without inhibitors was used as the experimental control. A general linear model using R software (R Version 2.15.0, Vienna, Austria) was applied to the experimental data in order to perform an analysis of variance (ANOVA) and multiple comparisons.

4.3.3 Analytical methods

Mycelia were collected by filtration and washed twice with distilled water. Cell mass was determined by drying under 65 °C to obtain a consistent weight. Glucose, xylose, acetate, furfural and HMF were determined by High Performance Liquid Chromatography (Shimadzu prominence), which equipped with a Bio-rad Aminex HPX-87H analytical column and a refractive index detector. The mobile phase was 0.005 $mol \cdot L^{-1}$ sulfuric acid with a flow rate of 0.6 $mL \cdot min^{-1}$and oven temperature at 65°C. HPLC grade glucose, xylose were purchased from Sigma-Aldrich, St. Louis, MO. Dried mycelia were ground in a mortar and used for lipid extraction. Total lipid was extracted (17) and its fatty acid profile determination was performed using an Agilent 6890N gas chromatograph equipped with an Agilent DB-23 column (30 m 250 um x 0.25 um) and a CTC PAL autosampler. The GC was connected to an Agilent 5975 single quadruple mass spectrometer equipped with a 70 eV electron ionization source (77). Protein content in the supernatant was determined using BCA kit from Sigma.

4.3.4 ^{13}C tracing for biomass and lipid synthesis on a synthetic hydrolysate

^{13}C-experiments were performed to provide fundamental information on *Mortierella* co-utilization of different carbon sources from lignocellulosic hydrolysate. The carbon sources were 10 g/L of glucose, 10g/L of xylose, 2g/L of acetate, and 1g/L of yeast extract (as nitrogen source). In addition, the medium contained 1g/L of furfural and 1g/L of HMF.

Three tracer experiments were performed using the same culture medium using ^{13}C-glucose, ^{13}C-xylose, or ^{13}C-acetate (Cambridge Isotope, MA). In each experiment, only one substrate of the major carbon sources was labeled in order to trace each carbon substrate's contribution to biomass and lipid synthesis. To reduce interference from residual carbon from the seed culture, we used a small inoculation ratio (<0.5%). The cultures were incubated for six days (until mid-growth phase) before harvesting for GC-MS analysis of isotopic labeling (n=2). Isotopomer analysis of proteinogenic amino acids analysis followed a previous published protocol (167). The cell mass samples were hydrolyzed in 6 M hydrochloric acid (HCl) at 100 °C for 24h. The resulting mixtures were then air-dried and derivatized with N-(tert-butyldimethylsily)-N-methyltrifluoroacetamide (TBDMS) prior to GC-MS analysis. For TBDMS-derivatized metabolites, the fragment $[M-57]^+$ from the entire amino acid was used for isotopomer analysis. The MS data are represented as m+0, m+1, and m+2…, which are the corresponding abundances of the unlabeled, singly-labeled, doubly-labeled fragments. For lipid isotopomer analysis, lipid extraction and transesterification was performed using a chloroform/methanol/H_2SO_4 solution (89). The resulting fatty acid methyl ester (fatty acid C14:0) was chosen for carbon distribution analysis, and the ratio of ^{13}C labeled carbons to total carbons of fatty acids was used to represent the relative contribution of the ^{13}C-substrate for fatty acid synthesis (159).

4.4 Results and Discussion

4.4.1 Effects of individual inhibitor on *M.isabellina* lipid and cell mass accumulation

When *M. isabellina* was cultured on the synthetic medium without inhibitors, which produced cell mass (9.6g/L), lipid yield (3.2g/L), and lipid content (33.7%, w/w) after two-day incubation. Addition of inhibitors could change fermentation performance (Fig. 7).

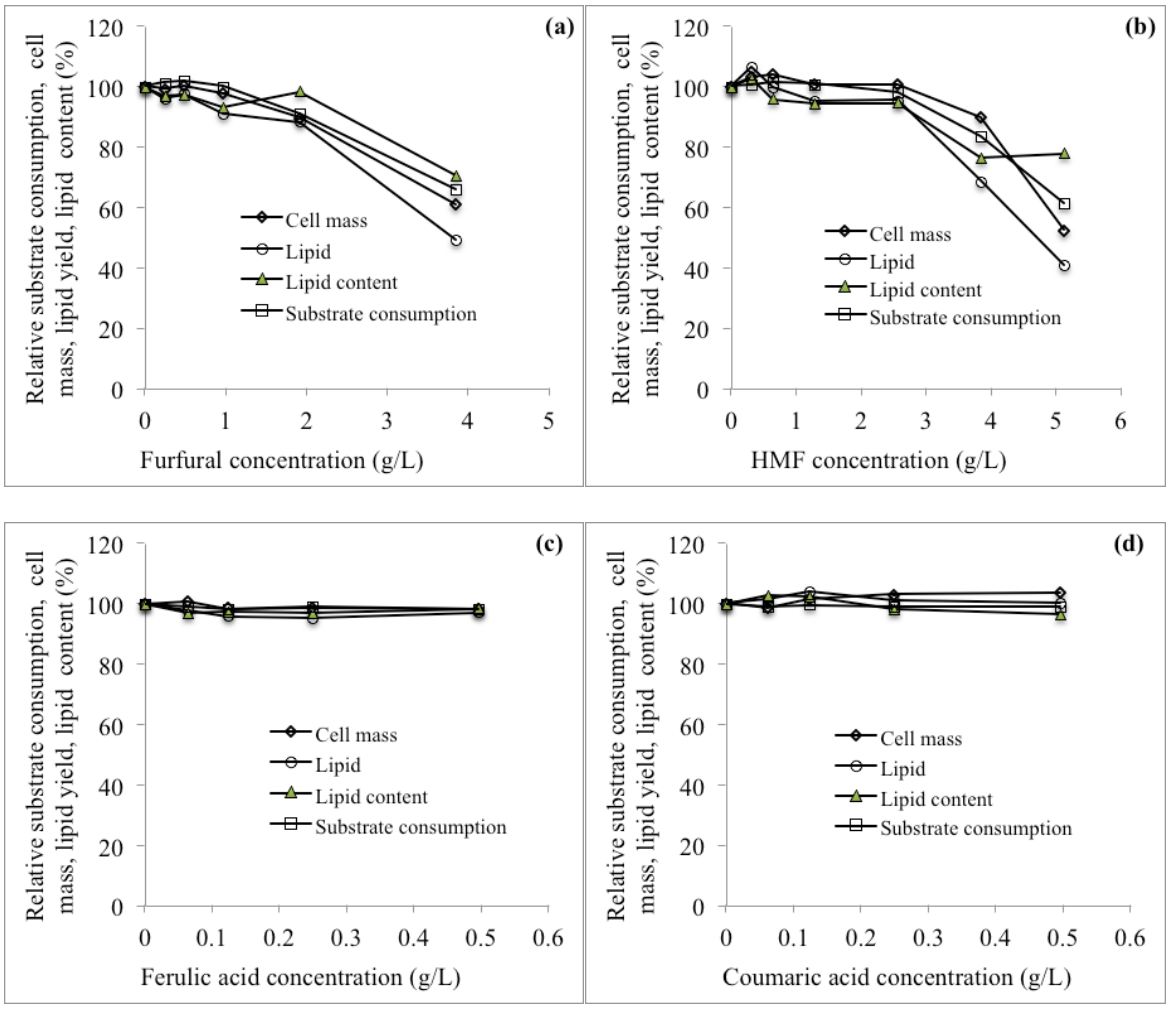

Figure 7 Effect of individual compound on cell growth and lipid accumulation. (a) furfural, (b) HMF, (c) ferulic acid, (d) coumaric acid

For furfural, there was no effect on cell growth and lipid accumulation under concentrations less than 1 g/L. The negative impact was observed when furfural concentration was increased to 1.9 g/L; the substrate consumption, cell mass and lipid yield were reduced by approximate 10% compared to the control. With further increase of furfural concentration to 3.8g/L, the inhibitory effect became more severe, relative substrate consumption, cell mass, lipid yield and lipid content was reduced to 60%, 56%, 50% and 70%, respectively (Fig. 7a). It has been reported that furfural, as a major inhibitor produced from acid pretreatment of lignocellulose (131), reduces cell growth and volumetric ethanol production during yeast ethanol fermentation. The furfural inhibition is

not only caused by furfural amount in the solution, but is also a function of cell density, culture conditions and aeration (107). Furfural influences cell growth by interfering with electron transport (150) and inhibiting glycolytic enzymes (10). It has also reported that furfural inhibits the activity of malic enzymes, the key enzyme providing NADPH for lipid biosynthesis in oleaginous microorganism such as *Mucor circinelloides* and *Aspergillus nidulans* (107, 119). Moreover, furfural may also repress the production of fatty acid synthesis precursors, such as acetyl-CoA, malonyl-CoA and the activity of related fatty acid synthase (13).

The inhibitory effect of HMF (a typical hexose decomposed byproduct during lignocellulose hydrolysis) on *M. isabellina* had similar trend with furfural. There was almost no influence on cell growth and lipid accumulation until HMF concentration reached 2.5g/L. At very high concentration (5 g/L) of HMF, the relative substrate consumption, cell mass growth, lipid yield and lipid content were decreased to 60%, 65%, 72% and 68%, respectively. (Fig.7b). HMF commonly interrupts with electron transfer and correspondingly influences the glycolysis and krebs cycle fluxes, thus affects the energy metabolism of the microorganisms (63, 108, 171).

The experimental data indicated that furfural was more toxic than HMF in terms of oleaginous fungal cell growth and lipid fermentation. Comparing to oleaginous yeasts (23, 64, 71), *M. isabellina* demonstrated much better tolerance to both furfural and HMF (Table 10). In our previous studies on lignocellulosic hydrolysates, furfural concentration in the broth was much higher than HMF (124-126), and thus the effect of furfural on *M.isabellina* lipid fermentation would be more profound compared with the effect of HMF.

Table 10 Inhibitory concentrations of furfural and HMF with various oleaginous microorganisms

Aldehydes	Strains	Concentration of 25% inhibition on lipid yield (g/L)	Concentration of 25% inhibition on lipid content (g/L)	Concentration of 25% inhibition on cell mass yield (g/L)
Furfural	*Trichosporon fermentans* CICC 1368	0.2	0.7	0.24
HMF	*Trichosporon fermentans* 1368	1.9	N.A	2.5
Furfural	*Cryptococcus curvatus* ATCC 20509	N.A	0.6	0.5
Furfural	*Rhodosporidium toruloides* Y4	N.A	N.A	0.5
Furfural	*Rhodosporidium toruloides* AS 2.1389	N.A	0.1	0.08
Furfural	*Trichosporon cutaneum* CCMCC 2.1374	N.A	1.0	0.8
Furfural	*Lipomuces starkeyl* CCMCC 2.1390	0.5	0.6	0.8
Furfural	*Mortierella isabellina* ATCC 42613	2.6	3.6	2.9
HMF	*Mortierella isabellina* ATCC 42613	3.5	4.5	4.2

No detectable inhibition was found under the tested concentrations for ferulic acid and coumaric acid (Fig. 7c,d). The phenolic compounds present in hydrolysates are dependent on the type of pre-treatment and the H/G/S ratio of the lignin in the biomass (88). Their concentration is much lower than weak acids and furan derivatives. Among them, coumaric acid and ferulic acid are produced by hydrolysis of esterified hemicellulose and lignin (27). Normally, these two acids are found at very low concentrations in lignocellulosic hydrolysates. In the hydrolysates, ferulic acid and coumaric acid concentrations ranged from 1.1-14.6 mg/L and 1.9-9 mg/L, separately. Therefore, these two acids as individual inhibitor would hardly affect lipid fermentation of *M.isabellima* at their likely concentrations in lignocellulosic hydrolyates.

4.4.2 Effect of different inhibitor combinations on cell growth and lipid fermentation

The lignocellulosic hydrolysate generally contains multiple inhibitors with the synergistic effect. Hu et.al., reported that when six inhibitors including 3g/L acetate, 1g/L furfural, 1.9g/L HMF, 0.152mg/L vanillin, 0.08mg/L syringaldehyde were added during lipid fermentation of *Rhodosporidium toruloides*, there was noticeable inhibition on both cell mass and lipid production. However, lipid content was almost identical to the control sample. The results indicated that the inhibition was mainly because of the cell growth delay (64). Zhao and co-workers investigated the synergistic effect of four inhibitors (acetic acid, formic acid, furfural and vanillin). They noticed that the synergistic effect of these compounds dramatically inhibited the cell mass concentration and sugar consumption, even the individual compounds were at low concentrations (176).

In this study, experiments were carried out to investigate the tolerance of *Mortierella* under combined effect of the four inhibitors (furfural, HMF, ferulic acid, coumaric acid) at their respective concentrations found in the hydrolysates. Fig. 8 indicated the relative cell mass, lipid yield, lipid content as well as substrate consumption of the cultures with and without inhibitors. If four representative inhibitors were presented in the fermentation broth, there was synergetic inhibition on *M. isabellina*, as indicated by longer lag phase, lower substrate consumption and lower lipid concentration compared to the control. The inhibitory effect increased significantly as the concentrations of these inhibitors were elevated. Furthermore, the inhibition was more profound at the initial culture (Fig. 8). With the extension of culture time, the inhibitory effect was alleviated. At the end of day one, culture of inhibitor combinations-1, 2, and 3 had a relative cell mass of 84%, 64%, and 33% (normalized to the control samples), respectively. The corresponding relative lipid yields were 80%, 35%, and 15%; and the corresponding related lipid contents were 95%, 55%, and 23%.

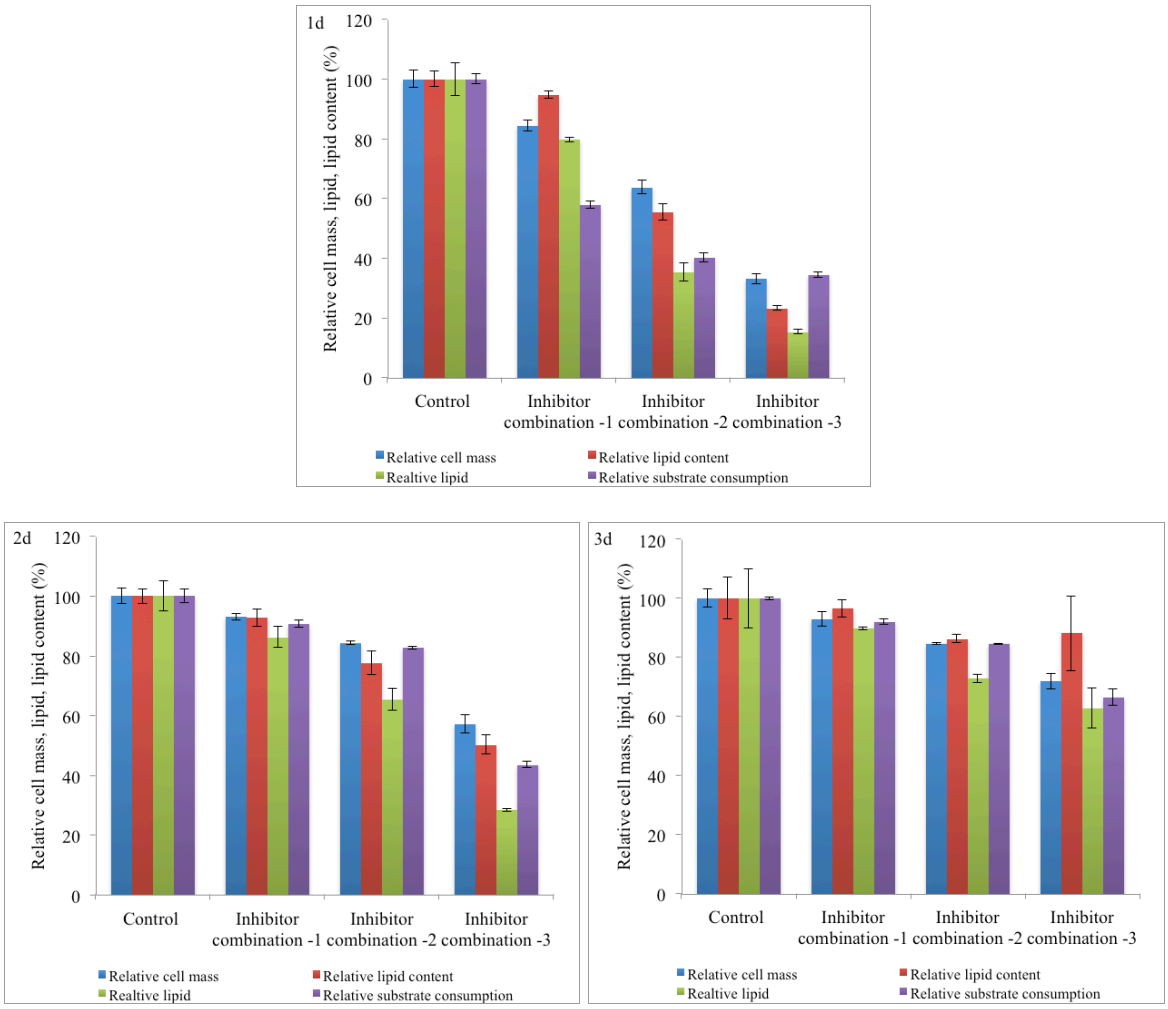

Figure 8 Effect of combination on the cell growth and lipid accumulation of *M. isabellina.* Inhibitor combination-1: 0.5g/L furfural, 0.1g/L HMF, 0.025g/L coumaric acid and 0.025g/L ferulic acid; Inhibitor combination-2: 1g/L furfural, 0.2g/L HMF, 0.05g/L coumaric acid and 0.05g/L ferulic acid; Inhibitor combination-3: 2g/L furfural, 0.4g/L HMF, 0.02g/L coumaric acid and 0.02g/L ferulic acid

With the increase of culture time (end of day 2), the relative cell mass from cultures (inhibitor combinations-1, 2, 3) increased to around 93%, 84% and 57%, respectively. The corresponding relative lipid yields also rose to about 86%, 65% and 28%; and the corresponding lipid contents were 92%, 77% and 50%. Compared with individual inhibitor effects (e.g. furfural at a concentration around 2g/L had minimal effect on cell growth), inhibitor cocktails have more reduction of cell mass and lipid yields.

4.4.3 Kinetics of *M.isabellina* fermentation on the selected inhibitor combination

The inhibitor combination (2g/L furfural, 0.4g/L HMF, 0.02g/L coumaric acid and 0.02g/L ferulic acid) was chosen to study the kinetics of carbon utilization and lipid accumulation because it exhibited the most negative influence on *M.isabellina*. Cell mass, lipid yield, as well as substrate (glucose, xylose and acetate) consumptions during the fermentation were demonstrated in Fig. 9. Despite a similar exponential growth pattern observed in both the control and experimental fermentation, there was a longer lag phase (around 20h) for the experimental fermentation as compared to the control (without inhibitors). The cell mass and lipid concentration from the control fermentation reached to 8.6 and 1.78 $g \cdot L^{-1}$, respectively, in the first 34 hours culture (Fig. 9a). The fermentation with inhibitor combination-3 started to consume the substrates and accumulate cell biomass at around 20h (Fig. 9b). With the culture progressed, the control fermentation continuously consumed substrate and accumulated cell mass and lipid. The cell mass and lipid concentration increased to 11.0, 4.14 $g \cdot L^{-1}$ at 70 h, respectively (Fig.9a). The fermentation with inhibitor combination-3 continuously accumulated cell mass and lipid after the 20h lag phase. The corresponding cell mass and lipid yield reached 9.28 and 3.39 g/L, respectively at 80 h (Fig. 9b).

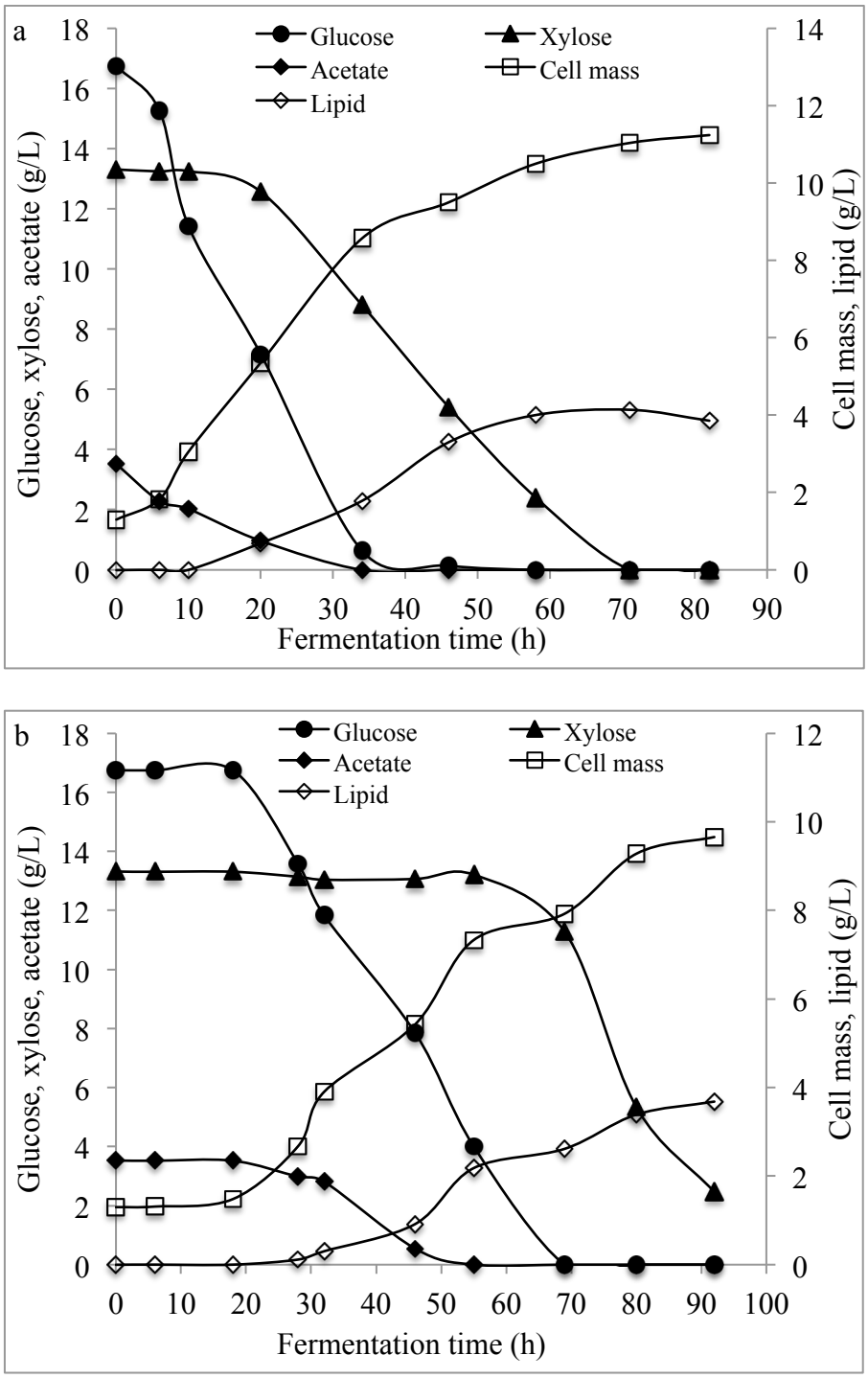

Figure 9 Effect of combination on substrate consumption, cell growth and lipid fermentation kinetics of *M.isabellina*. (a): without inhibitor, (b): with combined inhibitors

Table 11 reported cell growth and lipid accumulation at around 70h. The cell mass and lipid concentration of the control was significant higher compared with the the results from the experimental fermentation (~28% and 37% respectively). There was no statistical difference between the fermentation and control in terms of lipid content and lipid yields ($P > 0.05$).

Table 11 Cell growth and lipid production in the presence and absence of selected combined inhibitors

Samples	Cell mass (g/L)	Lipid (g/L)	Lipid yield (g/g substrate)	Lipid content (%)
Control	11.04±0.09	4.14±0.04	0.123±0.001	37.5±0.02
With combined inhibitors	7.93±0.28	2.62±0.28	0.118±0.02	33.08±4.7

*Data points are the average of two replicates with standard error
The inhibitor combination: 2g/L furfural, 0.4g/L HMF, 0.02g/L ferulic acid and 0.02g/L coumaric acid

To compare the effect of selected combined inhibitors on fatty acid composition of intracellular lipid of *M.isabellina,* fungal biomass samples were taken at different culture time and their fatty acid composition was determined and illustrated at Table 12. The fatty acid profiles of both fermentation conditions were dominated by oleic acid (C18:1) and palmitic acid (C16:0) with lower amounts of linolenic acid (C18:2) and steric acid (C18:0). During the cell growth under higher stress, there was a slightly increase in oleic acid content and decrease in steric acid, which indicated the accumulation of unsaturated oleic acid was due to the desaturation of saturated steric acid. This observation was consistent with previous report (120).

Table 12 Fatty acid composition of accumulated lipid with and without inhibitor

Treatment	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Others*
With inhibitors							
55h	28.0±0.0	0.6±0.0	12.3±0.0	46.3±0.1	8.0±0.1	1.6±0.02	3.2±0.05
69h	28.0±0.3	0.6±0.0	9.9±0.05	47.6±0.3	8.3±0.03	1.1±0.01	4.3±0.04
92h	27.5±0.1	0.6±0.01	8.7±0.08	50.0±0.1	9.3±0.03	1.3±0.03	2.7±0.1
Without inhibitors							
34h	27.7±0.0	1.2±0.05	6.0±0.04	51.4±0.0	9.2±0.07	2.3±0.02	2.3±0.04
57h	26.7±0.1	1.5±0.02	4.7±0.07	52.8±0.0	8.9±0.25	1.9±0.12	3.3±0.05
71h	24.3±0.0	1.4±0.06	5.7±0.02	54.0±0.2	9.2±0.28	2.7±0.02	2.6±0.05

*: others mean C14:0, C20:0, C22:0
Data points are the average of two replicates with standard error

4.4.4 ^{13}C tracer- experiments for *M.isabellina* carbon utilization during exponential growth phase

^{13}C-tracing experiments were performed using a synthetic hydrolysate medium, which studied the co-utilization of carbon sources during biomass growth stage. Figure 10 displayed ^{13}C labeling of four proteinogenic amino acids after cells were fed solely with ^{13}C labeled glucose, xylose, and acetate, respectively. By examining each carbon source separately, individual substrate's contribution towards cell mass synthesis was elucidated. Among the three substrates (glucose, xylose, acetate), glucose contributed most to cell mass synthesis, especially for alanine (65%). Glucose also contributed to synthesis of other amino acids, such as TCA cycle amino acid (Aspartate, 25%) and pentose phosphate pathway amino acid (Phenylalanine, 14.6%). Measurement of substrate consumption during cell growth showed significant use of glucose under inhibitor conditions, which suggested that a large fraction of glucose was necessary to push flux towards complete

oxidation to generate ATP and NAD(P)H to support cell energy metabolism under the stressed condition. In the carbon tracing experiment, xylose had a low contribution to cell mass growth once glucose and acetate were still available in the medium.

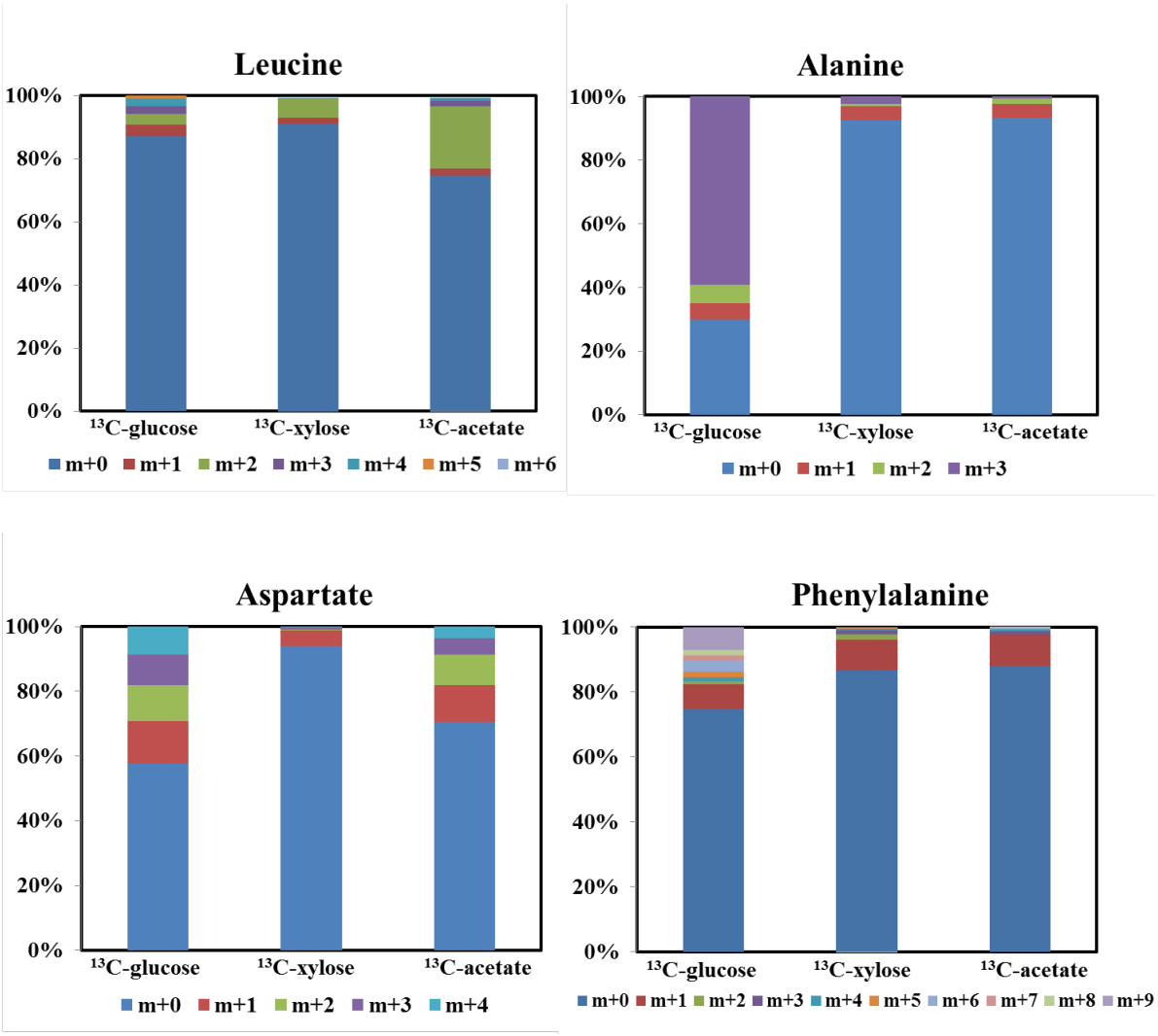

Figure 10 Isotopomer labeling of amino acids from the cultures with differently labeled substrates.
(1) The data determined from three separated tracer experiments, in which only one tracer of glucose, xylose, and acetate was fully labeled accordingly. The biomass samples were from the middle-log growth phase when all three substrates were present.

As indicated by substrate consumption, xylose utilization followed after the consumption of glucose and acetate. As for ^{13}C-tracing of acetate co-metabolism in *M.isabellina*,

acetate was found to be building blocks for leucine (8%) and TCA cycle amino acid (such as asparate, 15%). However, it had very little contribution to glycolysis-derived amino acid alanine (3%) and pentose phosphate pathway-derived amino acid phenylalanine (2%). Such observations demonstrate that acetate is mainly metabolized via the TCA cycle.

The ^{13}C tracers examined carbon flows to fungal lipid. The results illustrated that glucose and acetate contributed to almost 90% of the lipid accumulation in the six-day cultures (Fig.11).

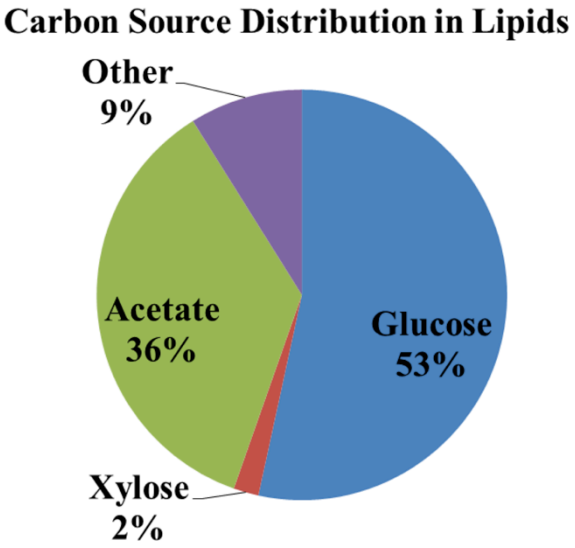

Figure 11 Carbon source distribution in lipids determined via 13C-carbon tracing analysis
(1) The cell mass sample was from the middle-log growth phase when all three substrates were present

Despite low acetate concentration in the medium (2g/L, one fifth of glucose fed), 36% of lipid synthesis was attributed to acetate, which was a significant portion as compared to 53% from glucose. In fungal species, pyruvate dehydrogenase (acetyl-CoA synthesis) and TCA cycle are in mitochondria, while lipid biosynthesis is in cytosol. In order to provide acetyl-CoA for lipid production, fungi have to transport citrate out of mitochondria, then use citrate lyase to degrade citrate to acetyl-CoA (133). The mitochondrial transport places a

Figure 11 (cont'd)

limit on lipid synthesis in cytosol. Adding acetate into the medium could alleviate this limitation. Fungal acetyl-CoA synthetase can directly convert acetate to produce acetyl-CoA in cytosol, which supports lipid production without mitochondrial transport limitations. Therefore, existence of acetate in lignocellulosic hydrolysate facilitates the fungal fermentation for lipid production.

4.5 Conclusions

M.isabellina has the potential to serve as a robust platform for producing value-added chemicals using abundant lignocellulosic resources. It showed excellent tolerance to inhibitors from lignocellulose hydrolysis such as furan derivatives furfural, HMF, and phenolic compounds. The inhibition delayed the substrate consumption and cell growth at the beginning of the fermentation; however, once the strain adapted to the toxic environment, its cell growth and lipid accumulation significantly improved. ^{13}C-carbon tracing indicated that *M. isabellina* could simultaneously utilize both glucose and acetate for cell mass growth and lipid accumulation with the existence of inhibitors. *M.isabellina* xylose utilization pathway, strongly repressed by glucose, is an important bottleneck in further improvement of fungal growth and lipid accumulation.

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

Microbial lipid offers an alternative to lipid based biofuel production. It is biodegradable, contributes no net carbon dioxide or sulfur accumulation to the environment and emits less gaseous pollutants compared to normal biodiesel (105). For efficient microbial lipid fermentation, it is necessary to select inexpensive and abundant carbon sources. Lignoellulose is such a resource, which plays an important role for future fuel and chemical biorefining. However, due to the recalcitrant nature of lignocellulose, it needs thermal/chemical/biological pretreatment and enzymatic hydrolysis to release sugars (glucose, xylose). Correspondingly, some byproducts such as furan derivatives are generated along with sugar release, which generally inhibit the cell metabolisms for both growth and product formation. Thus, finding proper microorganisms that have the ability to efficiently convert fermentable sugars into lipid with the existence of inhibitors is urgently needed to enable microbial biodiesel production on lignocellulosic materials. Our study successfully find a oleaginous fungus *M. isabellina* ATCC 42613, which is not only able to accumulate significant amounts of mycelia and lipid, but can also stably convert the major lignocellulosic sugars glucose and xylose as well as a byproduct acetate to cellular lipids at a relatively high conversion yield. The intracellular fatty acid profile and chemical properties of microbial lipid were ideal for biodiesel production. Further application on corn stover hydrolysates whole slurry lipid fermentation, without washing and detoxification, showed comparable results to synthetic hydrolysate without inhibitors, suggesting the promising potential of utilizing lignocellulose for lipid production using oleaginous fungal fermentation.

To date, there is quite limited information regarding the effect of inhibitors in

lignocellulosic hydrolysate on the cell growth and lipid accumulation of oleaginous fungi. Our study indicated that furfural was one major inhibitor, and there was synergetic inhibitory effect in the presence of different lignocellulose hydrolysis inhibitors. The inhibition was more profound at the early stage of the culture. Isotopic study could trace carbon contribution to cell growth and lipid biosynthesis, providing fundamental information on the direct utilization of different carbon sources from lignocellulosic hydrolysate. Our current study suggested that the oleaginous fungus *M. isabellina* could simultaneously utilize glucose and acetate for cell growth and lipid accumulation with the presence of inhibitors, once the glucose and acetate were at low level, the strain started to utilize xylose along with the culture. Acetate is highly assimilated for synthesis of lipid and amino acids from TCA cycle, it was twice more effective compared with glucose in terms of lipid accumulation. Large amount of glucose was consumed as energy source, not building blocks.

5.2 Future work

The following research may be useful for microbial lipid production from lignocellulose.

1. Kinetic modeling of *M.isabellina* lipid fermentation under multiple carbon sources.
2. Carbon source feeding strategy optimization to enhance lipid production performance, such as glucose and acetate co-feeding.
3. Techno-economic analysis and energy balance analysis to elucidate the large production possibility of microbial lipid from lignocellulose.Overexpression of the malic enzyme (one of the key lipid biosynthesis enzymes) of *M. isabellina* to possibly enhance its lipid accumulation,.

4. Bioreactor design such as applying micro-bubble technology to enhance oxygen mass transfer, thus hope to improve lipid production performance.
5. Carbon metabolism and fluxes to elucidate the effect of inhibitors on cell growth and lipid fermentation of *M.isabellina.* Such as the possible mechanism for the synergistic effect of these inhibitors and how their synergy influences the cell growth and lipid accumulation.

APPENDICES

APPENDIX A: DATA

A.1 CHAPTER 2 DATA

Table 11 Cell mass and lipid per consumed sugar for Figure 1

Consumed glucose (g/L)	Cell mass (g/L)	Lipid (g/L)
28.1	11.5	4.5
28.1	11.3	4.1
28.1	11.5	4.6
31.7	13.6	5.3
30.2	13.5	5.1
30.2	12.8	5.2
40.3	14.9	4.9
39.3	15.1	5.6
37.4	14.0	5.5
61.2	20.2	9.1
65.4	21.4	7.4
54.8	20.4	8.6
62.8	20.1	8.0
54.1	22.3	9.4
66.0	22.3	8.7
67.8	22.3	9.5
70.1	22.8	9.8
75.0	23.5	9.1

Table 11. (cont'd)

Consumed xylose (g/L)	Cell mass (g/L)	Lipid (g/L)
26.6	10.1	2.7
26.6	9.9	3.7
26.6	9.6	3.2
34.5	11.8	4.5
32.5	11.1	4.6
35.0	11.4	4.6
45.7	14.2	6.1
42.8	15.2	5.2
42.4	13.2	5.0
59.2	16.2	6.9
55.2	15.1	6.0
68.2	18.6	7.8
65.7	19.5	6.7
59.6	18.4	7.6
64.1	18.7	6.8
69.1	22.4	8.2
69.6	20.3	7.8
79.5	22.1	8.0

Table 12 Representative chemical properties of *M.isabellina* lipid for Figure 2

Glucose	Iodine value (g iodine/100g oil)	Saponification value (mg KOH/g oil)	High heating value (MJ/kg)
28.1	75.9	204.2	39.9
35.4	78.1	204.0	39.9
46	77.4	203.0	39.9
73.7	70.2	206.7	39.9
82.5	76.3	204.5	39.9
91.7	79.9	204.5	39.8

Xylose	Iodine value (g iodine/100g oil)	Saponification value (mg KOH/g oil)	High heating value (MJ/kg)
26.6	76.7	203.9	39.9
39.1	77.7	204.8	39.9
45.7	68.0	206.2	40.0
66.3	80.1	204.6	39.8
79.7	77.1	205.3	39.9
90.9	73.9	206.8	39.8

Table 13 Cell mass composition for Figure 3

Glucose (g/L)	Cell mass (g/L)	Lipid (g/L)	Carbohydrate (g/L)	Protein (g/L)
28.10	11.46	5.19	4.61	1.40
28.10	11.34	4.80	3.91	1.10
35.40	13.60	5.43	5.32	1.70
35.40	13.00	5.38	4.92	1.50
46.00	15.10	6.09	6.35	1.41
46.00	14.30	5.69	5.95	1.20
73.70	20.30	8.29	6.55	1.79
73.70	21.10	8.89	5.90	2.19
82.50	22.00	9.02	6.20	1.35
82.50	22.60	10.82	6.80	1.75
91.70	22.60	10.29	5.79	1.46
91.70	23.20	10.09	6.39	1.66

Table 13. (cont'd)

Xylose (g/L)	Cell mass (g/L)	Lipid (g/L)	Carbohydrate (g/L)	Protein (g/L)
26.60	9.60	3.39	3.95	1.01
26.60	10.00	4.19	4.35	1.20
39.10	11.20	4.78	4.01	1.46
39.10	11.60	4.99	4.45	1.48
45.70	13.60	5.14	5.44	1.29
45.70	14.80	6.14	6.44	1.69
66.30	16.10	7.27	6.24	1.41
66.30	18.90	8.08	7.44	1.21
79.70	18.60	7.64	6.20	1.38
79.70	19.20	8.24	6.80	1.20
90.90	22.30	8.66	5.79	1.11
90.90	20.90	9.06	6.39	1.31

A.2 CHAPTER 3 DATA

Table 14 Changes of sugar concentration during enzymatic hydrolysis for Figure 4

Enzymatic hydrolysis time (h)	Enzyme loading	Glucose (g/L)	Xylose (g/L)
0	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	3.39	10.43
0	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	1.78	10.19
0	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	3.39	10.43
0	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	1.78	10.19
0	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	3.39	10.43
0	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	1.78	10.19
17	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	27.87	16.18
17	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	28.56	15.87
17	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	23.75	12.83
17	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	25.09	13.62
17	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	13.91	10.28
17	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	14.21	10.66
28	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	29.09	16.43
28	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	29.94	16.70
28	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	25.11	14.01
28	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	25.43	14.37
28	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	14.95	9.62
28	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	16.22	10.75
48	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	29.69	17.60
48	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	29.33	17.86

Table 14. (cont'd)

Enzymatic hydrolysis time (h)	Enzyme loading	Glucose (g/L)	Xylose (g/L)
48	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	28.06	15.74
48	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	26.60	14.69
48	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	22.30	13.30
48	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	22.36	14.03
70	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	31.55	19.45
70	21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	29.62	18.42
70	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	28.30	16.56
70	10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	28.27	16.61
70	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	23.88	14.12
70	5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	21.18	12.89

Table 15 Combined glucose and xylose yield for Figure 5

Enzyme loading	Glucose and xylose of theoretical yield (%)	Glucose and xylose of biomass (g/g)
21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	76.63	45.90
21.2 mg Accellerase 1500 and 1.08 mg Accellerase XY	71.94	43.23
10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	67.19	40.38
10.6 mg Accellerase 1500 and 0.54 mg Accellerase XY	67.22	40.40
5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	56.91	34.20
5.4 mg Accellerase 1500 and 0.27 mg Accellerase XY	51.02	30.66

Table 16 *M.isabellina* cell growth and lipid accumulation for Figure 6

Time (h)	Glucose (g/L)	Xylose (g/L)	Acetate (g/L)	Cell mass (g/L)	Lipid (g/L)	Lipid content (%)
0	20.78	11.23	3.92	0.13	0.00	0.00
0	23.57	12.77	3.92	0.13	0.00	0.00
15	20.33	12.00	3.13	1.63	0.22	6.40
15	20.03	12.00	3.01	1.77	0.21	6.28
40	6.88	9.61	0.00	7.64	1.79	23.42
40	6.03	8.93	0.00	7.82	1.74	22.18
40	12.31	10.87	0.00	5.21	0.82	15.65
51	0.00	9.56	0.00	11.09	2.69	24.23
51	0.39	9.90	0.00	10.57	2.72	25.69
51	3.95	9.36	0.00	9.23	2.44	26.38
63	0.00	7.73	0.00	11.66	3.40	29.20
63	0.00	8.12	0.00	12.13	3.84	31.68
63	0.00	7.33	0.00	12.44	3.34	26.83
70.5	0.00	4.34	0.00	13.15	3.95	30.06
70.5	0.00	4.63	0.00	13.34	3.57	26.76
70.5	0.00	4.27	0.00	13.03	4.06	31.14
86	0.00	0.34	0.00	16.27	4.43	27.24
86	0.00	0.36	0.00	16.16	5.12	31.71
86	0.00	0.02	0.00	16.57	5.09	30.74
93	0.00	0.00	0.00	16.48	4.94	29.96
93	0.00	0.00	0.00	16.93	4.80	29.02
93	0.00	0.00	0.00	16.98	4.99	30.40
110	0.00	0.00	0.00	16.08	4.66	28.96
110	0.00	0.00	0.00	17.64	5.14	29.11
110	0.00	0.00	0.00	17.11	5.32	31.09

Table 16 (cont'd)

Time (h)	Glucose (g/L)	Xylose (g/L)	Acetate (g/L)	Cell mass (g/L)	Lipid (g/L)	Lipid content (%)
0	17.39	12.37	3.74	0.13	0.00	0.00
0	18.40	12.57	3.74	0.13	0.00	0.00
15	15.33	12.40	2.19	4.89	0.71	14.44
15	14.73	12.39	2.02	5.64	0.65	11.44
40	0.00	9.19	0.00	12.23	3.53	28.85
40	0.00	8.97	0.00	12.76	3.99	31.28
40	0.00	8.10	0.00	11.84	4.16	35.16
51	0.00	1.26	0.00	14.12	5.00	35.41
51	0.00	2.49	0.00	13.74	4.76	34.61
51	0.00	2.04	0.00	14.06	4.57	32.48
63	0.00	0.00	0.00	14.16	5.16	36.42
63	0.00	0.00	0.00	14.38	4.94	34.38
63	0.00	0.00	0.00	14.45	4.72	32.64
71	0.00	0.00	0.00	14.93	5.52	36.97
71	0.00	0.00	0.00	15.10	5.09	33.69
71	0.00	0.00	0.00	14.72	5.52	37.53
93	0.00	0.00	0.00	14.87	4.52	30.40
93	0.00	0.00	0.00	15.19	5.38	35.40

A.3 CHAPTER 4 DATA

Table 17 Individual inhibitor effect on cell growth and lipid formation for Figure 7

Furfural concentration (g/L)	Substrate consumed (g/L)	Cell mass (g/L)	Lipid content (%)	Lipid concentration (g/L)
0	22.25	9.72	30.92	3.01
0	22.03	9.46	36.42	3.45
0.24	21.98	9.62	30.99	2.98
0.24	22.85	9.41	34.30	3.23
0.48	22.57	9.44	31.21	2.95
0.48	22.47	9.76	34.38	3.36
0.96	22.29	9.21	30.42	2.80
0.96	21.96	9.51	32.42	3.08
1.92	19.29	8.23	30.38	2.50
1.92	20.96	8.95	35.89	3.21
3.84	14.38	5.85	30.84	1.80
3.84	14.78	5.82	23.78	1.38

Table 17 (cont'd)

HMF concentration (g/L)	Substrate consumed (g/L)	Cell mass (g/L)	Lipid content (%)	Lipid concentration (g/L)
0	22.25	9.72	30.92	3.01
0	22.03	9.46	36.42	3.45
0.32	22.31	9.90	31.85	3.15
0.32	22.33	9.89	37.47	3.71
0.64	22.67	9.89	29.82	2.95
0.64	22.25	10.03	34.82	3.49
1.28	22.27	9.74	31.02	3.02
1.28	22.38	9.58	32.63	3.13
2.56	21.66	9.67	30.71	2.97
2.56	21.84	9.62	33.05	3.18
3.84	18.66	8.64	25.39	2.19
3.84	18.37	8.58	26.02	2.23
5.12	13.78	5.07	25.39	1.29
5.12	13.36	4.96	27.14	1.35

Table 17 (cont'd)

Ferulic acid concentration (g/L)	Substrate consumed (g/L)	Cell mass (g/L)	Lipid content (%)	Lipid concentration (g/L)
0.000	23.59	10.59	30.92	3.28
0.000	23.28	10.25	36.42	3.73
0.064	23.24	10.50	30.99	3.25
0.064	23.70	10.52	34.30	3.61
0.124	23.87	10.34	31.21	3.23
0.124	23.69	10.17	34.38	3.50
0.248	23.59	10.01	32.71	3.28
0.248	23.58	10.53	32.42	3.41
0.496	23.52	9.56	30.38	2.90
0.496	23.21	10.88	35.89	3.91

Table 17 (cont'd)

Coumaric acid concentration (g/L)	Substrate consumed (g/L)	Cell mass (g/L)	Lipid content (%)	Lipid concentration (g/L)
0.000	23.59	10.59	30.92	3.28
0.000	23.28	10.25	36.42	3.73
0.062	23.54	10.45	31.85	3.33
0.062	23.56	10.08	37.47	3.78
0.123	23.91	10.54	34.10	3.60
0.123	23.49	10.60	34.82	3.69
0.248	23.51	10.83	33.31	3.61
0.248	23.05	10.59	32.79	3.47
0.496	23.90	11.00	32.94	3.62
0.496	23.66	10.55	32.16	3.39

Table 18 Combined inhibitor on cell growth and lipid production for Figure 8

Time (d)	Samples	Relative cell mass (%)	Relative lipid content (%)	Relative lipid yield (%)	Relative substrate consumption (%)
1	Control	102.02	101.78	103.80	101.10
1	Control	97.98	98.22	96.20	98.90
1	Inhibitor combination 1	85.57	93.86	80.29	58.59
1	Inhibitor combination 1	83.13	95.51	79.36	56.96
1	Inhibitor combination 2	62.03	53.59	33.23	39.15
1	Inhibitor combination 2	65.25	57.26	37.35	41.15
1	Inhibitor combination 3	32.03	23.65	15.72	35.02
1	Inhibitor combination 3	34.23	22.67	14.70	33.72
2	Control	101.79	101.68	103.47	101.54
2	Control	98.21	98.32	96.53	98.46
2	Inhibitor combination 1	92.33	90.75	83.76	89.97
2	Inhibitor combination 1	93.68	94.71	88.70	91.50
2	Inhibitor combination 2	84.60	80.31	67.92	82.91
2	Inhibitor combination 2	83.92	74.81	62.76	82.40
2	Inhibitor combination 3	55.04	52.44	28.62	42.80
2	Inhibitor combination 3	59.17	48.00	28.16	44.19
3	Control	102.12	104.95	107.06	100.26
3	Control	97.88	95.05	92.94	99.74
3	Inhibitor combination 1	91.34	98.66	90.02	91.46
3	Inhibitor combination 1	94.66	94.58	89.43	92.59
3	Inhibitor combination 2	84.82	87.16	73.85	84.57
3	Inhibitor combination 2	84.58	85.24	72.02	84.47
3	Inhibitor combination 3	73.60	79.32	58.03	68.31
3	Inhibitor combination 3	70.06	97.06	67.60	64.50

Table 19 *M.isabellina* cell growth and lipid accumulation for Figure 9

Time (h)	Glucose (g/L)	Xylose (g/L)	Acetate (g/L)	Cell mass (g/L)	Lipid (g/L)	Lipid content (%)
0.00	16.75	13.31	3.52	1.30	0.00	0.00
0.00	16.75	13.31	3.52	1.30	0.00	0.00
6.00	15.32	13.25	2.35	1.83	0.00	0.00
6.00	15.20	13.24	2.21	1.82	0.00	0.00
10.00	11.50	13.27	2.04	3.11	0.00	0.00
10.00	11.36	13.23	2.01	2.99	0.00	0.00
20.00	7.24	12.63	0.98	5.35	0.73	13.64
20.00	7.09	12.51	0.95	5.36	0.65	12.16
34.00	0.71	9.00	0.00	8.53	1.75	20.49
34.00	0.56	8.59	0.00	8.61	1.82	21.15
46.00	0.17	5.55	0.00	9.58	3.23	33.69
46.00	0.11	5.25	0.00	9.42	3.38	35.84
58.00	0.00	2.52	0.00	10.55	3.99	37.84
58.00	0.00	2.29	0.00	10.45	4.01	38.41
71.00	0.00	0.00	0.00	11.10	4.16	37.51
71.00	0.00	0.00	0.00	10.97	4.11	37.49
82.00	0.00	0.00	0.00	11.16	3.83	34.31
82.00	0.00	0.00	0.00	11.32	3.88	34.31

Table 19 (cont'd)

Time (h)	Glucose (g/L)	Xylose (g/L)	Acetate (g/L)	Cell mass (g/L)	Lipid (g/L)	Lipid content (%)
0.00	16.75	13.31	3.52	1.30	0.00	0.00
0.00	16.75	13.31	3.52	1.30	0.00	0.00
6.00	16.75	13.31	3.52	1.31	0.00	0.00
6.00	16.75	13.31	3.52	1.31	0.00	0.00
18.00	16.05	13.31	3.52	1.48	0.00	0.00
18.00	16.15	13.31	3.52	1.40	0.00	0.00
28.00	13.02	13.13	2.97	2.72	0.11	4.23
28.00	13.14	13.15	2.99	2.63	0.14	5.15
32.00	11.58	13.01	2.82	4.77	0.30	6.35
32.00	12.09	13.06	2.82	3.04	0.22	7.16
46.00	8.11	12.89	0.65	5.23	0.92	17.67
46.00	7.60	13.24	0.43	5.62	0.91	16.17
55.00	3.88	13.16	0.00	7.97	2.59	32.47
55.00	4.14	13.27	0.00	6.70	1.77	26.50
69.00	0.00	10.64	0.00	8.12	2.42	29.76
69.00	0.00	11.92	0.00	7.73	2.82	36.41
80.00	0.00	5.34	0.00	9.39	3.40	36.18
80.00	0.00	4.20	0.00	9.17	3.38	36.87
92.00	0.00	2.28	0.00	10.01	3.76	37.59
92.00	0.00	2.64	0.00	9.31	3.60	38.72

APPENDIX B: STATISTICAL ANALYSIS

B1. Statistical analysis for chapter 3 section 3.4.1

treat sugar

a 76.39
a 71.94
b 67.19
b 67.22
c 56.91
c 51.02

```
> d <- read.table(file.choose(),header=TRUE)
> d.aov <- aov(d$sugar~d$treat)
> summary(d.aov)
            Df Sum Sq Mean Sq F value Pr(>F)
d$treat      2  421.2  210.59   23.19  0.015 *
Residuals    3   27.2    9.08
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> TukeyHSD(d.aov)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = d$sugar ~ d$treat)

$`d$treat`
      diff       lwr        upr     p adj
b-a  -6.96 -19.55367  5.6336736 0.1975172
c-a -20.20 -32.79367 -7.6063264 0.0138138
c-b -13.24 -25.83367 -0.6463264 0.0438831
```

B2. Statistical analysis for chapter 4 section 4.4.3

treat	cell
a	11.10
a	10.97
b	8.12
b	7.73

```
> d <- read.table(file.choose(),header=TRUE)
> d.aov <- aov(d$cell~d$treat)
> summary(d.aov)
            Df Sum Sq Mean Sq F value  Pr(>F)
d$treat      1  9.672   9.672   228.9 0.00434 **
Residuals  2  0.084   0.042
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

treat	lipidcontent
a	37.5
a	37.5
b	29.8
b	36.4

```
> d <- read.table(file.choose(),header=TRUE)
> d.aov <- aov(d$lipidcontent~d$treat)
> summary(d.aov)
            Df Sum Sq Mean Sq F value Pr(>F)
d$treat      1  19.36   19.36   1.778  0.314
Residuals    2  21.78   10.89
```

treat	lipidyield
a	0.124
a	0.122
b	0.105
b	0.13

```
> d <- read.table(file.choose(),header=TRUE)
> d.aov <- aov(d$lipidyield~d$treat)
> summary(d.aov)
            Df    Sum Sq   Mean Sq F value Pr(>F)
d$treat      1 3.025e-05 3.025e-05   0.192  0.704
Residuals    2 3.145e-04 1.573e-04
```

B2. Statistical analysis for chapter 4 section 4.4.3 continued

treat	lipid
a	4.16
a	4.11
b	2.20
b	2.63

```
> d <- read.table(file.choose(),header=TRUE)
> d.aov <- aov(d$lipid~d$treat)
> summary(d.aov)
            Df Sum Sq Mean Sq F value Pr(>F)
d$treat      1 2.9584  2.9584   63.15 0.0155 *
Residuals    2 0.0937  0.0468
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

APPENDIX C: COMBINED HYDROLYSIS

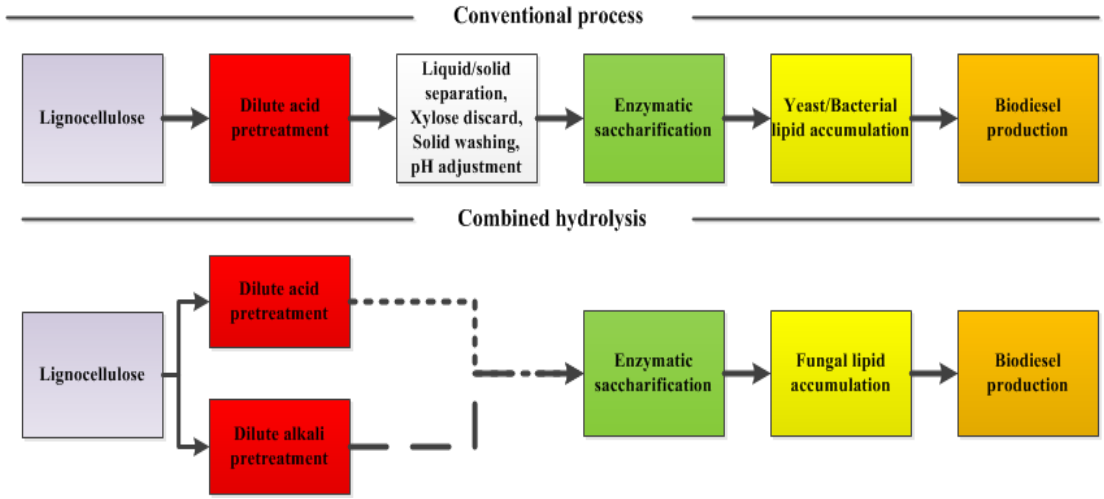

Figure 12 Combined hydrolysis of lignocellulose for microbial lipid production

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