วารสารวิจัย มข. | 16 (2) | กุมภาพันธ์ 2554

การคัดเลือกและเพาะเลี้ยงยีสต์ไขมันสูง เพื่อผลิตน้ำมันจากจุลินทรีย์

Isolation and Cultivation of Oleaginous Yeast for Microbial Oil Production

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บทคัดย่อ

การคัดเลือกยีสต์จากตัวอย่างดินและน้ำที่ปนเปื้อนน้ำมันที่เก็บในพื้นที่มหาวิทยาลัยขอนแก่นระหว่างฤดูฝน ด้วยเทคนิคเพาะเลี้ยงเพิ่มจำนวนได้ยีสต์ทั้งสิ้น 69 ไอโซเลท โดย 10 ไอโซเลทจัดเป็นยีสต์ไขมันสูงเนื่องจากสะสมลิพิดภายในเซลล์สูงกว่าร้อยละ 20 โดยน้ำหนักเซลล์แห้ง และพบว่ายีสต์ไอโซเลท OYS3 เจริญและสะสมลิพิดสูงเมื่อเพาะเลี้ยงในอาหารที่มีไนโตรเจนต่ำโดยให้ปริมาณเซลล์ 7.9 กรัม/ลิตร อัตราการเจริญจำเพาะ 0.258 ต่อวัน ปริมาณลิพิดร้อยละ 52.7 โดยน้ำหนักเซลล์แห้งเมื่อเพาะเลี้ยงในอาหารที่มีกลูโคส 90 กรัม/ลิตร ปริมาณ $(NH_4)_2SO_4$ 0.1 กรัม/ลิตร ที่ pH 5.0 ระยะเวลา 8 วัน การจัดจำแนกยีสต์ OYS3 โดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการเปรียบเทียบลำดับนิวคลีโอไทด์บริเวณ D1/D2 ของ 26S rDNA จัดเป็น *Candida viswanathii* ผลการเพาะเลี้ยงแบบกึ่งกะพบว่าให้ปริมาณเซลล์ 9.11 กรัม/ลิตร ปริมาณลิพิดร้อยละ 59.50 โดยน้ำหนักเซลล์แห้ง เมื่อศึกษาผลของแหล่งคาร์บอนอื่นพบว่ายีสต์ *C. viswanathii* OYS3 เจริญได้ดีในกลีเซอรอลและกากน้ำตาลโดยให้เซลล์ 7.8, 7.1 กรัม/ลิตร และปริมาณลิพิดร้อยละ 37.2 และ 39.4 โดยน้ำหนักเซลล์แห้งตามลำดับ เมื่อวิเคราะห์องค์ประกอบของลิพิดที่สกัดได้จาก *C. viswanathii* OYS3 ด้วยเครื่องแก๊สโครมาโทกราฟีพบว่าเป็นกรดไขมันชนิดสายยาวโดยมีกรดปาล์มิติก กรดสเตียริก และกรดโอเลอิกเป็นองค์ประกอบหลักเช่นเดียวกับที่พบในน้ำมันพืชซึ่งมีศักยภาพในการใช้เป็นวัตถุดิบในการผลิตไบโอดีเซลได้

Abstract

Yeasts were isolated from oil-contaminated soil and water samples, collected during the rainy season in the area of the KKU campus by the enrichment technique. A total of 69 isolates were obtained, and 10 isolates were preliminarily defined as oleaginous yeast with lipid accumulation of more than 20% of cellular dry weight (CDW). Among 10 yeast isolates, isolate OYS3 was found to accumulate the highest cellular lipid content. The yeast was identified by a molecular genetics technique based on sequence analysis of the variable D1/D2 domain of the large subunit (26S) ribosomal DNA and it was identified as *Candida viswanathii*. The optimal culture conditions for lipid accumulation by *C. viswanathii* OYS3 were obtained to be as follows: glucose 90 g/L; pH of 5.0;

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$(NH_4)_2SO_4$ 0.1 g/L, incubated at 30°C. Under these conditions, a biomass of 7.9 g/L, specific growth rate of 0.258 d^{-1}, and lipid content of 52.7 %CDW could be achieved after cultivation for 8 days. Initial study for flask fed-batch fermentation revealed a biomass of 9.11 g/L and lipid content of 59.50 %CDW. Among the carbon sources tested, pure glycerol supported the maximum biomass of 7.8 g/L with lipid content of 37.2 % CDW, followed by molasses of 7.1 g/L with lipid content of 39.4 %CDW. Gas chromatography analysis revealed that lipids from *C. viswanathii* OYS3 contained mainly long-chain fatty acids with 16 and 18 carbon atoms. The three major constituent fatty acids were palmitic acid, stearic acid, and oleic acid that are comparable to conventional vegetable oils, suggesting that microbial lipids from the *C. viswanathii* OYS3 could be used as potential feedstock for biodiesel production.

คำสำคัญ : ยีสต์ไขมันสูง น้ำมันจากจุลินทรีย์ ไขมันเซลล์เดียว ไบโอดีเซล

Keywords: oleaginous yeast, microbial oil, single cell oil (SCO), biodiesel

INTRODUCTION

Biodiesel is defined as a mixture of mono-alkyl esters of long chain fatty acids derived from a renewable lipid feedstock, such as vegetable oil or animal fat. Biodiesel fuels are attracting increasing attention worldwide as a blending component or a direct replacement for diesel fuel in vehicle engines (Fukuda et al., 2001). Nowadays, there has been an increasing interest in looking for new oil feedstock for biodiesel production. Among them, microbial oils, lipid produced from oleaginous microorganisms, are now considered as promising feedstock because of their similar fatty acid composition to that of vegetable oils, and because the culture of these microbe species is affected neither by seasons nor by climates and they can accumulate lipids within a short period of time as well as grow well on a variety of substrates (Hassan et al., 1996; Xue et al., 2006; Angerbauer et al., 2008; Li et al., 2008; Meng et al., 2009). Microbial oils, also referred to as single cell oils (SCO), are produced by many oleaginous microorganisms involving yeasts, moulds, and microalgae, which have the ability to accumulate lipids to over 20 % of their biomass. Some oleaginous yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp., *Lipomyces* sp. can accumulate intracellular lipids to levels exceeding 70% of their biomass under nutrient limitation conditions. The majority of those lipids are triacylglycerol (TAG) contained long-chain fatty acids that are comparable to conventional vegetable oils (Meng et al., 2009; Evan and Ratledge, 1984; Ratledge and Wynn 2002; Tehlivets et al., 2007). It is known that lipid production requires a medium with an excess of sugars and limited other nutrients, usually nitrogen (Evan and Ratledge, 1984; Montet et al., 1985). Thus, oleaginous potential is critically affected by the carbon-to-nitrogen (C/N) ratio of the culture or lipid production is restricted when cultivation is carried out in nitrogen-limited sugar-based media (Evan and Ratledge, 1984; Montet et al., 1985; Turcotte and Kosaric, 1989; Hassan et al., 1996). It has also been reported that

single cell oil production by oleaginous yeasts has many advantages due to their fast growth rate, high oil content and the resemblance of their TAG fraction to plant oil (Meng et al., 2009). The objective of this research is to isolate the oleaginous yeasts from the oil-contaminated soil and water using the glucose enrichment approach, then, the lipid production of the isolated oleaginous yeasts is investigated.

MATERIALS AND METHODS

Source of the oleaginous yeasts

The oil-contaminated samples were collected from canteen waste cooking oil-soaked samples in the area of KKU campus during the rainy season. Ten soil samples and 10 water samples were obtained for the isolation of yeasts.

Isolation and screening of oleaginous yeasts

A mass of 5.0 g of soil and 5.0 mL of water was added into a 250-mL flask containing 50 mL of glucose-enriched medium. The components of the medium were (g/mL): glucose 50, NH_4Cl 2.5, KH_2PO_4 7.0, $MgSO_4.7H_2O$ 1.0, $CaCl_2$ 0.2, $FeCl_3.6H_2O$ 0.0005, $CuSO_4.5H_2O$ 0.0005, $ZnSO_4.7H_2O$ 0.7, yeast extract 2.0 and supplemented with streptomycin 100 mg in a 250 - mL flask. Samples were incubated in an incubator shaker at 30°C for 2 days with shaking speed at 150 rpm. Ten-fold serial dilutions were made from the enriched-culture broths, 0.1 mL from each dilution ranging from 10^{-2} to 10^{-4} were spread onto YPG agar plates containing (g/L): glucose 20, peptone 10, yeast extract 10, agar 15. The plates were then incubated at 30°C for a few days. Yeasts were then isolated by cross streaking each isolate on the YPG agar plates, then purifying and maintaining onto YPG agar for further study. The isolated yeasts were initially cultivated onto YPG broth for 1 day at 30°C, then 5 mL of these culture were transferred to 250-mL flasks with 50 mL of nitrogen-limiting medium containing (g/L): glucose 70, $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.4, $MgSO_4.7H_2O$ 1.5, $ZnSO_4$ 0.0044, $CaCl_2$ 0.0025, MnCl2 0.0005, CuSO4 0.0003, and yeast extract 0.75. Samples were grown at 30°C in an incubator shaker at 150 rpm for 4 days. Duplicated samples were analyzed for cell dry weight, lipids, and residual glucose. The isolated oleaginous yeasts with lipid accumulation more than 20% cell dry weight (CDW) were used for further study. Study of some characteristics of the isolated yeasts was carried out according to the methods described in The Yeast, A Taxonomic Study (Kurtzman and Fell, 1998).

Identification and genetic characterization

In order to identify the isolated yeast, sequence analysis of the variable D1/D2 domain of the large subunit (26S) ribosomal DNA was performed. The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman and Robnett, 1998). The polymerase chain reaction (PCR) reactions were performed in a final volume of 100 µl reaction mixtures conditioning 100 ng of genomic DNA, 2.5 U of Taq polymerase, 20 mM of each dNTP, 40 mM

of each primer, 10 mM Tris-HCl and 1.5 mM MgCl2. The reaction was pre-denatured at 94°C for 5 min, then repeated for 30 PCR cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2.5 min, followed by the final extension at 72°C for 10 min. The amplified product was then purified and sequenced. The 26S sequence of the isolated yeast was used for a BLAST search in the EMBL/GenBank database.

Lipid production by the isolated oleaginous yeasts

Seed culture cultivation

The seed culture of isolated oleaginous yeasts with high cellular lipid content were prepared in the YPG medium at 30°C in an incubator shaker at 150 rpm. Time course of cell growth was investigated in 250-mL flask containing 50 mL of nitrogen-limiting medium supplemented with 70 g/L glucose, incubated in an incubator shaker at a shaking speed of 150 rpm at 30°C.

Effects of initial pH of culture medium

To study the effect of initial medium pH, the seed culture (10%, v/v) was inoculated into 250-mL flasks containing 50 mL of nitrogen-limiting medium supplemented with 70 g/L glucose with pH value adjusted from 4 to 8. The flasks were then incubated in an incubator shaker at a shaking speed of 150 rpm at 30°C.

Effects of nitrogen sources and nitrogen concentrations

The seed culture (10%, v/v) was inoculated into 250-mL flasks containing 50 mL of nitrogen-limiting medium supplemented with 70 g/L glucose with different nitrogen sources and concentrations. Initial $(NH_4)_2SO_4$ concentration of 0.1, 0.2, 0.3, 0.4, and 0.5 g/L were tested. The different nitrogen sources with concentration of 0.1 g/L ($(NH_4)_2SO_4$, urea, $NaNO_3$, yeast extract) were studied. The flasks were then incubated in an incubator shaker at a shaking speed of 150 rpm at 30°C.

Effects of glucose concentration of the medium

The seed culture (10%, v/v) was inoculated into 250-mL flasks containing 50 mL of nitrogen-limiting medium supplemented with glucose concentration of 40, 50, 60, 70, 80, 90, and 100 g/L. The flasks were then incubated in an incubator shaker at a shaking speed of 150 rpm at 30°C.

Fed-batch fermentation

Fed-batch fermentations were performed in 2.0 L flask containing 1.0 L of nitrogen-limiting medium with initial glucose concentration at 90g/L and inoculated with 10% (v/v) of seed culture. The cultures were incubated under continuous mixing using a magnetic stirrer at a speed of 300 rpm at 30°C. Twenty mL of 500 g/L glucose solution was fed when residual glucose was lower than 15 g/L.

Effects of different carbon sources of nitrogen-limiting medium

Flask cultures were performed in 250-mL flasks with 50 mL of nitrogen-limiting medium supplemented with different carbon sources such as glucose, pure glycerol, biodiesel-derived crude glycerol, molasses and distiller slop. The cultures were then incubated in an incubator shaker at a speed of 150 rpm at 30°C, for 8 days.

Determination of glucose and cell dry weight

Duplicate samples were analyzed for cell dry weight, and residual glucose. The culture broth (5 mL) was centrifuged at 5,000 rpm for 5 min. The supernatant was analyzed for glucose concentration according to the DNS method (Miller, 1959). Harvested biomass was washed twice with 5 mL of distilled water and then dried at 90°C to constant weight. The biomass was determined gravimetrically. Specific growth rate (μ) was calculated according to the equation: $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_2 and X_1 are the dry cell weight concentrations (g/L) at times t_2 and t_1, respectively. Growth yield or cell yield ($Y_{X/S}$) was calculated from the following equation: $Y_{X/S} = (X_2 - X_1) / (S_1 - S_2)$, where S_1 and S_2 are the glucose concentrations (g/L) at time t_1 and t_2, respectively.

Determination of lipid content

Duplicate samples were analyzed for cellular lipid concentration. The lipid content was obtained by the cellular lipid concentration (g) per 100 g of dry biomass. The total lipids were determined by the modified method of Kwon and Rhee (1986) with modifications. The fatty acid profile of the lipid was determined as fatty acid methyl esters (FAMEs) by the direct transesterification method with BF_3-methanol at 100°C for 45 min, reported by Lepage and Roy (1984). FAMEs samples were analyzed by gas chromatography (Shimadzu) equipped with a flame ionization detector (FID). The condition of GC analysis was as follows: FID 350 °C, N_2 carrier gas 40 mL/min, injection port temperature 230 °C, oven temperature 190 °C.

RESULTS AND DISCUSSION

The isolation and screening of oleaginous yeasts

In this preliminary study, 69 colonies with the morphology typical of yeast were isolated from the soil samples and water samples. All of the yeast colonies were tested for lipid accumulation on nitrogen-limiting medium supplemented with 70 g/L glucose, and 10 isolates (Figure 1) were preliminary defined as oleaginous yeast with lipid accumulation of more than 20% of cellular dry weight. After the preliminary screening on 10 yeast isolates, it was found that isolate OYS3 grows extremely well and accumulated the highest lipid content using glucose as carbon source under nitrogen-limited conditions (Table 1). Therefore, the yeast isolate OYS3 was used for further study. The preliminary identification revealed yeast OYS3 as an ascomycetous yeast with diazonium blue B (DBB) and urease negative test.

Identification and genetic characterization

BLAST analysis of the 26S rRNA gene sequence of the yeast isolate OYS3 revealed it to be a perfect match with that of the *Candida viswanathii* type strain. The alignment and comparison of the 26S sequence of the isolate to the published 26S rRNA sequences belonging to five reference strains of phenotypically close species of Candida confirmed the 99% correspondence to the *C. viswanathii* CBS 4024, *C. viswanathii* FBFY 16S, C. viswanathii ATCC 38835, *C. viswanathii* SN40 and *C. viswanathii* voucher LMSA2.08.001 type strains.

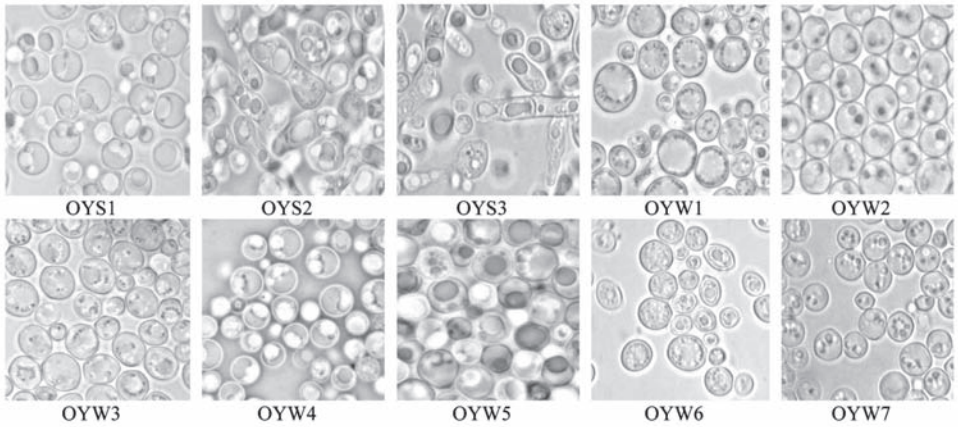

Figure 1. Microscopic morphologies of the 10 yeast isolates observed under a light microscope (1000X).

Table 1. Biomass and lipid accumulation of 10 isolated oleaginous yeasts cultivated in nitrogen-limiting medium at 30°C, 150 rpm shaking for 6 days.

Yeast isolate	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%CDW)	Consumed glucose (g/L)	Biomass yield (YX/S)	Lipid productivity (g/L/d)
OYS1*	7.2	1.89	26.2	54.3	0.133	0.315
OYS2	5.4	1.59	29.5	45.2	0.119	0.265
OYS3	8.4	3.06	36.4	57.1	0.147	0.510
OYW1**	4.2	1.02	24.4	51.1	0.082	0.170
OYW2	7.2	2.13	29.7	60.2	0.120	0.355
OYW3	6.0	1.71	28.4	57.1	0.105	0.185
OYW4	7.8	2.28	29.0	58.4	0.134	0.380
OYW5	9.1	2.55	28.0	59.2	0.154	0.425
OYW6	3.0	0.84	28.4	25.6	0.117	0.140
OYW7	9.0	2.04	22.7	57.8	0.156	0.340

* OYS represents the oleaginous yeast isolated from oil-contaminated soil,

** OYW represents the oleaginous yeast isolated from oil-contaminated water

Lipid production by *Candida viswanathii* OYS3

Growth and lipid production profile

The time course of cell growth, glucose utilization and lipid production of *C. viswanathii* OYS3 in 70 g/L glucose nitrogen-limiting medium are shown in Figure 2. It is apparent that glucose was used mainly for cell growth at the beginning of cultivation. Biomass, lipid content and utilized glucose gradually increased and lipid yield reached the maximum of 3.73 g/L or 43.2 %CDW at day 8. During the period between days 8 and 10, an apparent decrease in lipid content was observed. Similar changes were also observed in lipid content of *Trichosporon fermentans*, after exhaustion of the carbon source in the growth environment (Zhu et al., 2008).

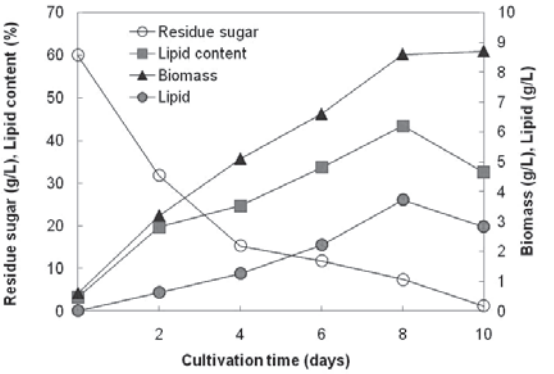

Figure 2. Time course of cell growth and lipid accumulation in *C. viswanathii* OYS3. Culture growth was performed in the nitrogen-limiting medium supplemented with 70 g/L glucose in an incubator shaker at 130 rpm, 30 °C for 10 days.

Effects of initial pH of medium

Effects of initial pH on cell growth and lipid accumulation of *C. viswanathii* OYS3 were investigated with pH ranging from 4.0 to 8.0. As shown in Table 2, maximum biomass of 8.4 g/L with cellular lipid content of 44.5%CDW, were achieved at pH 5.0.

Effects of nitrogen concentrations

$(NH_4)_2SO_4$ concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 g/L were used as the initial nitrogen source to investigate the effects on cell growth and lipid content. After 8 days of cultivation, higher initial nitrogen concentrations of the culture medium led to an increase in biomass concentration,

Table 2. Effect of initial pH medium on biomass and lipid accumulation of *C. viswanathii* OYS3.

pH	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%CDW)	Consumed glucose (g)	Specific growth rate (μ, d^{-1})	Biomass yield ($Y_{X/S}$, g/L)	Lipid productivity (g/L/d)
4	8.1	2.8	34.4	48.8	0.349	0.166	0.35
5	8.4	3.7	44.5	56.8	0.355	0.148	0.47
6	7.3	3.1	42.5	58.2	0.331	0.125	0.39
7	7.1	2.8	39.4	51.2	0.327	0.139	0.35
8	6.8	2.4	35.3	46.8	0.319	0.145	0.30

with the highest biomass of 10.11 g/L obtained by cultivation with an initial $(NH_4)_2SO_4$ of 0.5 g/L, as shown in Table 3. In the experimental data, an increase in the $(NH_4)_2SO_4$ concentration of the culture medium led to a decrease in lipid content of cells. *C. viswanathii* OYS3 had the highest total lipid content of 43.28 % CDW by cultivation with an initial $(NH_4)_2SO_4$ of 0.1 g/L. The lipid productivity decreased as $(NH_4)_2SO_4$ concentration increased from 0.1 to 0.5 g/L. Consequently, initial concentration of $(NH_4)_2SO_4$ at 0.1 g/L, was considered to be appropriate to achieve high lipid productivity. The growth rate and lipid accumulation of *C. viswanathii* OYS3 were strongly influenced by nitrogen concentration. The maximum lipid productivity obtained was 0.431 g/L/d when initial $(NH_4)_2SO_4$ concentration was 0.1 g/L.

Effects of different nitrogen sources

It has been reported that different nitrogen sources vary influence on microbial oil production. Therefore, effects of different nitrogen sources on cellular oil production of *C. viswanathii* OYS3 were tested. Effects of inorganic ($(NH_4)_2SO_4$, $NaNO_3$) and organic (urea, yeast extract) nitrogen sources with concentration of 0.1 g/L on biomass and lipid production of *C. viswanathii* OYS3 are presented in Figure 3. Among the nitrogen sources tested, yeast extract supported the maximum biomass of 12.4 g/L with lipid content of 49.19 % CDW. With respect to the low cost of nitrogen source, $(NH_4)_2SO_4$ was the best and the biomass of 8.10 g/L with lipid content of 43.09 %CDW, and lipid productivity of 0.436 g/L/d was obtained. Similar results were also observed in *Rhodosporidium toruloides* that there is more lipid accumulation when an organic nitrogen source is employed (Evan and Ratledge, 1984). This is consistent with the report of Huang et al. (1998) which reported that inorganic nitrogen sources were good for cell growth of *Mortierella isabellina*, but not suitable for oil production, while organic nitrogen sources were good for oil production, but not suitable for cell growth.

Table 3. Effects of $(NH_4)_2SO_4$ concentration on biomass, specific growth rate, lipid content and lipid productivity of *C. viswanathii* OYS3 on nitrogen-limiting medium with 70 g/L glucose, pH 5.0.

$(NH_4)_2SO_4$ concentration (g/L)	Biomass (g/L)	Lipid yield (g/L)	Lipid content (%CDW)	Consumed glucose (g)	Specific growth rate (μ, d^{-1})	Biomass yield ($Y_{X/S}$, g/L)	Lipid Productivity (g/L/d)
0.1	7.96	3.45	43.28	63.57	0.259	0.125	0.431
0.2	8.92	3.12	34.97	58.57	0.274	0.152	0.390
0.3	9.28	2.42	26.07	60.71	0.278	0.153	0.303
0.4	9.31	2.35	25.22	63.57	0.279	0.146	0.293
0.5	10.11	2.51	24.87	62.14	0.289	0.163	0.314

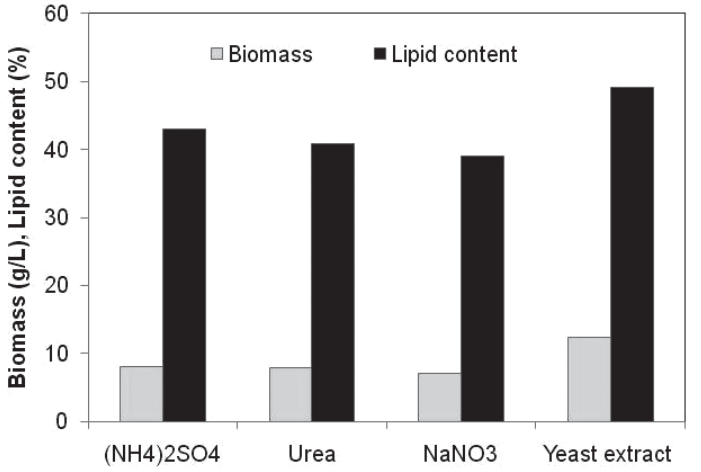

Figure 3. Effects of nitrogen sources on biomass, specific growth rate, lipid content and lipid productivity of *C. viswanathii* OYS3 on nitrogen-limiting medium with 70 g/L glucose, 0.1g/L nitrogen sources, pH 5.0.

Effects of glucose concentrations

To develop an improved cultivation technique for lipid production by *C. viswanathii* OYS3, batch flask cultures were investigated to determine the suitable glucose concentration of the initial medium. Therefore, to study glucose concentrations relative to carbon to nitrogen molar ratio (C/N ratio) on cell growth and lipid accumulation, the concentration of glucose at 40, 50, 60, 70, 80, 90 and 100 g/L or C/N ratio of 88 to 219, were investigated. As shown in Table 4, biomass decreased gradually with the increase of C/N ratio and the maximum biomass of 10.2 g/L with specific growth rate (μ) at 0.290 d^{-1} and cell yield ($Y_{X/S}$) at 0.290, at 40 g/L glucose or C/N ratio of 88, whereas the low biomass of 6.3 g/L with specific growth rate at 0.230 d^{-1} was obtained at 100 g/L glucose. Cellular lipid accumulation was quite low with 2.2 g/L or lipid content of 21.2 % CDW at the C/N ratio of 88, then showed a sharp increase when C/N ratio increased from 154 to 197, and reached the maximum of 52.7 % CDW at 197. Further increase in C/N ratio beyond 197 resulted in a slight drop in lipid content and biomass, suggesting that a considerable glucose inhibitory effect had occurred. Indeed, C/N ratio has been found to be the major impact factor for oil accumulation by the oleaginous microorganisms (Papanikolaou et al., 2004). When oleaginous organisms are grown with an excess of carbon and limited quantity of nitrogen, they may accumulate high concentration of cellular lipid. The growth of *C. viswanathii* OYS3 shows biomass increase followed by glucose concentration decrease and lipid production increased as nitrogen limitation occurred. In the cultivation of oleaginous yeast, when nitrogen was low in the medium, the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NAD-IDH) decreases or even disappears from the mitochondria of the oleaginous yeasts, then the tricarboxylic acid cycle was repressed, metabolism pathway

altered, and protein synthesis stopped and lipid accumulation activated (Evans et al., 1981; Botham and Ratledge, 1979; Palmieri et al., 1996).

Table 4. Effects of glucose concentrations (C/N ratio) on biomass, specific growth rate (μ), lipid content of *C. viswanathii* OYS3 when cultivated at 30°C for 8 days, pH 5.0, 150 rpm shaking speed.

Glucose (g/L)	C/N ratio	Biomass (g/L)	Specific growth rate (μ, d^{-1})	Biomass yield ($Y_{x/s}$, g/L)	Lipid yield (g/L)	Lipid content (%CDW)	Lipid productivity (g/L/d)
40	88	10.2	0.290	0.290	2.2	21.2	0.270
50	110	9.5	0.281	0.233	2.5	26.0	0.308
60	132	9.0	0.275	0.164	2.9	32.7	0.368
70	154	8.7	0.270	0.147	3.9	44.8	0.488
80	176	8.3	0.265	0.126	3.9	47.1	0.491
90	197	7.9	0.258	0.111	4.1	52.7	0.518
100	219	6.3	0.230	0.082	2.6	41.0	0.323

Fed-batch fermentation

The time courses of cell growth of *C. viswanathii* OYS3 in flask fed-batch cultivation for 12 days with initial glucose concentration at 90 g/L supplemented with 0.1 g/L $(NH_4)_2SO_4$, corresponding to C/N ratio of 197 are shown in Figure 4. Final biomass, lipid and lipid content were 9.11 g/L, 5.42 g/L and 59.5%CDW, respectively. Lipid productivity of 0.452 g/L/d was obtained. It is obvious that cell growth was fast during the initial stage, and biomass increased rapidly from 0.3 to 2.4 g/L within 2 days. The obtained result was not significantly different to the batch fermentation, suggesting that high concentration of glucose with lower level of nitrogen source could affect the cell growth. Because nitrogen source supported the cell growth, depletion of nitrogen may result in low biomass. Therefore, further fed-batch fermentation should be conducted with initial nitrogen-rich medium to obtain high biomass at the early stage of cell growth, and then high concentration of carbon source will be fed onto the culture broth for stimulation of the cellular lipid accumulation. Fed-batch fermentation modes have been widely applied for microbial lipid production. Pan et al.(1986) reported fed-batch fermentation of *Rhodotorula glutinis* with feeding medium containing 600 g/L glucose, 20 g/L yeast extract and 9 g/L MgSO4·7H2O, and cellular lipid content of 40 %CDW was obtained. A fed-batch flask cultivation of *Rhodosporidium toruloides* Y4 was investigated for 25 days and reached a biomass of 151.5 g/L and cellular lipid content of 48.0 % CDW (Li et al., 2007).

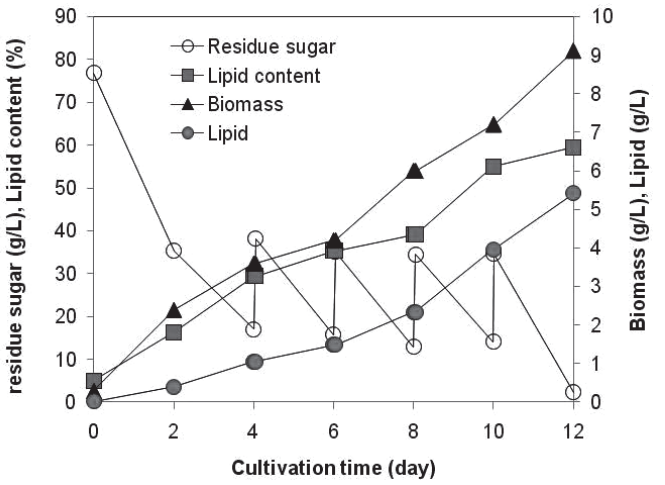

Figure 4. Fed-batch flask fermentation of *C. viswanathii* OYS3, pH 5.0, at 30°C for 12 days.

Effects of different carbon sources on the lipid production

Glucose is the most commonly used carbon source for microbial oil production. Therefore, it is important to use a low cost carbon source instead of glucose in order to reduce the cost of microbial oil production. Thus, different carbon substrates were used for oil production by *C. viswanathii* OYS3. After cultivation on nitrogen-limiting medium supplemented with 70 g/L glucose, 70 g/L xylose, 70 g/L pure glycerol, 70 g/L biodiesel-derived crude glycerol, 70 g/L molasses, and distiller slop (21.5 g/L reducing sugar) as a carbon substrate with pH 5.0, *C. viswanathii* OYS3 grew well on several types of organic carbon source (Figure 5), even on the medium supplemented with distiller slop, waste-disposal from molasses-based ethanol production plants. Among the carbon sources tested, pure glycerol supported the maximum biomass of 7.8 g/L with lipid content of 37.2 % CDW, followed by molasses of 7.1 g/L with lipid content of 39.4 %CDW. The obtained results suggest that oil production from *C. viswanathii* OYS3 can be performed with a lower cost production process. Moreover, it has been confirmed that *C. viswanathii* OYS3 can assimilate xylose which is the main component of hydrolysis of lignocelluloses. Li et al. (2010) reported that *Rhodotorula mucilaginosa* TJY15a could accumulate a large amount of lipid from hydrolysate of cassava starch. The cells contained 47.9 % (w/w) oil during batch cultivation, whereas 52.9 % (w/w) of lipid was obtained during the fed-batch cultivation. Zhao et al. (2010) found that *Rhodotorula mucilaginosa* TJY15a could accumulate 48.8 % (w/w) oil from hydrolysate of inulin and its cell dry weight reached 14.8 g/l during batch cultivation while it could accumulate 48.6 % (w/w) oil and 52.2 % (w/w) oil from hydrolysate of extract of Jerusalem artichoke tubers. It is known that the costs of microbial oil production are currently higher than those of vegetable oil but there are many methods to improve the low cost of microbial oil production processes. For example, the more economic carbon source should be

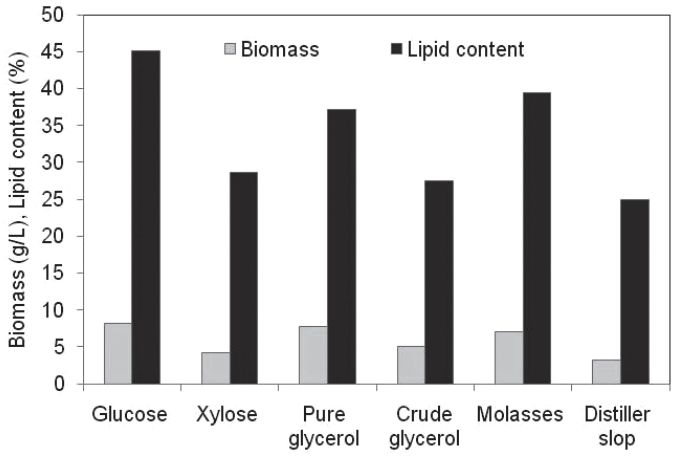

Figure 5. Effects of different carbon sources on growth and lipid accumulation of *C. viswanathii* OYS3.

employed to replace pure glucose such as sweet potato, new promoting plant oil, agro-industrial waste residues. In addition, potential and realistic progress in transforming of lignocelluloses to fermentable carbon sources might provide an optimal way to reduce the cost of microbial oils production. Process engineering that leads to a higher lipid production rate and cellular lipid content may also contribute in this regard. Thus, to realize the large-scale production of biodiesel from microbial oils, it is necessary to obtain a large amount of biomass and lipid content as well as a low cost of the cultivation process.

Fatty acid profile analysis

GC analysis showed that the lipid extracted from *C. viswanathii* OYS3 mainly contained triacylglycerols (TAG) as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), which is similar to that of vegetable oils, and the unsaturated fatty acids and saturated fatty acid amount to about 40.76% and 59.24% of total fatty acid, respectively (Table 5). Li et al. (2010) reported that the fatty acids from *Rhodotorula mucilaginosa* TJY15a were mainly composed of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2). Fatty acid of C16 and C18 and triglyceride (TG) dominated the crude lipid compounds of *Rhodotorula glutinis* cultivated on monosodium glutamate wastewater (Xue et al., 2008). Zhu et al. (2008) reported that the lipid of yeast *Trichosporon fermentans* mainly contains palmitic acid, stearic acid, oleic acid and linoleic acid and the unsaturated fatty acids amount to about 64% of the total fatty acids. The three major constituent fatty acids of *C. viswanathii* OYS3 were palmitic acid, stearic acid, and oleic acid that are comparable to vegetable oils. Therefore, based on these fatty acid profile data, microbial oil from *C. viswanathii* OYS3 may be used as potential feedstock for biodiesel production.

Table 5. Fatty acid profile of the extracted lipid of *C. viswanathii* OYS3, investigated in nitrogen-limiting medium supplemented with 90 g/L glucose.

Fatty acid type	Fatty acid profile	Fatty acid (%,w/w TFA*)
Saturated fatty acid	Myristic acid (C14:0)	0.54
	Palmitic acid (C16:0)	19.57
	Heptadecanoic acid (C17:0)	0.54
	Stearic acid (C18:0)	20.11
Unsaturated fatty acid	Palmitoleic acid (C16:1)	3.80
	cis-9-oleic acid (C18:1)	51.63
	Linoleic acid (C18:2)	3.26
	Linolenic acid (C18:3)	0.54

***TFA** represents the total fatty acids

Acknowledgements:

This research was supported by the Faculty of Science, Khon Kaen University.

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