Fuel 175 (2016) 64–74

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Microbial lipid production from SPORL-pretreated Douglas fir by *Mortierella isabellina*

S.M. Harde [a], Z. Wang [a], M. Horne [a], J.Y. Zhu [a,b], X. Pan [a,*]

[a] Department of Biological Systems Engineering, University of Wisconsin-Madison, 460 Henry Mall, Madison, WI 53706, USA
[b] USDA Forest Service, Forest Products Laboratory, Madison, WI 53726, USA

ARTICLE INFO

Article history:
Received 6 November 2015
Received in revised form 14 January 2016
Accepted 5 February 2016
Available online 12 February 2016

Keywords:
Forest residue
Microbial lipid
SPORL pretreatment
Mortierella isabellina
Detoxification

ABSTRACT

The solid substrate and spent liquor obtained after SPORL pretreatment of Douglas fir residues were evaluated as substrates for the production of intracellular microbial lipid by *Mortierella isabellina* NRRL 1757. The production of lipid was investigated in a batch fermentation using different strategies *viz*. separate hydrolysis and fermentation, quasi-simultaneous saccharification and fermentation (Q-SSF), and whole slurry (solid substrate and spent liquor) saccharification and fermentation. The maximum lipid yield (0.21 g/g sugar) was obtained with Q-SSF of the solid substrate. Nutrient supplementation was not necessary for lipid production from SPORL-pretreated Douglas fir. Detoxification of SPORL spent liquor with lime did remove the inhibitors and improved the fermentation for lipid production. The lipid produced had a similar fatty acid compositional profile to those of vegetable oil, suggesting that it could have potential as a substrate for biodiesel production.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Biodiesel has attracted significant attention as a sustainable and renewable alternative to the traditional fossil fuel. The most common feedstocks used in biodiesel production are vegetable oils from plants such as rapeseed, soybean, palm oil, sunflower, and other oleaginous crops and animal fats [1,2]. The major hurdle for biodiesel development and wide application is the high cost and limited supply of the raw material. In addition, consumption of a large amount of vegetable oils for biodiesel production would result in a shortage in edible oils and create food verses fuel debate [3].

Searching for new lipid resources is a key issue for sustainable biodiesel industry. Recently, researchers have evaluated microbial lipids as feedstock for biodiesel production, which are accumulated by oleaginous microorganisms (yeast, fungi, and algae) during fermentation on renewable resources [4]. The microbial lipids are mostly in the form of triacylglycerols (TAGs) and some in the form of free fatty acids (FFAs) [5] and have similar composition of fatty acids to that of vegetable oils [6]. In addition, the microbial lipids have many advantages such as shorter life cycle, less affection by venue and climate, less labor requirement, easier scaling-up, non-arable land usage, and high carbon to heteroatom ratios [7,8]. However, the microbial lipids are currently more expensive than vegetable oils and animal fats because of the high cost for feedstock and fermentation process [9].

Compared to biodiesel, the production of high-value lipids by the microorganisms is currently economically more attractive [10]. For example, plant-derived lipids contain very limited γ-linolenic acid, which has a high value. Several oleaginous microorganisms have been reported to produce microbial lipids containing γ-linolenic acid [11–13].

Recently, lignocellulosic biomass was considered as a promising and potentially inexpensive substrate for microbial lipid production as it is a renewable, sustainable, and the most abundant feedstock in nature. Various lignocellulosic substrates, including rice straw [3], sugarcane bagasse [14], wheat straw [15], corn stover [16–18], and rice hull [19] have been successfully used for microbial lipid production. A group of oleaginous microorganisms such as *Cunninghamella echinulata* [20], *Trichosporon fermentans* [3], *Cryptococcus curvatus* [15], *Mortierella isabellina* [19], *Thamnidium elegans* [21], *Rhodosporidium toruloides* [22], *Lipomyces starkeyi* [23], and *Rhodotorula glutinis* [24] were reported to be able to use lignocellulosic hydrolysates for microbial lipid production. Some of them have xylose-assimilated capacity. For example, Huang et al. [3] reported that *T. fermentans* could grow well and accumulate lipids efficiently not only on glucose but also on xylose, suggesting that it has the potential of converting lignocellulosic hydrolysate containing both glucose and xylose to microbial lipids.

Woody biomass such as forest residues is advantageous over herbaceous biomass for producing biofuels and bio-products.

* Corresponding author. Tel.: +1 6082624951; fax: +1 6082621228.
E-mail address: xpan@wisc.edu (X. Pan).

http://dx.doi.org/10.1016/j.fuel.2016.02.023
0016-2361/© 2016 Elsevier Ltd. All rights reserved.

Advantages of using the residues includes (1) relatively high bulk density which reduces transportation cost, (2) no need for long term storage because of flexible harvesting schedules [25], (3) easily available and renewable feedstocks [26], and (4) large quantities [27,28]. However, the forest residues have very strong recalcitrance to bioconversion to sugars due to high content of bark and juvenile wood from the tree tops and branches [29–31], especially those from softwoods such as the Douglas fir, as is studied here.

One of the efficient ways to reduce the recalcitrance of the forest residue is SPORL (sulfite pretreatment to overcome recalcitrance of lignocellulose) pretreatment [31–33]. SPORL pretreated woody biomass has better digestibility than other methods and would be much easier to scale up because all the unit operations are based upon those used in paper industry [34]. SPORL uses sulfite to pretreat biomass in acidic condition. During the pretreatment, most hemicelluloses and partial lignin are dissolved in the pretreatment liquor in the form of fermentable sugars and lignosulfonate, respectively, while cellulose is partially polymerized but retained in the pretreated solids. The cellulosic solid substrate is subsequently broken down to glucose by using cellulases. The spent liquor stream contains monomeric sugars, lignosulfonate, and other soluble products. It was found that lignosulfonate from SPORL process could enhance enzymatic hydrolysis of pretreated solids by preventing nonproductive binding of cellulase to lignin on solid substrate [35–37]. Fermentation of spent sulfite liquor from softwoods that contains a lignosulfonate and phenolic compounds with relatively low amounts of furans and acetic acid has been in industry practice, which suggests that strains of *Saccharomyces cerevisiae* can tolerate phenolic compounds. Literature on microbial lipid production using SPORL pretreated substrate is scant.

The objective of this study was to investigate microbial lipid production from SPORL pretreated Douglas fir using *M. isabellina*. The SPORL pretreated Douglas fir washed water insoluble solid (WIS) substrate, pretreatment spent liquors, and whole pretreated slurry (mixture of the substrate and spent liquor without separation and washing) were investigated separately for lipid fermentation. The inhibitors present in the spent liquors were examined, and different detoxification methods were evaluated to remove the inhibitors before the pretreated spent liquors were subsequently used for lipid fermentation.

2. Materials and methods

2.1. Materials

Douglas fir forest residue was collected from a regeneration harvest in a primarily Douglas fir stand on Mosby Creek southeast of Cottage Grove in Lane County, OR and owned by Weyerhaeuser Company. As described previously [38], road piles of forest residue were ground using a horizontal drum fixed-hammer grinder (Model 4710B, Peterson Pacific Corporation, Eugene, OR) equipped with a combination of 76 and 102 mm grates and then shipped by truck to Weyerhaeuser Company at Federal Way, WA. The moisture content of the residues measured at arrival was 43.9%. The residue was then screened using a gyratory screen (BlackClawson) equipped with a 44.5 mm (1.75 in.) diameter round-hole punched-plate top deck to remove oversized particles and a 3.2 mm (1/8 in.) clear-opening woven wire bottom screen (6 wires/in. mesh) to remove fines. The accept forest residues were then air-dried to a moisture content of 15%, labeled as FS-10, and were used for the SPORL pretreatment at USDA Forest Products Laboratory, Madison, WI.

Arabinose, galactose, glucose, xylose, mannose, and potato dextrose agar were purchased from Dot Scientific Inc., MI, USA. Yeast extract and hexanes were purchased from EMD, NJ, USA. Peptone, manganese sulfate, magnesium chloride, and copper sulfate were purchased from Amersco, OH, USA. Iodine was purchased from Renovating science, NY, USA. Chloroform and sodium sulfite were purchased from Acros, NJ, USA. Calcium hydroxide was purchased from Wards Science, NY, USA. Sodium hydroxide, disodium hydrogen phosphate, citric acid, and methanol were purchased from VWR, PA, USA. Furfural, acetic acid, levulinic acid, hydroxymethyl furfural (HMF), and cornstarch were purchased from Sigma Aldrich, MO, USA. Potassium hydrogen phosphate, potassium hydroxide, sodium thiosulfate, and sulfuric acid were purchased from Fisher Chemicals, NJ, USA. All fatty acid methyl esters were purchased from MP biomedical, OH, USA. Enzyme Cellic® CTec2 was provided by Novozymes North America (Franklinton, North Carolina), USA. All chemicals were used as received.

2.2. Experimental procedure for microbial lipid production

Douglas fir residue FS-10 was pretreated using the SPORL method. The pretreated whole slurry was separated into wet solid and spent liquor by pressing in a screen box. The moisture of the solid fraction was determined gravimetrically by oven drying the collected wet sample. The wet solid sample was thoroughly washed with deionized water to remove soluble solids until pH of the filtrate became neutral, and the washed biomass was used for further experiments. Both the washed water insoluble solids (WIS) and spent liquor were used for lipid fermentation. Chemical compositions of the WIS and spent liquor were analyzed according to NREL protocol (Laboratory analytical procedure, NREL/TP-510-42618). Different strategies were employed for microbial lipid production, (a) enzymatic saccharification followed by fermentation of WIS obtained from SPORL pretreated Douglas fir, (b) detoxification of spent liquor followed by fermentation, (c) quasi-simultaneous saccharification and fermentation of WIS and, (d) saccharification and fermentation of whole SPORL pretreated slurry. Overall schematic flow diagram of this study is represented in Fig. 1.

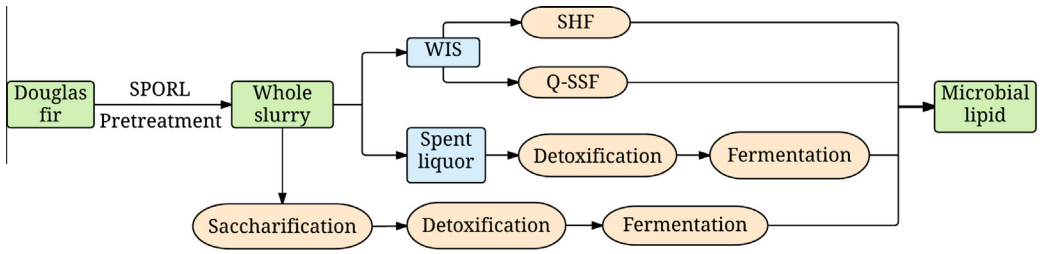
2.3. SPORL substrate production

SPORL pretreatment of Douglas fir was conducted using a 390-L rotating wood pulping digester. Briefly, digester was externally heated using a steam jacket and rotated at 2 rpm for mixing. Both pretreatments were conducted at 140 °C using 50 kg FS-10 in oven-dry (od) with a liquor to wood ratio (L/W, v/w) of 4:1, and targeted total SO_2 loading of 80 g/L and combined (with magnesium oxide) SO_2 loading of 11 g/L in the pretreatment liquor. Magnesium oxide was used as metal base. These chemical loadings were to simulate the chemistry in a commercial sulfite pulp mill in the US that recovers magnesium in operation. The temperature ramping and reaction time at 140 °C were 32 and 60 min for C-t60 and 36 and 120 min for C-t120, respectively. Mixing in the 390-L digester was achieved through liquor circulation. After the chemical treatment, the wood chips remained intact and were disk-milled with the pretreatment spent liquor after neutralization to pH 6.0 to facilitate milling and material transport. The two whole slurries had total solid contents of 21.88 ± 0.17% and 21.02 ± 0.24%, respectively, for C-t60 and C-t120. The whole slurries were further processed to obtain the WIS and spent liquor fractions from C-t60 and C-t120 as described in Section 2.2.

2.4. Screening of microorganisms for maximum lipid production using synthetic medium

2.4.1. Microorganism and inoculum preparation

Two yeast strains *viz. C. curvatus* NRRL Y-1511 and *R. toruloides* NRRL Y-1091 and two fungi *viz. M. isabellina* NRRL 1757 and *Chaetomium globosum* NRRL 1870 were obtained from the Agricultural



Note:
WIS: Washed water insoluble solid; SHF: Separate hydrolysis and fermentation;
Q-SSF: Quasi-simultaneous saccharification and fermentation.

Fig. 1. Overall flow diagram of the process.

Research Service (ARS) Culture Collection, Illinois, USA. Initially, strains were cultivated from lyophilized preparation using YM broth (g/L; yeast extract 3, malt extract 3, peptone 5, and dextrose 10) at 30 °C for 5 days. Then, yeasts were grown on YPDA (g/L; yeast extract 10, peptone 20, dextrose 20, and agar 20); fungi were grown on potato dextrose agar at 30 °C for 5 days; and they were finally stored at 4 °C. All strains were sub-cultured and transferred every 2 months. The yeast cell suspension (1 mL) and fungal spore suspension (1 mL) were inoculated in 50 mL YPD in 125-mL Erlenmeyer flasks and grown for 24 h at 30 °C. After 24 h, the prepared inoculum (10% v/v; 2×10^6 spores/mL) was used for further production experiments.

2.4.2. Medium

The modified medium reported by Yu et al. [39] was used as a production medium and YPD for the inoculum preparation. The inoculum medium (YPD) contained yeast extract 10, peptone 20, and dextrose 20 (in g/L, pH 6.5 ± 0.2). The standard production medium contained carbon source 40 (glucose, xylose or mixture of both), manganese sulfate 0.003, magnesium sulfate 0.4, copper sulfate 0.0001, potassium dihydrogen phosphate 2.0, and yeast extract 5.6 (in g/L). The medium was adjusted to pH 5.5 with 0.1 N NaOH. After preparation, the medium was autoclaved at 121 °C for 20 min. Batch fermentations were carried out in 125-mL Erlenmeyer flasks with 25 mL of production medium. It was inoculated (10% v/v) with 24-h actively growing seed culture and then incubated for 168 h at 30 °C. All experiments were performed on the basis of total sugars (arabinose, galactose, glucose, xylose, and mannose). Samples were withdrawn at 24-h intervals and analyzed for dry cell weight (DCW), residual sugars, and lipid concentration. All experiments were performed in triplicates, and means and standard deviations were reported.

2.5. Enzymatic saccharification followed by fermentation of washed water insoluble solids (WIS) obtained from SPORL pretreated Douglas fir

The principal component of SPORL pretreated washed solids was cellulose. Hence, Cellic® CTec2 was used in order to complete saccharification of cellulose. Enzymatic saccharification of the WIS of both C-t60 and C-t120 were conducted at 10% and 15% (w/v) in 100-mL of 50 mM citrate buffer (pH 5.5) on an incubator shaker (New Brunswick Scientific, 4200, NJ, USA) at 50 °C and 200 rpm. pH 5.5 was used because saccharification of lignocellulose can be enhanced by reducing nonproductive cellulose binding to lignin [35–37]. The Cellic® CTec2 loading was 14.6 FPU/g glucan (based on glucan in the washed solids). Samples from enzymatic hydrolysate were withdrawn (1 mL) at 3, 6, 24, 48, and 72 h and centrifuged at 13000 g for 10 min for glucose analysis. All experiments were performed in duplicates and mean values and standard deviations were reported.

Enzymatic hydrolysates of WIS of C-t60 and C-t120 were further used for lipid production using *M. isabellina*. Fermentation of both hydrolysates was performed with and without supplementation of nutrients. The pH of the hydrolysates media was adjusted to 5.5 and autoclaved at 121 °C for 20 min. Batch fermentation was performed as explained in Section 2.4.2. All experiments were performed in triplicates, and means and standard deviations were reported.

2.6. Detoxification of spent liquor from SPORL pretreated Douglas fir

Overliming (calcium hydroxide treatment) was evaluated for the detoxification of spent liquor of C-t60 and C-t120. In the treatment by overliming, the spent liquor was placed on a magnetic stirrer and heated to 50 °C. $Ca(OH)_2$ was added gradually and mixed by using magnetic stirrer until the pH reached 9 to 10. The hydrolysate was then maintained at 50 °C for 30 min [40] and then centrifuged and vacuum filtered to remove the generated solids. The overlimed filtrate was adjusted to pH 5.5 with 0.1 N HCl and used for lipid fermentation. Detoxified spent liquors were analyzed for all sugars and inhibitors. The amount of total sulfite before and after detoxification of spent liquors was determined through titration [41].

2.7. Fermentation of detoxified spent liquor

Spent liquors of C-t60 and C-t120 were detoxified with overliming (Section 2.6) prior to lipid fermentation. Detoxified liquor was autoclaved at 121 °C for 20 min. It was inoculated (10% v/v) with 24-h seed culture and incubated for 168 h at 30 °C. No additional nutrients were applied during lipid fermentation. All experiments were performed on the basis of total sugars (arabinose, galactose, glucose, xylose, and mannose). Samples were withdrawn at 24-h intervals and analyzed for dry cell weight (DCW), residual sugars, and lipid concentration. All experiments were performed in triplicates, and means and standard deviations were reported.

2.8. Quasi-simultaneous saccharification and fermentation of WIS

Quasi-simultaneous enzymatic saccharification and fermentation (Q-SSF) of the WISs from C-t60 and C-t120 were carried out in 125-mL Erlenmeyer flasks in a shaker incubator. Enzymatic hydrolysis was conducted at 10% solids using Cellic® CTec2 at 14.6 FPU/g of glucan. The pH of the media was controlled by adding citric acid-phosphate buffer (50 mM) of pH 5.5. The WIS was liquefied within approximately 24 h at 50 °C and 200 rpm. The liquefied samples were analyzed. The samples were then cooled down to 35 °C; and the shaker speed was reduced to 180 rpm; and the

samples were inoculated (10% v/v) with 24-h seed culture and incubated for 168 h. No additional nutrients were applied during fermentation. All experiments were performed on the basis of total sugars (arabinose, galactose, glucose, xylose, and mannose). Samples were withdrawn at 24-h intervals and analyzed for residual sugars and lipid concentration. All experiments were performed in triplicates, and means and standard deviations were reported.

2.9. Saccharification and fermentation of SPORL pretreated slurry

Saccharification of whole pretreated biomass slurry of samples C-t60 and C-t120 were carried out without separation of solids from liquor in 500-mL Erlenmeyer flasks in a shaker incubator. The pH of the slurry was adjusted to 6.2 using solid calcium hydroxide and was controlled at pH 5.5 by adding citric acid-phosphate buffer (50 mM) of pH 5.0. The enzymatic hydrolysis was conducted at 10% solids (w/v, solid loading adjusted using buffer) and with enzyme Cellic® CTec2 at 14.6 FPU/g of glucan. Saccharification was carried out for 72 h at 50 °C and 200 rpm. The compositions of the liquefied samples were analyzed after 72-h saccharification. Hydrolysates of sample C-t60 and C-t120 were centrifuged to get clear liquor and treated with overliming (Section 2.6) prior to lipid fermentation. It was autoclaved at 121 °C for 20 min. It was inoculated (10% v/v) with 24-h seed culture and incubated for 216-h at 30 °C. No additional nutrients were applied during lipid fermentation. All experiments were performed on the basis of total sugars (arabinose, galactose, glucose, xylose, and mannose). Samples were withdrawn at 24-h intervals and analyzed for dry cell weight (DCW), residual sugars, and lipid concentration. All experiments were performed in triplicates, and means and standard deviations were reported.

2.10. Lipid production using sulfite adapted M. isabellina

To develop the tolerance of the *M. isabellina* strain to sulfite toxicity associated with SPORL pretreatment of Douglas fir, the original *M. isabellina* strain was inoculated into a medium containing a low concentration of sulfite (0.2 g/L). Once the strain grew well in this medium, it was then inoculated into a medium with a slightly higher concentration of sulfite (0.4 g/L) and on up in iterations of 0.2 g/L each time. Till the date, *M. isabellina* has achieved resilience in sulfite concentrations up to 2.2 g/L. The lipid production using the sulfite-adapted strain was then tested in media with sulfite (2.0 g/L) and without sulfite. The samples were analyzed for dry cell weight (DCW), residual sugars, and lipid concentration. All experiments were performed in triplicates, and means and standard deviations were reported.

2.11. Analytical method

Yeast cells and fungal spores were harvested by centrifugation at 13000 g for 10 min and washed three times with water. Washed yeast cells and fungal spores were dried overnight at 105 °C and used for determining dry cell weight (DCW) in the culture (g/L) and lipid concentration. The supernatant of cultured broth was used for residual sugar analysis. Total lipid was analyzed by extracting lipid with a mixture of methanol and chloroform [42]. In brief, for mycelia disruption with acid lysis, dried biomass was digested with 1 mL of 4 M hydrochloric acid (HCl) at 78 °C in water bath for 2 h. Further, total lipids were extracted by adding 1 mL chloroform/methanol (2:1, v/v) solution into the sample. The solvent was then removed from the extract until constant weight to give the total cellular lipids. The lipid concentration was expressed as the dry mass fraction of the lipid extracted from the cells in per liter fermentation broth (g/L).

The sugar analysis was performed using high performance ion chromatography (Dionex ICS-3000) equipped with an integrated amperometric detector, as described before [43]. Samples were separated using CarboPac™ PA1 (4 × 250 mm) column. The flow rate of eluent (water) was 0.7 mL/min, and the column temperature was maintained at 30 °C. Sugar contents were quantified by running a standard curve ranging from 5 to 40 ppm for all sugars (arabinose, galactose, glucose, xylose, and mannose). The sugars concentrations were measured in triplicate.

Lipids in dry mycelium were converted to fatty acid methyl esters (FAMEs) by a two-step reaction [44]. The fatty acid profile was determined by measuring the corresponding FAMEs composition using GC (GC-2014, Shimadzu chromatograph, USA). The GC was equipped with a flame-ionization detector and a ZB-Wax Plus column (30 m × 0.32 mm × 0.5 μm) (Phenomenex, USA). The injector was kept at 240 °C, with an injection volume of 1 μL by split injection mode (ratio at 10:1). The oven temperature was kept constant at 200 °C. The detector temperature was set at 250 °C. Helium was used as the carrier gas.

3. Results and discussion

3.1. Compositional analysis of SPORL pretreated Douglas fir

Two fractions i.e. WIS and spent liquor, after SPORL pretreatment, were recovered from each of the samples (C-t60 and C-t120). The samples were analyzed for chemical composition as the raw material influences the bioconversion process, which further decides the approaches to be followed for the processing of the material. The total insoluble solid content of whole slurry was found to be 11.75% and 12.87% for C-t60 and C-t120, respectively. The compositions of the WIS samples are listed in Table 1. The chemical compositions showed that WIS of both samples were rich in glucan and were 69.40% and 56.92% for C-t60 and C-t120, respectively. Hemicelluloses were minor in the WIS with trace amounts of arabinan and galactan and small amounts of xylan and mannan. Lignin in WIS as Klason lignin was 21.02% and 36.44% for C-t60 and C-t120, respectively. The removal of hemicelluloses enriched glucan and lignin in WIS. The WIS of C-t120 had lower cellulose and hemicellulose contents and higher lignin content than C-t60 due to extended pretreatment of 120 min, which dissolved more cellulose and hemicelluloses and thereby enriched lignin in the WIS.

The compositional analyses of spent liquors from C-t60 and C-t120 are shown in Table 2. The results indicated that spent liquors were abundant with both pentoses and hexoses. Majority of the sugars were mannose as Douglas fir is softwood, followed by xylose and glucose. Significant amount of galactose and arabinose were detected as well. These detected hemicellulose sugars

Table 1
Compositional analysis of washed water insoluble solids (WISs) of sulfite pretreated Douglas fir forest residue FS-10.

	C-t60 (% w/w)	C-t120 (% w/w)
Arabinan	ND	0.02 ± 0.02
Galactan	0.03 ± 0.00	0.02 ± 0.03
Glucan	69.40 ± 2.07	56.92 ± 0.88
Xylan	2.05 ± 0.21	0.53 ± 0.01
Mannan	2.59 ± 0.11	0.36 ± 0.25
AIL	21.02 ± 1.49	36.44 ± 0.69
ASL	0.98 ± 0.08	1.03 ± 0.01
Total	96.09 ± 0.56	95.33 ± 0.49

ND: not detectable; C-t60: 1 h pretreated biomass; C-t120: 2 h pretreated biomass; AIL: acid insoluble lignin; ASL: acid soluble lignin; results are mean ± standard deviation (SD) of three determinations.

Table 2
Compositional analysis of spent liquor of sulfite pretreated Douglas fir residue FS-10.

	C-t60		C-t120	
	Before detoxification (g/L)	After detoxification (g/L)	Before detoxification (g/L)	After detoxification (g/L)
Arabinose	2.9 ± 0.0	1.9 ± 0.2	2.6 ± 0.1	1.9 ± 0.1
Galactose	7.7 ± 0.1	4.9 ± 0.3	7.3 ± 0.3	4.8 ± 0.2
Glucose	9.0 ± 1.0	8.9 ± 0.5	11.6 ± 0.7	10.9 ± 0.4
Xylose	11.8 ± 0.1	7.8 ± 0.2	13.2 ± 0.1	8.5 ± 0.3
Mannose	24.2 ± 0.3	21.0 ± 0.8	23.5 ± 0.7	20.7 ± 0.9
Furfural	0.30 ± 0.03	0.01 ± 0.02	0.53 ± 0.07	0.02 ± 0.01
HMF	0.03 ± 0.04	0.02 ± 0.01	0.08 ± 0.02	0.03 ± 0.01
Formic acid	0.95 ± 0.03	0.34 ± 0.02	0.71 ± 0.10	0.60 ± 0.05
Acetic acid	4.83 ± 0.54	4.12 ± 0.14	5.24 ± 0.24	4.90 ± 0.31

C-t60: 1 h pretreated biomass; C-t120: 2 h pretreated biomass; results are mean ± SD of three determinations.

were in agreement with the compositions of the pretreated WISs in Table 1 that most hemicelluloses were removed during pretreatments. The concentrations of the sugars were higher in C-t120 than in C-t60, in agreement with the results in Table 1, i.e., more hemicelluloses and cellulose were removed from C-t120 than from C-t60 due to extended pretreatment. HMF and furfural were detected in the spent liquors. Their concentrations, however, were low because of the low pretreatment temperature of 140 °C. Slightly higher furan concentrations in C-t120 spent liquor suggested that extended pretreatment resulted in more sugar degradations.

3.2. Screening of microorganisms for maximum lipid production using synthetic medium

C. curvatus NRRL Y-1511, *R. toruloides* NRRL Y-1091, *M. isabellina* NRRL 1757, and *C. globosum* NRRL 1870 were screened for maximum lipid production using glucose, xylose, and mixture of both as a carbon source. Fermentation was carried out for 168 h and samples of all strains were analyzed for biomass concentrations i.e. DCW, lipid concentration, and relative proportion of fatty acids. The results are summarized in Table 3. All strains showed the significant lipid production on the sugars, but *M. isabellina* NRRL 1757 produced maximum lipids on both glucose and xylose as a carbon source. The DCW and the lipid concentration using *M. isabellina* were 20.0 g/L and 9.5 g/L, respectively, when glucose was the sole carbon source. When the xylose was the carbon source, however DCW and the lipid concentration were 15.5 g/L and 10.1 g/L, respectively. When the mixture of glucose and xylose was used, the lipid concentration was similar to those achieved with glucose, but slightly less than that with xylose. Lipid yield found in this study (0.25 g/g using xylose as a substrate) was higher than that reported in the literature using *M. isabellina*. Up-to-date, the highest conversion yield (g/g of sugars) of microbial lipid by *M. isabellina* was 0.22 g/g in bioreactor trials [45]. Thus, in approximate terms, it will take four tons of sugar to make one ton of lipids under ideal growth conditions. Papanikolaou and Aggelis [46] reported that maximum theoretical yield of lipid could be achieved more with the xylose as a substrate (0.34 g/g) than with glucose as a substrate (0.32 g/g), assuming that oleaginous microorganisms utilize exclusively the phosphoketolase pathway for xylose assimilation.

Lipids produced by *M. isabellina* were transesterified, and the resultant corresponding fatty acid methyl esters were analyzed by GC. It was found that the lipids contained 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); the unsaturated fatty acids amounted to about 72%. The most abundant fatty acids produced were oleic acid (C18:1), followed by palmitic acid (C16:0). The similar pattern was observed with the fatty acids produced by other strains (*C. curvarus*, *C. globosum*, and *R. toruloides*) in the present study. Gao et al. [47] reported that *M. isabellina* produced oleic acid (C18:1) as the predominant fatty acid, followed by palmitic acid (C16:0). Papanikolaou and Aggelis [46] found that oleic acid (C18:1) was the predominant fatty acid of the lipids produced by different yeasts. Similar fatty acid composition to that of vegetable oils suggests that lipids produced by *M. isabellina* are a suitable substrate for biodiesel production [48]. The results above suggested that *M. isabellina* performed better in lipids production than other strains investigated, and thereby it was selected for further fermentation experiments for lipid production from SPORL pretreated Douglas fir.

Table 3
Lipid production on different carbon sources by various microorganisms.

Strain	Carbon source	DCW (g/L)	Lipid (g/L)	Relative proportion of the fatty acids (% w/w)						
				C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Cryptococcus curvatus NRRL Y-1511	Glucose [a]	13.2 ± 0.1	2.6 ± 0.1	0.8	18.3	0.7	12.0	54.0	11.9	2.0
	Xylose [a]	13.3 ± 0.1	5.1 ± 0.3	1.2	15.8	0.6	11.2	58.6	11.4	2.0
	Glucose [b] + Xylose [b]	12.6 ± 0.3	6.1 ± 0.2	1.1	16.3	0.8	10.7	61.6	7.8	1.4
Mortierella isabellina NRRL 1757	Glucose [a]	20.0 ± 0.4	9.5 ± 0.2	2.0	16.9	1.3	9.9	63.4	4.8	1.3
	Xylose [a]	15.3 ± 0.6	10.1 ± 0.1	1.2	18.4	1.1	12.9	59.6	6.4	0.1
	Glucose [b] + Xylose [b]	18.8 ± 0.7	9.5 ± 0.9	1.0	17.2	1.3	8.8	64.9	6.6	0.0
Chaetomium globosum NRRL 1870	Glucose [a]	18.6 ± 0.4	4.65 ± 0.2	0.6	22.2	1.1	9.1	20.5	45.7	0.6
	Xylose [a]	16.3 ± 0.5	5.4 ± 0.2	0.9	20.1	1.3	10.7	53.1	13.5	0.1
	Glucose [b] + Xylose [b]	16.0 ± 0.6	8.7 ± 0.4	0.8	19.2	1.6	5.4	60.1	12.6	0.08
Rhodosporidium toruloides NRRL Y-1091	Glucose [a]	15.1 ± 0.8	4.7 ± 0.5	1.0	16.2	0.8	12.9	64.2	3.9	0.6
	Xylose [a]	3.9 ± 1.2	1.7 ± 0.1	ND	29.7	ND	26.0	24.7	19.3	ND
	Glucose [b] + Xylose [b]	4.2 ± 6.0	2.1 ± 0.1	1.1	20.2	1.2	14.0	52.1	5.2	5.8

[a] Fermentation using the carbon source 40 g/L.
[b] Fermentation using the carbon source 20 g/L; ND: not detectable; 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 – Linoleneic acid; results are mean ± SD of three determinations.

A very few literatures deal with the production of microbial lipid by *C. curvatus* strain NRRL Y-1511 growing on different types of sugars, and only recently such a study was carried out using this oleaginous strain [49]. The authors demonstrated the interplay between the biosynthesis of intracellular total sugars and lipid synthesis for oleaginous yeast strains. They also found that *C. curvatus* NRRL Y-1511 showed remarkable growth and intracellular total sugar release during growth on sugar-based media (media containing substrates such as lactose and sucrose), while this strain produced lower quantities of microbial lipids, which is consistent with the observations in this study. Many researchers have used the *M. isabellina* strain for lipid production using the substrates such as glucose, raw glycerol, sweet sorghum, wheat straw, and rice hull. The microbial lipid production using different carbon sources by *M. isabellina* is summarized in Table 4 [19,20,45,50–54]. Chatzifragkou et al. [45] reported the lipid production by *M. isabellina* using commercial glucose, fructose, sucrose, and molasses, and the lipid contents found were 74, 61, 9 and, 54% w/w, respectively. This indicates that *M. isabellina* has the potential to use different low-cost sugars.

3.3. Enzymatic saccharification followed by fermentation of washed WIS

Enzymatic hydrolysis of WIS was carried out to evaluate the effectiveness of SPORL pretreatment in removing recalcitrance of Douglas fir. Cellic® CTec2 activity was estimated in our laboratory and was found to be 146 FPU/mL. Hydrolysis efficiency (%), defined as the percentage of glucan in WIS enzymatically hydrolyzed to glucose. Hydrolysis was carried out at 10% w/v and 15% w/v of WIS loading for 72 h at pH 5.5 and 50 °C with enzyme loading of 14.6 FPU/g glucan. For sample C-t60 (Fig. 2a), the glucose release was 85.4% and 70.3% in 24 h with a titer of 65.2 g/L and 80.5 g/L at 10% and 15% WIS loading (w/v), respectively. Hydrolysis efficiency further increased to 97.1% at 10% solid loading and 91.7% with 15% solid loading in 72 h. For sample C-t120 (Fig. 2b), the glucose release was 77.5% and 65.4% in 24 h with titer of 48.5 g/L and 61.5 g/L at a washed solid loading of 10% and 15%, respectively. Hydrolysis efficiency further increased to 90.8% at 10% solid loading and 84.2% with 15% solid loading in 72 h. These results suggested that the SPORL pretreatment was effective for maximal saccharification of Douglas fir residue. It has been observed that with the increase in WIS loading from at 10% to 15%, the corresponding glucose yield was reduced for both samples. This might be due to insufficient exposure of substrate to enzymes and end product inhibition. The hydrolysability of C-t120 was poorer than that of C-t60 because of the former had higher lignin content (Table 1).

Sugar released from the 72-h hydrolysis of C-t60 (78.1 g/L) and C-t120 (65.9 g/L) at 10% substrate loading was further utilized for lipid fermentation using *M. isabellina*. In order to determine the effect of nutrient supplementation on the production of lipid, experiments were carried out with (mentioned in Section 2.4.2) and without nutrients using batch fermentation. DCW, lipid concentration, and lipid yield using enzymatic hydrolysate (78.1 g/L of total sugars) with nutrients were found to be 23.7 g/L, 14.9 g/L, and 0.19 g/g of sugars, respectively, after 96-h fermentation, whereas those without nutrients were 25.5 g/L, 14.4 g/L, and 0.18 g/g of sugars after 120-h fermentation (Fig. 3a). Lipid production with and without nutrients reached the maximum after 96 h and 120 h fermentation, respectively, and decreased thereafter. Longer time fermentation was needed probably because the microorganism needed more time to acclimatize in the media without nutrients. The fermentation profiles using sample C-t120 with and without nutrients is shown in Fig. 3b. DCW, lipid concentration, and lipid yield using enzymatic hydrolysate (65.9 g/L of total sugars) were 23.8 g/L, 11.0 g/L, and 0.16 g/g of sugars after

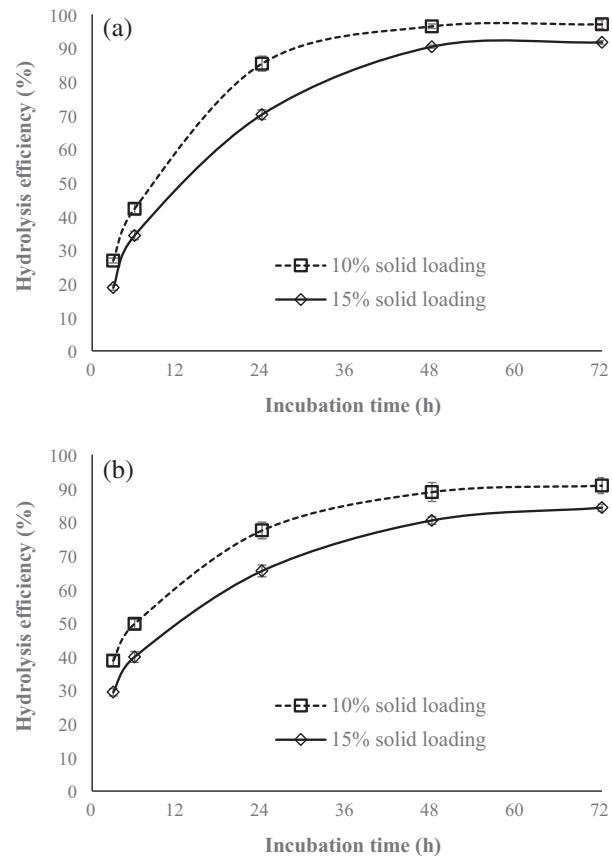


Fig. 2. Time-dependent enzymatic saccharification of WISs obtained from sulfite pretreated Douglas fir residue FS-10 with Cellic® CTec2, (a) C-t60 and (b) C-t120.

Table 4
Lipid production by *M. isabellina* from various feedstocks.

Microorganisms	Feedstocks	Lipid (g/L)	Lipid content (% w/w)	Lipid yield (g/g)	References
M. isabellina ATHUM2935	Glycerol	3.3	53.2	0.04	[20]
M. isabellina ATHUM2935	Starch	3.7	35.6	0.15	[50]
M. isabellina ATHUM2935	Glycerol	4.4	51	0.08	[51]
M. isabellina ATHUM2935	Sweet sorghum	9.3	43	0.10	[52]
M. isabellina ATHUM2935	Rice hull	3.6	64.3	0.14	[19]
M. isabellina NRRL 1757	Wheat straw	3.7	34	0.14	[53]
M. isabellina ATHUM2935	Sweet sorghum	10	11	0.19	[54]
M. isabellina ATHUM2935	Molasses	5.1	54	0.085	[45]
M. isabellina NRRL 1757	Douglas fir	17	[a]	0.21	Present study

[a] Lipid content was unable to determine because DCW was overlapped by the lignocellulosic biomass.

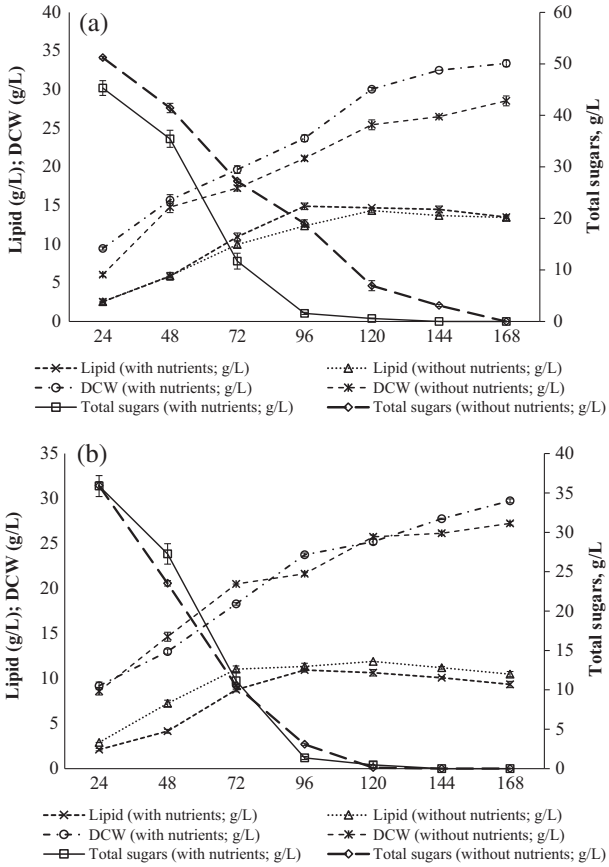


Fig. 3. Lipid production using the enzymatic hydrolysate obtained from WISs of sulfite pretreated Douglas fir residue FS-10 in the batch culture of *M. isabellina* NRRL 1757, (a) with and without nutrients (C-t60) and (b) with and without nutrients (C-t120).

96-h fermentation with nutrients and 25.7 g/L, 11.9 g/L, and 0.18 g/g of sugars after 120-h fermentation without nutrients, respectively.

The results above indicated that lipid yields with and without nutrient supplementation were comparable for both C-t60 and C-t120. The fermentation took a long time without nutrients to show the yield comparable to that of with nutrients. Though DCW was increasing, lipid concentration was found to decline after exhaustion of sugars. This might be because of utilization of the intracellular stored lipids for cell biomass growth after the depletion of sugars.

3.4. Fermentation of detoxified spent liquor

Lipid fermentation of spent liquors of C-t60 and C-t120 were performed using *M. isabellina* NRRL 1757. The microorganisms could neither grow nor produce lipids using both spent liquors though the liquors contain a good amount of fermentable sugars such as mannose, xylose, and glucose (Table 2). The probable inhibitors, i.e. sulfite, HMF, furfural, and organic acids, in spent liquors may be a possible reason for the inhibition of the growth of the microorganism. SPORL pretreatment of Douglas fir used 80 g/L total SO_2, and a certain amount of the chemical remained in the spent liquor in the form of SO_2, HSO_3^-, or $SO_3^=$ (total sulfite) after the pretreatment. The sulfite was suspected as the key inhibitor as the concentrations of HMF, furfural, and organic acids were relatively low (Table 2). Sulfite could inhibit the growth of microorganisms in different ways. The sulfite ion could enter the cell membrane and disrupt the normal metabolic activity of microbial cells [55]. Therefore, sulfite content in the spent liquors was quantified by using iodimetry method. Total sulfite content of C-t60 and C-t120 spent liquors was found to be 5.04 and 4.41 g/L, respectively. This could have a significant effect on lipid fermentation.

Detoxification of the spent liquors to remove sulfite was believed to be beneficial for fermentation. After lime treatment, sulfite content was decreased from 5.04 g/L to 1.26 g/L for C-t60 and from 4.41 g/L to 1.76 g/L for C-t120, respectively, and fermentation went on successfully. The spent liquor of C-t60 with 55.6 g/L of total sugars produced 16.05 g/L of DCW, 8.4 g/L of the lipid, and 0.18 g/g lipid yield after 168-h fermentation (Fig. 4a). All sugars were utilized after 168-h fermentation. Further increase in fermentation time, some quantities of previously stored lipids were degraded (lipid turnover) because of the complete exhaustion of sugars from the fermentation medium. Papanikolaou and Aggelis [46] thoroughly discussed the lipid turnover and concluded that after exhaustion or decrease in the uptake rate of the carbon source in the fermentation medium, the oleaginous microorganisms, as a general rule, consume their own lipid reserves. The spent liquor of C-t120 with 58.2 g/L of total sugars produced 11.6 g/L of DCW, 7.7 g/L of the lipid, and 0.16 g/g lipid yield after 168-h fermentation (Fig. 4b). Lipid production from C-t120 spent liquor was comparatively less than that from C-t60 spent liquor perhaps due to the presence of more sulfite after detoxification. All sugars were utilized after the 168-h fermentation. Detoxification with lime successfully removed the inhibition of sulfite. In addition, other

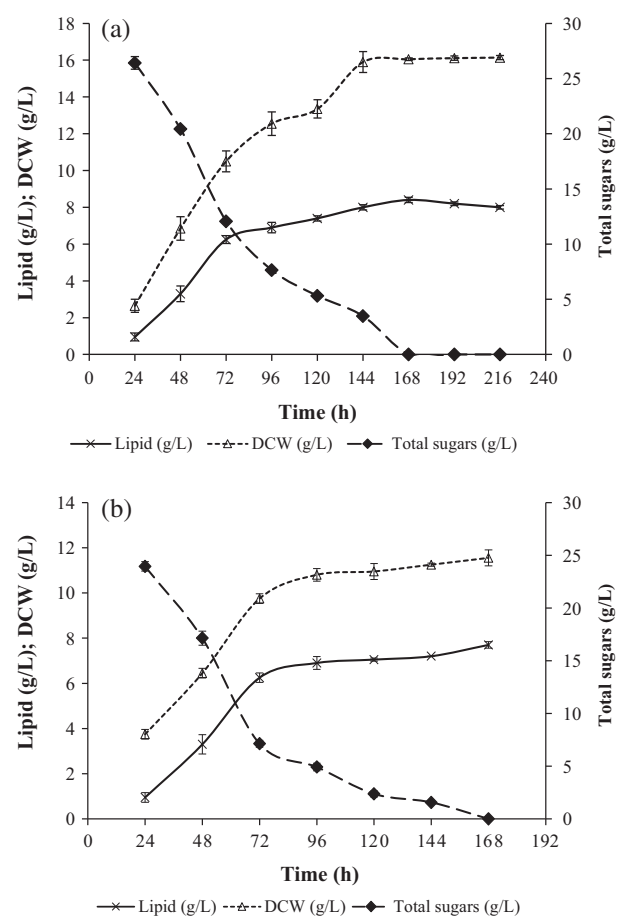


Fig. 4. Effect of detoxification of spent liquor obtained from the sulfite pretreatment of Douglas fir residue FS-10 on the lipid production in batch culture of *M. isabellina* NRRL 1757, (a) Detoxified liquor (C-t60) and (b) Detoxified liquor (C-t120).

inhibitors such as HMF, furfural, lignosulfonate, and organic acids were also removed though their inhibition might be minor. The results suggested that overliming was essential and suitable for lipid fermentation of the SPORL spent liquors. The detoxifying effect of overliming was attributed to both the precipitation of toxic components and the instability of some inhibitors at high pH [56]. Ruan et al. [57] reported that *M. isabellina* could tolerate high concentrations of HMF and furfural during lipid production. They also reported that acetate in the hydrolysate could act as a carbon source rather than an inhibitor. Huang et al. [58] reported that *T. coremiiforme* could grow well and accumulate more microbial lipid on the detoxified hydrolysates by overliming and absorption.

3.5. Quasi-simultaneous saccharification and fermentation of WIS (Q-SSF)

Q-SSF of WISs of C-t60 and C-t120 were carried out at 10% solid loading. The washed solids were first liquefied for 24 h prior to *M. isabellina* inoculation. For example, total sugars released from C-t60 WIS after 24 h was 79.2 g/L. Glucan saccharification and glucose utilization was simultaneous after inoculation. Total sugars were completely utilized after 168 h, and lipid production reached maximum. The lipid concentration and the lipid yield were found to be 17.0 g/L and 0.21 g/g, respectively (Fig. 5a). Q-SSF produced higher lipid yield compared with separate hydrolysis and fermentation (SHF) of the same WIS. DCW was not reported as it overlapped with the lignocellulosic biomass. Total sugars released from C-t120 after 24 h was 52.76 g/L. The lipid concentration and the lipid yield were 11.7 g/L and 0.18 g/g, respectively (Fig. 5b). Lipid yields were calculated by considering theoretical maximum release of sugars from the WISs, 82.2 g/L and 64.2 g/L, for C-t60 and C-t120, respectively. Simultaneous saccharification and fermentation (SSF) without pre-hydrolysis for liquefying WIS was also carried out to evaluate the cell biomass growth and lipid production from WIS. Unfortunately, *M. isabellina*

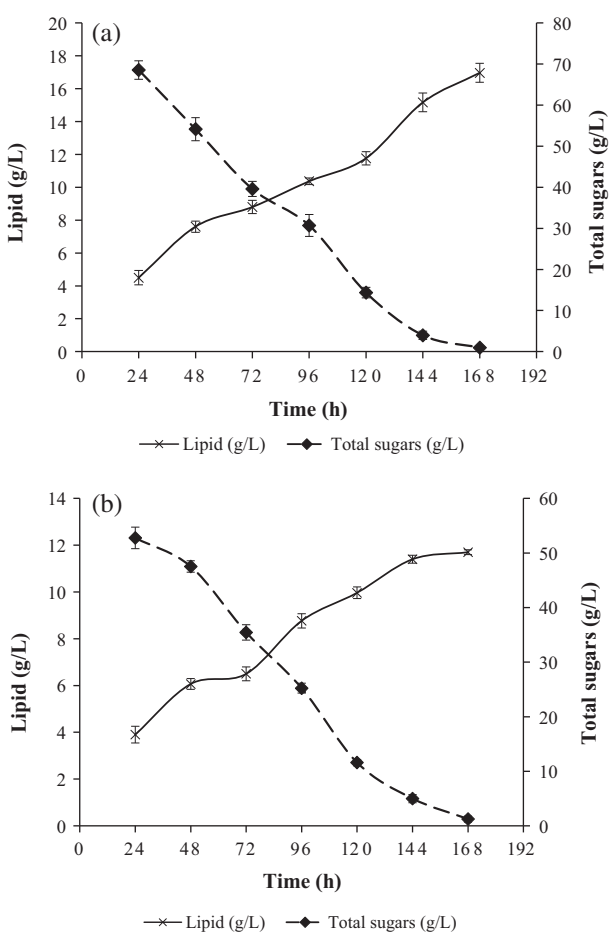


Fig. 5. Quasi-simultaneous saccharification and fermentation of WISs in batch culture of *M. isabellina* NRRL 1757, (a) C-t60 and (b) C-t120.

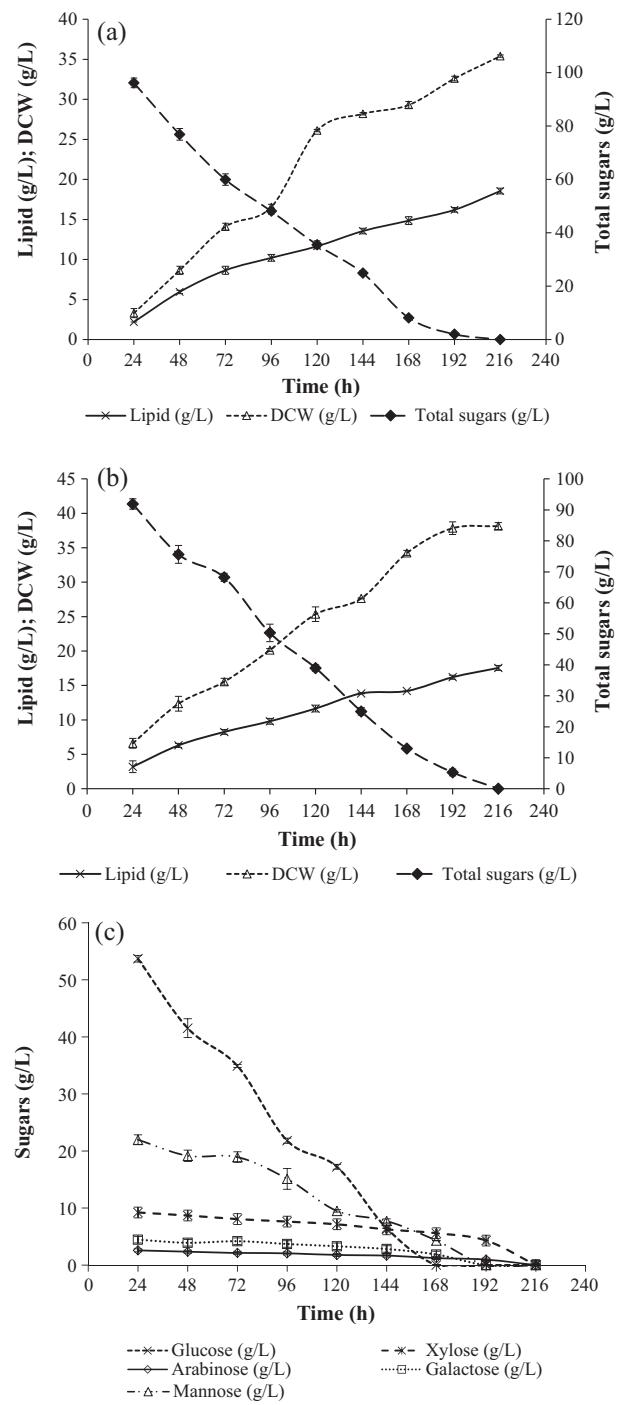


Fig. 6. Saccharification and fermentation of SPORL pretreated whole slurry of Douglas fir residue FS-10 in batch culture of *M. isabellina* NRRL 1757, (a) C-t60 (b) C-t120 and, (c) kinetics of individual sugar of C-t120.

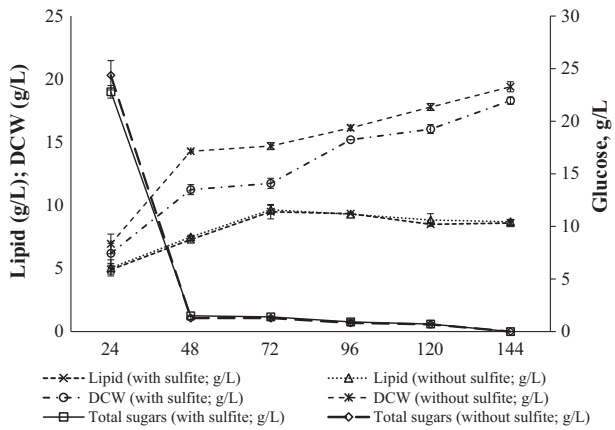


Fig. 7. Lipid production using the synthetic glucose in the batch culture of sulfite adapted *M. isabellina* NRRL 1757.

was not able to grow without liquefaction. These results suggested that Q-SSF was an efficient way for lipid production from WIS.

3.6. Lipid production from the whole slurry of SPORL pretreated Douglas fir

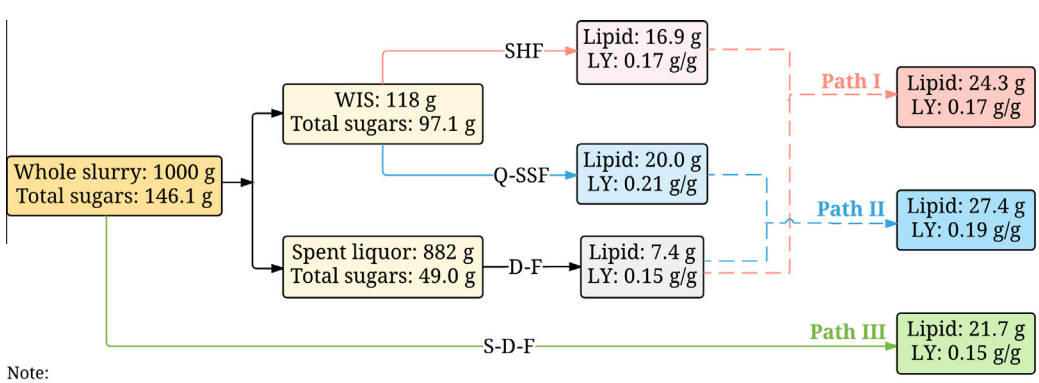
To simplify process integration and to simultaneously use the sugars from both solid and spent liquor, enzymatic saccharification of the SPORL pretreated whole slurry of C-t60 and C-t120 was separately carried out at 10% solid loading for 72 h. After detoxification with lime, the enzymatic hydrolysate of each whole slurry was then used for lipid fermentation. DCW, lipid concentration, and the lipid yield from the enzyme hydrolysate of C-t60 with 108.5 g/L of total sugars were 35.4 g/L, 18.55 g/L, and 0.17 g/g of sugars, respectively, after 216-h fermentation (Fig. 6a), whereas those from the enzyme hydrolysate of C-t120 with 93.34 g/L of total sugars were 38.2 g/L, 17.6 g/L, and 0.18 g/g, respectively, after 216-h fermentation (Fig. 6b). Fig. 6c shows the kinetics of individual sugars (arabinose, galactose, glucose, xylose, and mannose) during the fermentation of enzyme hydrolysate of C-t120. The sugar consumption profile indicated that all sugars were consumed simultaneously, not sequentially. The C6 sugars were utilized completely after 192-h (glucose was utilized completely after 168-h, mannose and galactose after 192-h), followed by C5 sugars (xylose and arabinose were utilized completely after 216-h). Complete utilization of sugars and maximal lipid production were observed after 216-h fermentation. This sugar utilization profile suggests that *M. isabellina* has preference to C6 sugars over C5 sugars. Sugar concentrations in enzymatic hydrolysates of washed water insoluble solid (78.1 g/L and 65.9 g/L for C-t60 and C-t120, respectively) and detoxified spent liquors (55.6 g/L and 58.2 g/L for C-t60 and C-t120, respectively) were less as compared to enzymatic hydrolysates of slurry (108.5 g/L and 93.34 g/L for C-t60 and C-t120, respectively). The difference in sugar concentrations showed that microorganism needs more time to utilize all sugars for lipid fermentation. Gao et al. [47] reported the highest cell biomass and lipid concentration after 220-h fermentation on a medium containing 100 g/L xylose.

3.7. Lipid production using sulfite adapted M. isabellina

As discussed above, sulfite is one of the fermentation inhibitors to *M. isabellina* present in spent SPORL pretreatment liquor, and therefore detoxification was necessary before the fermentation. To improve the sulfite tolerance of *M. isabellina* and potentially avoid the detoxification process, the strain was adapted by repetitively growing it in media containing sulfite as mentioned in Section 2.10. After 14-week adaptation, it was observed that *M. isabellina* was able to tolerate 2.2 g/L sulfite in this synthetic medium. The performance of the sulfite-adapted *M. isabellina* was then evaluated for lipid productivity in a synthetic medium. As shown in Fig. 7, the lipid productions using the sulfite-adapted *M. isabellina* in the media with sulfite (2.0 g/L) and without sulfite were compared. The DCW (18.3 g/L) and lipid production (8.6 g/L) using the synthetic glucose (40 g/L) by the sulfite-adapted *M. isabellina* in the medium with sulfite (2.0 g/L) was found to be comparable with the results obtained in the medium without sulfite (19.4 g/L and 8.7 g/L, respectively). The results suggest that the sulfite-adapted *M. isabellina* could tolerate the sulfite toxicity, and the detoxification process using calcium hydroxide could be potentially avoided for the fermentation of the spent SPORL pretreatment liquor.

3.8. Preliminary mass balance of lipid production from SPORL pretreated Douglas fir

Maximum lipid yield (0.21 g/g sugars) was obtained with Q-SSF from the WIS of C-t60 slurry, which is higher than the reported ones, summarized in Table 4 and below. Gao et al. [47] reported the DCW 28.8 g/L and lipid yield 0.18 g/g sugars using 100 g/L xylose by *M. isabellina*. Ruan et al. [59] reported a lipid yield of 0.15 g/g sugars from a corn stover hydrolysate using *M. isabellina*.



Note:
WIS: Washed water insoluble solid; SHF: Separate hydrolysis and fermentation;
Q-SSF: Quasi-simultaneous saccharification and fermentation;
D-F: Detoxification followed by fermentation; S-D-F: Saccharification, detoxification and fermentation;
LY: Lipid yield (g lipid/ g total sugars).

Fig. 8. Preliminary mass balance of the lipid production using SPORL pretreated Douglas fir residue FS-10.

Matsakas et al. [60] reported a lipid yield of 0.13 g/g sugars using sweet sorghum stalk hydrolysate by *L. starkeyi* CBS 1807. Simultaneous utilization of glucose and xylose for lipid production using *T. cutaneum* has been investigated, and maximum lipid yield was reported to be 0.20 g/g sugars [61]. Liu et al. [62] reported the practical feasibility of simultaneous saccharification and microbial lipid fermentation (SSF) of corn stover by oleaginous yeast *T. cutaneum* and lipid yield was reported to be 0.13 g/g sugars in the 5 L bioreactor. The authors also reported that cellulase enzyme could be partially recycled in the SSF.

Preliminary mass balance of the overall process for producing lipid from SPORL pretreated Douglas fir using three paths is summarized in Fig. 8. Lipid yield reported was calculated by dividing total lipids produced by total initial sugars present in the starting materials (whole slurry, WIS, or spent liquor) without considering sugar loss during the process. One kilogram of whole slurry of C-t60 (146.1 g total sugars) contained 118 g WIS (97.1 g total sugars) and 882 g spent liquor (49.0 g total sugars). From the WIS, 16.9 g and 20.0 g lipids were produced by SHF and Q-SSF with the yields of 0.17 and 0.21 g lipid/g total sugars, respectively. It is apparent that Q-SSF gave higher lipid yield than SHF. From the spent liquor, 7.4 g lipids were produced with a yield of 0.15 g lipid/g total sugars after detoxification and fermentation (D-F). If combining the lipid productions from both WIS and spent liquor, Path I (SHF of WIS and D-F of spent liquor) produced 24.3 g of lipids with an overall yield of 0.17 g lipids/g of total sugars, while Path II (Q-SSF of WIS and D-F of spent liquor) generated 27.4 g lipids with the lipid yield of 0.19 g/g of total sugars. When the whole slurry was saccharified and fermented through the S-D-F process (Path III), lipid production and lipid yield were found to be 21.7 g and 0.15 g/g of total sugars, respectively. The results indicated that the total lipids produced from whole slurry (path III) were less than those from other two paths (Path I and path II) where WIS and spent liquor were processed separately. Though lipid production and lipid yield were higher with Paths I and II, it would be economically attractive to use whole slurry saccharification and fermentation (Path III), as this process reduced the number of operations and thereby capital and operational costs for lipid production.

4. Conclusion

In the present study, washed water insoluble solids (WIS), spent liquor, and whole slurry from SPORL pretreated Douglas fir forest residue were successfully utilized for microbial lipid production using *M. isabellina* NRRL 1757. This strain could utilize pentose and hexose simultaneously to accumulate intracellular lipid with good lipid yield. Over liming of spent liquors removed the inhibitors and facilitated successfully lipid fermentation. Maximal lipid yield (0.21 g/g total sugars) was obtained with Q-SSF from the WIS. Separately processing WIS and spent liquor yielded more total lipids, but the whole slurry processing avoids liquor and solid separation and would reduce capital and operational costs for lipid production. This work also contributes toward efficient utilization of renewable forest residue as a resource for microbial lipid production. The continuation of this project will focus on the development of *M. isabellina* strain to tolerate the sulfite present in the spent liquor after SPORL pretreatment, which will reduce the number of the process steps and save the cost of detoxification.

Acknowledgements

This work was supported by WARF (Wisconsin Alumni Research Foundation) and the Graduate School at the University of Wisconsin-Madison.

References

[1] Pinzi S, Leiva-Candia D, López-García I, Redel-Macías MD, Dorado MP. Latest trends in feedstocks-for biodiesel production. Biofuel Bioprod Bioref 2013;8:126–43.
[2] Leiva-Candia DE, Pinzi S, Redel-Macías MD, Koutinas A, Webb C, Dorado MP. The potential for agro-industrial waste utilization using oleaginous yeast for the production of biodiesel. Fuel 2014;123:33–42.
[3] Huang C, Zong MH, Wu H, Liu QP. Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. Bioresour Technol 2009;100(19):4535–8.
[4] Koutinas AA, Papanikolaou S. Biodiesel production from microbial oil. In: Luque R, Campelo J, Clark JH, editors. Handbook of biofuels production – processes and technologies. Cambridge, UK: Woodhead Publishing; 2011. p. 177–98.
[5] Cohen Z, Ratledge C, editors. Single cell oils. Champaign (IL, USA): AOCS Press; 2005.
[6] Zhu LY, Zong MH, Wu H. Efficient lipid production with *Trichosporon fermentans* and its use for biodiesel preparation. Bioresour Technol 2008;99(16):7881–5.
[7] Li Q, Du W, Liu D. Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol 2008;80:749–56.
[8] Chen X, Li ZH, Zhang XX, Hu FX, Ryu DDY, Bao J. Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. Appl Biochem Biotechnol 2009;159:591–604.
[9] Koutinas AA, Chatzifragkou A, Kopsahelis N, Papanikolaou S, Kookos IK. Design and technoeconomic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel 2014;116:566–77.
[10] Ratledge Wynn J. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 2002;51:1–51.
[11] Hiruta O, Futamura T, Takebe H, Satoh A, Kamisaka Y, Yokochi T, et al. Optimization and scale-up of gamma-linolenic acid production by *Mortierella ramanniana* MM 15-1, a high gamma-linolenic acid producing mutant. J Ferment Bioeng 1996;82:366–70.
[12] Papanikolaou S, Aggelis G. Lipids of oleaginous yeasts. Part II. Technology and potential applications. Eur J Lipid Sci Technol 2011;113:1052–73.
[13] Papanikolaou S, Aggelis G. *Yarrowia lipolytica*: a model microorganism used for the production of tailor-made lipids. Eur J Lipid Sci Technol 2010;112:639–54.
[14] Tsigie YA, Wang CY, Truong CT, Ju YH. Lipid production from *Yarrowia lipolytica* Po1g grown in sugarcane bagasse hydrolysate. Bioresour Technol 2011;102(19):9216–22.
[15] Yu X, Zheng Y, Dorgan KM, Chen S. Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour Technol 2011;102(10):6134–40.
[16] Zhang J, Hu B. Microbial lipid production from corn stover via *Mortierella isabellina*. Appl Biochem Biotechnol 2014;174(2):574–86.
[17] Xue YP, Jin M, Orjuela A, Slininge P, Dien BS, Dale BE, et al. Microbial lipid production from AFEX™ pretreated corn stover. RSC Adv 2015;5:28725–34.
[18] Gong Z, Shen H, Yang X, Wang Q, Xie H, Zhao ZK. Lipid production from corn stover by the oleaginous yeast *Cryptococcus curvatus*. Biotechnol Biofuels 2014;7:158.
[19] Economou CN, Aggelis G, Pavlou S, Vayenas D. Single cell oil production from rice hulls hydrolysate. Bioresour Technol 2011;102(20):9737–42.
[20] Fakas S, Papanikolaou S, Batsos A, Galiotou-Panayotou M, Mallouchos A, Aggelis G. Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. Biomass Bioenerg 2009;33(4):573–80.
[21] Zikou E, Chatzifragkou A, Koutinas AA, Papanikolaou S. Evaluating glucose and xylose as cosubstrates for lipid accumulation and γ-linolenic acid biosynthesis of *Thamnidium elegans*. J Appl Microbiol 2013;114:1020–32.
[22] Wiebe MG, Koivuranta K, Penttilä M, Ruohonen L. Lipid production in batch and fed-batch cultures of *Rhodosporidium toruloides* from 5 and 6 carbon carbohydrates. BMC Biotechnol 2012;12:26.
[23] Zhao X, Kong X, Hua Y, Feng B, Zhao Z. Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. Eur J Lipid Sci Technol 2008;110:405–12.
[24] Easterling ER, French WT, Hernandez R, Licha M. The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*. Bioresour Technol 2009;100(1):356–61.
[25] Zhu JY, Pan XJ. Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation. Bioresour Technol 2010;101:4992–5002.
[26] National Research Council. Renewable fuel standard: potential economic and environmental effects of U.S. Biofuel Policy; 2011. <http://www.nap.edu/catalog.php?record_id=13105>, ISBN 978-0-309-18751-0.
[27] Gan J, Smith CT. Availability of logging residues and potential for electricity production and carbon displacement in the USA. Biomass Bioenerg 2006;30:1011–20.
[28] Perlack RD, Stokes BJ. DOE. 2011. U.S. Billion-ton update: biomass supply for a bioenergy and bioproducts industry. Oakridge National Laboratory. DEAC05-00R22725; 2011.
[29] Leu SY, Gleisner R, Zhu JY, Sessions J, Marrs G. Robust enzymatic saccharification of a Douglas-fir forest harvest residue by SPORL. Biomass Bioenerg 2013;59:393–401.

[30] Yamamoto M, Niskanen T, Iakovlev M, Ojamo H, van Heiningen A. The effect of bark on sulfur dioxide–ethanol–water fractionation and enzymatic hydrolysis of forest biomass. Bioresour Technol 2014;167:390–7.
[31] Zhang C, Zhu JY, Gleisner R, Sessions J. Fractionation of forest residues of Douglas-fir for fermentable sugar production by SPORL pretreatment. Bioenergy Res 2012;5:978–88.
[32] Zhang C, Houtman CJ, Zhu JY. Using low temperature to balance enzymatic saccharification and furan formation in SPORL pretreatment of Douglas-fir. Process Biochem 2014;49:466–73.
[33] Zhu JY, Pan XJ, Wang GS, Gleisner R. Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine. Bioresour Technol 2009;100:2411–8.
[34] Zhu JY, Chandra MS, Gu F, Gleisner R, Reiner R, Sessions J, et al. Using sulfite chemistry for robust bioconversion of Douglas-fir forest residue to bioethanol at high titer and lignosulfonate: a pilot-scale evaluation. Bioresour Technol 2015;179:390–7.
[35] Wang ZJ, Lan TQ, Zhu JY. Lignosulfonate and elevated pH can enhance enzymatic saccharification of lignocelluloses. Biotechnol Biofuels 2013;6:9.
[36] Wang Z, Zhu JY, Fu Y, Qin M, Shao Z, Jiang J, et al. Lignosulfonate-mediated cellulase adsorption: enhanced enzymatic saccharification of lignocellulose through weakening nonproductive binding to lignin. Biotechnol Biofuels 2013;6:156.
[37] Zhou H, Lou H, Yang D, Zhu JY, Qiu X. Lignosulfonate to enhance enzymatic saccharification of lignocelluloses: role of molecular weight and substrate lignin. Ind Eng Chem Res 2013;52:8464–70.
[38] Cheng J, Leu SY, Zhu JY, Gleisner R. High titer and yield ethanol production from undetoxified whole slurry of douglas-fir forest residue using pH-profiling in SPORL. Biotechnol Biofuels 2015;8:22.
[39] Yu X, Zheng Y, Xiong X, Chen S. Co-utilization of glucose, xylose and cellobiose by the oleaginous yeast *Cryptococcus curvatus*. Biomass Bioenrg 2014;71:340–9.
[40] Mohagheghi A, Ruth M, Schell DJ. Conditioning hemicellulose hydrolysates for fermentation: effects of overliming pH on sugar and ethanol yields. Process Biochem 2006;41:1806–11.
[41] Ning Z. Handbook for analysis of food ingredients. Beijing: China Light Industry Press; 1998.
[42] Li Z, Zhang L, Shen X, Lai B, Sun S. A comparative study on four method of fungi lipid extraction. Microbiol 2001;6(28):72–5.
[43] Shuai L, Yang Q, Zhu JY, Lu FC, Weimer PJ, Ralph J, et al. Comparative study of SPORL and dilute acid pretreatments of spruce for cellulosic ethanol production. Bioresour Technol 2010;101:3106–14.
[44] O'Fallon JV, Busboom JR, Nelson ML, Gaskins CT. A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. J Anim Sci 2007;85(6):1511–21.
[45] Chatzifragkou A, Fakas S, Galiotou-Panayotou M, Komaitis M, Aggelis G, Papanikolaou S. Commercial sugars as substrates for lipid accumulation in *Cunninghamella echinulata* and *Mortierella isabellina* fungi. Eur J Lipid Sci Technol 2010;112:1048–57.
[46] Papanikolaou S, Aggelis G. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. Eur J Lipid Sci Technol 2011;113:1031–51.
[47] Gao D, Zeng J, Zheng Y, Yu X, Chen S. Microbial lipid production from xylose by *Mortierella isabellina*. Bioresour Technol 2013;133:315–21.
[48] Tao J, Dai CC, Dai Q, Zhao M. The conversion efficiency and economic feasibility of microbial energy. J Microbiol 2006;26(6):48–54.
[49] Tchakouteu SS, Chatzifragkou A, Kalantzi O, Koutinas AA, Aggelis G, Papanikolaou S. Oleaginous yeast *Cryptococcus curvatus* exhibits interplay between biosynthesis of intracellular sugars and lipids. Eur J Lipid Sci Technol 2015;117:657–72.
[50] Papanikolaou S, Galiotou-Panayotou M, Fakas S, Komaitis M, Aggelis G. Lipid production by oleaginous Mucorales cultivated on renewable carbon sources. Eur J Lipid Sci Technol 2007;109:1060–70.
[51] Papanikolaou S, Fakas S, Fick M, Chevalot I, Galiotou-Panayotou M, Komaitis M, et al. Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: production of 1,3-propanediol, citric acid and single cell oil. Biomass Bioenerg 2008;32:60–71.
[52] Economou CN, Aggelis G, Pavlou S, Vayenas DV. Modeling of single-cell oil production under nitrogen-limited and substrate inhibition conditions. Biotechnol Bioeng 2011;108:1049–55.
[53] Zeng J, Zheng Y, Yu X, Yu L, Gao D, Chen S. Lignocellulosic biomass as a carbohydrate source for lipid production by *Mortierella isabellina*. Bioresour Technol 2013;128:385–91.
[54] Economou ChN, Makri A, Aggelis G, Pavlou S, Vayenas DV. Semi-solid state fermentation of sweet sorghum for the biotechnological production of single cell oil. Bioresour Technol 2010;101:1385–8.
[55] Barnett D. Sulphites in foods: their chemistry and analysis. Food Technol Ass Aust 1985;37:503–5.
[56] Palmqvist E, Hagerdal BH. Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxificaton. Bioresour Technol 2000;74:17–24.
[57] Ruan Z, Hollinshead W, Isaguirre C, Tang YJ, Liao W, Liu Y. Effects of inhibitory compounds in lignocellulosic hydrolysates on *Mortierella isabellina* growth and carbon utilization. Bioresour Technol 2015;183:18–24.
[58] Huang C, Chen XF, Xionga L, Yang XY, Chen XD, Ma LL, et al. Microbial oil production from corncob acid hydrolysate by oleaginous yeast *Trichosporon coremiiforme*. Biomass Bioenerg 2013;49:273–8.
[59] Ruan Z, Zanotti M, Archer S, Liao W, Liu Y. Oleaginous fungal lipid fermentation on combined acid- and alkali-pretreated corn stover hydrolysate for advanced biofuel production. Bioresour Technol 2014;163:12–7.
[60] Matsakas L, Sterioti AA, Rova U, Christakopoulos P. Use of dried sweet sorghum for the efficient production of lipids by the yeast *Lipomyces starkeyi* CBS 1807. Ind Crops Prod 2014;62:367–72.
[61] Hu C, Wu S, Wang Q, Jin G, Shen H, Zhao ZK. Simultaneous utilization of glucose and xylose for lipid production by *Trichosporon cutaneum*. Biotechnol Biofuels 2011;4:25.
[62] Liu W, Wang Y, Yu Z, Bao J. Simultaneous saccharification and microbial lipid fermentation of corn stover by oleaginous yeast *Trichosporon cutaneum*. Bioresour Technol 2012;118:13–8.