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SOPHOROLIPID PRODUCTION FROM LIGNOCELLULOSIC BIOMASS FEEDSTOCKs

by

Abdul Samad

B. Tech., Kathmandu University, Nepal, 2009

A Thesis

Submitted in Partial Fulfillment of the Requirements for the

Master of Science

Department of Civil and Environmental Engineering

Southern Illinois University Carbondale

December, 2015

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THESIS APPROVAL

SOPHOROLIPID PRODUCTION FROM LIGNOCELLULOSIC BIOMASS FEEDSTOCKs

by

Abdul Samad

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in the field of

Civil and Environmental Engineering

Approved by:

Dr. Yanna Liang, Chair

Dr. Da Chen

Dr. Lichang Wang

Graduate School

Southern Illinois University Carbondale

August 21, 2015

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AN ABSTRACT OF THE THESIS OF

ABDUL SAMAD, for the Master of Science degree in CIVIL AND ENVIRONMENTAL ENGINEERING, presented on August 21, 2015 at Southern Illinois Carbondale.

TITLE: SOPHOROLIPID PRODUCTION FROM LIGNOCELLULOSIC BIOMASS FEEDSTOCKs

ADVISOR: Dr. Yanna Liang

The present study investigated the feasibility of production of sophorolipids (SLs) using yeast *Candida bombicola* grown on hydrolysates derived lignocellulosic feedstock either with or without supplementing oil as extra carbon source. Several researchers have reported using pure sugars and various oil sources for producing SLs which makes them expensive for scale-up and commercial production. In order to make the production process truly sustainable and renewable, we used feedstocks such as sweet sorghum bagasse, corn fiber and corn stover.

Without oil supplementation, the cell densities at the end of day-8 was recorded as 9.2, 9.8 and 10.8 g/L for hydrolysate derived from sorghum bagasse, corn fiber, and corn fiber with the addition of yeast extract (YE) during fermentation, respectively. At the end of fermentation, the SL concentration was 3.6 g/L for bagasse and 1.0 g/L for corn fiber hydrolysate. Among the three major sugars utilized by *C. bombicola* in the bagasse cultures, glucose was consumed at a rate of 9.1 g/L-day; xylose at 1.8 g/L-day; and arabinose at 0.98 g/L-day. With the addition of soybean oil at 100 g/L, cultures with bagasse hydrolysates, corn fiber hydrolysates and standard medium had a cell content of 7.7 g/L; 7.9 g/L; and 8.9 g/L, respectively after 10 days. The yield of SLs from bagasse hydrolysate was 84.6 g/L and corn fiber hydrolysate was15.6 g/L. In the

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same order, the residual oil in cultures with these two hydrolysates was 52.3 g/L and 41.0 g/L. For this set of experiment; in the cultures with bagasse hydrolysate; utilization rates for glucose, xylose and arabinose was recorded as 9.5, 1.04 and 0.08 g/L-day respectively. Surprisingly, *C. bombicola* consumed all monomeric sugars and non-sugar compounds in the hydrolysates and cultures with bagasse hydrolysates had higher yield of SLs than those from a standard medium which contained pure glucose at the same concentration. Based on the SL concentrations and considering all sugars consumed, the yield of SLs was 0.55 g/g carbon (sugars plus oil) for cultures with bagasse hydrolysates.

Further, SL production was investigated using sweet sorghum bagasse and corn stover hydrolysates derived from different pretreatment conditions. For the former and latter sugar sources, yellow grease or soybean oil was supplemented at different doses to enhance sophorolipid yield. 14-day batch fermentation on bagasse hydrolysates with 10, 40 and 60 g/L of yellow grease had cell densities of 5.7 g/L, 6.4 g/L and 7.8 g/L, respectively. The study also revealed that the yield of SLs on bagasse hydrolysate decreased from 0.67 to 0.61 and to 0.44 g/g carbon when yellow grease was dosed at 10, 40 and 60 g/L. With aforementioned increasing yellow grease concentration, the residual oil left after 14 days was recorded as 3.2 g/L, 8.5 g/L and 19.9 g/L. For similar experimental conditions, the cell densities observed for corn stover hydrolysate combined with soybean oil at 10, 20 and 40 g/L concentration were 6.1 g/L, 5.9 g/L, and 5.4 g/L respectively. Also, in the same order of oil dose supplemented, the residual oil recovered after 14-day was 8.5 g/L, 8.9 g/L, and 26.9 g/L. Corn stover hydrolysate mixed with the 10, 20 and 40 g/L soybean oil, the SL yield was 0.19, 0.11 and 0.09 g/g carbon. Overall, both hydrolysates supported cell growth and sophorolipid production.

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The results from this research show that hydrolysates derived from the different lignocellulosic biomass feedstocks can be utilized by *C. bombicola* to achieve substantial yields of SLs. Based upon the results revealed by several batch-stage experiments, it can be stated that there is great potential for scaling up and industrial scale production of these high value products in future.

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Yanna Liang, for her guidance and mentorship throughout my graduate study. The assistance she provided was pivotal to the success of this research. I would also like to thank my graduate committee members, Dr. Da Chen for providing his lab facility and valuable time during my research and Dr. Lichang Wang for the direction and advice.

I am grateful to colleagues of my lab, Yi Cui and Kim Jarosz for their help at different stages of my research. Special thanks to Ji Zhang for helping me with HPLC studies.

Most importantly, I wish to thank my parents, Mr. Karim Baksh Sheikh and Mrs. Farida Sheikh for their unfettered support throughout my academic endeavor. I would also like to express my gratitude to all my friends who supported me with their advice and wishes.

Finally, I express my deepest appreciation to the entire faculty, staff and colleagues of the Southern Illinois University's Civil and Environmental Engineering Department for their help and guidance.

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CHAPTER 1

LITERATURE REVIEW

1.1. Biosurfactants

During different phases of the growth, microorganisms can produce various metabolites which can be designated as primary or secondary. Some of these metabolites, known as biosurfactants, produced by certain bacteria, yeast and fungi have been reported to lower the interfacial tension. These biosurfactants, oftentimes, extracellular products secreted by the host cells, are low molecular weight compounds that consist of a hydrophilic part comprising of an acid, peptide cations or anions, mono or di or polysaccharides, and a hydrophobic part consisting of unsaturated or saturated hydrocarbon chains (Banat, 2010).

1.1.1. Types of Biosurfactants

Among biosurfactants, the glycolipids are the most studied ones as they have high efficiency with good biodegradability (Kosaric, 2014) and can be derived from renewable resources. Dembitsky et al. (2004) has listed more than 250 glycolipids with their biological activities and chemical structures. Glycolipids have both carbohydrate and aliphatic acids or hydroxyaliphatic parts. These are again differentiated into: Rhamnolipids, Trehalolipids, Cellabiolipids, and Sophorolipids.

Rhamnolipids (Fig. 1) have been reported to be produced by *Pseudomonas aeruginosa*, in which one or two molecules of α-L-rhamnose are linked to one or two molecules of 3-hydroxydecanoic acid via *O*-glycosidic bond. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (Kosaric, 2014).

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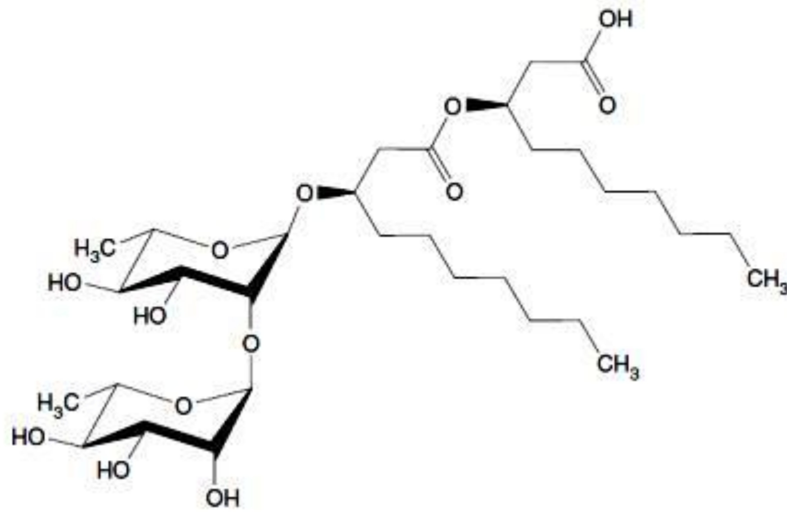

Figure 1- Structure of typical Rhamnolipid produced from *Pseudomonas aeruginosa* (Adapted from Kosaric, 2014)

Trehalolipids (Fig. 2) have disaccharide trehalose acylated at C-6 and C-6' with mycol acids. These mycol acids are long-chained, α-branched 3-hydroxyl fatty acids. Trehalolipids production have been associated with *Mycobacterium* (Goren, 1972), *Arthrobacter* (Suzuki, Tanaka, Matsubara, & Kinoshita, 1969), *Rhondococcus* (Peng, Liu, Wang, & Shao, 2007) species.

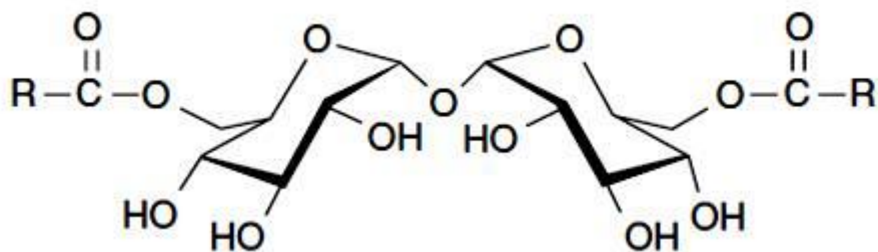

Figure 2- Structure of Trehalolipids (Adapted from Kosaric, 2014)

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Cellobiolipids (Fig. 3) have been reported to be produced by fungus such as *Pseudozyma flocculosa* and *Ustilago maydis*. The disaccharide cellobiose is glycosidically bonded to the terminal hydroxyl group of a 15, 16-dihyoxypalmitin acid (ustiligin acid A), or a 2, 15, 16-trihydroxypalmitin acid (ustilagin acid B). Sometimes the acyl or acetyl groups at 6' and 2'' position may substitute the cellobiose (Bölker, Basse, & Schirawski, 2008).

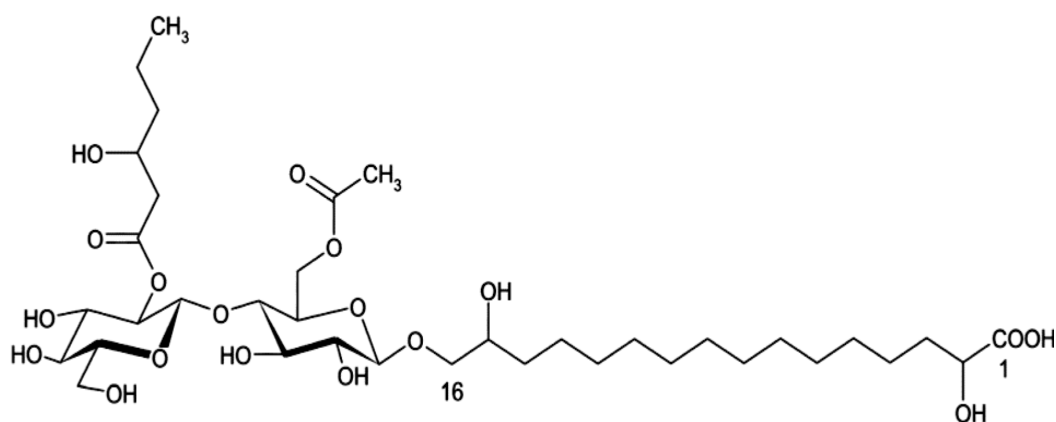

Figure 3-Structure of Cellobiolipids (Adapted from Bölker, Basse, & Schirawski, 2008)

Sophorolipids (Fig. 4) are either in lactonic form or the open acid form of the disaccharide sophorose (Nunez, 2001). These lipids are mainly produced by yeast, *Candida (Stramerella) bombicola and C. apicola*. The hydrophobic part of sophorolipid (SL) consists of a glycosidically bound 17-hydroxyleic acid that is generally linked lactonically with the 4''position of sophorose, as well as acetyl residue in the 6' and 6'' positions.

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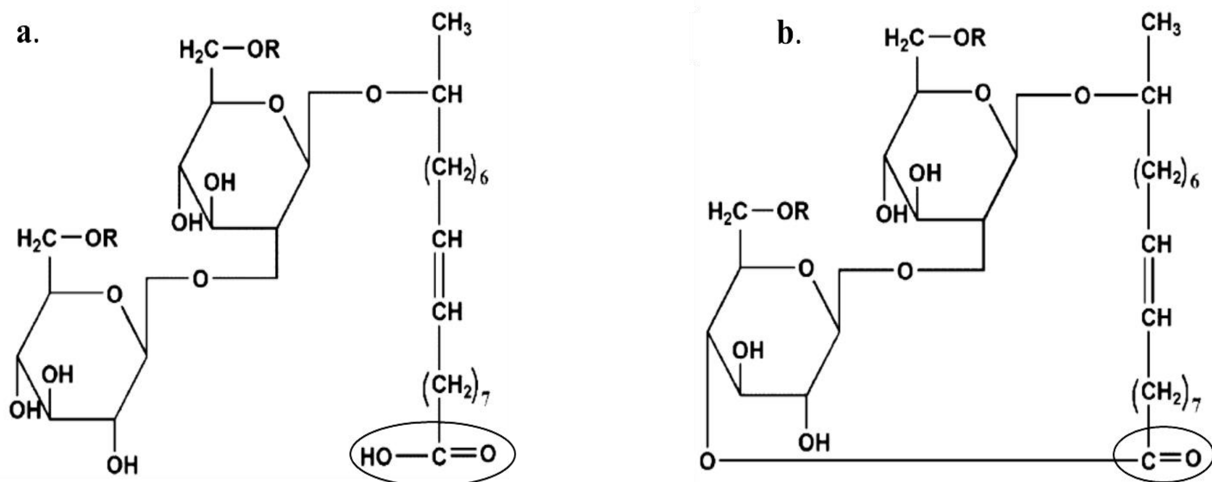

Figure 4- Structures of Sophorolipids, (a) acidic form, and (b) lactonic form (Adapted from Ma, 2011)

1.2. Sophorolipid Production and Characterization

This section will mainly focus on the sophorolipid production using different microorganisms, methodologies used, carbon sources, sophorolipid yield, and characterization reported by different authors.

1.2.1. Sophorolipid Producing Microorganisms, Media and Methods

Sophorolipids which are surface-active compounds, have been reported to be synthesized by different yeast species. Gorin et al. (1961) first reported production of SLs using *Torulopsis magnolia* (currently known as *Candida apicola*), a yeast strain, isolated from the petal portion of sow thistle. This yeast was grown in medium with glucose (20%), yeast extract (YE) (1.25%), and urea (0.2%). These SLs were found to have *2-O-β-D-* glucopyranosyl-*D*-glucopyranose units bonded *β*-glycosidically to *17-L*-hydroxyoctadecanoic or *17-L*-hydroxy-*9*-octadecenoic acids, with partly acetylated sugar moieties.

Jones et al. (1967) reported chemical and spectrometric results of sophorolipids produced by yeast strain *Torulopsis gropengiesseri*. The yeast was grown in a medium consisting of a 10%

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aqueous solution of glucose containing YE (0.5%), urea (0.1 %), potassium dihydrogen phosphate (0.1 %), magnesium sulphate (0.3%) with different supplement like methyl 9-octadecenoate, heptadecane, n-hexadecane, and methyl hexadecanoate.

Tulloch et al. (1968) studied *Rhodotorula* (*Candida) bogoriensis,* the yeast strain, isolated from the leaf surface of the shrub *Randia malleifera*. This yeast was grown in the medium containing glucose (8%), dialyzed YE (0.4%), KH_2PO_4 (0.1 %), and $MgSO_4.7H_2O$ (0.02%) and the SL yield of 0.5-1 g/L was reported by the authors.

Chen et al. (2006) reported SL yield of 320 g/L of using *Wickerhamiella domercqiae* Y_{2A}, a yeast strain isolated from an oil-containing wastewater sample. The authors observed 17-L-(-oxy)-octadecanoic acid 1,4''-lactone 6',6''-diacetate, which is similar major component of the SLs produced by *C. apicola and C. bombicola*.

Li et al. (2013) studied *Wickerhamiella domercqiae var. sophorolipid* CGMCC 1576 strain and observed 47 g/L SL production from the fermentation medium containing glucose-80 g/L, YE -3 g/L, KH_2PO_4 -1 g/L, $Na_2HPO_4.12H_2O$ - 1 g/L and $MgSO_4.7H_2O$ - 0.5 g/L with rapeseed oil or fish oil at 4 % (v/v).

Konishi et al. (2008) reported SL production from *Candida batistae* and quantified it to be 6 g/L after 3 days of cultivation in a medium containing glucose- 50 g/L, olive oil- 50 g/L, YE -1 or 5 g/L, $NaNO_3$- 3 g/L , KH_2PO_4 -0.5 g/L, and $MgSO_4.7H_2O$ - 0.5 g/L. The authors also reported 60% of the SLs produced were acidic.

Spencer et al. (1970) described production of extracellular hydroxyl acid fatty acid sophorosides using a novel yeast species, *Torulopsis bombicola*. The authors reported that the yeast utilized relatively few carbon compounds and formed a mannan having a proton magnetic resonance spectrum similar to the spectra of galactomannans of *Torulopsis apis*, *T. nodaensis*

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and *T. magnolia.* Later, Rosa and Lachance (1998) described *Stramerella (Torulopsis) bombicola* as *Candida bombicola* based on the studies related to 18 S rDNA identity and reproductive function. *C. bombicola* ATCC 22214 has been reported to be a very efficient strain for SL production due to its non-pathogenic nature and high yields, as large as 400 g/L (G. Pekin, Vardar-Sukan, F., & Kosaric, N, 2005).

1.2.2. Sophorolipid Production using C. bombicola

Rau et al. (1996) conducted extended fed-batch fermentation for 200 hours using *C. bombicola* on production medium containing glucose -161 g/L and oelic acid - 45 g/L. The SL production was reported to be 180 g/L. Later, Rau et al. (2001) studied both fed- batch and two-stage continuous process for SL production using rapeseed oil or oleic acid in combination with glucose. For fed-batch process, a production rate of 57 g/L-day was reported by using glucose-100 g/L, refined rapeseed oil- 100 g/L, $(NH_4)_2SO_4$–.4 g/L, KH_2PO_4- 1 g/L, $(MgSO_4)_2$ SO_4, corn-steep liquor- 5 g/L. And for the continuous process, oleic acid, YE and NH_4Cl were used instead of rapeseed oil, corn steep liquor and $(NH_4)_2SO_4$ to achieve a productivity of 76 g/L-day.

Casas and Garcia-Ochoa et al. (1999) conducted fed-batch studies on *C. bombicola* to know the influences of glucose concentration, properties of oil and YE concentration on production of SLs. The researchers used different combinations of the three substrates at different concentrations. First, to study the effect of glucose, the researchers used 100 g/L, 200 g/L and 300 g/L of glucose with sunflower oil and YE concentrations fixed at 100 g/L and 5 g/L. Second, they used 100 g/L of glucose, 5 g/L of YE with 100 g/L of lipid source (included sunflower, olive, corn, coconut or grapeseed or palmitic acid) to study the influence of lipid source on SL production. Third, to study the influence of YE, they used 100 g/L of glucose, 100 g/L of sunflower oil with 1, 2, 5, 10 or 20 g/L of YE. The highest SL production of 75 g/L was

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observed when glucose -100 g/L, sunflower oil-100 g/L, YE-1 g/L were used as production medium.

Ashby et al. (2005) performed fed-batch studies using *C. bombicola* to synthesize SL. The researchers conducted comparative study between pure glycerol and bio-diesel Cco-product stream (BCS) as carbon sources. It has been reported that SL concentration from pure glycerol was 9 g/L and 60 g/L form BCS.

Bhangale et al. (2014) used castor oil (10% w/v) with glucose (10% w/v) or glycerol (15% w/v) for fermentative production of SLs. The authors have reported a SL content of 6.1 g/L from the combination of castor oil and glucose and 2.7 g/L from the combination of castor oil and glycerol.

Pekin et al. (2005) performed fed batch studies using Turkish corn oil and honey. Initially, the authors used a combination of corn oil and glucose for batch run and after the consumption of glucose, corn oil feed with 10% (w/v) of cheap market honey was used. The authors reported SL concentration of more than 400 g/L which still remains as one of the highest reported till date.

Different carbon and other nutrient sources have been used to investigate the SL production and yield by different authors as summarized in Table 1.

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Table 1: An overview of Sophorolipid Production Methods, Production and Yields

Production Method	SL-Production (g/L)	C-Yield (g/g)	Time (h)	Volumetric Production (g/L/h)	References
10% glucose 10% animal fat	120	0.58	68	2.4	Deshpande and Daniels (1995)
10% glucose 10.5% safflower oil	137		192	0.7	Zhou and Kosaric (1993)
10% glucose 10.5% rapeseed oil	160		216	0.7	Zhou and Kosaric (1995)
30% rapeseed oil 10% deproteinized whey concentrate lactose not consumed	280		280	1	Daniel et al. (1998)
Single cell-oil from *Cryptococcus curvatus* grown on deproteinized whey concentrate 40% rapeseed oil added after single cell oil consumption	422		145 410	0.8-1.0	Daniel et al. (1998b)
Turkish corn oil Glucose Honey added when glucose depleted	>400	>0.6	436	>0.9	Pekin et al. (2005)
Fed batch of glucose and octadecane	175	0.33	165	1.1	Davlia et al. (1994)
Fed batch of glucose and rapeseed FAEE (Fatty Acid Ethyl Ester)	340	0.65	165	2.1	
Fed batch of glucose and rapeseed oil	255	0.53	165	1.1	
11% glucose 10% soybean oil fed batch	120	0.6	110	1.1	Lee and Kim (1993)
10% glucose 10% sunflower oil resting cells	120	0.6	200	0.6	Casas and Garcia-Ochoa (1999)

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Table 1 (Continued)

Glucose Oleic acid Fed batch Crystals if limited oil feeding	180		200	0.9	Rau et al. (1996)
Glucose Rapeseed oil Fed batch	300	0.68	125	2.4	Rau et al. (2001)
4% oleic acid and 10% glucose at start additional fed batch focus on aeration (50–80 mM O_2/L/h)	350		>week	1-1.5	Guilmanov et al. (2002)
Glucose: 30–40 g/L Rapeseed oil	365		192	1.9	Kim et al. (2009)
Glucose Tallow fatty acid residue	120	0.41	240	0.5	Felse et al. (2007)
Glucose Rapeseed oil High dry cell weight	200		54	3.7	Gao et al. (2012)

1.2.3. Sophorolipid Characterization

Nunez et al. (2001) performed a reversed phase high performance liquid chromatographic method combined with atmospheric pressure chemical ionization mass detection (LC/APCI-MS). The method was developed for the analysis of sophorolipids produced by *C. bombicola* when grown on fatty acid mixtures. The author reported the sophorolipids were mainly lactonic (C16:0, C18:0, C18:1, C18:2 and C18:3) and dominantly C18:0.

Daverey et al. (2009) used partially purified sophorolipids recovered from cheap fermentative medium containing sugarcane molasses, YE, urea, and soybean oil using *C. bombicola*. The SL was first identified and characterized by Fourier-transform infrared (FT-IR) spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) using $CDCl_3$ as the solvent.

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Consequently, they were confirmed by liquid chromatography–mass spectroscopy (LC-MS) The author reported a mixture of both lactonic (C18:0, C18:1, C18:2, and C16:0) and acidic sophorolipids (C18:0, C18:1, C18:2, and C16:0).

Rebeiro et al. (2012) reported the use of high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) and HPLC with electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) methods to separate and identify sophorolipids (SLs) produced by *C.* (*Starmerella) bombicola.* For the production medium used, the author reported a mixture of 18 and 16 carbon hydroxy fatty acid SLs.

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CHAPTER 2

SOPHOROLIPID PRODUCTION FROM BIOMASS HYDROLYSATES

2.1. Introduction

Compared with petroleum based detergents, surfactants produced by microorganisms, biosurfactant, is biodegradable, has low toxicity and is compatible with the environment (Kitamoto, Isoda, & Nakahara, 2002). Several microorganisms are known as glycolipid producers. These microbes include: rhamnolipid-producing pathogenic bacterium *Pseudomonas aerugisosa*, cellobiose lipids-producing fungus *Pseudozyma flocculosa* and *Ustilago maydis* and sophorolipid-producing *Candida (Starmerella) bombicola*. Among these producers, *C. bombicola* has attracted the greatest attention due to: (1) this yeast strain is non-pathogenic; (2) it has high productivity of sophorolipids. The highest one reported is 400 g/L with a yield of 0.6 g sophorolipids/g substrate (G. Pekin, Vardar-Sukan, & Kosaric, 2005); and (3) sophorolipids have been commercially used in several products: Sophoron, a dishwasher detergent by the Japanese company Saraya; defatting sprayers by the Belgian company Ecover; and sophorolipid-based cosmetics by the French company Soliance (Ciesielska et al., 2013).

SLs are a group of compounds that are composed of a sophorose head whose reducing end is connected to a terminally or sub-terminally hydroxylated fatty acid through a beta-glycosidic bond (Ciesielska et al., 2014). Sophorose is a glucose disaccharide with an unusual β-1, 2 bond and can be acetylated on the 6' and/or 6'' positions. The carboxylic end of the fatty acid is either free (acidic or open form) or internally esterified at the 4'' or in some rare cases at the 6' or 6'' position (lactonic or close form). The hydroxyl fatty acid itself is generally either C16 or C18 and can have one or more unsaturated bonds. As a result, SLs produced by *C. bombicola* is a mixture of related molecules with differences in the fatty acid part (chain length, saturation,

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position of hydroxylation) and the lactonization and acetylation pattern. Through using a gradient elution high performance liquid chromatography (HPLC) and evaporative light scattering for detection, over 20 different sophorolipids are identified (A. M. Davila, Marchal, Monin, & Vandecasteele, 1993).

As unpurified and crude mixtures, besides their role as emulsifiers, sophorolipids have been used as a bacteriocidal agent to treat acne, dandruff, and body odors (Mager, Röthlisberger, & Wzgner, 1987). In addition, they are claimed to trigger several beneficial events in terms of protection of hair, skin, thus making them attractive as components in cosmetic, hygienic, and pharmaco-dermatological products (Maingault, 1999) . It is believed that sophorolipids can stimulate the dermal fibroblast metabolism and collagen neosynthesis (Borzeix Conçaix, 1999), inhibit free radical and elastase activity, possess macrophage-activating and fibrinolytic properties, and act as desquamating (i.e., eliminating the surface portion of the protective layer of the epidermis as part of the wound healing process) and depigmenting agents (Hillion, Marchal, Stoltz, & Borzeix, 1998) (Maingault, 1999). Furthermore, crude sophorolipid mixtures are found to trigger cell differentiation instead of cell proliferation and inhibit protein kinase C activity of the human promyelocytic leukemia cell line HL60 (Isoda, Kitamoto, Shinmoto, Matsumura, & Nakahara, 1997).

Production of sophorolipids necessitates two kinds of substrates, a sugar and a lipid source. Over the years, various sugars, such as glucose, sucrose, galactose, and lactose have been tested (Casas & García-Ochoa, 1999; Daniel, Reuss, & Syldatk, 1998; Daverey & Pakshirajan, 2010; Gao, Falkeborg, Xu, & Guo, 2013). A broad range of oil sources, such as: corn, canola, safflower, sunflower, olive, rapeseed, grape seed, palm, coconut, fish, soybean, waste frying oil, and waste streams from biodiesel production has been evaluated (Ashby & Solaiman, 2010;

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Gupta & Prabhune, 2012; Imura et al., 2013; Kim, Yun, & Kim, 2009; H. Li, X. J. Ma, S. Wang, & X. Song, 2013; Otto et al., 1999). The highest SL yield of 400 g/L was obtained when corn oil and honey served as the carbon sources (G. Pekin et al., 2005).

However, the current production of sophorolipids through use of glucose and other pure sugars is not truly sustainable and renewable. Thus, for this study, we aimed to evaluate the feasibility of producing sophorolipids from lignocellulosic feedstocks. We chose sweet sorghum bagasse and corn fiber in light of: 1) their abundance globally; and 2) they are both pre-processed during extraction of juice and oil/carbohydrates/proteins, respectively. Based on our previous study, a simple pretreatment using dilute sulfuric acid (0.5%) at 121ºC for 1 h releases 83.2% and 86.5% of theoretically available sugars out of corn fiber and sorghum bagasse, respectively (Yanna Liang, Kimberly Jarosz, Ashley T Wardlow, Ji Zhang, & Yi Cui, 2014). Considering the complicated compositions of bagasse and corn fiber hydrolysates which contain both sugar and non-sugar compounds, we sought to answer these four questions: 1) Could *C. bombicola* grow on the two hydrolysates? 2) If yes, how would the cells handle five-carbon sugars and the potentially toxic degradation products in the hydrolysates? 3) What would be the yield of sophorolipids on these two hydrolysates? and 4) Could lignocellulosic hydrolysates replace pure glucose in producing sophorolipids?

2.2. Material and Methods

2.2.1. Microorganism and inoculum preparation

Candida bombicola (ATCC 22214) was maintained on agar plates containing (per liter): glucose, 100 g; yeast extract, 10 g; urea, 1g; and agar, 20 g. Colonies were transferred to fresh plates every 6 weeks and were used to start inoculum in the same medium but without agar.

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After 2 days growth at 25°C in a shaking incubator set at 120 rpm, the inoculum culture was used to inoculate different samples as described below.

2.2.2. Pretreatment of sorghum bagasse and corn fiber

The same sorghum bagasse and corn fiber that were studied in our previous works (Choudhary et al.; Yanna Liang, Perez, Goetzelmann, & Trupia, 2014; Y. Liang, Tang, Siddaramu, Choudhary, & Umagiliyage; Y. Liang, Tang, Umagiliyage, et al.; Yesuf J., 2012) were used in this research. Based on National Renewable Energy Laboratory (NREL)'s protocol (Sluiter et al., 2004), the sorghum bagasse contained approximately 36.9 $\pm$ 1.6% cellulose, 17.8 $\pm$ 0.6% hemicellulose, and 19.5 $\pm$ 1.1% lignin (Choudhary et al.). The corn fiber consisted of 17.2 $\pm$ 1.8 % of cellulose, 27.6 $\pm$ 1.7 % of xylan and galactan and 12.1 $\pm$ 0.7 % of arabinan (Yanna Liang, Ivan Perez, et al., 2014). Pretreatment of both biomass materials followed our published procedure exactly (Yanna Liang, Kimberly Jarosz, et al., 2014). Briefly, bagasse or corn fiber (10 g dry weight) mixed with 100 mL 0.5% sulfuric acid was autoclaved at 121°C for 1 h. Following pretreatment, the slurry was centrifuged at 4,000 $\times$ g for 10 min. The resulting liquid phase after pH adjustment to 5.5 through use of NaOH was used in the experiments detailed below.

2.2.3. Fermentation of C. bombicola on cellulosic hydrolysates

C. bombicola inoculum was used to inoculate hydrolysates at two different conditions: 1) only hydrolysates derived from pretreatment without any oil supplements. For this testing condition, corn fiber hydrolysates added with yeast extract (10 g/L) were also studied; 2) hydrolysates supplemented with soybean oil (100 g/L) and pure glucose to ensure a total glucose concentration of 100 g/L. For this second condition, controls with standard medium which contained (per liter): glucose, 100 g; yeast extract, 10 g; urea, 1 g; and soybean oil, 100 g were

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also investigated. All experiments were conducted in Erlenmeyer flasks comprising 10% of the inoculum with a total volume of 40 mL for the first test and 50 mL for the second. All flasks were cultivated in a shaking incubator set at 25ºC and 120 rpm. At different time points, a volume of 1 mL sample was withdrawn and used for observing cells under microscope to check for contamination and measurements of cell dry weight and sophorolipid concentrations.

2.2.4. Analysis

2.2.4.1. Sophorolipid extraction

For 1 mL samples taken at different time points, the whole sample was subjected to extraction using equal volume of ethyl acetate twice. The top (ethyl acetate) layer was then extracted by two volumes of hexane for residual oil. The remaining ethyl acetate fraction was proceeded with solvent evaporation. Sophorolipids were determined gravimetrically. Following ethyl acetate extraction, the water phase in the original samples was centrifuged at 10,000 g for 5 min. The liquid layer was used for HPLC analysis. The pellet was freeze-dried and used to determine cell dry weight.

To measure concentrations of sophorolipids and residual oil in the final-day cultures, the remaining samples at the termination of different experiments were freeze-dried first. The dried material was then extracted by ethyl acetate for five days in a shaking incubator set at 120 rpm. Following this extraction, ethyl acetate was evaporated from the liquid phase through use of a Rotovap. Once ethyl acetate disappeared, hexane was added to extract remaining oil. Both sophorolipids and residual oil were quantified gravimetrically.

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2.2.4.2. Sophorolipid characterization

Detection of sophorolipids was performed by liquid chromatography-tandem quadrupole mass spectrometry (LC-MS/MS). Separation of sophorolipid compounds was achieved using an Agilent 1260 HPLC equipped with a Phenomenex Luna® $C_{18}(2)$ column (100 mm, 2 mm i.d., 3 µm particle diameter). The mobile phases consisted of water spiked with 0.5% formic acid (v/v) (A) and acetonitrile (B). The mobile phase flow rate was 0.2 mL/min and the following gradient was employed: 5% B ramped to 70% B in 3 min (linear) and then ramped to 80% B in 12 min (linear), followed by a linear increase to 95% B in 3 min (held for 8 min) and then a change to 5% in 1 min (held for 3 min). The HPLC was interfaced with a 3200 Q Trap® triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex; Toronto, Canada) equipped with a TurboIonSpray® electrospray ionization (ESI) probe operated in multiple reaction monitoring (MRM) mode for quantitative determination.

2.2.4.3. HPLC

Concentrations of monomeric sugars (glucose, xylose, arabinose) and non-sugar compounds (formic acid, acetic acid, levulinic acid, and 5-hydroxymethyfurfural (5-HMF)) in samples were determined by HPLC (Shimadzu Scientific Instrument, Inc. Columbia, MD, USA) with a refractive index detector. An Aminex HP87 column (5 µm, 30 cm ×4.6 mm, Bio-Rad, CA, USA) was used in an oven set at 50°C. Sulfuric acid at 0.005 M was used as the mobile phase with a flow rate of 0.6 mL/min. The injection volume was 20 µL. Concentrations of the aforementioned chemicals were calculated based on calibration curves built for each compound using external standards. Before HPLC analysis, all samples were filtered through 0.2 µm filters to remove any potential particles.

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2.3. Results and Discussion

Extensive discussions of dilute acid pretreatment of sorghum bagasse and corn fiber have been provided in our previous study (Yanna Liang, Kimberly Jarosz, et al., 2014). According to results revealed by that research, the pretreatment condition of using 0.5% sulfuric acid at 121ºC for 1 h yields the highest sugar recovery compared with pretreatment using 2% sulfuric acid and/or at 134ºC. Thus, for this work, we adopted the best pretreatment scheme but focused on understanding the behavior of *C. bombicola* on cellulosic hydrolysates.

2.3.1. Fermentation of C. bombicola on cellulosic hydrolysates without oil supplementation

As shown by Fig. 5, *C. bombicola* grew well on both hydrolysates tested. By day 8, the cell density was 9.2, 9.8 and 10.8 g/L for hydrolysate derived from sorghum bagasse, corn fiber, and corn fiber with the addition of yeast extraction during fermentation, respectively. We tested the effect of adding yeast extract since it was found that an oleaginous yeast strain, *Cryptococcus curvatus* does not grow on corn fiber hydrolysate alone and requires the presence of a minimal medium which contains yeast extract (Yanna Liang, Ivan Perez, et al., 2014). But for *C. bombicola*, the addition of this nutrient resulted in slightly higher cell yield. The overall cell density for the three tested samples, however, did not differ significantly from a statistical standpoint.

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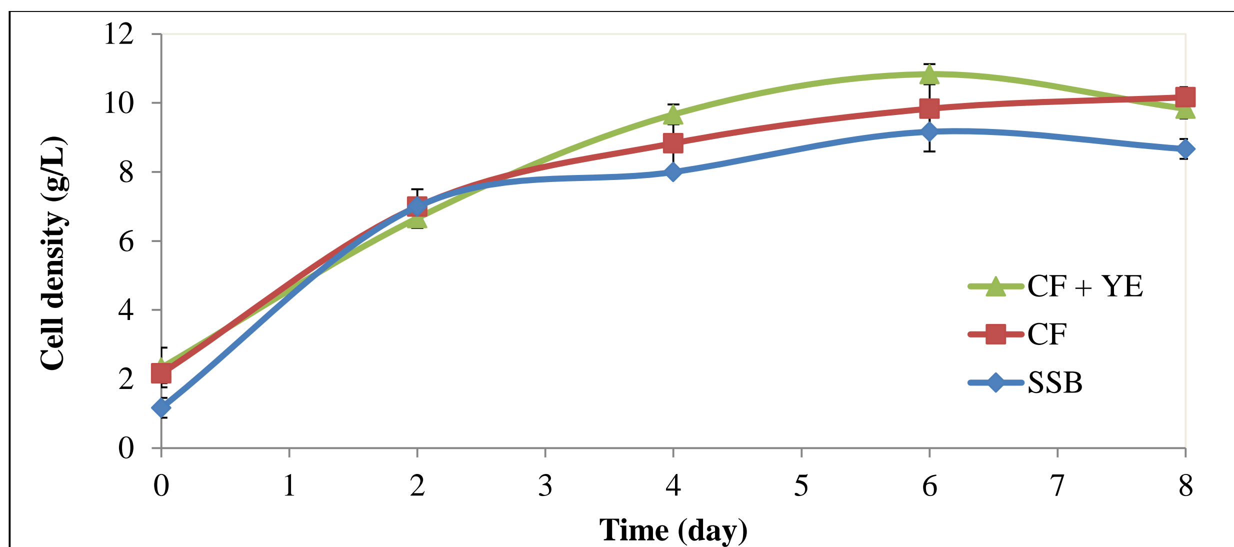

Figure 5: Growth of *C. bombicola* on different hydrolysate samples. Corn fiber (CF) + yeast extract (YE) (Δ), corn fiber (CF) (■), and sweet sorghum bagasse (SSB) (♦).

As cells grew, concentrations of both sugar and non-sugar compounds decreased with time (Fig. 6). The sorghum bagasse hydrolysate had a total monomeric sugar concentration of 42.9 g/L. In the fermentation culture, the total concentration of glucose, xylose and arabinose was 38.6 g/L due to dilution caused by the addition of inoculum at 10% of the final volume. Among the three sugars, glucose was consumed at a rate of 9.1 g/L-day and disappeared after two days (Fig. 6a). Xylose was used simultaneously with glucose, but the utilization rate of 1.8 g/L-day was much lower than that of glucose. Arabinose which had a very small concentration of 0.98 g/L in the cultures was consumed by *C. bombicola*, too. Thus, this is the first study to demonstrate that five carbon sugars, xylose and arabinose can be utilized by this special yeast strain. Besides sugars, the fermentation cultures also contained non-sugar compounds introduced by the bagasse hydrolysate: 5-HMF, 1.6 g/L; acetic acid, 1.0 g/L; levulinic acid, 0.2 g/L; and formic acid, 0.16 g/L (Fig. 6b). Interestingly, in 8 days, *C. bombicola* utilized all of these compounds which are often found to cause significant inhibition to other fermenting

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microorganisms, such as ethanol producing yeast and bacteria (Klinke, Thomsen, & Ahring, 2004; Palmqvist & Hahn-HÃ¤gerdal, 2000; Palmqvist & Hahn-Hägerdal, 2000; Parawira & Tekere, 2011).

The corn fiber hydrolysates after pretreatment had a total sugar concentration of 43.2 g/L which was similar to that of the bagasse hydrolysate. In the fermentation cultures, the total sugar concentration of 38.9 g/L was contributed by glucose, 12.2 g/L; xylose, 19.0 g/L; and arabinose, 7.7 g/L (Fig. 6c). The high concentration of arabinose was due to the high percentage of arabinan in corn fiber. Similar to results demonstrated in Fig. 6a, *C. bombicola* consumed glucose and xylose at the same time with glucose being completely depleted in 2 days. The only non-sugar compound in corn fiber hydrolysate was acetic acid, which was in agreement with our previous study (Yanna Liang, Kimberly Jarosz, et al., 2014). This acid at a concentration of 2.2 g/L was exhausted in 4 days (Fig. 6d).

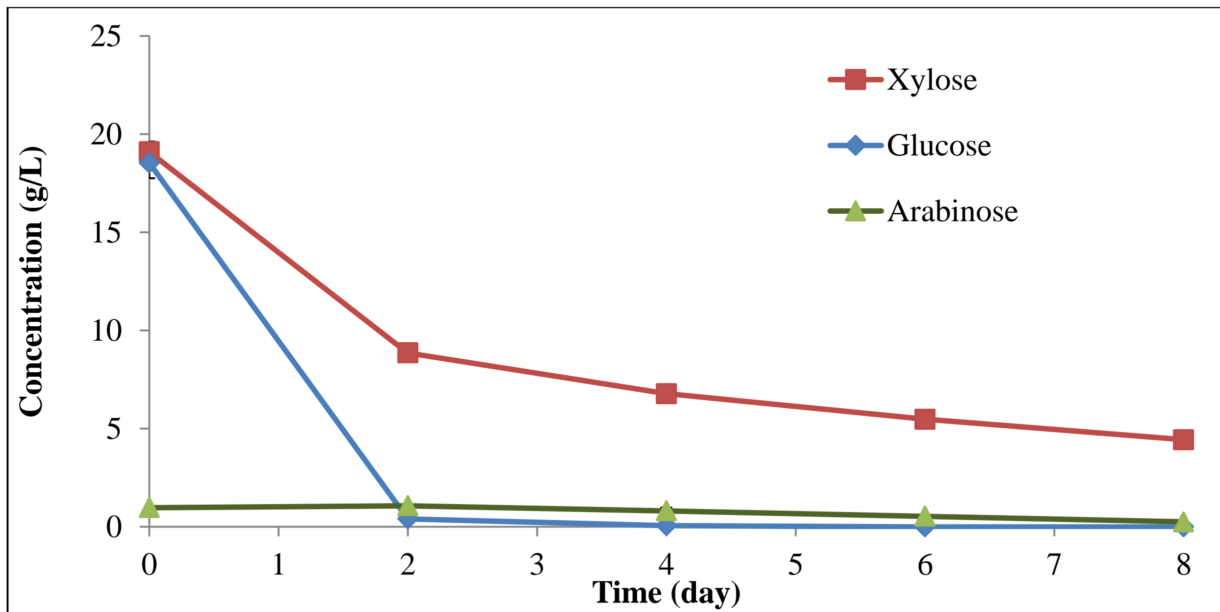

Figure 6 (a): Compound utilization by *C. bombicola* during fermentation. Sorghum bagasse: sugar compounds Xylose (■), glucose (♦), arabinose (Δ).

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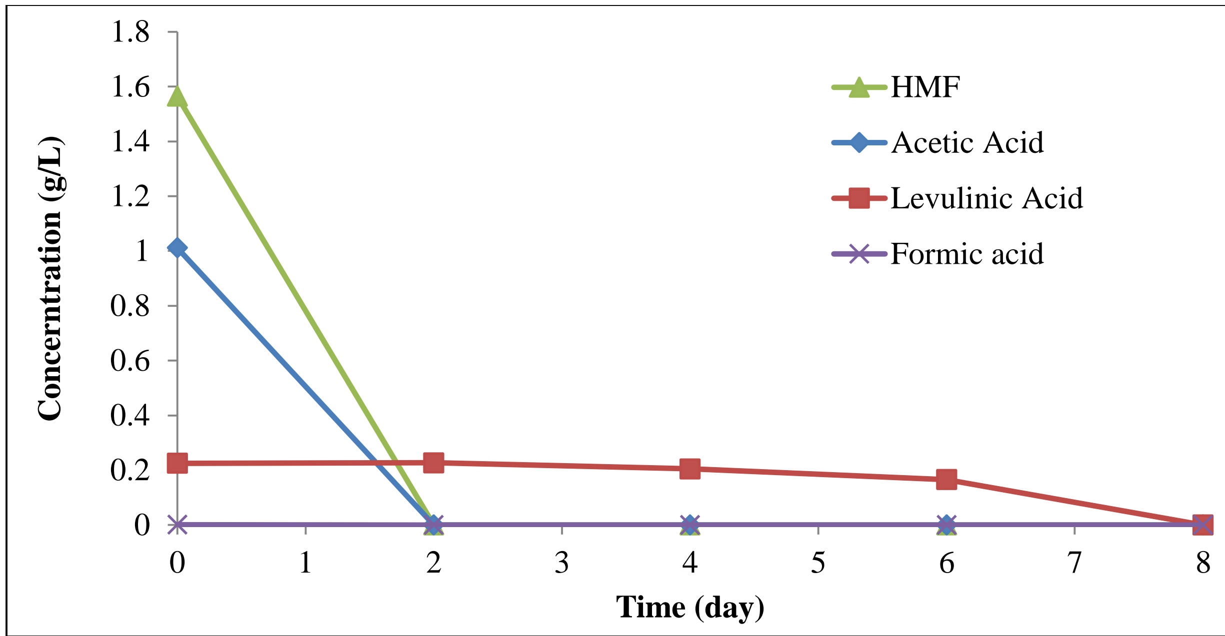

Figure 6 (b): Compound utilization by *C. bombicola* during fermentation. Sorghum bagasse: nonsugar compounds HMF (Δ), acetic acid (♦), levulinic acid (■), formic acid (×).

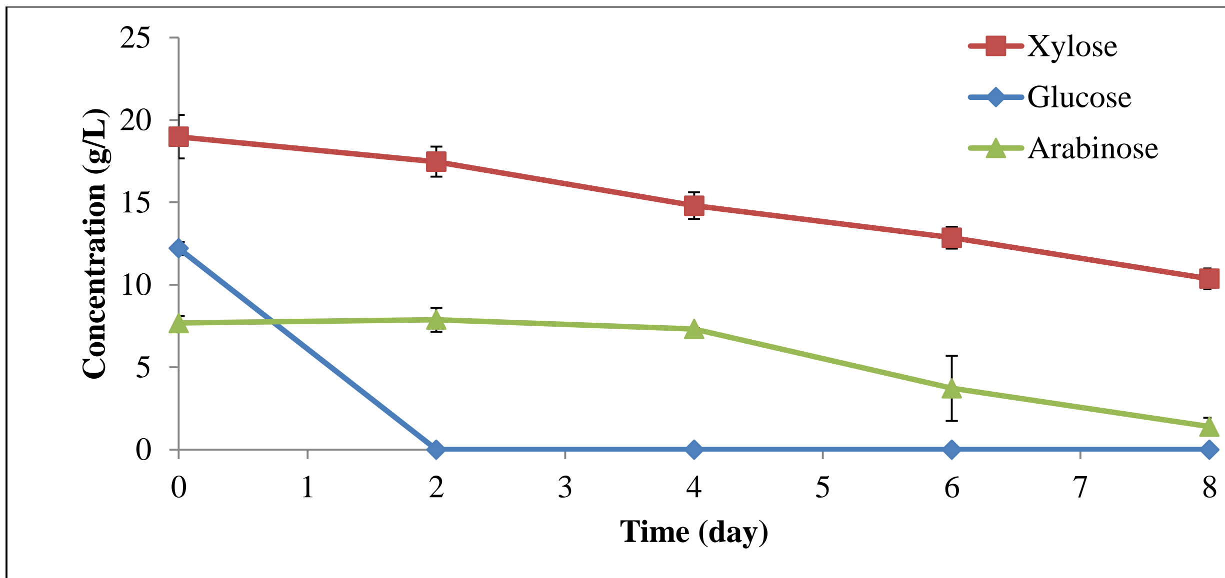

Figure 6 (c): Compound utilization by *C. bombicola* during fermentation. Corn Fiber: sugar compounds Xylose (■), glucose (♦), arabinose (Δ).

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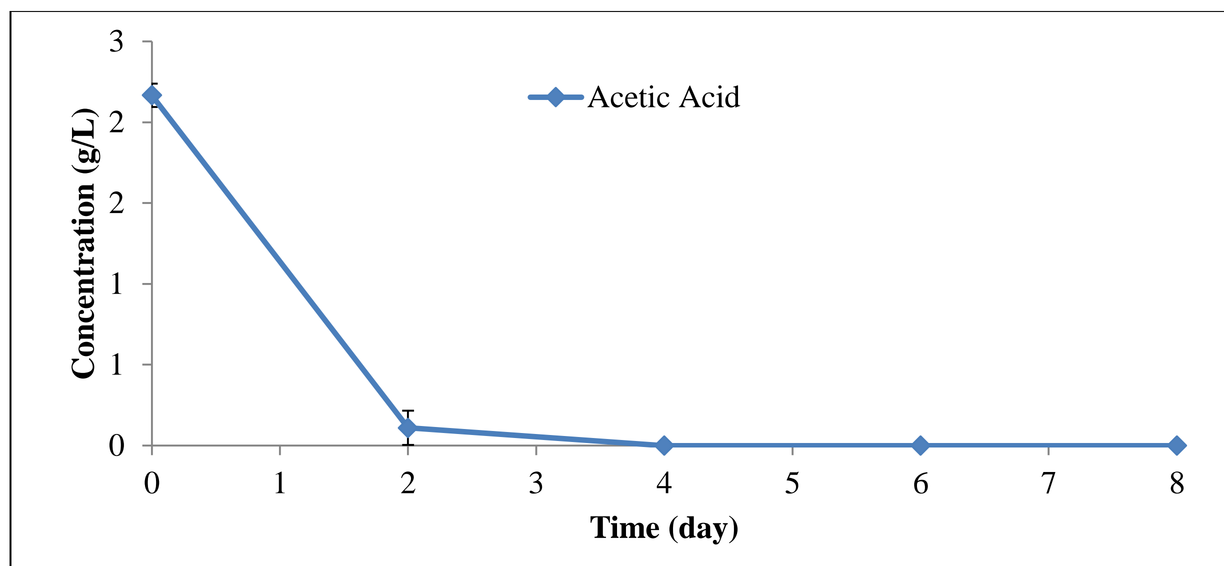

Figure 6 (d): Compound utilization by *C. bombicola* during fermentation. Corn fiber: nonsugar compounds HMF (Δ), acetic acid (♦), levulinic acid (■), formic acid (×).

After 8-day fermentation, sophorolipids were observed in all tested cultures. The concentration was 3.6 ± 0.6 g/L, 1.0 ± 0.1 g/L and 0.4 ± 0.05 g/L for cultures with bagasse hydrolysates, corn fiber hydrolysates and corn fiber hydrolysates supplemented with yeast extract, respectively (Fig. 7). Between hydrolysates from bagasse and corn fiber, sorghum bagasse gave the highest yield of sophorolipids. Adding yeast extract did not promote sophorolipids production although it led to a slightly better cell growth (Fig. 5). These results are consistent with previous observations that: 1) *C. bombicola* does produce sophorolipids on sugars only and synthesizes fatty acids through the *de novo* synthetic pathway; and 2) the yield of sophorolipids is low under this condition (Van Bogaert et al., 2007).

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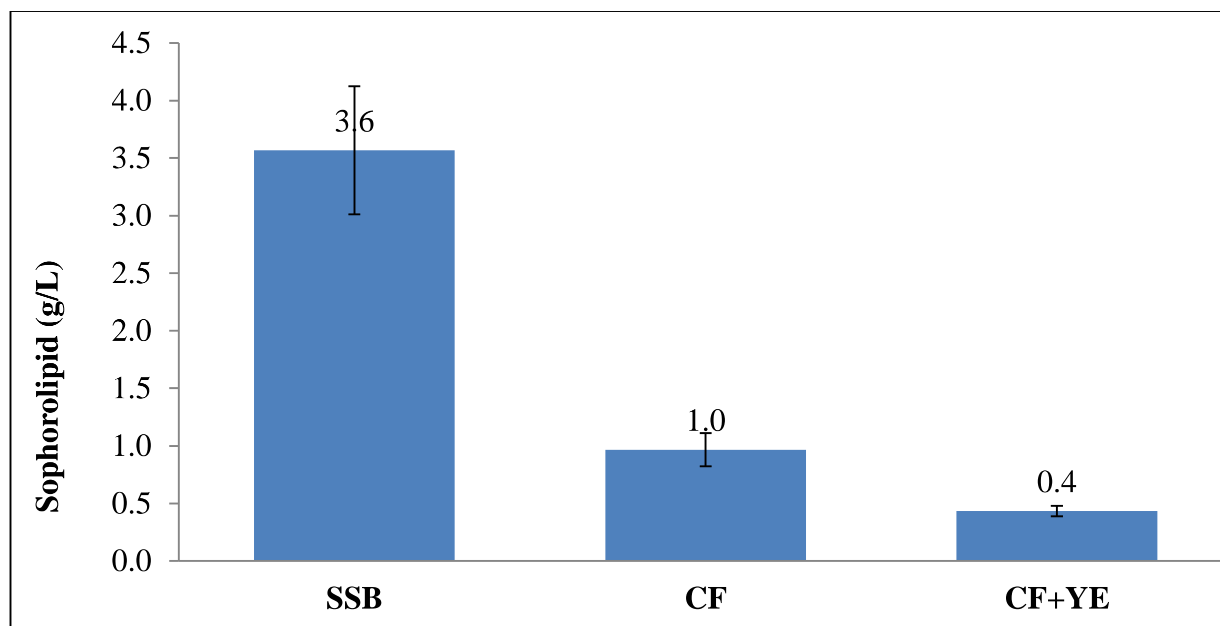

Figure 7: Concentration of sophorolipids after an 8-day fermentation.

2.3.2. Fermentation of C. bombicola on cellulosic hydrolysates supplemented with soybean oil

To increase sophorolipids yield, a hydrophobic carbon source, such as: oil, fatty acid, or alkane needs to be supplemented. Throughout literature, the majority of researchers use an oil source at 100 g/L and a hydrophilic carbon sources, generally glucose, at the same concentration. Besides these two carbon sources, yeast extract (10 g/L) and urea (1 g/L) are also included in the fermenting medium. In order to evaluate whether the complex nature of cellulosic hydrolysates, especially the degradation products and many other unidentified compounds by HPLC can inhibit sophorolipid production, we tested the performance of *C. bombicola* in three setups as described above. All samples had a total glucose and soybean oil concentration of 100 g/L. For the standard medium which served as controls, yeast extract (10 g/L) and urea (1 g/L) were also added.

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By the end of the 10-day fermentation, cell density, sophorolipid concentration and residual oil concentration were determined. As indicated by Fig. 8: 1) cultures with bagasse hydrolysates, corn fiber hydrolysates and standard medium had a cell content of 7.7 $\pm$ 0.1 g/L; 7.9 $\pm$ 0.2 g/L; and 8.9 $\pm$ 0.2 g/L, respectively. Time series data for cell growth revealed no statistically significant difference among the samples (Fig. 9a); 2) the sophorolipid concentration for the three setups in the same order was 84.6 $\pm$ 5.6 g/L; 15.6 $\pm$ 0.7 g/L; and 24.1 $\pm$ 0.1 g/L and 3) the residual oil concentration was 52.3 $\pm$ 1.5 g/L; 41.0 $\pm$ 1.8 g/L; and 58.0 $\pm$ 3.0 g/L for the three sets of samples. Thus, apparently, the bagasse hydrolysates led to the highest production of sophorolipids while corn fiber hydrolysates resulted in a less yield of sophorolipids compared to those of the controls.

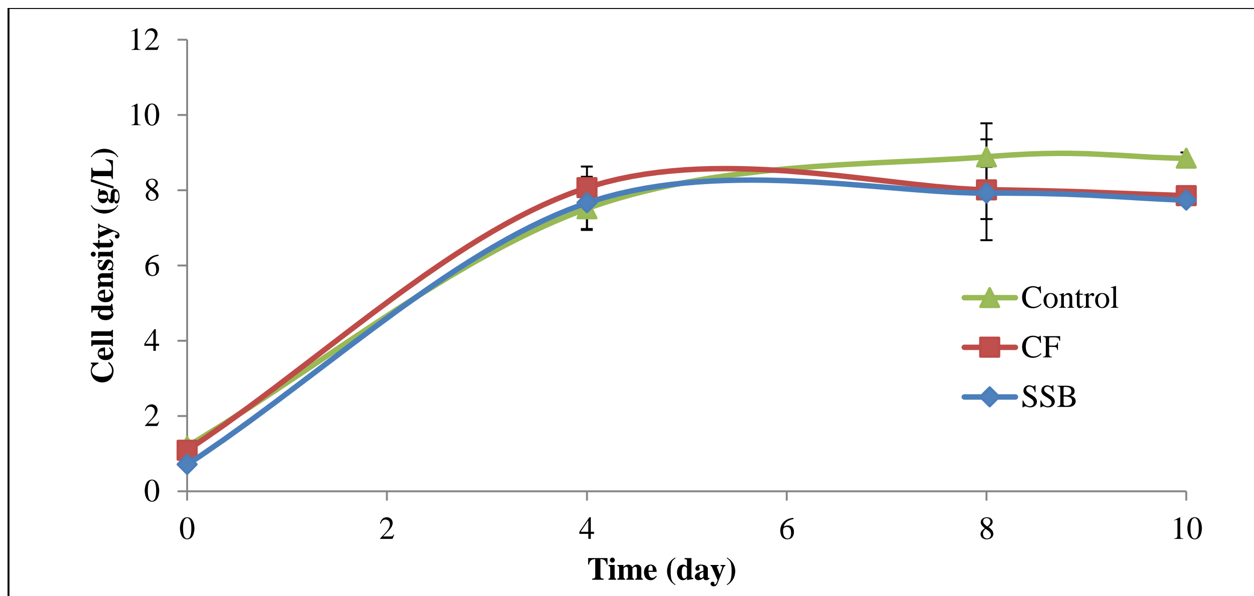

Figure 8 (a): Time series of cell growth. Control (Δ), corn fiber (CF) (■), sweet sorghum bagasse (SSB) (♦).

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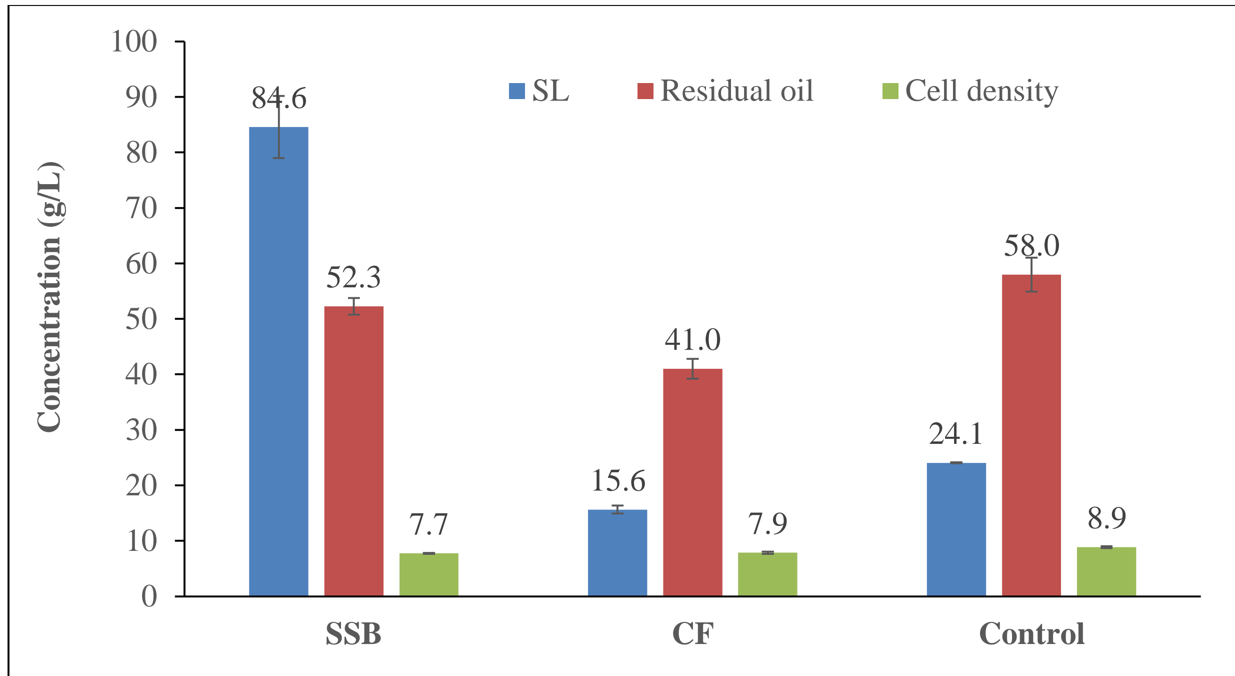

Figure 8 (b): Concentrations of sophorolipids, and residual oil after a 10-day fermentation.

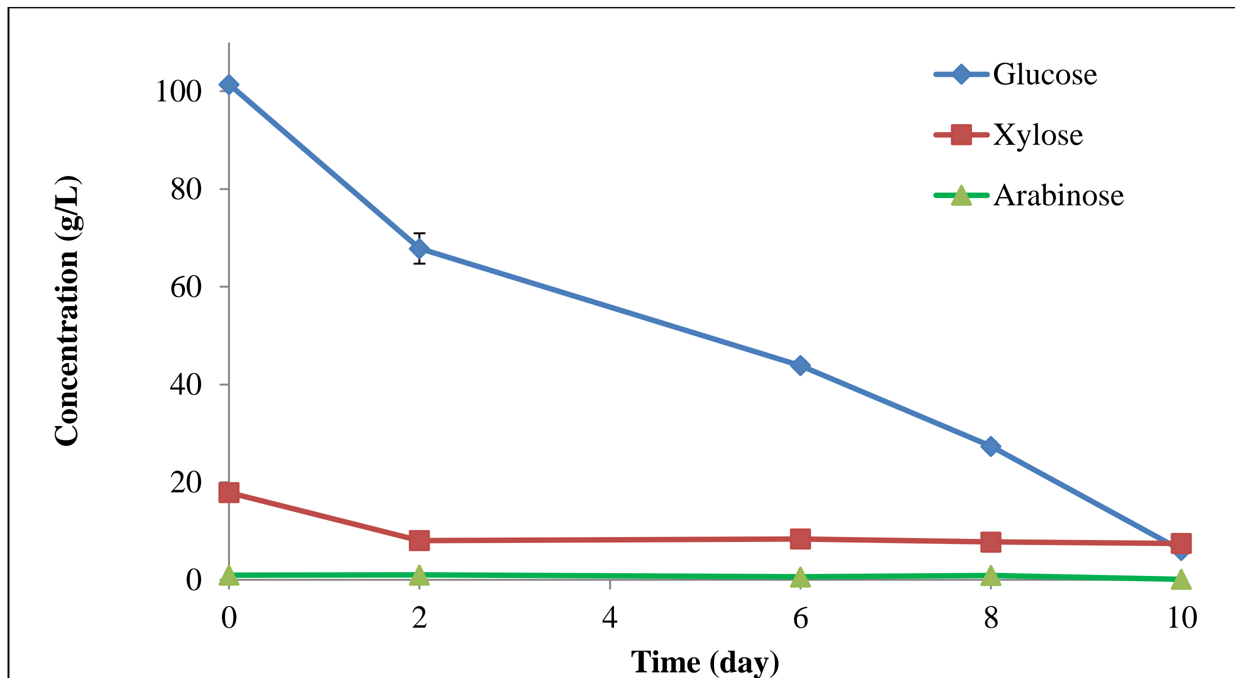

Figure 9 (a): Sugar utilization during fermentation. (a); glucose (♦), xylose (■), arabinose (Δ).

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Closer look of the samples containing bagasse hydrolysates demonstrated that: 1) *C. bombicola* again utilized all three sugars simultaneously (Fig. 9b). Glucose was consumed at a rate of 9.5 g/L-day which was higher than 9.1 g/L-day we observed previously. Utilization rates of xylose and arabinose as 1.04 and 0.08 g/L-day were much lower than our previous results described above. This could be due to the presence of glucose at high concentrations; 2) accumulation of sophorolipids did not start until cells enter stationary phase which was after day 2 (Fig. 9c). This agrees with other researchers' report that sophorolipid production is not growth associated (Rau, Hammen, Heckmann, Wray, & Lang, 2001). During this phase, cell density basically remained the same while sophorolipids concentration increased dramatically. Thus, it can be assumed that all sugars consumed during this phase was used to synthesize sophorolipids.

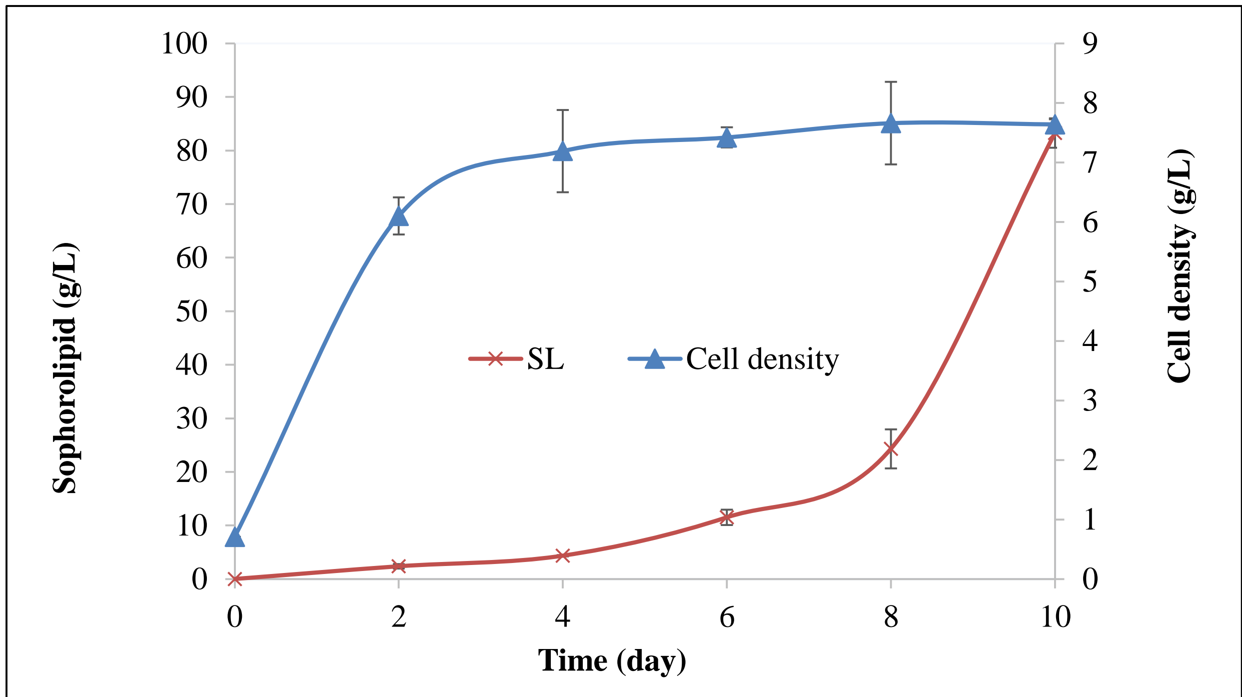

Figure 9(b): SL production starts when cells enter stationary phase. sophorolipid (SL) (■), cell density (Δ).

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Although we were able to obtain time series curves for cell growth, sugar utilization and sophorolipids accumulation, it was impossible to generate such a curve for soybean oil in the cultures. This impossibility lies in the fact that soybean oil tends to float at the surface of the cultures and it was difficult to withdraw representative samples at different time points. Thus, only residual oil concentrations in the final-day samples can be used for analysis. Based on these concentrations and considering all sugars consumed, the yield of sophorolipids was 0.55 g/g carbon (sugars plus oil) for cultures with bagasse hydrolysates. Higher yields of 0.64 (Anne-Marie Davila, Marchal, & Vandecasteele, 1992), 0.68 (A-M Davila, Marchal, & Vandecasteele, 1997) and 0.73 (Rau et al., 2001) g/g substrate have been reported for *C. bombicola*. But it needs to be noted that all of these numbers are from studies conducted at fed-batch fermentation mode which aims to enhance production of sophorolipids. In light of the fact that higher sophorolipids were produced from bagasse hydrolysates than those from pure glucose, we expect a much higher production when the fermentation process is optimized.

Sophorolipids extracted from cultures containing bagasse hydrolysates were analyzed by HPLC-MS-MS. As shown by Fig. 10, the recovered sophorolipids was a mixture and consisted of acidic sophorolipids with C18 fatty acid chains and lactonic sophorolipids with either C16 or C18 fatty acids. Detailed characterization of each sophorolipid molecule is ongoing.

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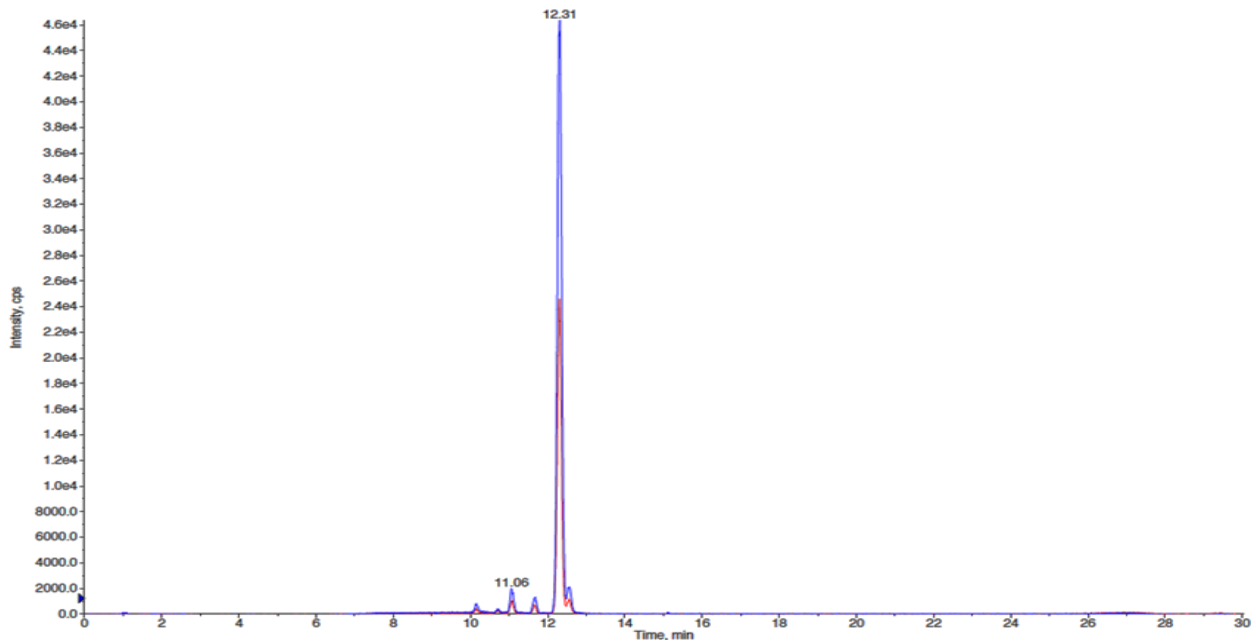

Figure 10 (a) Sophorolipid identification: Standard SL. The LC-MS/MS chromatogram of the 1′,4″-sophorolactone 6′,6″-diacetate, a reference standard from Sigma.

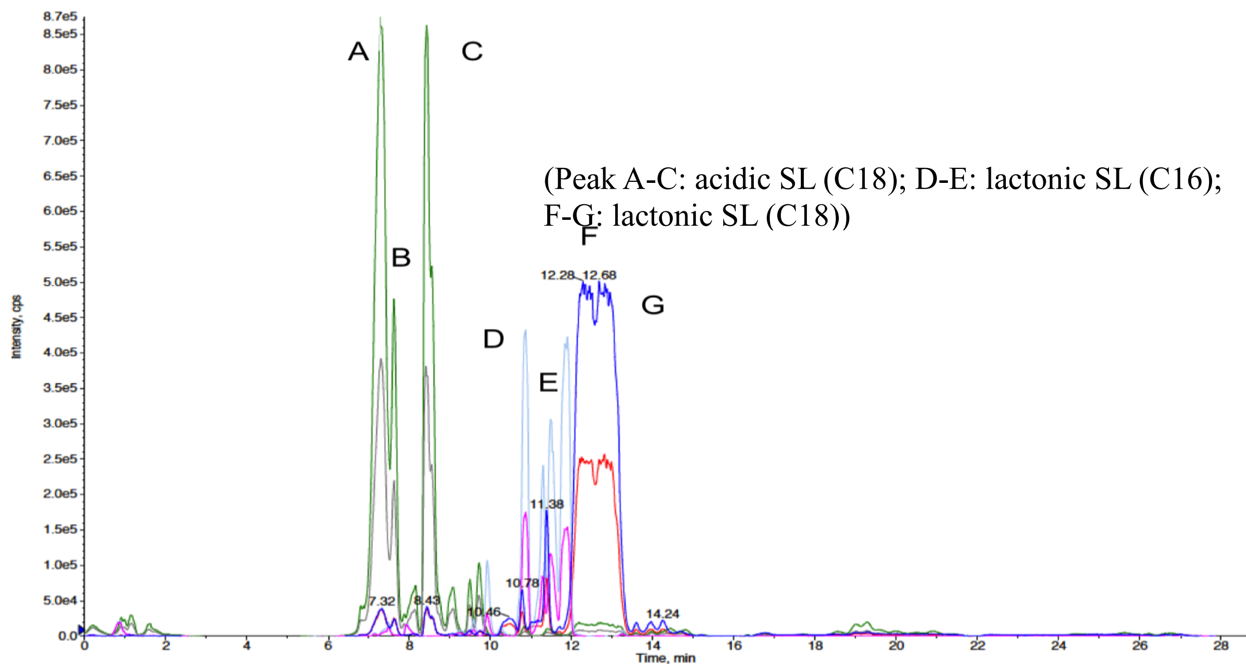

Figure 10 (b): Sophorolipid identification: SL profile. The LC-MS/MS chromatogram of sophorolipids extracted from cultures with bagasse hydrolysates.

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2.4. Conclusion

This is the first study to demonstrate that sophorolipids can be produced from sorghum bagasse and corn fiber through the process of pretreatment and fermentation. *C. bombicola* was able to consume all monomeric sugars and non-sugar compounds. Cultures with bagasse hydrolysates had the highest concentration of sophorolipids of 84.6 g/L, which was higher than those from cultures containing corn fiber hydrolysates or a standard medium. The overall yield of sophorolipids on bagasse hydrolysates was 0.55 g/g carbon. Results from this study open the door for producing a biosurfactant from truly sustainable resources and warrant further efforts to increase the yield of sophorolipids from lignocellulosic biomass.

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CHAPTER 3

SWEET SORGHUM BAGASSE AND CORN STOVER SERVING AS SUBSTRATES FOR PRODUCING SOPHOROLIPIDS

3.1. Introduction

Biosurfactants, synthesized naturally by microorganisms, have many advantages over those that are chemically synthesized from petrochemical or oleo-chemical sources (Desai, 1997). These advantages include low or no toxicity and biodegradability. In addition, if biosurfactants are produced from renewable resources, then these bioproducts are sustainable and environmentally friendly. Over the years, biosurfactants with different structures, such as: glycolipids, lipopeptides, lipoproteins, fatty acids, phospholipids, neutral lipids, polymeric biosurfactants, and particulate biosurfactants have been identified from various microbial cultures. Among these, glycolipids are commonly isolated and widely studied. Glycolipids include three subgroups: sophorolipids, rhamnolipids, and trehalolipids (Gautam & Tyagi, 2006).

Sophorolipids are a group of compounds that comprise two components: a sophorose head (a dimeric sugar residue) and a hydroxylated fatty acid (Ciesielska et al., 2014) and are synthesized by several non-pathogenic yeast strains, *Candida bombicola*, *C. apicola*, *C. batistae*, *Wickerhamiella domericqiae* and *Rhodotorula bogoriensis* (Morya, Park, Kim, Jeon, & Kim, 2013). As surface active biocompounds, sophorolipids have replaced part of their chemical counterparts in household/laundry detergents and are consumed at around 10 million tons per year (Van Bogaert et al., 2007). In addition, due to their structural diversity and related physicochemical variability, sophorolipids have been used in the fields of cosmetic, hygienic, medical and pharmaco-dermatological (Hillion et al., 1998) (Maingault, 1999) (Isoda et al., 1997). Furthermore, through chemical reactions, such as acid hydrolysis, sophorolipids can be

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converted to inducers of cellulases (Huang, 2013, 2014) which have broad applications in producing biofuels from renewable biomass feedstocks.

Conventionally, sophorolipids are produced by yeast strains grown on both sugar and oil feedstocks. The highest yield was reported to be 422 g/L when deproteinized whey and rapeseed oil were used as substrates for *C. bombicola* (Daniel et al., 1998). The second highest concentration of 400 g/L was obtained when the same yeast strain was cultivated on honey and corn oil in a fed-batch mode (G. Pekin et al., 2005). To make sophorolipids' production process even greener and more sustainable, we have investigated the feasibility of producing these biosurfactants from hydrolysates developed from sweet sorghum bagasse and corn fiber (Samad, Zhang, Chen, & Liang, 2015). Both hydrolysates, although contain a wide variety of chemicals that are considered to be inhibitory to microbial fermentation, supported growth of *C. bombicola*. Significant production of sophorolipids by this yeast strain was observed when soybean oil was provided.

For the purpose of lowering the production cost, in this study, we further studied the yield of sophorolipids when waste restaurant oil or yellow grease was supplemented as a source of oil. The reasons for selecting yellow grease are two-fold: 1) yellow grease, compared to soybean oil or other oil sources, is much cheaper. The current market price is around $1/gallon while soybean oil costs approximately three times higher; and 2) according to US EPA's estimate, US hotels and restaurants generate 3 billion gallons (11.4 billion L) of waste oil per year (www.epa.gov/region9/waste/biodiesel/questions.html). Thus, the volume of yellow grease is great enough for producing sophorolipids through yeast fermentation. Certainly, yellow grease can be used to generate biodiesel or other kinds of biofuels. But since sophorolipids have much

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higher market values than those of biofuels, use of yellow grease for producing sophorolipids would be much more attractive from the perspectives of investment.

Besides evaluating sophorolipids yield on hydrolysates of sweet sorghum bagasse and yellow grease, we also aimed to study sophorolipids production from hydrolysates derived from corn stover. This is to consider the abundance of corn stover as a potential biomass feedstock. More importantly, corn stover hydrolysates developed from a novel process was used for this work. To the best of our knowledge, no studies have been performed on using these hydrolysates for sophorolipid production.

3.2. Material and Methods

3.2.1. Microorganism and Inoculum Preparation

C. bombicola (ATCC 22214) was routinely maintained on agar plates at 4°C. The plates contained (per liter): glucose, 100 g; yeast extract, 10 g; urea, 1 g; and agar, 20 g. Yeast colonies were transferred to fresh plates every 6 weeks. To start an inoculum, colonies were introduced to an autoclaved medium with the same composition as the agar plates but without the presence of agar. The inoculum culture was incubated at 25 °C in a shaker set at 120 rpm for 2 days before use.

3.2.2. Pretreatment of Sorghum Bagasse

Bagasse of sweet sorghum which was studied in our previous works (Choudhary et al.; Yanna Liang, Ivan Perez, et al., 2014; Y. Liang, Tang, Siddaramu, et al.; Y. Liang, Tang, Umagiliyage, et al.; Yesuf J., 2012) was used in this study. But instead of using a 10% solid loading during acid pretreatment, a bagasse loading of 20% was adopted for the purpose of increasing total sugar concentrations in the derived hydrolysates. Briefly, pretreatment of

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sorghum bagasse was conducted by mixing 20 g bagasse (dry weight) with 100 mL of 0.5 % sulfuric acid followed by autoclaving at 121 °C for 1 h. Following pretreatment, the slurry was centrifuged at 4,000×g for 10 min. The acidic supernatant thereafter was adjusted to a pH of 6.0 using NaOH (Y. Liang, K. Jarosz, A. T. Wardlow, J. Zhang, & Y. Cui, 2014) and was used in the following studies.

3.2.3. Pretreatment of Corn Stover

Deacetylated Disc Refined (DDR) corn stover hydrolysate was provided by National Renewable Research Laboratory (NREL). In short, corn stover harvested at Hurley County (South Dakota, United States) was first knife milled to pass through a 19 mm (0.75 inch) round screen (Chen, 2014). The screened corn stover was then added to a 1,900-L paddle mixer along with a dilute 0.1 M sodium hydroxide solution to achieve a solid slurry of 8% (w/w). After the slurry was heated to 80 °C and held for 2 h, the liquor was allowed to drain and the solid was rinsed and pumped to a continuous screw press (Vincent Corp. Model CP10, Tampa, Florida, United States) for dewatering to between 45 and 50% (w/w) of total solids. The deacetylated corn stover was then mechanically refined in a commercial scale disc refiner (Sprout model 401 36-inch). Corn stover particles after the deacetylation and disc refining processes were hydrolyzed by cellulase and hemicellulase (Cellic® CTec3 and HTec3, Novozymes) at 48 ºC for 96 h. The liquid portion after enzymatic hydrolysis was used in yeast fermentation as described below.

3.2.4. Fermentation of C. bombicola on Cellulosic Hydrolysates

Regarding hydrolysates derived from sorghum bagasse, yellow grease at 10 g/L, 40 g/ L or 60 g/L was supplemented. The yellow grease used was from the cafeteria at Southern Illinois University Carbondale, where soybean oil was used for frying. For each yellow grease

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concentration tested, two replicate cultures were established. Each culture included a certain volume of yellow grease, 10% of yeast inoculum and a different volume of bagasse hydrolysates to ensure that the total volume in each 250-mL Erlenmeyer flask was 50 mL. Two control cultures containing (per liter): glucose, 100 g; yeast extract, 10 g; urea, 1 g; and yellow grease, 40 g were also set up at the same time. In terms of flasks containing corn stover hydrolysates, soybean oil at 10 g/L, 20 g/ L or 40 g/L was added.

All flasks were cultivated in a shaking incubator set at 25 °C and 120 rpm. At different time points, a 1-mL sample was withdrawn from each culture for checking possible contamination and for measuring cell dry weight and sophorolipid concentrations.

3.2.5. Analysis

3.2.5.1. Sophorolipids and Residual Oil Extraction

Two different volumes, the 1-mL samples and the remaining volume of the last day cultures, were used for extractions of sophorolipids and residual oil through liquid-liquid extraction. For both types of samples, equal volume of ethyl acetate as that of the sample was used twice for sophorolipids extraction. Ethyl acetate layer at the top was then subjected to extraction by using two volumes of hexane for residual oil. The remaining ethyl acetate fraction and the hexane-soluble were proceeded with solvent evaporation. The dry weights of sophorolipids and residual oil were determined gravimetrically. Subsequently after ethyl acetate extraction, the water phase in the original samples was centrifuged at 10,000g for 5 min. The resulting liquid layer was used for HPLC analysis. The pellet was used for determining cell dry weight through drying in an oven at 105 °C overnight.

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3.2.5.2. Yellow grease characterization

Compared to soybean oil, the yellow grease looked fairly turbid. To determine the true oil content, yellow grease was extracted with equal volume of hexane for three times. After phase separation, the hexane layer was evaporated to dryness in a Rotavap. Weight of the residue was used to calculate oil content. The remaining non-hexane soluble was centrifuged at 5,000 g for 10 min. The liquid portion was weighed and dried to get moisture content while the solid portion was weighed to obtain solid content. In addition, representative samples of the yellow grease and soybean oil was reacted with methanol and sulfuric acid for transesterification following procedures reported previously. The resulting FAME was analyzed by GC (Y. Liang, Sarkany, et al.).

3.2.5.3. High-Performance Liquid Chromatography

All samples were filtered through hydrophilic 0.2-μm filters beforehand to eliminate any potential particles. The HPLC (Shimadzu Scientific Instrument, Inc. Columbia, MD, USA) with a refractive index detector was used to determine the concentrations of monomeric sugars (glucose, xylose, and arabinose) and non-sugar compounds (formic acid, acetic acid, levulinic acid, and 5-hydroxymethylfurfural (5-HMF), furfural) in samples. Chemical separation was carried out using an Aminex HP87 column (5 μm, 30 cm×4.6 mm, Bio-Rad, CA, USA) set at 50 °C. Freshly prepared 0.005M sulfuric acid was used as the mobile phase with a flow rate of 0.6 mL/min. A 20 μL sample injection volume was used. All concentrations of the chemicals under investigation were calculated based on calibration curves established for each compound using external standards.

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3.3. Results and Discussion

3.3.1. Yellow grease and soybean oil

The yellow grease used in this study had a density of 914.7 g/mL. Through hexane extraction, it was found that the yellow grease contained 99.4% of hexane-soluble, 0.13% of solid and 0.46% of moisture. This yellow grease sample had basically the same fatty acid profile as that of the soybean oil used (Table 2). For both oil samples, C18:1n7 (cis-vaccenic acid), C16:0 (palmitic acid), C18:3n4 (isomer of linolenic acid) and C18:0 (stearic acid) were the dominant fatty acids. Thus, apparently, even though after repeated use and the resulting yellow grease looked cloudy, the process of frying did not change the fatty acid composition of the oil. The composition of yellow grease used in this study is different from those reported (Shah, 2007). This could be due the fact that different oil was used for frying.

Table 2: Fatty acid profile of soybean oil and yellow grease used in this study

Fatty acids	Soybean oil		Yellow grease	
	Average	STDEV	Average	STDEV
C18:1n7	56.15	4.55	56.44	4.65
C16:0	24.85	1.01	24.32	1.94
C18:3n4	12.80	0.75	12.25	1.02
C18:0	4.46	6.31	5.38	7.61
C20:1n9	0.48	0.00	0.46	0.03
C20:5n3	0.22	0.01	0.20	0.00
C14:0	0.20	0.04	0.21	0.02
C18:4n3	0.19	0.13	0.06	0.05
C22:5n3	0.17	0.13	0.20	0.00
C22:6n3	0.08	0.01	0.09	0.00
C20:4n3	0.08	0.01	0.11	0.02
C20:4n6	0.08	0.05	0.04	0.01
C18:3n3	0.04	0.02	0.04	0.00

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3.3.2. Fermentation using C. bombicola on sorghum bagasse hydrolysates with yellow grease supplementation

As described above, the sorghum bagasse hydrolysate used in this study was derived from pretreating bagasse using 0.5% sulfuric acid at 20% solid loading for 1 h. The resulting hydrolysate contained 36.4 g/L of glucose, 37.9 g/L of xylose and 1.7 g/L of arabinose with a total sugar concentration of 76.1 g/L. These concentrations were almost twice of those obtained when pretreating SSB at a 10% solid loading with other conditions remaining the same (Samad et al., 2015).

When yellow grease was supplemented at three concentrations, 10, 40 or 60 g/L, *C. bombicola* grew well (Fig. 11). The log phase typically lasted for two days and was followed by a long stationary phase. For all three yellow grease doses tested, glucose was consumed rapidly in four days. Xylose and arabinose were both utilized by the cells, but at much slower rates compared with that of glucose. As we reported before, all monosaccharides were consumed simultaneously. At later stages, sophorolipids were produced. The biggest jump took place between day 12 and day 14 for all cultures.

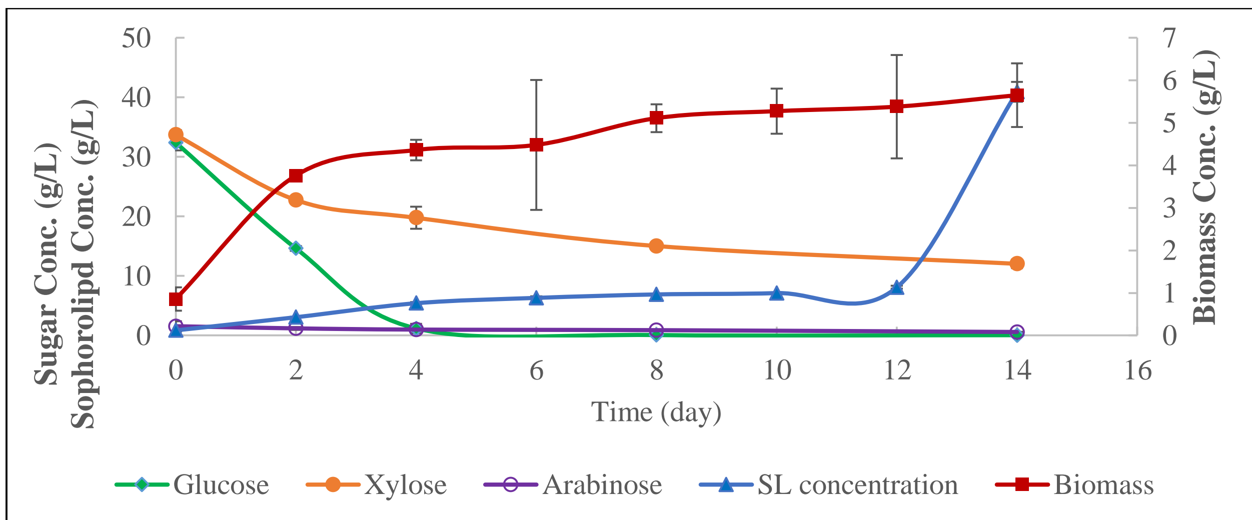

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Figure 11 (a): Growth profile on SSB hydrolysate with yellow grease at 10 g/L

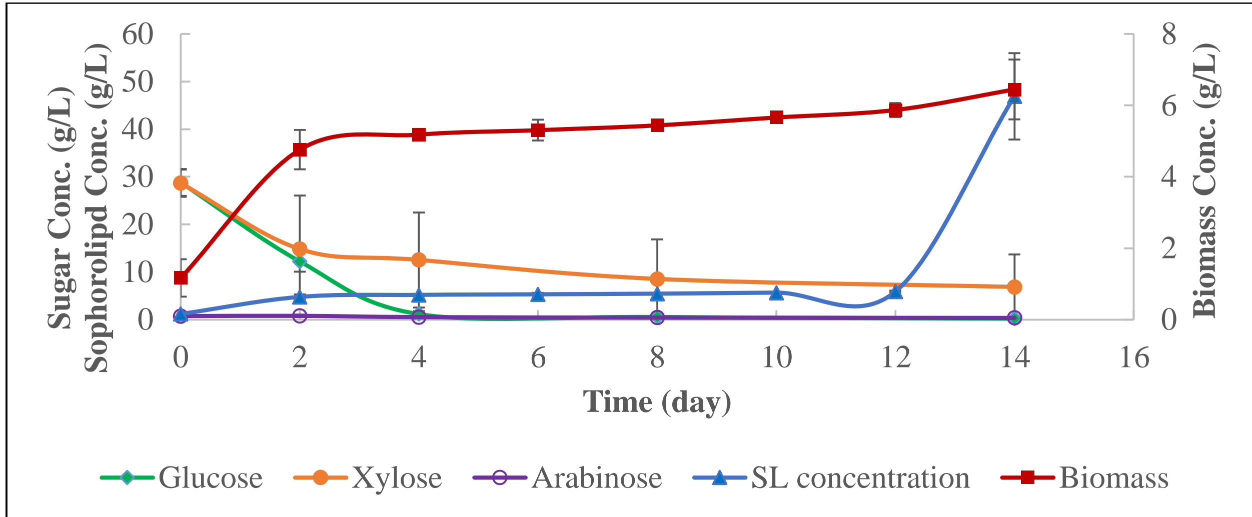

Figure 11(b): Growth profile on SSB hydrolysate with yellow grease at 40 g/L

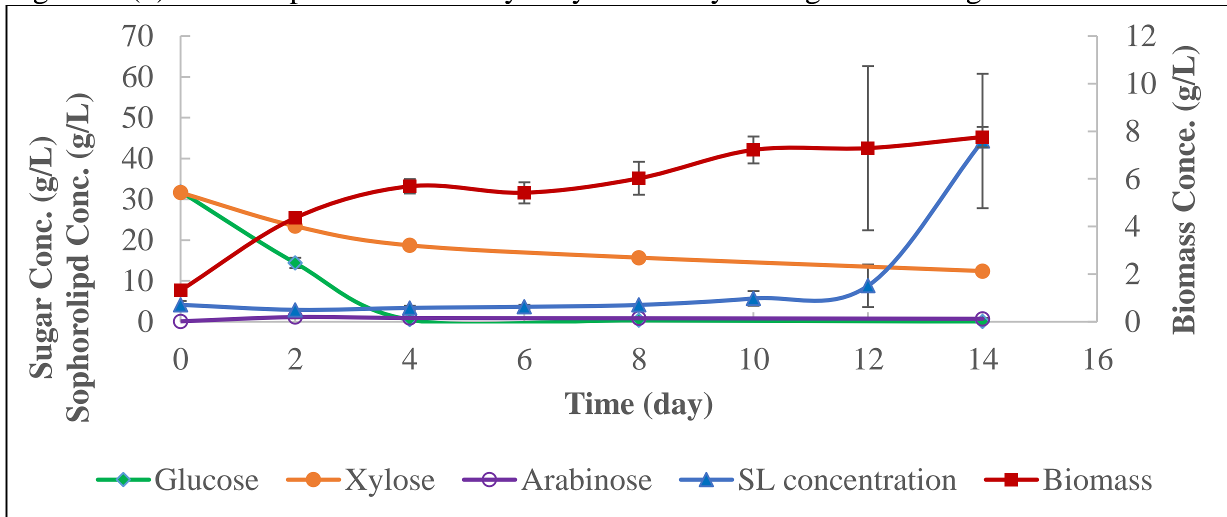

Figure 11 (c): Growth profile on SSB hydrolysate with yellow grease at 60 g/L

With 10, 40 and 60 g/L of yellow grease, the final day cell density was 5.7 ± 0.8 g/L, 6.4 ± 0.8 g/L and 7.8 ± 0.4 g/L, respectively (Fig. 12). Thus, higher yellow grease concentration resulted in an increase of cell growth, but the difference was not statistically significant. At day 14, content of sophorolipids was 40.9 ± 1.7 g/L, 46.9 ± 9.1 g/L, and 44.3 ± 16.5 g/L for yellow

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grease at 10, 40, and 60 g/L, respectively. These sophorolipids concentrations were higher than 34 g/L reported when *C. bombicola* was cultivated on a standard medium with yellow grease added in a step-wise fashion to a fermentor (Shah, 2007). Thus, compared with the standard medium which comprised 100 g/L glucose, 10 g/L of yeast extract, and 1 g/L of urea, sorghum bagasse hydrolysate appeared to be a better substrate, which is consistent with what we reported previously (Samad et al., 2015). In addition, based on other publications on *C. bombicola* (A-M Davila et al., 1997; Morya et al., 2013; Solaiman, Ashby, Zerkowski, & Foglia, 2007), yield of sophorolipids on bagasse hydrolysates could be improved substantially if a fed-batch instead of batch feeding scheme and/or better control of pH and oxygen level are adopted.

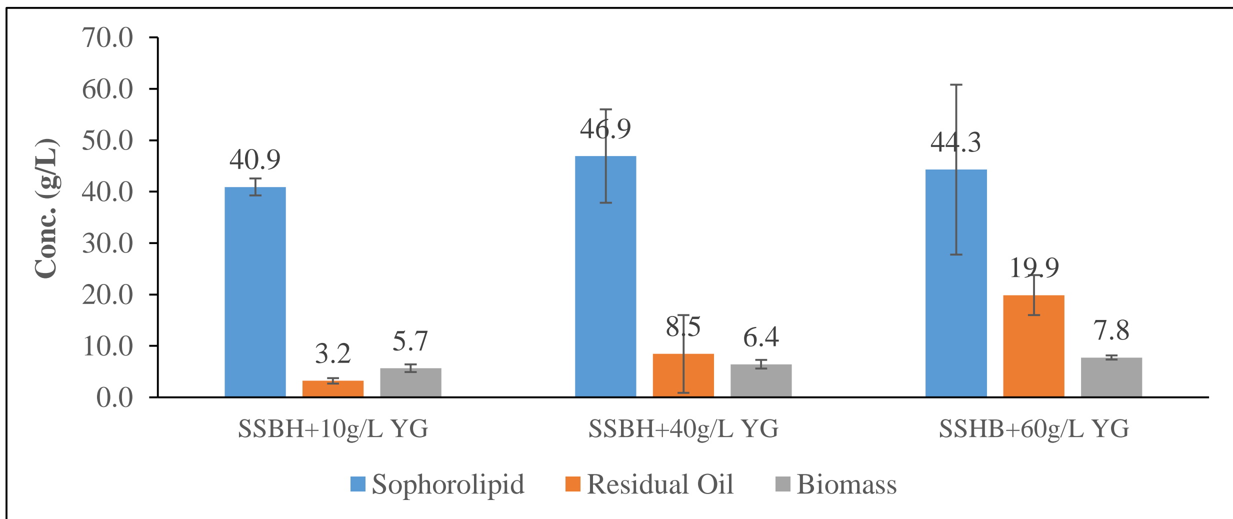

Figure 12: Comparison of parameters when SSB served as the substrate

With increasing yellow grease concentration from 10 to 60 g/L, the residual oil left after 14 days was 3.2 $\pm$ 0.5 g/L, 8.5 $\pm$ 7.6 g/L and 19.9 $\pm$ 3.9 g/L. Yield of sophorolipids (g sophorolipids/g carbon) was calculated by dividing sophorolipids produced with total consumption of sugars and oils. As shown in Fig. 13, with increasing yellow grease concentrations, the yield decreased from 0.67 $\pm$ 0.05 to 0.61 $\pm$ 0.13 and to 0.44 $\pm$ 0.18 g

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sophorolipids/ g carbon. The yield difference between cultures with 10 and 40 g/L of yellow grease was not significant. However, when yellow grease concentration was 60 g/L, sophorolipid yield was significantly lowered. This could be due to the impurities in yellow grease. During frying, at least three kinds of reactions take place: hydrolytic, oxidative, and thermolytic (Mittelbach & Enzelsberger, 1999). As a result, various chemicals, such as alkanes, alkenes, aldehydes, hydrocarbons, and semialdehydes can be present in yellow grease, which might have affected sophorolipid production and secretion.

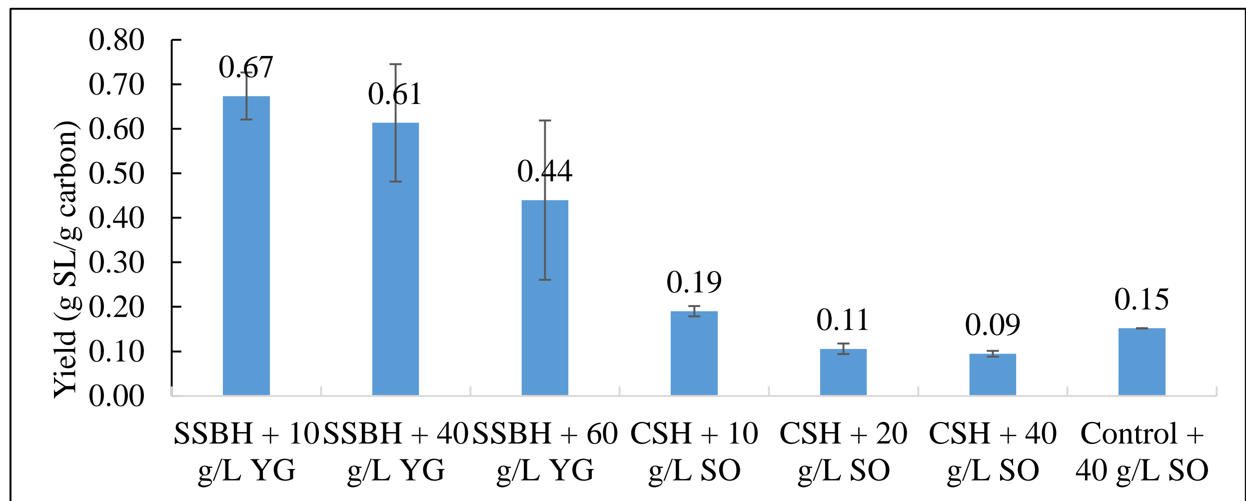

Figure 13: Yield of sophorolipids

The large standard deviations for some results reported here was due to the fact that the presence of yellow grease led to non-homogenous cultures, especially at higher concentrations. Thus, sample withdrawal at different time points might contribute to inconsistent loss of yellow grease and eventually resulted in variations in final day results. But even with some large difference between duplicate samples, it is obvious that *C. bombicola* utilized sugars in SSB hydrolysates and yellow grease efficiently and had the highest sophorolipid yield of 0.67 g/ g

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carbon at the lowest concentration of yellow grease. This yield is comparable with those reported. For example, an overall consumption of 140 g/L rapeseed oil and 300 g/L of glucose led to a yield coefficient of 0.68 (Rau et al., 2001). A similar yield of 0.64 was demonstrated when rapeseed ethyl ester of 184 g/L and glucose of 304 g/L was consumed (Anne-Marie Davila et al., 1992). For these two cases, a fed-batch cultivation was employed for producing large quantity of sophorolipids.

3.3.3. Fermentation using C. bombicola on corn stover hydrolysates with soybean oil supplementation

Corn stover hydrolysate obtained through the DDR process contained 87.3 g/L of glucose, 59.4 g/L xylose and 3.1 g/L of arabinose. The total concentration of monomeric sugars were 149.8 g/L. With this high sugar concentrations and for all three tested doses of soybean oil, *C. bombicola* utilized all three sugars simultaneously during the first two days when exponential growth took place (Fig. 14). After two days when cells enter stationary phases, however, consumption of xylose and arabinose was extremely slow. Same phenomenon was observed for controls where only glucose and xylose at concentrations similar to those in corn stover hydrolysates and 40 g/L of soybean oil were supplemented. Thus, the slow utilization of pentose sugars should not be owing to the complexity of chemicals in the corn stover hydrolysate. Rather, high sugar concentration or substrate inhibition might be the explanation. Currently, we are conducting experiments to understand this observation.

For this part of the study, we chose to use soybean oil instead of yellow grease considering: 1) use of corn fiber hydrolysate for sophorolipid production resulted in less than one-fifth of that from sorghum bagasse hydrolysates under similar experimental conditions; and 2) unlike bagasse hydrolysate which was tested on soybean oil already (Samad et al., 2015), this

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is the first study to investigate sophorolipid production on corn stover hydrolysates. For batch fermentation, since the initial high concentration of an oil feedstock did not lead to high yield of sophorolipids as demonstrated above for bagasse hydrolysate, we sought to study three lower oil doses between 10 and 40 g/L.

As shown in Fig. 14 and 15, *C. bombicola* grew rapidly under four tested conditions. By the end of day 14, the biomass concentration was 6.1 ± 0.2 g/L, 5.9 ± 0.9 g/L, 5.4 ± 0.1 g/L and 5.8 ± 0.1 g/L for soybean oil concentration at 10, 20, 40 g/L and the control where glucose (80 g/L), xylose (55 g/L) and soybean oil (40 g/L) were added, respectively. Statistically, no difference in terms of cell growth could be detected. In the same order, concentration of sophorolipids were 17.3 ± 1.5 g/L, 10.7 ± 0.8 g/L, 9.6 ± 0.4 g/L and 16.1 ±0.7 g/L. Thus, with increasing soybean oil concentration, a decreasing yield of sophorolipids was observed. Comparing the two conditions where content of soybean oil of 40 g/L was the same and total sugar concentrations were similar, corn stover hydrolysates yielded less sophorolipids than those with pure glucose and xylose. Hence, unknown compounds in corn stover hydrolysate might contribute to this lower sophorolipid yield.

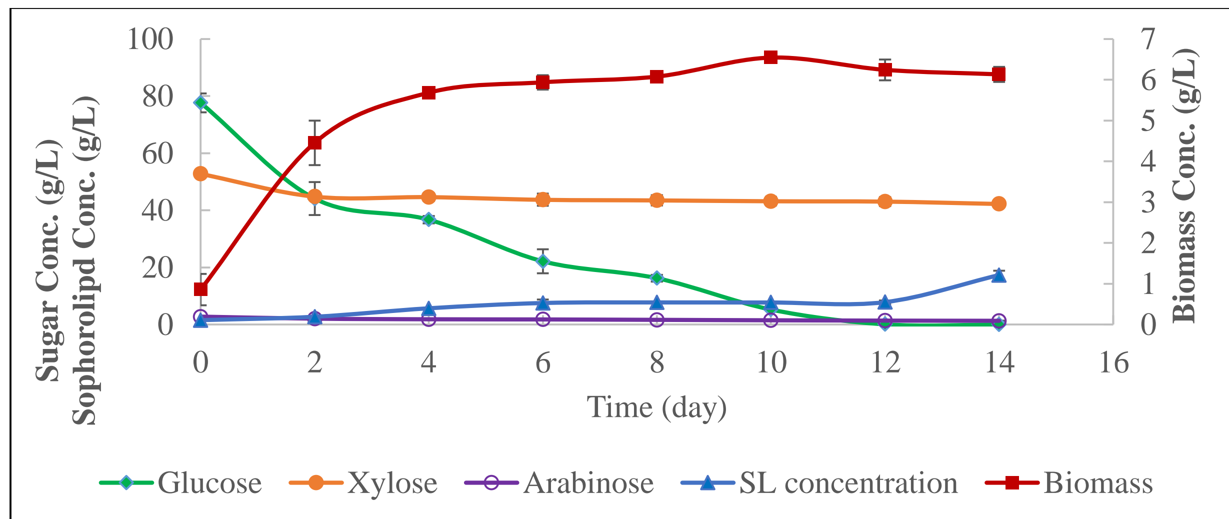

Figure 14(a): Growth profile on DDR hydrolysate and soybean oil at 10 g/L

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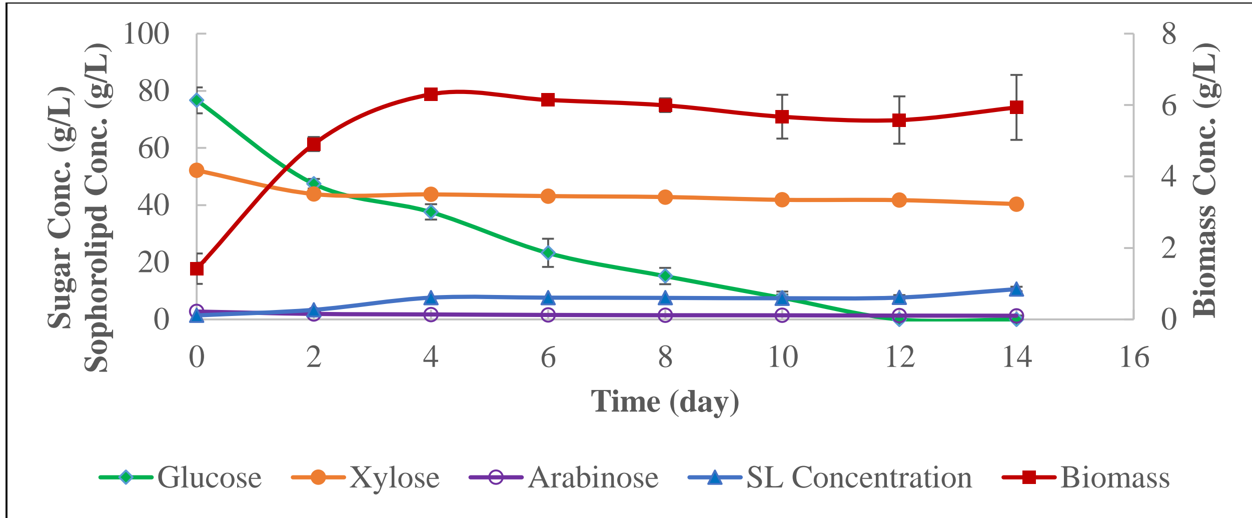

Figure 14(b): Growth profile on DDR hydrolysate and soybean oil at 20 g/L

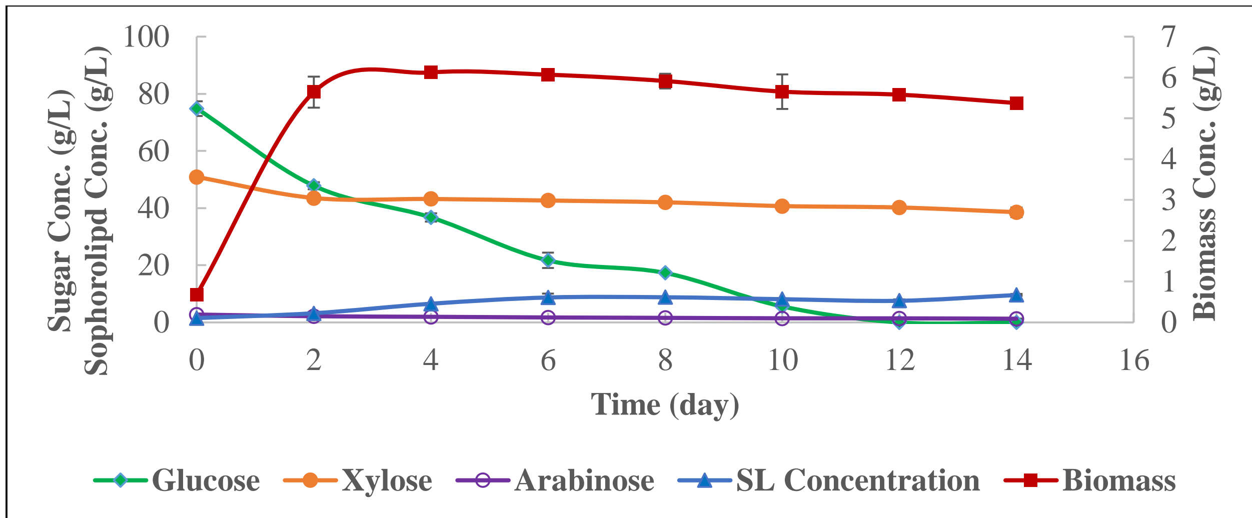

Figure 14(c): Growth profile on DDR hydrolysate and soybean oil at 40 g/L

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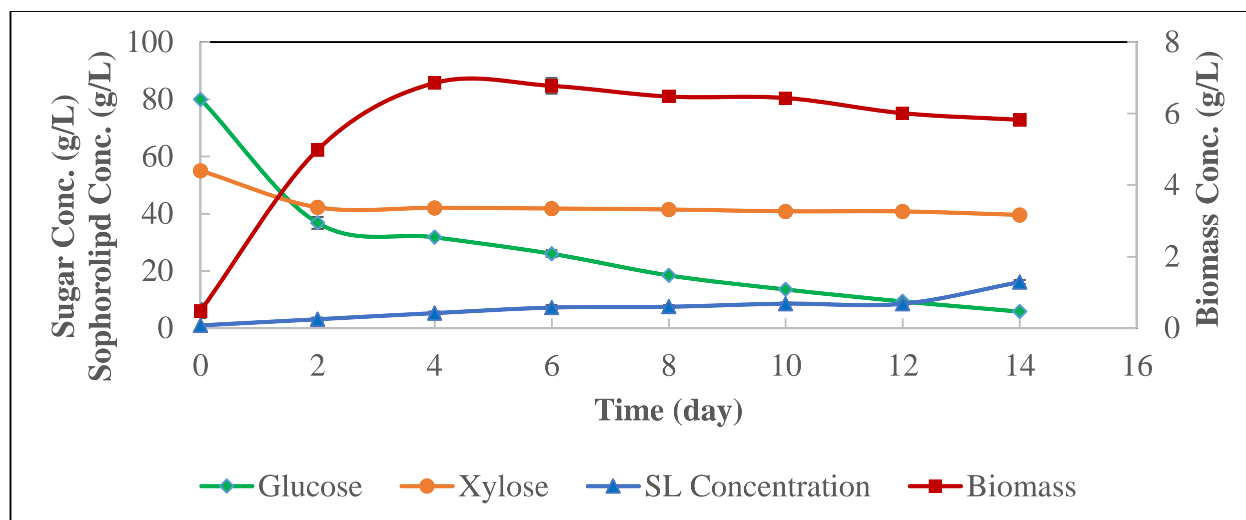

Figure 14(d): Growth profile on glucose and soybean oil at 40 g/L

Similar to cells grown on bagasse hydrolysates and yellow grease as described above, *C. bombicola* did not consume all soybean oil during the 2-week cultivation period. Residual oil left was 8.5 ± 1.9 g/L, 8.9 ± 4.0 g/L, and 26.9 ± 1.5 g/L for oil content of 10, 20, and 40 g/L, respectively. For the controls with pure glucose and xylose, the residual oil was 24.0 ± 5.6 g/L. Considering all carbons (sugars and oil) utilized, yield of sophorolipids was 0.19 ± 0.01, 0.11 ± 0.01, 0.09 ± 0.0, and 0.15 ± 0.0 (Fig. 13). Compared to those observed when *C. bombicola* grew on sorghum bagasse hydrolysates and yellow grease, these yield were much lower. These could be due to: 1) substrate inhibition at high sugar concentrations which led to slow uptake of sugar, especially pentoses. To solve this issue, fed-batch cultivation where hydrolysates are fed intermittently may lessen the inhibition from high sugar concentration; and 2) unknown compounds in the corn stover hydrolysate which might inhibit production and release of sophorolipids. The rationale for pretreating corn stover using DDR is to avoid formation of potential fermentation inhibitors, such as acetic acid, formic acid, furfural and HMF, etc. Although these compounds were not present in the studied corn stover hydrolysates based on our

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HPLC analysis, other compounds that could potentially exist and impact yield of sophorolipids negatively need to be further investigated.

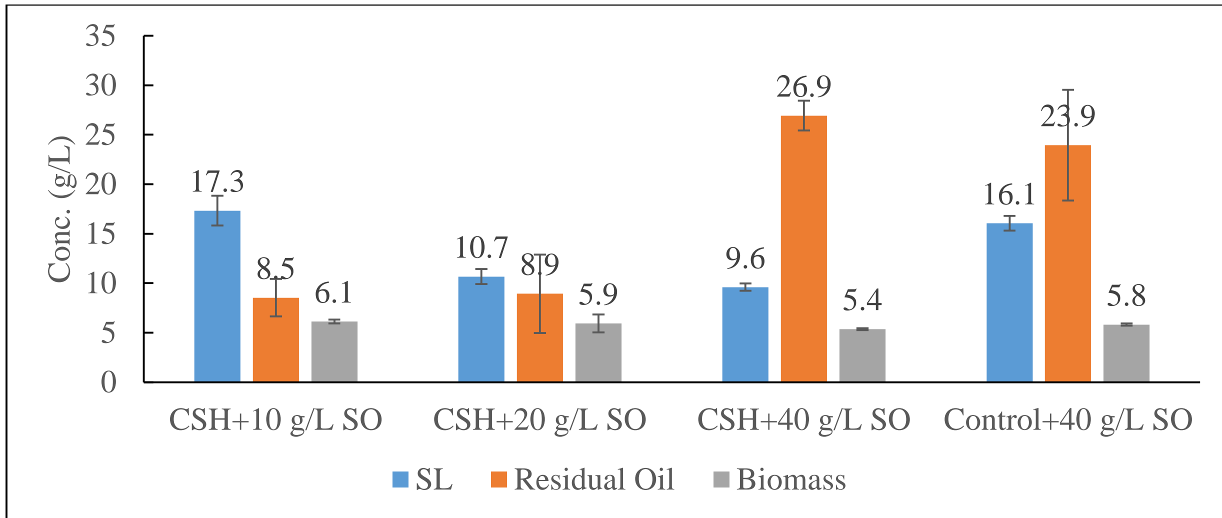

Figure 15: Comparison of parameters when corn stover hydrolysate served as the substrate

3.4. Conclusion

Comparing hydrolysates derived from sorghum bagasse and corn stover through different pretreatment schemes, bagasse hydrolysate together with yellow grease resulted in the highest yield of sophorolipids of 0.67 g/g carbon. Thus, based on results from this batch study, bagasse hydrolysates developed from dilute acid pretreatment appears to be a better substrate for sophorolipid production. Further research in terms of scale up in well-controlled fermenters need to be conducted to enhance the yield and production rate of sophorolipids on biomass hydrolysate.

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CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

4.1. Conclusions

The objectives of this thesis were to (i) investigate whether the biosurfactant can be produced from different production media (hydrolysates) especially using lignocellulosic feedstocks; (ii) determine the yield of SLs from the feedstocks with or without any oil or waste oil source, and (iii) determine composition of SLs produced from the substrates under investigation. For the aforementioned purpose, yeast *C. bombicola* was grown on hydrolysates derived from sweet sorghum bagasse, corn fiber and corn stover. Also the hydrolysates were tested for additional carbon source mainly soybean oil or yellow grease. All the studies were conducted using batch fermentation. The research has been summarized in two publications included in chapter 2 and chapter 3, respectively.

The studies on pretreated sweet sorghum bagasse and corn fiber demonstrated that *C. bombicola* could utilize monomeric sugars as well as non-sugar compounds to produce SLs. This is the first study to report SL production from those hydrolysates. Time series data analysis revealed that the yeast cells were able to synthesize more SLs when they reached the stationary phase. Compared to corn fiber hydrolysate and standard glucose media, the highest concentration of SLs, 84.6 g/L, was detected for cultures with bagasse hydrolysates. The overall yield of sophorolipids from bagasse hydrolysates was 0.55 g/g carbon source which is moderately lower than previously reported yields. HPLC-MS-MS study revealed that SLs recovered from the hydrolysates consisted of acidic SLs with C18 fatty acid chains and lactonic SLs with either C16 or C18 fatty acids.

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Sorghum bagasse and corn stover hydrolysates derived through different pretreatment schemes were investigated under different culture conditions. The study revealed that bagasse with yellow grease as additional carbon source had the highest yield of SLs of 0.67 g/g carbon source. Although corn stover with disc refined deacetylated pretreatment and bagasse hydrolysates developed from dilute acid pretreatment contained similar sugar concentrations, the bagasse hydrolysate appears to be a better substrate for sophorolipid production.

4.2. Recommendations

All the studies were conducted under batch fermentation using Erlenmeyer flasks in an incubator, thus, it is highly recommended to use more controlled environment such as bioreactor where various parameters- temperature, dissolved oxygen, pH, etc. can be well monitored and controlled during the fermentation. One of the challenges for residual oil measurement at different time points was that oil or yellow grease tend to float on the surface of the culture, therefore, the withdrawal of culture requires very careful handling. Sophorolipids recovered from the extraction of final day culture from bagasse hydrolysates were mainly studied, therefore, cultures of other hydrolysates such as corn fiber and corn stover is also recommended for sophorolipid characterization. Also, it is recommended to perform economic analysis for the overall production cycle of sophorolipids to know the cost and benefits of the inputs, processes, products.

4.3. Engineering Significance

It has been known from several literatures that biosurfactants such as sophorolipids have several advantages over chemically derived surfactant in terms of eco-toxicity and applications. The cost competitiveness for commercial production of sophorolipids using standard production

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sources has been one of the major issues. The results obtained from our studies show a promising way of producing biosurfactant from lignocellulosic biomass.

4.4. Future Research

Based upon the studies performed, further research can be conducted to enhance the yield and production rate of SLs on biomass hydrolysate by scaling up in well-controlled fermenters. Most literatures have recorded higher production rate by adding the carbon source and other nutrients during different phase(s) of fermentation, thus, further research using fed-batch can be performed using the biomass hydrolysates studied in this research. Also, the effect of various parameters (i) substrate conditions such as sugar or oil contents and (ii) physical conditions such as pH, Temperature, DO; can be explored to investigate whether they can enhance or reduce the production rate of SLs. Further, sophorolipid purification and characterization can be studied to enhance the recovery of certain types of sophorolipids. Several studies have reported using biosurfactant for remediation of soil and water contaminated by hydrocarbons; SLs recovered from the hydrolysates under investigation can also be studied for its remediation properties.

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Publications:

Samad, A., Zhang, J., Chen, D., & Liang, Y. (2015). Sophorolipid production from biomass hydrolysates. *Appl Biochem Biotechnol, 175*(4), 2246-2257. doi: 10.1007/s12010-014-1425-x

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