Oil Production by the Oleaginous Yeast *Lipomyces starkeyi* using Diverse Carbon Sources

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Producing microbial oils *via* oleaginous yeast fermentation has drawn broad attention in the biodiesel industry. The oleaginous yeast *Lipomyces starkeyi* utilizing diverse carbon sources including glucose, xylose, glycerol, and willow wood sawdust (WWS) hydrolysate for the biosynthesis of oils in its cell growth were investigated in this study. High carbon/nitrogen ratios within the glucose media significantly increased the lipid content of *Lipomyces starkeyi* cells and modified the fatty acid composition of lipids, promoting the accumulation of C16:0 fatty acids and saturated fatty acids (C16:0 and C18:0). The accumulation of C18 fatty acids (C18:0, C18:1, and C18:2) and unsaturated fatty acids (C16:1, C18:1, and C18:2) was restricted. When crude glycerol and WWS hydrolysate were used as the sole carbon sources for *L. starkeyi* fermentation, the dry cell weight, lipid content, and lipid productivity were 9.1 g/L, 46.2%, and 4.2 g/L, respectively, for glycerol, and 8.2 g/L, 42.7%, and 3.5 g/L, respectively, for the hydrolysate solution. This study provides useful information for producing oils with *L. starkeyi* fermentation using glycerol and WWS hydrolysate as the primary or secondary carbon substrates.

Keywords: Acid hydrolysis; Fermentation; Lipomyces starkeyi; Microbial oil; Willow wood sawdust hydrolysate

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INTRODUCTION

As crude oil prices continue to increase and the world's oil supply becomes more and more uncertain, it is becoming increasingly important to search for renewable substitutes. Biodiesel could be one such alternative, but using plant oils or animal fat as feedstock for producing biodiesel is often controversial because of the large land area required for their production, their potential competition with food production, and their high cost. Using microbial oils as feedstock for producing biodiesel has great potential (Papanikolaou and Aggelis 2011b). In contrast to plant oils and animal fats, the fatty acid compositions of microbial oils with similar composition and energy value are fit for producing biodiesel, but microbial oils offer unique advantages like a short production period and easy scale-up in industry (Li *et al*. 2007; Li *et al*. 2008). Moreover, microbial oils usually contain medically and dietetically important polyunsaturated fatty acids, which could be broadly used in various dietetics and cosmetology productions (Ratledge 1993; Dyal and Narine 2005). However, the current cost of biodiesel or diet products converted from microbial oils still is high, for the high cost of media for cell culture and low efficiency

of producing microbial oils (Ratledge and Cohen 2008; Papanikolaou and Aggelis 2011b; Ratledge 2011). In attempts to reduce the cost of microbial culture media for microbial oil production, researchers have investigated the use of agro-industrial residues including whey permeate, sewage sludge, waste glycerol, sugar cane molasses, and monosodium glutamate wastewater as inexpensive culture media (Akhtar *et al*. 1998; Angerbauer *et al.* 2008; Papanikolaou *et al*. 2008; Xue *et al.* 2008; Wu *et al*. 2011). However, using lignocellulosic biomass as a raw material appears to be a better strategy for the cost-effective preparation of oils because of its universal availability and high biomass (Parajo *et al*. 1996). Studies have demonstrated that there is great potential for the effective use of microbial oils derived from lignocellulosic hydrolysate as the culture medium for oleaginous microorganisms (Zhao *et al.* 2008; Zhu *et al.* 2008; Fakas *et al.* 2009; Huang *et al*. 2009; Pirozzi *et al*. 2012).

The prospect of using biomass from woody trees such as poplar, willow, and eucalyptus as feedstock for energy production has received much attention recently because these trees grow quickly and are short rotation coppices (SRCs) in many countries (Mola-Yudego 2010). Willow, for example, was planted across 14,000 ha of agricultural lands in the southern and central areas of Sweden for energy production (Mola-Yudego and González-Olabarria 2010). Compared to other SRC species, willow has greater potential as a biomass feedstock for energy production because of its easy propagation, fast growth, high yield, low cost, and high efficiency for energy storage (Karp and Shield 2008). The use of woody trees in energy production *via* bioethanol and biodiesel production requires that structural polysaccharides (*i.e.*, cellulose and lignin) are hydrolyzed to generate soluble sugars. Soluble sugars derived from the hydrolysis of these polysaccharides can serve as nutritious substrates for oleaginous microorganisms that produce microbial oils (Ma and Hanna 1999).

As much as 50% of the dry cell weight of oleaginous microorganisms like *Rhodosporidium* sp., *Rhodotorula* sp., *Lipomyces* sp., *Yarrowia lipolytica*, and *Lipomyces starkeyi* mostly consists of intracellular lipids (Zhao *et al.* 2008). The microbial oils derived from these oleaginous microorganisms are an ideal feedstock for biodiesel production (Ratledge and Wynn 2002). In particular, *L. starkeyi*, originally isolated from soil, offers great potential in producing microbial oils by fermentation because it is able to produce more than 60% lipids of its cell dry weight and to utilize a variety of carbon sources such as glucose, xylose, and their derivatives. Using *L. starkeyi* fermentation for producing microbial oils as biodiesel feedstock in industry has drawn great attention (Angerbauer *et al.* 2008; Zhao *et al.* 2008; Huang *et al.* 2011; Lin *et al.* 2011). In addition, crude glycerol is the main byproduct of methyl ester-based biodiesel. How to most efficiently reuse crude glycerol is an important question to the biodiesel industry. Using crude glycerol as a carbon source for the production of microbial oils for biodiesel feedstock could be promising, though glycerol can be used as substrates for producing citric acid, 1,3-propanediol, and acetic acid (Papanikolaou and Aggelis 2002; Papanikolaou *et al*. 2008; Chatzifragkou *et al*. 2011). However, little is known about whether or not *L. starkeyi* can utilize crude glycerol or soluble carbon sources derived from the hydrolysis of lignocellulosic biomass to produce lipids. In this study, acid hydrolysis was performed on willow biomass to convert a lignocellulosic material into fermentable sugars. Further, the feasibility of using willow wood sawdust (WWS) hydrolysate or glycerol as carbon sources for microbial oil production *via L. starkeyi* fermentation was explored. This study provides valuable information regarding the production of microbial oil with glycerol or lignocellulosic hydrolysate as the feedstock.

EXPERIMENTAL

Materials

WWS used in this study was collected from Chang Qing sawmills, Shandong, China. The WWS was screened to remove oversized particles, sun-dried, and sieved to remove particles larger than 0.5 mm in diameter. The screened WWS was homogenized and stored in polypropylene bags at ambient temperature until further use. Aliquots from the homogenized WWS were oven-dried to a constant weight at 105 ˚C and cooled in desiccators to room temperature before they were used in experiments (Rafiqul and Sakinah 2012). All chemicals, including crude glycerol, were commercially obtained.

Hydrolysis Experiments

A 500-mL, high-pressure autoclave (FCFD05-30, Yantai Jianbang Chemical Mechanical Co. Ltd., Shandong, China) was used for the two-step dilute sulfuric acid hydrolysis. WWS (15 g, on an oven-dry basis) mixed with 2% (w/w) sulfuric acid solution at a liquid-to-solid ratio (LSR) of 10 g/g was hydrolyzed for 1h at 130 ˚C in an autoclave (Rafiqul and Sakinah 2012). After the first step of the hydrolysis, the solid residue was collected by filtration, washed, and dried prior to the second step of the hydrolysis. Then, 15 g of solid residue was hydrolyzed with 4% (w/w) sulfuric acid solution at a LSR of 10 g/g at 150℃for 1 h as described by Jiang *et al.* (2012). After the pH was adjusted to 5.0 by the addition of calcium oxide (CaO) powder, the hydrolyzate solutions were filtered and concentrated using a rotary evaporator (Buchi R-210, Switzerland) at 60 ˚C to achieve a sugar concentration of approximately 80 g/L. The concentrated hydrolysate solutions (200 mL) were detoxified for 2 h at 50 ˚C and shaken at 200 rpm in a 500-mL flask containing 5 g of activated charcoal (Sangon Biotech Co. Ltd., Shanghai). Finally, after removing the charcoal by filtration, the sugar concentration was adjusted to 36 g/L for fermentation.

Microorganism and Media

The oleaginous yeast *L. starkeyi* AS 2.1560 was purchased from the China General Microbiological Culture Collection Center (CGMCC). The culture was maintained on a YPD agar slant at 4 ˚C and subcultured twice a month. The seed medium was composed of glucose (20 g/L), peptone (10 g/L), and yeast extract (10 g/L) at pH 6.0. The fermentation medium was composed of sugar or glycerol (36 g/L), KH_2PO_4 (12.5 g/L), $Na_2HPO_4 \cdot 12H_2O$ (2.5 g/L), $(NH_4)_2SO_4$ (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (2.5 g/L), $CaCl_2 \cdot 2H_2O$ (0.25 g/L), yeast extract containing 9.7% (w/w) of nitrogen and 35.6% (w/w) of carbon (0.9 g/L), and 0.625 mL of trace element solution with pH 5.0. The trace-element-solution contained (g/L): $FeSO_4 \cdot 7H_2O$ 16; $MnSO_4 \cdot H_2O$ 4; $Al_2(SO_4)_3 \cdot 18H_2O$ 5.52; $CoCl_2 \cdot 6H_2O$ 2.92; $ZnSO_4 \cdot 7H_2O$ 0.8; $Na_2MoO_4 \cdot 2H_2O$ 0.8; $CuCl_2 \cdot 2H_2O$ 0.4; H_3BO_3 0.2 and KI 1.6 in 5 N hydrochloric acid. To adjust different the ratios of carbon and nitrogen (C/N) in fermentation media, the concentration of the N source, *i.e.* $(NH_4)_2SO_4$, was adjusted to 3.6, 1.6, 0.6, 0.3, 0.1, and 0 g/L, resulting in the C/N ratios imposed 20, 40, 80, 120, 160, and 200, respectively.

Fermentation Experiments in Flasks

For seed preparation, a full loop of *L. starkeyi* from a fresh slant tube was inoculated in a 250-mL flask with 50 mL of fresh seed medium in a rotary shaker running at 200 rpm. Shaking-flask cultures were carried out in 250-mL flasks loaded with 50 mL of fermentation medium. The culture was initiated with 5% (v/v) of 24-h-old seeding cultures and incubated in a rotary shaker running at 200 rpm and 30 ºC for 120 h.

Analytical Methods

The chemical components and composition of the biomass materials were analyzed with a fiber analyzer (ANKOM 200, USA) using the Van Soest Fiber Analysis System and an organic elemental analyzer (Elementar Analysensysteme GmbH D-63452 Hanau, Germany). The 3,5-dinitrosalicylic acid method was used to analyze the content of reducing sugar by using spectrophotometry as described by Miller (1959). The content of glycerol in the medium was measured with the Glycerol Enzymatic Test Kit (R-Biopharm, Germany). Sugar composition was assayed by an HPLC apparatus (LC-20A, Shimadzu, Japan) fitted with a refractive index detector and an Aminex HPX-87P column (Bio-Rad, USA) at 80 °C using water as the mobile phase. The mobile phase flow rate was 0.4 mL/min. Acetic acid, furfural, and 5-hydroxymethyl-furfural were analyzed by an HPLC UV detector at 280 nm.

Total lipids of cultured cells were extracted as described by Zhao *et al.* (2008). Briefly, yeast cells were harvested from 20 mL culture broth and dried at 105 °C to constant weight to give the dry cell weight (DCW, g/L). In parallel, yeast cells harvested from 20 mL culture medium were digested with 4 M HCl at 78 °C for 1 h, and then the total lipids were extracted using the chloroform/methanol (1:1, v/v) method. The extracts were washed with 0.1% NaCl and distilled water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*, and the residue was dried at 105 °C overnight to give the total cellular lipid. The lipid content was expressed by the percentage of lipid in the DCW.

For fatty acid composition determination, fatty acid methyl esters (FAMEs) reactions were performed. FAMEs were prepared by heating the dry lipid materials at 85 °C for 1 h in 2% (v/v) sulfuric acid in methanol. The fatty acid composition was determined by HP6890GC/5973MS gas chromatography mass spectrometry (Agilent Technologies, USA). The analytical conditions of GC using an HP-5MS column (Agilent Technologies, USA, 30 m×0.25 mm×0.25 μm) were as follows: flame ionization detector (FID) at 250 °C; temperature raised from 150 °C to 260 °C at a rate of 3 °C /min; and a split ratio of 10:1. Helium was used as the carrier gas with a flow rate of 1 mL/min. The sample size was 0.2 μL. The mass spectrometry conditions were as follows: mass range of 35 to 500 amμ; electron impact (EI) of 70 eV; ion source temperature of 230 °C; transfer line temperature of 250 °C; and quadrupole temperature of 150 °C.

RESULTS AND DISCUSSION

Chemical Components of WWS and its Hydrolysate

Prior to the hydrolysis of WWS, the chemical components and composition of WWS were determined. Cellulose, hemicellulose, and lignin were the main biopolymer components of WWS. Cellulose accounted for 54.4%, hemicelluloses accounted for 19.3%, and lignin accounted for 16.3% (Table 1) of its mass. Combined, carbon, oxygen, and hydrogen accounted for 99.4% (Table 1) of its mass. These results are similar to those of previous reports (Sinag *et al.* 2009; Rafiqul and Sakinah 2012).

The sugars in the WWS hydrolysate were produced by two-step dilute sulfuric acid hydrolysis (Table 2). The major sugar products were xylose (15.5 g/L) from the first step of the hydrolysis and glucose (21.9 g/L) from the second step of the hydrolysis. After mixing the hydrolysates from both hydrolyses, the ratio of glucose to xylose was 1.4:1.

Table 1. Main Components and Elemental Composition of WWS

Components	(wt.%)	Elements	(wt.%)
Cellulose	54.4±0.6	Carbon	47.1±0.1
Hemicellulose	19.3±0.7	Oxygen	46.6±2.4
Lignin	16.3±0.5	Hydrogen	5.7±0.2
Extractives	9.0±0.2	Nitrogen	0.3±0.03
Ash	0.2±0.02	Sulfur	0.2±0.1
Total	99.2	Total	99.9

Data are presented as average values from triplicate experiments.

Table 2. Sugar Composition of WWS Hydrolysate

Compound	First-step hydrolysate (g/L)	Second-step hydrolysate (g/L)
Arabinose	1.1±0.02	1.9±0.01
Cellobiose	0.3±0.01	ND
Galactose	0.9±0.01	0.2±0.02
Glucose	2.6±0.01	21.9±0.05
Xylose	15.5±0.2	0.7±0.01

Data are average values from triplicate experiments. Abbreviation: ND (not detected).

Cell Growth and Lipid Accumulation

Studies have shown that lipid accumulation with in *L. starkeyi* strongly depends on the carbon/nitrogen (C/N) ratio (Angerbauer *et al*. 2008). To determine the influence of different C/N ratios on cell growth and lipid accumulation in *L. starkeyi* AS 2.1560, the dry cell weights (DCWs) and lipid accumulation yields of *L. starkeyi* AS 2.1560 on fermentation media containing different C/N ratios (200, 160, 120, 80, 40, and 20) were compared. In previous reports (Angerbauer *et al*. 2008), fermentation media with a pH value of 5.0 were used for *L. starkeyi* cell growth. Maximum DCWs were attained after 144 (cultured with C/N ratios of 20, 40, and 80) or 120 (cultured with C/N ratios of 120, 160, and 200) h of culture. This corresponds to the end of the fifth or sixth day of culture, as shown in Fig. 1. Higher C/N ratios slightly sped up the growth of *L. starkeyi*, but DCWs did not differ significantly among cultures with different C/N ratios, suggesting that the C/N ratio within fermentation media does not significantly influence the growth of *L. starkeyi* (Fig. 1). The lipid content of cultured cells increased significantly with increased C/N ratios. The highest lipid content was 48.6% at a C/N ratio of 200, and the lowest lipid content was 23.7% at a C/N ratio of 20 (Fig. 1). Similarly, the lipid productivity at C/N ratios of 20, 40, 80, 120, 160, and 200 were 2.8 g/L, 4.0 g/L, 5.0 g/L, 5.8 g/L, 5.5 g/L, and 5.4 g/L, respectively. The maximum lipid productivity (5.8 g/L) was achieved after 120 h of fermentation in broth with a C/N ratio of 120. These results showed that the higher C/N ratios were able to promote lipids accumulation when C/N ratios were from 20 to 120, but when C/N ratios were too high (such as 160 or 200) the promotion of C/N ratio was limited. Thus, it was concluded that the optimum C/N ratio for lipid accumulation within *L. starkeyi* cells was 120. In subsequent experiments, a C/N ratio of 120 was used. Such a C/N ratio yielded a lipid content of 47%, equivalent to a productivity of 5.8 g/L. These results are similar to those for other oleaginous yeasts such as *Yarrowia lipolytica* (Papanikolaou *et al*. 2009) and *Thamnidium elegans* (Papanikolaou *et al*. 2010; Chatzifragkou *et al*. 2011).

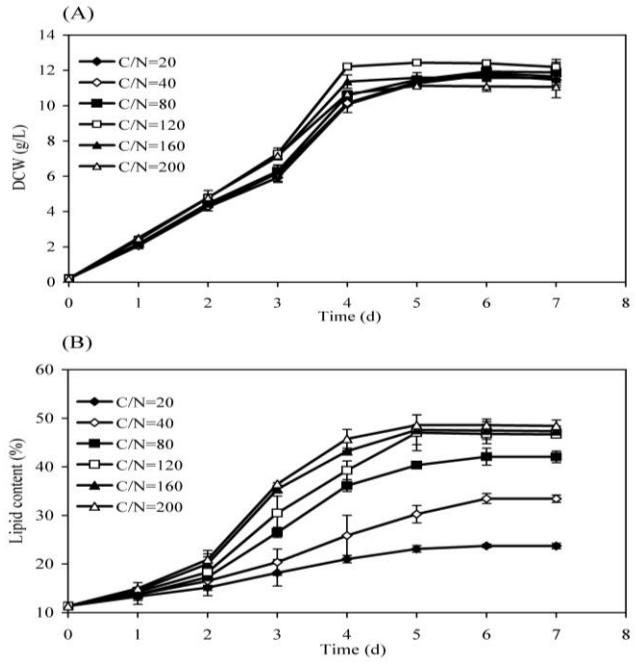

Fig. 1. Changes of dry cell weight and lipid accumulation with different C/N ratios in cell growth of *Lipomyces starkeyi*. (A): Changes of dry cell weight (DCW); (B) Changes of lipid accumulation

To further investigate the effects of C/N ratio on fatty acid composition, the fatty acid compositions of lipids accumulated after 120 h (high C/N ratios of 120, 160, and 200) or 144 h (low C/N ratios of 20, 40, and 80) of culture were examined. Lipids accumulated in *L. starkeyi* during fermentation consisted mainly of fatty acids such as palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) (see Fig. 2).

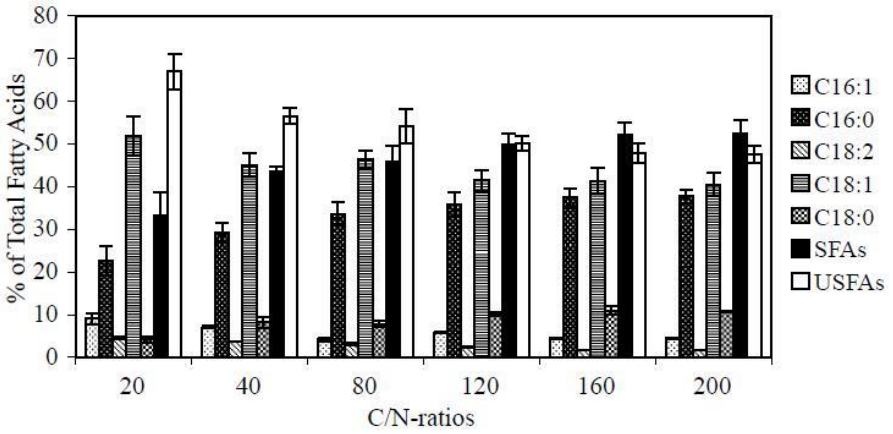

Fig. 2. Effects of the C/N ratio on the fatty acid methyl esters produced by *Lipomyces starkeyi* when cultured with glucose as the carbon source. Data are average values from triplicate experiments. SFAs: saturated fatty acids; USFAs: unsaturated fatty acids.

The proportion of SFAs is 52.5% when the C/N ratios were 160 or 200. This result means that higher C/N ratios promoted the accumulation of saturated fatty acids (SFAs, including C16:0 and C18:0) and correspondingly decreased the accumulation of

unsaturated fatty acids (USFAs, including 16:1, C18:1, and C18:2). Compared to other oleaginous yeasts, the present results obtained seem to be, to our knowledge, novel. However, it is uncertain whether this finding is a species-specific phenomenon in *L. starkeyi*. Usually, lipids with a high proportion of SFAs are better feedstocks for biodiesel production (Liu and Zhao 2007). Potentially adjusting the C/N ratio of media could allow for the production of optimized microbial oils for biodiesel products by *L. starkeyi* fermentation. Besides, the compositions of SFAs in *L. starkeyi* oils are similar to cocoa buffer (Papanikolaou and Aggelis 2010; Papanikolaou and Aggelis 2011b), implying that it may be a potential way for producing cocoa buffer substitutes in food industry.

Lipid Accumulation using Glycerol and xylose as the Sole Carbon Source

To examine the cell growth and lipid accumulation of *L. starkeyi* with crude glycerol and xylose as the carbon source, the DCWs and lipid accumulation yields using glycerol, xylose, and glucose as the sole carbon sources (with similar nitrogen-limited conditions) were compared. Results showed that the DCWs cultured using glycerol as the sole carbon source reached a maximum lipid productivity of 9.1 g/L after 168 h of culture (after the seventh day of culture). The lipid content of cells was 46.2% (Fig. 3), yielding a lipid productivity of 4.2 g/L (Table 3).

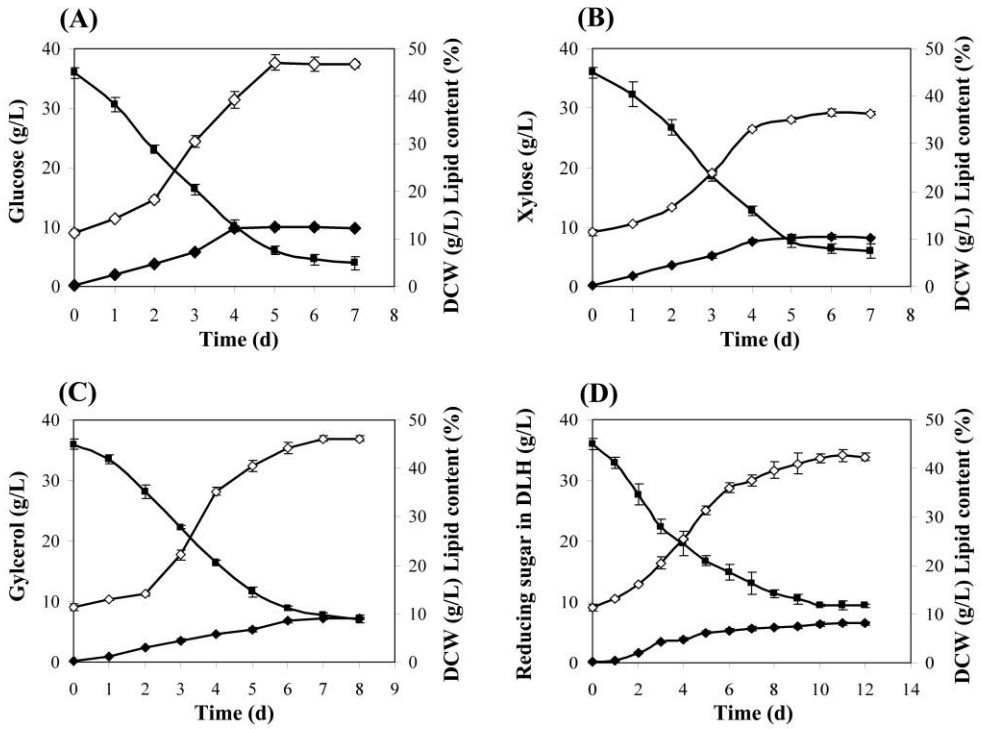

Fig. 3. Cell growth (dry cell weight, DCW) and lipid accumulation of *Lipomyces starkeyi* using different carbon sources in flasks: (A) glucose; (B) xylose; (C) glycerol; (D) detoxified liquid hydrolysate (DLH). Symbols: ■ denotes the consumed carbon sources (sugars or glycerol); ◆ denotes the accumulation of DCW; ◇ denotes the lipid content (%).

Table 3. Production of Oils and Fatty Acid Composition by *Lipomyces starkeyi* using Different Carbon Resources

Carbon resource	Time (h)	DCW (g/L)	Oil content (%)	Lipid (g/L)	16:1 (%)	16:0 (%)	18:2 (%)	18:1 (%)	18:0 (%)	SFAs (%)	USFAs (%)
Glucose	120	12.4	47.0	5.8	5.8	35.7	2.5	41.5	10.2	49.9	50.1
Xylose	144	10.4	36.6	3.8	5.5	38.6	1.8	43.7	7.0	48.9	51.1
Glycerol	168	9.1	46.2	4.2	4.0	35.8	4.1	43.8	7.5	47.8	52.2
DLH	264	8.2	42.7	3.5	9.6	35.4	2.5	39.4	6.3	48.2	51.8

Data are average values from triplicate experiments. Abbreviations: DCW (dry cell weight), DLH (detoxified liquid hydrolysate), SFAs (saturated fatty acids), USFAs (unsaturated fatty acids).

The accumulation processes of DCW (lipid and carbon uptake from medium during fermentation) were similar when glucose, xylose, and glycerol were used as the sole carbon sources (Fig. 3A, 3B, and 3C, respectively). Compared to the cell growths and lipid accumulations of *L. starkeyi* cultured on medium with xylose (DCW, 10.4 g/L and lipid content, 36.6%) and glucose (DCW, 12.4 g/L and lipid content, 47.0%) as the sole carbon sources, the DCW using glycerol as the carbon source was the lowest and glucose was proved to be an adequate substrate for growth and lipid production (Table 3). However, its lipid content (46.2%) was significantly higher than that of the cells cultured with xylose (36.6%) and comparable to that of the cells cultured with glucose (47.0%). The lipid productivity of cells cultured with glycerol were slightly higher (4.2 g/L) than those of cells cultured with xylose (3.8 g/L) and significantly lower than that of cells cultured with glucose (5.8 g/L) due to their low DCW (see Fig. 4), correspondingly the lipid conversion yield of glycerol (0.15 g/g) was slightly higher than xylose (0.13 g/g) but lower than glucose (0.18 g/g). Compared to other oleaginous yeasts such as *Mortierella isabellina* (with a lipid conversion yield of 0.09 g/g when glycerol was used as the sole carbon sources), *Mortierella ramanniana* (with a lipid conversion yield of 0.12 g/g when glycerol was used as the sole carbon sources) (Chatzifrakou *et al.* 2011), and *Yarrowia lipolytica* (with a lipid conversion yield of 0.13 g/g when glycerol was used as the sole carbon sources) (Papanikolaou and Aggelis 2002), the lipid conversion yield of *L. starkeyi* was significantly higher.

There was not a significant difference between the fatty acid compositions of the lipids accumulated using glycerol, glucose, and xylose as the sole carbon sources (Table 3), suggesting the fatty acids composition of oils accumulated with different carbon sources was reliable in *L. starkeyi* (though the proportion of different fatty acids could be switched with different C/N ratios). While testing the unconsumed carbon sources that remained in media after cell growth had reached the stable period, it was found that there were 3.9 g/L, 5.9 g/L, and 7.1 g/L of unconsumed carbon sources that remained in the glucose, xylose, and glycerol media, respectively. This further confirmed that the major carbon sources were consumed during cell growth and oil accumulation. Usually, many yeasts such as *Saccharomyces cerevisiae* and *Scheffersomyces spartiniae* are not able to use xylose as sole carbon source for cell metabolism (Kurtzman and Suzuki 2010; Yuan *et al*. 2011). The current study confirmed that *L. starkeyi* was able to use xylose for cell growth and oil accumulation. Also, we noted that the generated lipid productivity (8.9 g/L) using xylose as the sole carbon source in *Thamnidium elegans* (Zikou *et al*. 2013) was higher than our result. Due to the different media applied in these experiments, it is difficult to compare the lipid productivity between *T. elegans* and *L. starkeyi*. But, these studies clearly

demonstrated that xylose was able to be used as carbon sources for producing microbial oils. Generally, xylose was converted into xylulose through a two-step reduction and oxidation, and xylulose was further converted into glucose through the pentose phosphate pathway in the catabolism of xylose in yeasts (Papanikolaou and Aggelis 2011a; Winkelhausen and Kuzmanova 1998). The cell growth on xylose resulting in lower DCW and SCO production than that on glucose, implying that the metabolic utilization of xylose might probably be through the pentose-phosphate pathway and not through the phospho-ketolase reaction in *L. starkeyi*. However, the mechanism of xylose catabolism in *L. starkeyi* is not clear yet.

As mentioned above, crude glycerol is the main byproduct of methyl ester-based biodiesel. Reusing crude glycerol as substrates for generating microbial oils would be promising in industry. The current study showed that *L. starkeyi* was able to efficiently convert glycerol to lipids, resulting in a 46.2% of oil content (correspondingly, the lipid productivity was 4.2 g/L). Compared to other oleaginous yeasts such as *Rhodotorula glutinis*, *Cunninghamella echinulate*, and *Mortierella isabellina*, the lipid productivity of *L. starkeyi* was similar when glycerol was used as the sole carbon sources (Eastering *et al.* 2009; Fakas *et al.* 2009; Saenge *et al.* 2011). The cell growth on the glycerol media, however, seemed to be suppressed because its cell growth rate was slower and its DCW is relatively lower than on the glucose and xylose media. Possibly, the slower growth rate of oleaginous yeasts on the glycerol media resulted from the metabolic pathways of glycerol catabolism differed from glucose and xylose. Overall, the current results demonstrated that *L. starkeyi* can use glycerol and xylose as the sole carbon source for lipid accumulation during the cell growth.

Cell Growth of and Lipid Accumulation within *L. starkeyi* using Hydrolysate as the Carbon Source

To compare the cell growth and oil accumulation efficiency of *L. starkeyi* using WWS hydrolysate as the carbon source, the hydrolysate solution generated was adjusted to pH 5.0 and concentrated to 36 g/L sugars before it was used for fermentation (Fig. 4).

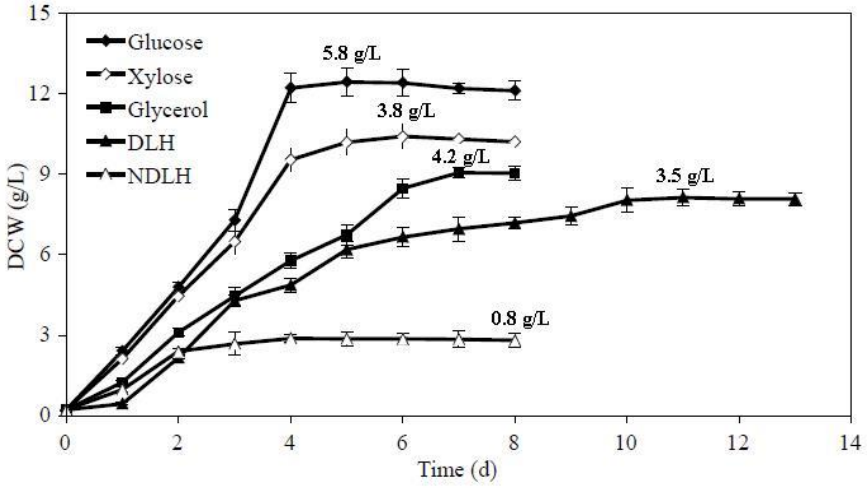

Fig. 4. Dry matter accumulation (dry cell weight, DCW) and maximum lipid concentrations of *Lipomyces starkeyi* using different carbon sources. The numbers above the lines denote the maximum oil concentrations. NDLH: non-detoxified liquid hydrolysate; DLH: detoxified liquid hydrolysate.

This sugar concentration was similar to the optimum sugar concentration for *L. starkeyi* growth when using glucose as the carbon source. The dry cell weight (2.9 g/L) and the lipid productivity (0.8 g/L) were low, even after 168 h of culture (Fig. 4). Only about 12 g/L of sugars were consumed after 168 h of culture. This means that cell growth was inhibited when the raw, concentrated hydrolysate solution was used for fermentation. Unsurprisingly, the chemical compositions of WWS hydrolysate are complex mixtures of various soluble carbon sources. Several degraded products (such as acetic acid, furfural, 5-hydroxymethyl-furfural, and water-soluble lignin) generated during the acid hydrolysis process could inhibit the cell growth of *L. starkeyi*. In particular, acetic acid, furfural and 5-hydroxymethyl-furfural derived during the WWS hydrolysis usually were potential inhibitors, resulting in the suppression of cell growth (Sun and Cheng 2002). Further, the concentration of acetic acid, furfural and 5-hydroxymethyl-furfural were measured, resulting in approximate 4.53 g/L (acetic acid), 0.06 g/L (furfural) and 0.78 g/L (5-hydroxymethyl-furfural), respectively.

To remove or reduce the cell growth inhibitors in the WWS hydrolysate, the raw, concentrated hydrolysate solution was detoxified *via* active charcoal adsorption. After using charcoal adsorption, the concentrations of potential inhibitors were significantly decreased up to about 4.17 g/L (acetic acid), 0.01 g/L (furfural) to 0.03 g/L (5-hydroxymethyl-furfural). However, the amount of fermentable sugars also decreased, resulting in the loss of fermentable sugars from 80 g/L to 58.9 g/L. When the detoxified liquid hydrolysate were directly used as carbon sources, the cell growth and lipid production of *L. starkeyi* were significantly improved, as depicted in Fig. 3. The DCW increased steadily from the second day to the seventh day of culture, reaching its maximum of 8.2 g/L on the tenth day of culture. At that point, the lipid content of the cells was 42.7%, yielding a lipid productivity of 3.5 g/L (Fig. 4). The total sugar concentration of broth on the tenth day of culture was 9.4 g/L, indicating that about 26.6 g/L of sugars, total, were consumed by the tenth day of culture. This means that *L. starkeyi* was able to use detoxified WWS hydrolysate as a carbon source for growth and lipid accumulation even though the chemical composition of WWS hydrolysate is complex. The DCW and lipid productivity of cells using detoxified WWS hydrolysate were 8.2 g/L and 3.5 g/L, respectively. These results showed that the cell growth and lipid accumulation of *L. starkeyi* with different carbon sources varied, though *L. starkeyi* was able to use diverse carbon sources. Probably, the effects and utilization efficiency of different carbon sources on the cell growth and lipid accumulation in *L. starkeyi* were resulted from the metabolic pathways of different carbon sources during the cell growth and lipids biosynthesis.

Compared to the cultivation of *L. starkeyi* using xylose as the carbon source, the lipid content (42.7%) was higher, but both the DCW (8.2 g/L) and lipid productivity (3.5 g/L) were lower (Table 3). The fatty acid composition of lipids accumulated by *L. starkeyi* using WWS hydrolysate as the carbon source is similar to that when using other carbon sources (Table 1). Yu *et al.* (2011) reported DCW (14.7 g/L) and lipid productivity (4.6 g/L) of *L. starkeyi* (using hydrolysate of wheat straw with acid hydrolysis as the carbon source) higher than our results. The most likely reason is the composition of WWS hydrolysate differed from the hydrolysate of wheat straw. This difference can be explained by the different chemical compositions of wheat straw and WWS. This likely resulted in differing concentrations of *L. starkeyi* cell growth inhibitors. In contrast to the oleaginous *Mortierella isabellina* using hydrolysate as carbon sources for lipid accumulation, its lipid productivity (3.6 g/L, Economou *et al.* 2011) was similar to our results. This experiment, using hydrolysate solution as the carbon source, was far from optimal for cell growth and

lipid accumulation within *L. starkeyi*, but it did provide proof of concept for utilizing lignocellulosic biomass for microbial oil production. However, in spite of the easy availability and low cost of lignocellulosic biomass from woody trees, the hydrolysis process of lignocellulosic biomass is expensive and time-consumed. Using the hydrolysate solution of lignocellulosic biomass as carbon sources for producing microbial oils in industry may not be a feasible way because of the technical limit of lignocellulosic biomass hydrolysis. Improving hydrolysis techniques to lower cost and to reduce the amount of inhibitors generated during hydrolysis and optimizing cell growth and lipid accumulation conditions are pivotal next steps toward the efficient use of lignocellulosic biomass for the production of microbial oil.

CONCLUSIONS

1. *L. starkeyi* can use diverse carbon sources, including xylose, glycerol, and willow wood saw dust hydrolysate solution, to produce microbial oils by fermentation.

2. A high C/N ratio in fermentation media promotes the accumulation of saturated fatty acids in microbial oils.

3. This study provides a promising method for producing microbial oils *via L. starkeyi* fermentation fueled by diverse carbon sources.

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REFERENCES CITED

Akhtar, P., Gray, J. I., and Asghar, A. (1998). "Synthesis of lipids by certain yeast strains grown on whey permeate," *Journal of Food Lipids* 5(4), 283-297.

Angerbauer, C., Siebenhofer, M., Mittelbach, M., and Guebitz, G. M. (2008). "Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production," *Bioresour. Technol*. 99(8), 3051-3056.

Chatzifragkou, A., Makri, A., Belka, A., Bellou, S., Mavrou, S., Mastoridou, M., Mystrioti, P., Onjaro, G., Aggelis, G., and Papanikolaou, S. (2011). "Biotechnological conversions of biodiesel derived waste glycerol by yeast and fungal species," *Energy* 36(2), 1097-1108.

Dyal, S. D., and Narine, S. S. (2005). "Implications for the use of *Mortierella* fungi in the industrial production of essential fatty acids," *Food Res. Int.* 38(4), 445-467.

Easterling, E.R., French, W.T., Hernandez, R., and Licha, M. (2009). "The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*," *Bioresour. Technol.* 100(1), 356-361.

Economou, C. N., Aggelis, G., Pavlou, S., and Vayenas, D. V. (2011). "Single cell oil production from rice hulls hydrolysate," *Bioresour. Technol.* 102(20), 9737-9742.

Fakas, S., Papanikolaou, S., and Batsos, A., Galiotou-Panayotou, M., Mallouchos, A., and Aggelis, G. (2009). "Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*," *Biomass Bioenerg*. 33(4), 573-580.

Huang, C., Zong, M. H., Wu, H., and Liu, Q. P. (2009). "Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*," *Bioresour. Technol*. 100(19), 1797-1801.

Huang, L. H., Zhang, B., Gao, B. Y., and Sun, G. P. (2011). "Application of fishmeal wastewater as a potential low-cost medium for lipid production by *Lipomyces starkeyi* HL," *Environ. Technol.* 32(16), 1975-1981.

Jiang, L. Q., Fang, Z., Guo, F., and Yang, L. B. (2012). "Production of 2,3-butanediol from acid hydrolysates of *Jatropha* hulls with *Klebsiella oxytoca*," *Bioresour. Technol*. 107, 405-410.

Karp, A., and Shield, I. (2008). "Bioenergy from plants and sustainable yield challenge," *New Phytol.* 179(1), 15-32.

Kurtzman, C. P., and Suzuki, M. (2010). "Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*," *Mycoscience* 51(1), 2-14.

Li, Y. H., Zhao, Z. B., and Bai, F. W. (2007). "High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture," *Enzyme Microbiol. Technol.* 41(3), 312-317.

Li, Q., Du, W., and Liu, D. H. (2008). "Perspectives of microbial oils for biodiesel production," *Appl. Microbiol. Biotechnol.* 80(5), 749-756.

Lin, J. T., Shen, H. W., Tan, H. D., Zhao, X., Wu, S. G., Hu, C. M., and Zhao, Z. B. K. (2011). "Lipid production by *Lipomyces starkeyi* cells in glucose solution without auxiliary nutrients," *J. Biotechnol.* 152(4), 184-188.

Liu, B., and Zhao, Z. (2007). "Biodiesel production by direct methanolysis of oleaginous microbial biomass," *J. Chem. Technol. Biot*. 82(8), 775-780.

Ma, F. R., and Hanna, M. A. (1999). "Biodiesel production: A review," *Bioresour. Technol.* 70(1), 1-15.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31(3), 426-428.

Mola-Yudego, B. (2010). "Regional potential yields of short rotation willow plantations on agricultural land in Northern Europe," *Silva. Fenn.* 44(1), 63-76.

Mola-Yudego, B., and González-Olabarria, J. R. (2010). "Mapping the expansion and distribution of willow plantations for bioenergy in Sweden: Lessons to be learned about the spread of energy crops," *Biomass Bioenerg*. 34(4), 442-448.

Papanikolaou, S., and Aggelis, G. (2002). "Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture," *Bioresour. Technol.* 82(1), 43-49.

Papanikolaou, S., and Aggelis, G. (2010). "*Yarrowia lipolytica*: A model microorganism used for the production of tailor-made lipids," *Eur. J. Lipid Sci. Tech.* 112(6), 639-654.

Papanikolaou, S., and Aggelis, G. (2011a). "Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production," *Eur. J. Lipid Sci. Tech.* 113(8), 1031-1051.

Papanikolaou, S., and Aggelis, G. (2011b). "Lipids of oleaginous yeast. Part II: Technology and potential applications," *Eur. J. Lipid Sci. Tech.* 113(8), 1052-1073.

Papanikolaou, S., Chatzifragkou, A., Fakas, S., Galiotou-Panayotou, M., Komaitis, M., Nicaud, J. M., and Aggelis, G. (2009). "Biosynthesis of lipids and organic acids by *Yarrowia lipolytica* strains cultivated on glucose," *Eur. J. Lipid Sci. Tech.* 111(12), 1221-1232.

Papanikolaou, S., Diamantopoulou, P., Chatzifragkou, A., Philippoussis, A., and Aggelis, G. (2010). "Suitability of low-cost sugars as substrates for lipid production by the fungus *Thamnidium elegans*," *Energ. Fuel.* 24, 4078-4086.

Papanikolaou, S., Fakas, S., Fick, M., Chevalot, I., Galiotou-Panayotou, M., Komaitis, M., Marc, I., and Aggelis, G. (2008). "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.* 32(1), 60-71.

Parajo, J. C., Dominguez, H., and Dominguez, J. M. (1996). "Charcoal adsorption of wood hydrolysates for improving their fermentability: Influence of the operational conditions," *Bioresour. Technol*. 57(2), 179-185.

Pirozzi, D., Yousuf, A., Zuccaro, G., Aruta, R., and Sannino, F. (2012). "Synthesis of biodiesel from hydrolyzates of *Arundo Donax*," *Environ. Eng. Manag. J*. 11(10), 1797-1801.

Rafiqul, I. S. M., and Sakinah, A. M. M. (2012). "Kinetic studies on acid hydrolysis of *Meranti* wood sawdust for xylose production," *Chem. Eng. Sci.* 71, 431-437.

Ratledge, C. (1993). "Single-cell oils-have they a biotechnological future?" *Trends Biotechnol.* 11(7), 278-284.

Ratledge, C. (2011). "Are algal oils realistic options for biofuels?" *Eur. J. Lipid Sci. Tech.* 113(2), 135-136.

Ratledge, C., and Cohen, Z. (2008). "Microbial and algal oils: Do they have a future for biodiesel or as commodity oils," *Lipid Technology* 20(7), 155-160.

Ratledge, C., and Wynn, J. P. (2002). "The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms," *Adv. Appl. Microbiol.* 51, 1-51.

Saenge, C., Cheirslip, B., Suksaroge, T. T., and Bourtoom, T. (2011). "Efficient concomitant production of lipids and carotenoids by oleaginous red yeast *Rhodotorula glutinis* cultured in palm oil mill effluent and application of lipids for biodiesel production," *Biotechnol. Bioprocess Eng.* 16(1), 23-33.

Sinag, A., Gulbay, S., Uskan, B., and Gullu, M. (2009). "Comparative studies of intermediates produced from hydrothermal treatments of sawdust and cellulose," *J. Supercrit. Fluid.* 50(2), 121-127.

Sun, Y., and Cheng, J. (2002). "Hydrolysis of lignocellulosic materials for ethanol production: A review," *Bioresour. Technol.* 83(1), 1-11.

Winkelhausen, E., and Kuzmanova, S. (1998). "Microbial conversion of D-xylose to xylitol," *J. Ferment. Bioeng.* 86(1), 1-14.

Wu, H., Li, Y. Y., Chen, L., and Zong, M. H. (2011). "Production of microbial oil with high oleic acid content by *Trichosporon capitatum*," *Appl. Energ*. 88(1), 138-142.

Xue, F. Y., Miao, J. X., Zhang, X., Luo, H., and Tan, T. W. (2008). "Studies on lipid production by *Rhodotorula glutinis* fermentation using monosodium glutamate wastewater as culture medium," *Bioresour. Technol.* 99(13), 5923-5927.

Yu, X. C., Zheng, Y. B., Dorgan, K. M., and Chen, S. L. (2011). "Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid," *Bioresour. Technol*. 102(10), 6134-6140.

Yuan, T. Z., Ren Y., Meng, K., Feng, Y., Yang, P. L., Wang, S. J., Shi, P. J., Wang, L., Xie, D. X., and Yao, B. (2011). "RNA-Seq of the xylose-fermenting yeast *Scheffersomyces stipitis* cultivated in glucose or xylose," *Appl. Microbiol. Biotechnol.* 92(6), 1237-1249.

Zhao, X., Kong, X. L., Hua, Y. Y., Feng, B., and Zhao, Z. B. (2008). "Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*," *Eur. J. Lipid Sci. Technol.* 110(5), 405-412.

Zhu, L. Y., Zong, M. H., and Wu, H. (2008). "Efficient lipid production with *Trichosporon fermentans* and its use for biodiesel preparation," *Bioresour. Technol.* 99(16), 7881-7885.

Zikou, E., Chatzifragkou, A., Koutinas, A. A., and Papanikolaou, S. (2013). "Evaluating glucose and xylose as cosubstrates for lipid accumulation and gamma-linolenic acid biosynthesis of *Thamnidium elegans*," *J. Appl. Microbiol.* 114(4), 1020-1032.

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