ความเป็นไปได้ในการผลิตลิพิดของยีสต์ไขมันสูง *Rhodotorula mucilaginosa* ที่ได้จากดินป่าชายเลนบนอาหารเลี้ยงเชื้อที่เหมาะสม เพื่อเป็นแหล่งวัตถุดิบทางเลือกสำหรับการผลิตไบโอดีเซล

Possibility of Soil Mangrove-Derived Oleaginous Yeast *Rhodotorula mucilaginosa* on Nutritionally Optimized Medium for Lipid Production for Alternative Biodiesel Feedstock

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บทคัดย่อ

ลิพิดที่สกัดได้จากยีสต์ไขมันสูงกำลังได้รับการพิจารณาให้สามารถใช้เป็นแหล่งวัตถุดิบเพื่อการผลิตไบโอดีเซลได้ การศึกษาครั้งนี้ ได้ทำการคัดแยกและคัดเลือกยีสต์ไขมันสูงจากตัวอย่างดินที่เก็บจากป่าชายเลนในเขตภาคตะวันออกของประเทศไทย ได้ยีสต์ทั้งหมด 51 ไอโซเลท แล้วนำมาทดสอบบนอาหารเลี้ยงเชื้อจำกัดปริมาณไนโตรเจน และมีกลูโคส 50 กรัมต่อลิตร เป็นแหล่งคาร์บอน โดยมียีสต์ 23 ไอโซเลทพบการสะสมลิพิดภายในเซลล์ และเมื่อนำมาทดสอบบนอาหารเลี้ยงเชื้อที่มีไนโตรเจนต่ำและมีกลูโคส 50 กรัมต่อลิตร เป็นแหล่งคาร์บอน พบยีสต์ไอโซเลท SMY40 สะสมลิพิดภายในเซลล์สูงสุดร้อยละ 29.44 โดยน้ำหนักแห้ง จัดเป็นยีสตส์ไขมันสูงและจัดจำแนกได้เป็น *Rhodotorula mucilaginosa* SMY40 ผลการศึกษาสภาวะของอาหารเลี้ยงเชื้อที่เหมาะสมต่อการเพิ่มประสิทธิภาพการเจริญและการผลิตลิพิดของ *R. mucilaginosa* SMY40 พบสภาวะที่ดีที่สุดให้ปริมาณเซลล์ 4.40 กรัมต่อลิตร ปริมาณลิพิด 1.76 กรัมต่อลิตร และปริมาณลิพิดภายในเซลล์ร้อยละ 40.00 โดยน้ำหนักแห้ง คือ อาหารเลี้ยงเชื้อYNB w/o aa-AS 1.67 กรัมต่อลิตรที่มีกลูโคส 70 กรัมต่อลิตร เป็นแหล่งคาร์บอนและมียูเรีย 10.0 กรัมต่อลิตร เป็นแหล่งไนโตรเจน ค่าความเป็นกรดด่างเริ่มต้นเท่ากับ 4.7 บ่มเพาะแบบเขย่าที่ 150 รอบต่อนาทีอุณหภูมิ 30 องศาเซลเซียส ระยะเวลาเพาะเลี้ยง 120 ชั่วโมง องค์ประกอบของลิพิดที่สกัดได้จาก *R. mucilaginosa* SMY40 มีกรดไขมันชนิดสายยาวของคาร์บอน 16 และคาร์บอน 18 เป็นองค์ประกอบหลัก ได้แก่ กรดปาล์มิติกร้อยละ 20.21 กรดปาล์มิโตเลอิกร้อยละ 2.40 กรดสเตียริกร้อยละ 5.85 กรดโอเลอิกร้อยละ 51.64 และกรดไลโนเลอิกร้อยละ 12.17 เช่นเดียวกับที่พบในน้ำมันพืช ซึ่งจากผลการทดลองทำให้ทราบว่า น้ำมันที่สกัดได้จาก *R. mucilaginosa* SMY40 สามารถนำไปใช้เป็นวัตถุดิบสำหรับการผลิตไบโอดีเซลได้

คำสำคัญ : *Rhodotorula mucilaginosa* ยีสต์ไขมันสูง ดินป่าชายเลน น้ำมันที่สกัดได้ วัถตุดิบสำหรับผลิตไบโอดีเซล

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Abstract

Lipid-derived from oleaginous yeast is now being considered as promising for biodiesel feedstock. In this study, oleaginous yeasts were screened and isolated from soil mangrove samples collected in the eastern region of Thailand. A total of fifty one isolates were obtained, and preliminarily examined as potential lipid producer by cultivating in a nitrogen-limiting medium containing 50 g/L of glucose as a sole carbon source. Only twenty three isolates were observed the accumulation of intracellular lipid granule. It was found that the yeast strain SMY40 accumulated the highest lipid content 29.44% of their dry biomass weight, which was defined as oleaginous yeast and identified to be a *Rhodotorula mucilaginosa*. Optimization of nutritional parameters and culture condition were carried out to improved biomass and lipid production of *R. mucilaginosa* SMY40.Under the optimal condition; 1.67 g/L YNB w/o aa-AS with initial pH of 4.7 containing 70 g/L glucose and 10.0 g/L urea, incubation temperature at 30°C, orbital shaking speed at 150 rpm for 120 h of cultivation time, 4.40 g/L of dry biomass, 1.76 g/L of cellular lipid accumulation and up to 40.00%of accumulated lipid of total dry biomass weight were produced. The produced lipid composition from *R. mucilaginosa* SMY40 contained the high proportion of C16 and C18 fatty acids. The extracted lipids were mainly 20.21% palmitic acid (C16:0), 2.40% palmitoleic acid (C16:1), 5.85% stearic acid (C18:0), 51.64% oleic acid (C18:1) and 12.17% linoleic acid (C18:2) thatiscomparable to conventional vegetable oils. The results suggest that the extracted lipids of *R. mucilaginosa* SMY40 could be used as feedstock for biodiesel production.

Keywords: *Rhodotorula mucilaginosa*, oleaginous yeast, soil mangrove, extracted lipid, biodiesel feedstock

Introduction

Biodiesel, which is one of the alternative and eco-friendly fuels, has received increasing attention that can be produced from vegetable oils, animal fats and waste-cooking oils through transesterification. Nowadays, the production of biodiesel from oil crops and animal fats has been raised significantly, it cannot realistically satisfy the global demand for economically and environmentally sustainable (Chang *et al.,* 2013). Recently, more researchers have attempted to use microbial oils as alternative for biodiesel production because the lipid composition is similar to that of vegetable oils. Moreover, microbes have much higher growth rates and productivity than the conventional forestry and agricultural crops (Wang *et al.,* 2014). Microbial oils, also referred to as single cell oils (SCOs), are the oils produced by oleaginous microorganisms such as bacteria, yeasts, molds and microalgae. They can accumulate lipids in their cells to more than 20% of their dry biomass weight. In general, yeasts and molds can accumulate much more lipids than bacteria and microalgae (Meng *et at.,* 2009). Some oleaginous yeast such as *Rhodotorula* sp., *Rhodosporidium* sp., *Lipomyces* sp., *Cryptococcus curvatus*, *Yarrowia lipolytica* have been reported to accumulate intracellular lipids level exceeding 50% of their dry biomass weight under nitrogen-limiting cultivation (Sriwongchai *et al.,* 2013). The yeast oils are suitable for biodiesel feedstock, due to the majority of lipids

are triacylglycerol (TAG) contained long chain saturated fatty acid and saturation degree (Meng *et at.,* 2009; Tanimura *et al.,* 2015). The advantages of yeast oils that involves the cell ability to accumulate lipid within a short period; to produce lipids from various substrates such as; sugars, organic acids, hydrocarbons, fats and vegetable oils, glycerol and crude glycerol and nutritional residues from agriculture and industry; and to process with less labor required, less affection by venue, season and climate, and easier to scale up (Zhu *et. al.,* 2008; Gong *et al.,* 2013). Although, screening for optimal oleaginous yeasts from various ecological systems has been studied such as soils (Pan *et al.,* 2009)and forest soils (Chang *et al.,* 2013), waste of palm oil mill and biodiesel plant (Kitcha & Cheirsilp, 2011), mangrove ecosystem (Wang *et. al.,* 2014), marine fish *Synechogobius hasta* (Li *et al.,* 2010) and sea water (Wang *et at.,* 2008). There has not been studied on oleaginous yeast screening from soil mangrove ecosystem in eastern region of Thailand and nutritional optimization for lipid accumulation.Therefore, the first purpose of this research was to screen lipid accumulating yeasts in mangrove soil obtained from the five provinces belonging to the eastern region of Thailand. After that, the second purpose was to investigate the potential of selected oleaginous yeast from these mangrove soil samples to produce lipids for biodiesel production under optimized culture condition.

Methods

Media and Yeast isolation

Forty-seven soil samples of mangrove forests, in eastern coast of Thailand (35°C, March 2015 to September2015); Chachoengsao (13.6903°N, 101.0780°E), Chanthaburi (12.5704°N, 101.8985°E), Chonburi (13.3811°N, 100.9892°E) Samutprakarn (13.5738°N, 100.5521°E) and Trat (12.2254°N, 102.3693°E) Province, were collected at the depth 5-10 cm from soil surface. The samples were kept at 4°C until the further experimentation. One g of each mangrove soil sample was enriched by transferring to 25 mL YEPD medium, containing (in g/L): glucose 20.0, yeast extract 10.0, peptone 20.0 and agar 15.0, and incubated on 150 rpm of orbital shaker speed at room temperature for 24 h. Each enriched cultures were used for preparing the 10-fold serial dilution and then spread on the YEPD agar. The cultivation was carried on 30°C for 48 h. The retrieved yeast colonies were selected and then purified by cross streak technique. The selected yeast isolates were maintained in YEPD slant. The medium for screening and optimized the yeast strains containing (in g/L): glucose 50.0, NH_4NO_3 5.0, yeast nitrogenous base without amino acid and ammonium sulfate (YNB w/o aa-AS) medium 1.67 with initial of pH 4.7 (Sriwongchai *et al*., 2013). In all experiments this medium was modified with respect to type of carbon sources, carbon concentrations, type of nitrogen sources and nitrogen concentrations in order to promote lipid accumulation in their yeast cells.

Oleaginous yeast screening and evaluation of lipid production

The isolated yeast colonies were primarily screened for their lipid droplets by Sudan Black B staining (Pan *et al.,* 2009). Then the potential yeast strains were selected and inoculated into 10-mL test tubes containing 5 mL YNB w/o aa-AS medium containing 20.0 g/L glucose as a sole carbon source with initial pH of 4.7. The pre-culture was grown at 30°C with orbital shaking at 150 rpm for 48 h. In test tube cultures, 3-mL of inoculation was transferred to 27 mL YNB w/o aa-AS medium containing 50.0 g/L glucose as sole carbon source and grown for 120 h under the same condition as above. The lipid extraction was performed as describes below. In this experiment we found that the yeast strain *R. mucilaginosa*SMY40 contained the highest amount of total lipid. Therefore the oleaginous yeast strain *R. mucilaginosa* SMY40 was selected for lipid-producing investigation.

Seed preparation and flask batch cultivation

A loopful of cells from single colony on YEPD agar plate was aseptically transferred to 50 mL of YNB w/o aa-AS medium containing 20.0 g/L glucose as carbon source in a 125-mL Erlenmeyer flask. The cultures were incubated at 30°C shaking at 150 rpm for 48 h. After preculture ($2.0 \times 10^7 - 3.0 \times 10^8$ cells/mL),5-mL of seed inoculums (10% v/v) were introduced to 45 mL of YNB w/o aa-AS media that contained 50.0 g/L of different carbon sources (glucose, sucrose, xylose, and soluble starch), different carbon concentrations (30.0, 50.0 70.0 and 100.0 g/L), different nitrogen sources (NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$ and urea) and different nitrogen concentrations (5.0, 10.0, 10.5 and 20.0 g/L) and grown at 30°C with orbital shaking at 150 rpm for 120 h. All experiments were conducted in triplicate.

Determination of dry biomass

The cell dry matter was determined according to Sriwongchai *et al.* (2013). 10-mL of the cultures was centrifuged at 6,000xg for 10 min. The pellet was washed three times with deionized water and dried at 80°C until constant weight (typically 24 h). The dry cell weight was determined gravimetrically.

Lipid extraction and determination of lipid content

Total lipids were extracted from whole yeast cells by the method of Folch *et al.* (1957) with some modifications and according to Sriwongchai *et al.* (2013). A known wet weight 100- to 1000-mg of the pellet was extracted with 3.75 mL of chloroform/methanol solution (2:1, v/v), the mixture was vortexed for 15 min at room temperature. To this was added 1.25 mL of chloroform. The mixture was vortexed for 1 min followed by addition of 1.25 mL of 1 M NaCl to the mixture and vortexed again for 1 min. The mixture was centrifuged at 3000 x g for 15 min. The combined extract was evaporated by drying at 60°C as described by Xue *et al.* (2008)and lipid weight was determined gravimetrically.

Determination of reducing sugar concentrations

To determine the amount of residual glucose, the glucose content in the supernatant was analyzed by the dinitrosalicylic acid (DNS) method as described by Miller (1959), with glucose as a standard solution.

Fatty acid composition analysis

The total lipids were saponified and methyl esterified to yield methyl esters of fatty acid which were analyzed using an Agilent 6890N/5973 GC/MSD fitted with a capillary column (30.0 m × 0.32 m × 0.25 μm) under the following conditions: Initial oven temperature at 45°C ramped to 260°C at the rate of 2°C/min.; injector temperature and detector temperature were held at 250°Cand280°C, respectively, and carrier gas (helium) flow rate of 2 mL/min (Sriwongchai *et al.,* 2012). The peaks of fatty acids were identified by comparing their retention times to those of known standard materials. The quantification was determined by the technique of internal standardization.

Identification and oleaginous yeast strain characterization

The biochemical and physical characteristics of selected oleaginous yeast strain SMY40 was performed using the methods described by Kurtzman and Fell (2000). The total genomic DNA of the yeast strain SMY40 was isolated and purified by using the methods described by Sambrook *et al*. (1989). Amplification and sequencing of D1/D2 26s rDNA sequences from the yeast were performed. The primer used for the polymerase chain reaction (PCR) were NL1 (5'-CATATCAATAAGCGGAGGAAAAG-3')and NL4 (5'-GTCCGTGTTTCAAGACGG-3') (Kurztman and Robnett, 1998)and performed according to the methods described by Leesing and Nantaso (2011). Briefly, 100 ul reaction mixtures conditioning 100 ng of genomic DNA, 2.5U of Taq polymerase, 20 mM of each dNTP, 40 mM of each primer, 10 mMTris-HCl and1.5 mM $MgCl_2$. The reaction was pre-denature at 94°C for 5 min, then repeated for 30 PCR cycles with denature 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. For identification, the obtained sequence was used for a BLAST search in the Gen Bank database.

Statistical analysis

Experiments were performed in triplicates and the data were analyzed using one way analysis (one-way ANOVA). Means were compared using Tukey's test at 95% confidence interval ($p < 0.05$).

Results and Discussion

Screening and isolation of the oleaginous yeast

The preliminary study, fifty-one colonies with morphology typical of yeast were isolated from forty seven soil mangrove samples. All colonies of yeast were tested for lipid accumulation by Sudan Black B staining, twenty-three isolates as a potential lipid biomass producer were found positive showing fat globules within their cells and selected for further experiment (Table 1). Usually, oleaginous microorganism isolation is examined by Sudan Black B staining to determine fat droplet structure in oleaginous microorganism cells (Thakur *et al.,* 1989). However, this method only roughly indicates the presence microbial lipids and lends many false-positive results (Li *et al.,* 2009; Li et *al.,* 2012). The oleaginous yeast screening was enriched in high C/N medium and lipid-accumulating ability of

the yeast strain is expressed as lipid content after 96 h or 4 days of cultivation (Tanimura *et al.,* 2014). Then, these isolates were confirmed the lipid accumulation ability by growing on YNB w/o aa-AS medium containing 50.0 g/L glucose as sole carbon source and 5.0 g/L NH_4NO_3 as nitrogen source and grown at 30°C with orbital shaking at 150 rpm for 120 h. Among twenty-three isolates, ten of them SMY04, SMY07, SMY11, SMY23, SMY26, SMY35, SMY39, SMY40, SMY41 and SMY44 lend high lipid yield with lipid content higher 20% of their dry biomass weight as listed in Table 2, especially, isolates SMY40 accumulated highest lipid content up to 29.44% of their dry biomass weight. Therefore, the yeast isolate SMY40 was employed for further study. After the preliminary screening and evaluation of lipid production, by sequencing its 26S rDNA, of dry biomass weight isolate was identified, the result showed high sequence similarity (99%) with the type strain of *R.mucilaginosa*SMY40. Wang *et al.* (2014) have screened and isolated the oleaginous yeast from 12 species of mangrove plants of mangrove ecosystem in China, and they found the oleaginous yeast strain *Aureobasidium pullulans* var. *melagnogenum* P10 could accumulated lipid in their cells as 54.2 g per 100 g of dry biomass weight. In addition, the oleaginous yeast strain *R.mucilaginosa*TJY15a was isolated from skin of marine fish and contained the lipid content 30.4% of their dry biomass weight (Li *et al.,* 2010).

Lipid production of *R.mucilaginosa* SMY40 on nutritionally optimized medium

The commonly used carbon source for the lipid production from oleaginous microorganisms is glucose. *R. mucilaginosa* TJY15a has been reported to assimilate glucose, xylose, sucrose and cassava starch hydrolysate (Li *et al.,* 2010) but there has been no study on directly soluble starch assimilation. So in this study, *R. mucilaginosa* SMY40 was examined on different carbon sources such as glucose, xylose, sucrose and soluble starch with initial concentration at 50.0 g/L for growth and lipid production. The carbon source was significant on growth and lipid production of *R. mucilaginosa* SMY40. The maximum dry biomass of 3.53 g/L and lipid yield of 1.19 g/L (lipid content as 33.71% of their dry biomass weight) was obtained on YNB w/o aa-AS medium containing glucose as carbon source, followed by sucrose (3.17 g/L and 0.81 g/L) and xylose (2.88g/L and 0.61 g/L), respectively. The amount of carbon source consumed by *R. mucilaginosa* SMY40 as 44.12 g/L of glucose, 27.16 g/L of xylose and 30.29 g/L of sucrose (data was not shown),while soluble starch was not assimilated(Fig. 1A). Conversely, hydrolysate of starch could be easily use as carbon source for lipid production and growth of oleaginous microorganisms. There were reported that the optimal concentration of hydrolysate of cassava starch at 2.0% (w/v) the oleaginous yeast strain *R. mucilaginosa* TJY15a could accumulate 45.9% of their dry biomass weight of oils in its cells and dry cell mass reached 10.9 g/L (Li *et al.,* 2010), while the alga *Chlorella protothcoides* could accumulate oils with starch hydrolysate as carbon source and grew well when compare to glucose as carbon source (Han *et al.,* 2006). Therefore glucose was chosen for all following experiments.

Table 1 Fat globules examination by Sudan Black B staining.

Isolates	Fat globules	Isolates	Fat globules
SMY01	+	SMY27	+
SMY02	-	SMY28	-
SMY03	+	SMY29	-
SMY04	+	SMY30	-
SMY05	-	SMY31	+
SMY06	-	SMY32	-
SMY07	+	SMY33	-
SMY08	-	SMY34	-
SMY09	+	SMY35	+
SMY10	+	SMY36	-
SMY11	+	SMY37	-
SMY12	-	SMY38	-
SMY13	-	SMY39	+
SMY14	-	SMY40	+
SMY15	+	SMY41	+
SMY16	-	SMY42	-
SMY17	+	SMY43	-
SMY18	+	SMY44	+
SMY19	-	SMY45	-
SMY20	-	SMY46	-
SMY21	-	SMY47	-
SMY22	-	SMY48	+
SMY23	+	SMY49	-
SMY24	-	SMY50	+
SMY25	+	SMY51	-
SMY26	+		

+: showing fat globules

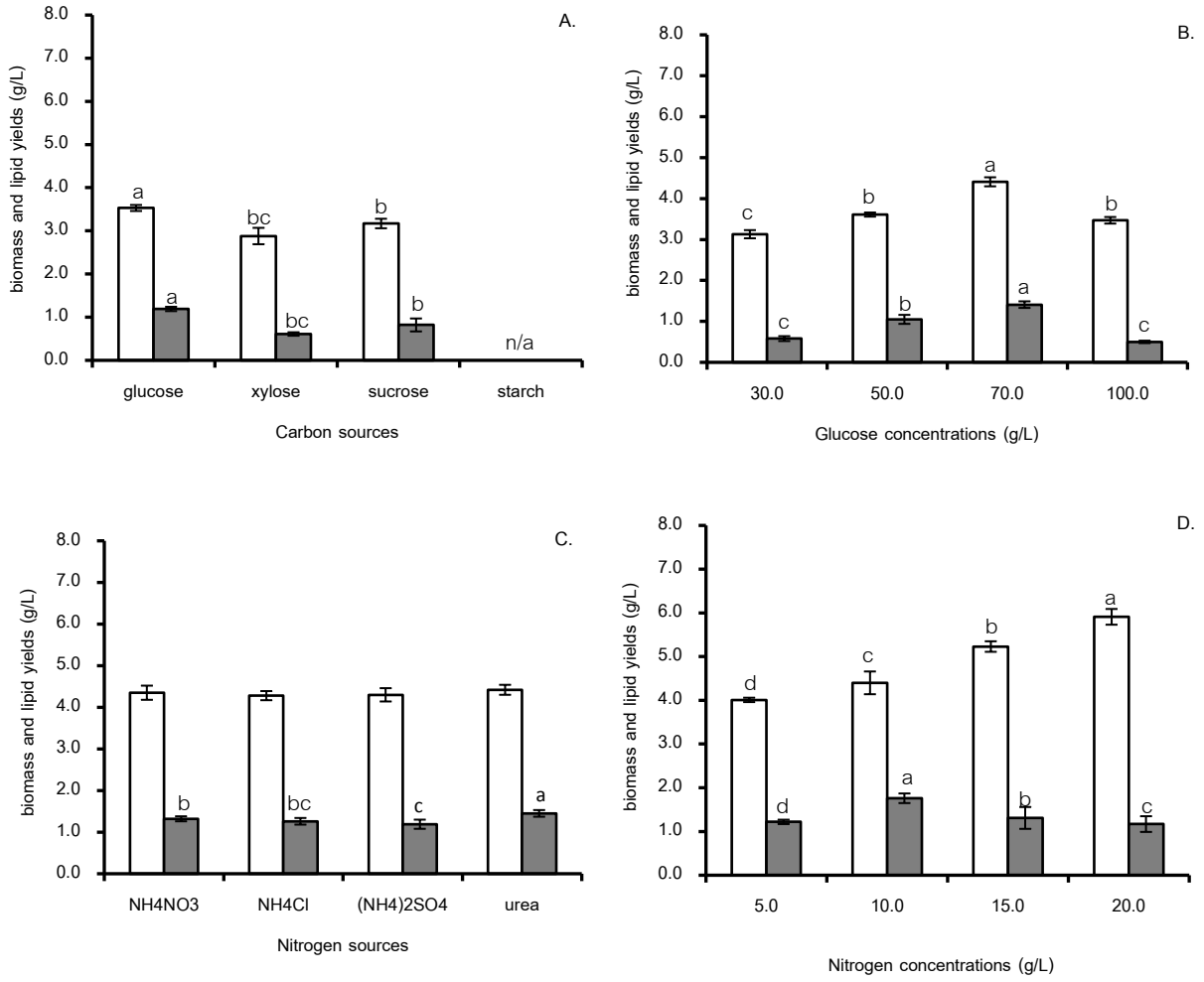
Table 2 Lipid content of twenty-three yeast isolates.

Isolates	Lipid content (%, w/w)	Isolates	Lipid content (%, w/w)
SMY01	8.11 ± 0.13	SMY25	17.70 ± 0.13
SMY03	8.21 ± 0.37	SMY26	23.21 ± 0.18
SMY04	21.24 ± 0.08	SMY27	8.04 ± 0.21
SMY07	20.10 ± 0.52	SMY31	10.06 ± 0.28
SMY08	14.12 ± 0.41	SMY35	22.12 ± 0.94
SMY09	9.41 ± 0.17	SMY39	21.05 ± 1.01
SMY10	6.11 ± 0.05	SMY40	29.44 ± 0.98
SMY11	26.23 ± 0.43	SMY41	20.11 ± 0.85
SMY15	13.76 ± 0.44	SMY44	23.22 ± 0.94
SMY17	12.01 ± 0.81	SMY48	18.31 ± 0.41
SMY18	17.82 ± 0.26	SMY50	16.07 ± 0.37
SMY23	20.61 ± 0.03		

In order to, the selection of the optimum concentrations of glucose as sole carbon source, four concentrations were used as 30.0, 50.0, 70.0 and 100.0 g/L. Batch flask cultivation was investigated relative to carbon to nitrogen molar ratio (C/N molar ratio) as 8, 13, 19 and 27, respectively, on growth and lipid production of *R.mucilaginosa*SMY40. As shown in Fig. 2B, the carbon concentration was significant on growth and lipid production of *R. mucilaginosa* SMY40. The maximum yield 4.01 g/L of dry biomass and 1.44 g/L of lipid was obtained with the glucose concentration of 70.0 g/L at the C/N molar ratio of 19, followed by 50.0 g/L of glucose at the C/N molar ratio of 13 (3.61 g/L and 1.15 g/L), 30.0 g/L of glucose at the C/N molar ratio of 8 (3.27 g/L and 0.78 g/L) and 100.0 g/L of glucose at the C/N molar ratio of 26 (3.13 g/L and 0.50 g/L), respectively. Lipid yield of *R. mucilaginosa* SMY40 was low with 30.0 g/L at the C/N molar ratio of 8 and then sharply increased when glucose concentration increasing from 50.0 to 70.0 g/L or at C/N molar ratio of13 to 19, and reach the maximum of lipid content 35.91% of their dry biomass weight. Increment of glucose concentration at 100.0 g/L in YNB medium or at the C/N molar ratio of 26 resulted in slight drop in lipid content and biomass of *R. mucilaginosa* SMY40, suggesting that a considerable glucose inhibitory effect had occurred as in the yeast strain *Cryptococcus viswanathii* OYS3 when glucose concentration increased to 100.0 g/L at the C/N molar ratio of 219, lipid accumulation and growth significantly decreased (Leesing and Nantaso, 2011). Therefore 70.0 g/L of glucose was chosen for all following experiments.

It has been reported that the different nitrogen sources also had varied influence on lipid production. Both inorganic nitrogen sources and organic nitrogen sources can be used for lipid production of the oleaginous yeast. We next investigated the effect of different nitrogen sources on growth and lipid production of *R. mucilaginosa* SMY40, the use of 4.86 g/L NH_4Cl and 8.18 g/L $NH_4(SO_4)_2$ as inorganic nitrogen source and 3.75 g/L urea as organic nitrogen source were replaced of NH_4NO_3. Among the nitrogen sources examined for *R. mucilaginosa* SMY40 strain, there was significant difference on lipid production but no difference on growth. The maximum yield 1.71 g/L of lipid (lipid content as 39.85% of their dry biomass weight) was obtained from the medium containing urea as nitrogen source, followed by NH_4NO_3 (1.62 g/L), NH_4Cl (1.46 g/L) and $NH_4(SO_4)_2$ (1.29 g/L), respectively, as for the biomass yield ranged from 4.28 g/L to 4.35 g/L (Fig. 1C). Similar results were also observed in the two oleaginous yeast strains *Y. lipolytica* JDC335 and *Y. lipolytica* DSM70561 grown on medium supplementing urea as nitrogen source and lend more lipid yield (Sriwongchai *et al.,* 2013). Huang *et al.* (1998) reported that inorganic nitrogen sources were good for cell growth, but not suitable for oil production, while organic nitrogen sources were good for oil production, but not suitable for cell growth of *Mortierella isabellina*, *R. mucilaginosa* TYJ15a has been found to accumulate more lipids when an organic nitrogen source was employed (Li *et al.,* 2010). Likewise the oleaginous yeast strains *C. curvatus* O3 (Zhang *et al.,* 2011), *C. curvatus* OYS3 (Leesing and Nantaso, 2011) and *Rhodosporidium toruloides* (Evan and Ratledge, 1984) accumulated more biomass and lipids when an organic nitrogen source was included in the medium.

Indeed, C/N ratio had been found to be the major impact factor for oil accumulation by the oleaginous microorganism (Papanikolaou *et al.,* 2004). When oleaginous microorganisms are grown with the excess of carbon and limiting-nitrogen condition, they may accumulate high concentration of intracellular lipid (Li *et al.,* 2010). In the subsequent studies, we use urea as a sole nitrogen source. The effect of varying urea concentrations of 5.0, 10.0, 15.0, and 20.0 g/L with relative to carbon to nitrogen mole ratio (C/N molar ratio) as 14, 7, 5 and 3.5, respectively, were used to investigate on lipid production and growth of *R. mucilaginosa* SMY40. The concentration of urea was significant on lipid production and growth of *R. mucilaginosa* SMY40. The maximum lipid yield 1.76 g/L (lipid content as 40.00% of their dry biomass weight) was obtained from the presence of 10.0 g/L of urea at the C/N molar ratio of 7, whereas, biomass was 4.40 g/L. The lipid production led to a decrease while, biomass slightly increased when urea concentration led to increase. At 5.0 g/L of urea showed in drop biomass and lipid yield (Fig. 1D).In the cultivation of oleaginous yeast, when nitrogen was low in the medium, the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NAD-IDH) decrease from mitochondria of the oleaginous yeast, then the tricarboxylic acid cycle was repressed, metabolism pathway altered, and protein synthesis stopped and lipid accumulation activated (Evans *et al.,* 1981). After nitrogen depletion, significant fat droplet quantities were accumulated inside the fungal mycelia of *M. isabellina* grown on high-sugar content media according to our results (Papanikolaou *et al.,* 2004).



* n/a = not applicable

Figure 1 Dry cell weight and cellular lipid of *R. mucilaginosa* SMY40 on YNB w/o aa-AS medium with initial pH of 4.7incubation temperature at 30°C and orbital shaking at 150 rpm for 120 h A) carbon sources; B) glucose concentrations; C) nitrogen sources; and D) nitrogen concentrations. Symbols: (☐) dry cell weight g/L and (■) cellular lipid g/L. Different alphabets (a, b, c, and d) indicated significant difference ($P \leq 0.05$).

Fatty acid composition of *R.mucilaginosa* SMY40 cells

The predominant fatty acid in the extracted lipids from the yeast *R. mucilaginosa* SMY40 cells grown on nutritionally optimized medium were transmethylated and analyzed by GC-FID, the results showed that the predominant fatty acids consisted of 20.21% palmitic acid (C16:0), 2.40% palmitoleic acid (C16:1), 5.85% stearic acid (C18:0), 51.64% oleic acid (C18:1) and 12.71% linoleic acid (C18:2), respectively (Table 3). In an earlier study, Li *et al.* (2010) reported high percentages of C16:0 and C 18:1 (85.8% w/w), especially C18:1 (63.5% w/w) among

the cellular lipid accumulated by *R. mucilaginosa* TJY15a cells grown on cassava starch hydrolysate. In another study, C18:1 was reported to be the predominant fatty acid (55.6% w/w) accumulated by *R. mucilaginosa* IIPL32 cells grown on sugarcane baggage derived pentose rich hydrolysis medium (Dasgupta *et. al.,* 2017). The lipids from *A. pulluans* var. *melanogenum* P10 also contain mainly long-chain fatty acids with 16 and 18 carbon (Wang *et al.,* 2014). This means that the fatty acid composition of *R. mucilaginosa* SMY40 was similar to that of the other oleaginous yeast (Table 3). In addition to, the major constituent fatty acids of the cellular lipid of *R. mucilaginosa* SMY40 closed to palm oil (Moser, 2008), corn oils, sunflower oils (Ramos *et al.,* 2009), and jatropha oils (Thiru *et al.,* 2011).

Table 3 Fatty acid composition (% of total fatty acids) of the extracted lipid from *R. mucilaginosa* SMY40 grown on nutritionally optimized medium.

Source	Relative proportion of fatty acids(%, w/w)					References
	C16:0	C16:1	C18:0	C18:1	C18:2	
R. mucilaginosa SMY40	20.21	2.40	5.85	51.64	9.17	This study
R. mucilaginosa TJY15a	22.3	1.8	5.2	63.5	5.7	Li *et al.* 2010
R. mucilaginosa IIPL32	13.4	12.9	3.8	55.6	18.9	Dasgupta *et al.* 2017
A. pulluans var. *melanogenum* P10	26.7	1.7	6.1	44.5	21.0	Wang *et al.* 2014
C. curvatus ATCC 20509	29.5	1.0	18.4	42.2	6.3	Yu *et al.* 2014
Palm oils	44		5	39	11	Moser 2009
Corn oils	6.5		0.14	65.6	25.2	Ramos *et al.* 2009
Sunflower oils	6.4		1.3	22.0	66.2	Ramos *et al.* 2009
Jatropha oils	0.4		0.2	56.0	26.0	Thiru *et al.* 2011

Conclusions

The yeast *R. mucilaginosa* SMY40 derived from soil mangrove was found to be able to produce lipid in its cells. The optimal medium for cell growth and lipid production was investigated, the oleaginous yeast *R. mucilaginosa* SMY40 showed the maximum lipid production of 40.00% of their dry biomass under the optimized medium of 70.0 g/L of glucose, 10.0 g/L of urea with initial of pH 4.7 and incubation temperature at 30°C shaking at 150 rpm for 120 h. The compositions of fatty acids in the extracted lipid were 20.21% palmitic acid (C16:0), 2.40% palmitoleic acid (C16:1), 5.85% stearic acid (C18:0), 51.64% oleic acid (C18:1) and 12.17% linoleic acid (C18:2). So these results suggest that *R. mucilaginosa* SMY40 have potential for lipid production and could be used as feedstock for biodiesel production.

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