Evaluation of Sugarcane Leaves as a Substrate for Production of Palmitoleic Acid Using *Cyberlindnera subsufficiens* NG8.2

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Oil was produced from sugarcane leaves hydrolysate (SLH) in the newly isolated oleaginous yeast *Cyberlindnera subsufficiens* NG8.2, using a two-stage cultivation method. The SLH contained sugars derived from both the dilute acid pretreatment of leaves and subsequent enzymatic hydrolysis (16.6 g/L of glucose and 15.87 g/L of xylose). The *Cyberlindnera subsufficiens* NG8.2 produced oil containing 18.73 wt% of palmitoleic acid, with a 0.99-g/L oil yield when grown in the SLH. Removal of phosphate from the SLH by $Ca(OH)_2$ treatment (SLH-P) resulted in an increased oil yield of 1.38 g/L, but the palmitoleic acid content of the oil decreased to 12.45 wt%. Supplementation of the SLH-P with 3.12 mM of Mg^{2+} increased the palmitoleic acid content in the oil to 15.80 wt% and the oil yield to 1.58 g/L, with a palmitoleic acid yield of 2.09 mg/g sugarcane leaves. Thus, sugarcane leaves are a promising feedstock for palmitoleic acid production using *Cyberlindnera subsufficiens* NG8.2.

Keywords: Sugarcane leaves; Palmitoleic acid; Cyberlindnera subsufficiens; $Ca(OH)_2$ treatment

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INTRODUCTION

Thailand is the world's fourth-largest sugarcane (*Saccharum* spp.) producer, with approximately 132 million metric tons of sugarcane cultivated in crop year 2019/2020 (Office of the Cane and Sugar Board 2020). This generated approximately 22.44 million metric tons of sugarcane leaves (0.17 tons leaves per ton sugarcane) (Department of Alternative Energy Development and Efficiency 2013). Most of these leaves were burnt before cane harvesting to prevent the farmers from being cut by the sharp edges of the leaves, making the cane harvesting easier. However, the burning of sugarcane leaves is a major cause of air pollution in Southeast Asia where this practice of cane harvesting is used. Value creation with the sugarcane leaves may reduce the amount of leaves that are burnt. Sugarcane leaves are high in cellulose, which can be converted to fermentable sugar. Therefore, they can serve as a low-cost fermentable sugar resource for production of various high-value products. Sugarcane leaves have previously been evaluated as feedstocks for the production of bioethanol (Jutakanoke *et al*. 2012) and yeast oil (Pranimit *et al*. 2019).

When grown under an overabundance of carbon but a deficiency of an essential nutrient (most often, nitrogen or phosphate), some yeasts that express cytosolic adenosine

triphosphate (ATP) citrate lyase (ACL) divert the carbon from energy production *via* the tricarboxylic acid (TCA) cycle to lipid biosynthesis. Under a nitrogen-limiting condition, the carbon flux is initiated by adenosine monophosphate (AMP) deaminase liberation of ammonia from AMP to be utilized as a nitrogen source. Reduction of the cellular AMP level inhibits the activity of isocitrate dehydrogenase, the enzyme responsible for conversion of isocitrate to α-ketoglutaric acid in the TCA cycle, and this results in citrate accumulation due to the equilibrium concentration between isocitrate and citrate by isocitrate aconitase in the mitochondria. The accumulated mitochondrial citrate is transported to the cytoplasm. In the cytoplasm, the citrate is then converted to acetyl coenzyme A (acetyl-CoA) and oxaloacetate by ACL, and the acetyl-CoA is then catalyzed to malonyl-CoA by acetyl-CoA carboxylase. The malonyl-CoA is catalyzed through a series of reactions, then channeled into the lipid biosynthesis pathway. Meanwhile, the oxaloacetate produced is catalyzed to malate by malate dehydrogenase, and then malic enzyme (ME) converts the malate to pyruvate (Ageitos *et al*. 2011; Probst *et al*. 2016).

Under a phosphate-limited condition, the reduction in AMP is initiated by AMP degradation in response to the need for inorganic phosphate in the cell. This reduces the isocitrate dehydrogenase activity, which is involved in citrate transportation from the mitochondria to the cytoplasm and lipid biosynthesis, as described above (Wang *et al*. 2018). Phosphate limitation is, therefore, effectively similar to nitrogen limitation in terms of stimulating microbial lipid production (Wu *et al*. 2010). Most of the lipids accumulate in the cell as triacylglycerols (TAG) and as discrete oil droplets (Ageitos *et al*. 2011; Probst *et al*. 2016).

Yeasts that accumulate oil to more than 20 wt% of dry cell weight (DCW) are defined as oleaginous yeasts (Ageitos *et al*. 2011). In general, dominant fatty acids of oleaginous yeast oils are palmitic acid, stearic acid, and oleic acid (Meesters *et al.* 1996) which are similar to those of common plant oils (Papanikolaou and Aggelis 2011). However, oils of some oleaginous yeasts including *Pichia segobiensis* SSOH12 (Schulze *et al*. 2014), *Candida krusei* DBM 2163, and *Yarrowia lipolytica* CCY 29-26-36 (Kolouchová *et al*. 2015) contained high concentration of palmitoleic acid (C16:1) or omega-7 fatty acid. These yeast oils have gained high interest due to broad applications of the palmitoleic acid in medicine and cosmetics (Řezanka *et al*. 2013; Kolouchová *et al*. 2015).

The palmitoleic acid prevents B-cell apoptosis and type-2 diabetes (Morgan and Dhayal 2010) and reduces LDL cholesterol in blood vessels (Griel *et al*. 2008), leading to prevention of brain and cardiovascular diseases. Moreover, palmitoleic acid also acts as a growth inhibitor of Gram-positive bacteria (Wille and Kydonieus 2003). Sea buckthorn and macadamia nuts are only two plants with naturally high concentrations of palmitoleic acid, at approximately 12 wt% to 22 wt% (Kolouchová *et al*. 2015). But their availability is limited and quality of the oils is depended upon locality including climatic and seasonal conditions. The usage of yeast for oil production provides better benefits over plants as yeasts grow fast without effects from changing of climatic or seasonal condition, they do not require land space for plantation compared with plant cultivation, and it is easier to expand production (Sitepu *et al.* 2014). In addition, yeasts can be cultured in various carbon sources, including sugars derived from lignocellulose, and the oil so produced can be manipulated to have different fatty acid profiles (Kitcha and Cheirsilp 2011).

In this study, it is reported that sugarcane leaf hydrolysate (SLH) is a potential sustainable, renewable raw material for the production of palmitoleic acid by

Cyberlindnera (*Cy.*) *subsufficiens* NG8.2 when the SLH is treated with $Ca(OH_2)$ and supplemented with Mg^{2+}.

EXPERIMENTAL

Microorganism

Cyberlindnera subsufficiens NG8.2 was isolated from the soil at Ngao waterfall, Ranong province, Thailand. It was identified by molecular operational taxonomic unit (MOTU) and species inference by comparing their D1/D2 domain (500–600 bp) of 26S rRNA gene sequence with those deposited in the NCBI GenBank database using the BLASTn program as reported by Hoondee *et al.* (2019). The D1/D2 domain sequence of the *Cy. subsufficiens* NG8.2 was submitted to the GenBank database with the accession number of LC602814. For short-term storage, *Cy. subsufficiens* NG8.2 was maintained by keeping the culture grown on yeast malt extract (YM) agar (10 g/L of glucose, 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of Bacto peptone, and 2 g/L of agar, pH 5.5) at 30 °C for 48 h in a refrigerator. Long-term storage of the culture was performed by lyophilization.

Sugarcane Leaves

Sugarcane (*Saccharum officinarum* L. CSB06-2-15) leaves were collected from the Sugarcane and Sugar Industry Promotion Center, Chonburi province, Thailand. They were sun dried, cut, hammer milled, and sieved to a particle size of 20 mesh to 40 mesh. The particles were then dried at 60 °C until reaching a constant weight and then kept in a desiccator. The chemical composition of the sugarcane leaves, as analyzed by the methods of the Technical Association of the Pulp and Paper Industry (TAPPI T221 om-02 2002; TAPPI T203 cm-99 2009; TAPPI T222 om-15 2015), is shown in Table 1.

Table 1. Chemical Composition of the Sugarcane Leaves

Component	Percentage of Dry Weight (DW) (wt%)
Cellulose	38.8
Hemicellulose	23.5
Lignin	13.8
Ash	11.4

Oil Production by *Cy. subsufficiens* NG8.2

Oil production in a high C/N medium

Production of oil by *Cy. subsufficiens* NG8.2 was performed by a two-stage cultivation method due to the different nutrient requirements in the growth and oil production phases (Lin *et al.* 2014). For the first stage (cell propagation), the *Cy. subsufficiens* NG8.2 was inoculated into YM broth (50 mL) at the initial optical density at 660 nm (OD_{660nm}) of 0.8 in a 250-mL Erlenmeyer flask and incubated at 30 °C and 200 rpm for 24 h. The resultant culture (approximately OD_{660nm} of 2.3) was transferred at 10 vol% into 50 mL of fresh YM medium and incubated at the same conditions for 48 h. The cells were then collected by centrifugation (4 °C, 9803 g, 10 min). For the second stage (oil production), the obtained cell pellet was washed twice with sterile distilled water and transferred into 50 mL of oil production medium (OPM) with a high C/N molar ratio (50 g/L of glucose, 0.1 g/L of yeast extract, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of $(NH_4)_2SO_4$,

0.1 g/L of NaCl, and 0.1 g/L of $CaCl_2 \cdot 2H_2O$, pH 5.5) in a 250-mL Erlenmeyer flask and incubated at 30 °C and 200 rpm for 6 d. At the indicated time, cells were harvested by centrifugation (4 °C, 9803 g, 10 min), washed twice with distilled water, dried by lyophilization, and weighed to obtain the DCW of the resultant cell biomass.

Evaluation of the intracellular oil accumulation

The intracellular oil accumulated in the lyophilized cells was analyzed as reported by Folch *et al*. (1957), except with minor modification. In brief, the lyophilized cells (1.0 g DCW) were suspended in 20 mL of a 2:1 (v/v) chloroform : methanol mixture and sonicated at 37 kHz (Elmasonic E60H, Elma Schmidbauer GmbH, Singen, Germany) at room temperature for 30 min prior to being centrifuged (4 °C, 5416 g, 40 min). Sodium chloride solution at 0.73% (w/v) was mixed with the resultant supernatant to obtain a 2:1:0.8 (v/v/v) chloroform : methanol : aqueous saline mixture and then centrifuged (4 °C, 774 g, 10 min). The lower phase was collected, dried by evaporation at room temperature, and weighed. The oil content (wt% DCW) was the amount of oil extracted from 100 g of cells DCW, while the oil yield (g/L) was calculated from the oil content divided by 100 and then multiplied by the cell biomass (g/L).

Fatty acid composition analysis

The extracted *Cy*. s*ubsufficiens* NG8.2 oil was directly converted to fatty acid methyl esters, extracted by hexane, and analyzed by gas chromatography using a flame ionization detector system (6890N, Agilent Technologies, Santa Clara, CA, USA) as previously described (Pranimit *et al*. 2019).

Preparation of the Sugarcane Leaves Hydrolysate (SLH)

The SLH was prepared as previously described (Jutakanoke *et al*. 2012). In brief, the dried sugarcane leaf particles at 6% or 12% (w/v) were suspended in 1.5% (w/v) of sulfuric acid and autoclaved at 121 °C and 103 kPa for 30 min. The pretreated slurry (pretreated sugarcane leaves suspended in pretreatment hydrolysate) was adjusted to a pH of 5.0 and further saccharified by Accellulase^{TM}1500 (2500 carboxymethyl cellulose (CMC) U/g, Genecor International Inc., New York, NY, USA) at 50 °C and 125 rpm for 6 h. Then, the solid residue was separated by filtration through a cotton sheet and centrifugation (4 °C, 9803 *g*, 20 min). The resultant filtrate (SLH) was adjusted to a pH of 5.5 and filter-sterilized through a 0.22-µm membrane filter. The SLH obtained from the 6% (w/v) sugarcane leaf loading (6%-SLH) was used for inoculum medium preparation, while that from the 12% (w/v) sugarcane leaf loading (12%-SLH) was used as the OPM. The 12%-SLH was reported to contain furfural and HMF at 0.13 g/L and 0.3 g/L, respectively (Pranimit *et al.* 2019), which were lower than reported toxic level (Chen *et al.* 2009; Yu *et al.* 2011). Figure 1a shows a schematic diagram of SLH preparation.

Removal of Phosphate from the SLH

Phosphate in the 12%-SLH was removed by the modified method of Yu *et al.* (2011). Calcium hydroxide ($Ca(OH)_2$) was added into the 12%-SLH until the pH reached 10, and the mixture was further incubated at 50 °C with 125-rpm mixing for 30 min. After centrifugation (4 °C, 9803 *g*, 20 min), the supernatant (phosphate-removed SLH, SLH-P) was harvested, adjusted to a pH of 5.5, and filter sterilized through a 0.22-µm membrane filter (Fig. 1b).

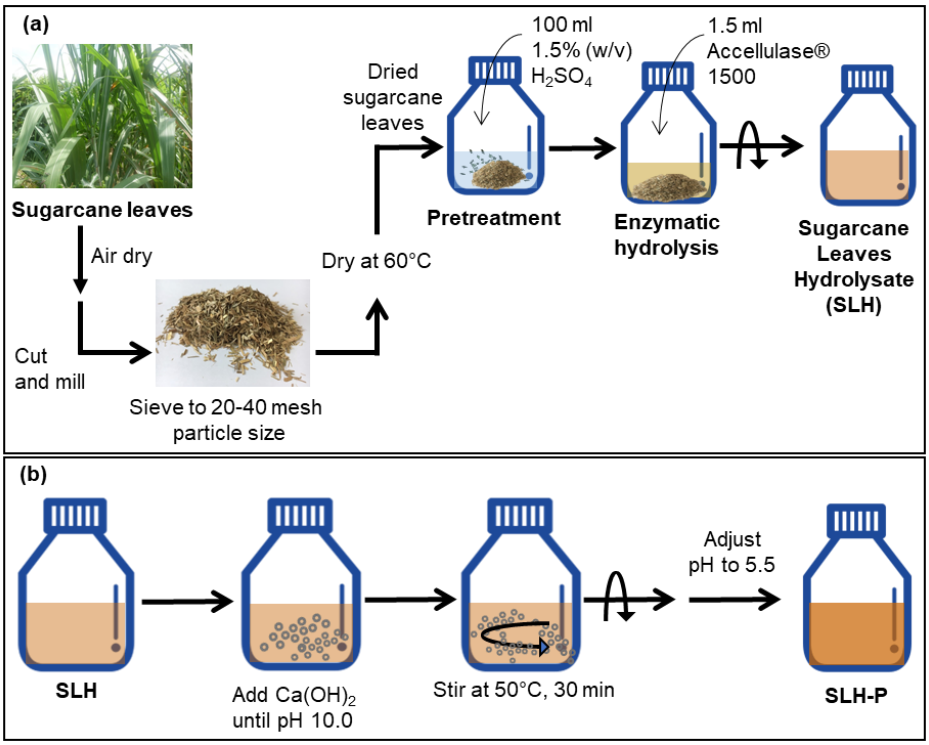


Fig. 1. Schematic diagram of the preparation of (a) SLH and (b) SLH-P

Oil Production by *Cy. subsufficiens* NG8.2 in 12%-SLH

Cyberlindnera subsufficiens NG8.2 oil was produced using the same method as above, except that 6%-SLH was used as the inoculum medium by supplementing with 0.3% (w/v) of yeast extract and 0.3% (w/v) of peptone, and 12%-SLH was used as the OPM.

Maximization of the oil production by Cy. subsufficiens NG8.2 in 12%-SLH

Optimal C/N and C/P molar ratios of an OPM are important factors for maximization of yeast oil production (Papanikolaou and Aggelis 2011). The SLH had low C/N molar ratio. Increase of the C/N molar ratio by removing of nitrogen is difficult. Therefore, C/P molar ratio of the SLH was increased by removing of phosphate. Soluble phosphate can be removed by precipitation with metal ions, such as Ca^{2+} and Mg^{2+} (Kolouchová *et al*. 2016). Treatment with $Ca(OH)_2$ (over-liming) is a popular method for removing soluble phosphate by precipitating it as $Ca_3(PO_4)_2$ (Xia *et al*. 2016; Bao *et al*. 2018). This approach has been used to remove phosphate from several lignocellulosic hydrolysates (Wu *et al*. 2010; Bao *et al*. 2018; Zhou *et al*. 2019). Thus, the SLH was treated with $Ca(OH)_2$ to increase the C/P molar ratio. The concentration of Mg^{2+} and initial pH of OPM, which were reported to have an influence on oleaginous yeast oil production, were also investigated (Chen *et al*. 2013). The activity of ACL, a key enzyme in *de novo* lipid accumulation, is dependent on the presence of Mg^{2+} (Amaretti *et al*. 2010). The optimum pH for oil production depends on the yeast strain and type of carbon source (Angerbauer *et al*. 2008). Generally, the optimal pH for oil production in yeasts ranges from 5 to 6 (Madani *et al*. 2017).

Maximization of the oil production by *Cy. subsufficiens* NG8.2 in the 12%-SLH was performed sequentially by 1) increasing the C/P molar ratio of the 12%-SLH by removal of phosphate (*i.e*., using SLH-P); 2) increasing the C/P molar ratio of the SLH-P

by adding glucose at 5 g/L or 10 g/L; 3) increasing the Mg^{2+} concentration of the selected-C/P-molar-ratio SLH-P by adding $MgSO_4 \cdot 7H_2O$ at 0.5 g/L, 0.75 g/L, or 1 g/L; and 4) varying the initial pH (4.5, 5.5, or 6) of the SLH-P containing the selected concentration of Mg^{2+}.

Palmitoleic acid yield (mg/g DW sugarcane leaves) was calculated by multiplication of the palmitoleic acid content (wt%) with the oil yield (g/L) and then divided by 12.

Analytical Procedures

The concentrations of glucose and xylose were analyzed using a biochemistry analyzer (YSI 2700 Select, YSI Incorporated, Yellow Springs, OH, USA). Total nitrogen was analyzed by the Kjeldahl method (Kjeldahl 1883), while the concentrations of galactose, arabinose, and cellobiose were determined by high-performance liquid chromatography using a monosaccharide column (Rezex™ RPM, Phenomenex, Torrance, CA, USA) and an evaporative light scattering detector (Agilent Technologies, Santa Clara, CA, USA) at the Scientific and Technological Research Equipment Centre, Chulalongkorn University, Bangkok, Thailand. The orthophosphate concentration was determined by the ascorbic acid method 4500-PE, as reported by Rice *et al*. (2012), while the concentrations of calcium, magnesium, potassium, zinc, copper, iron, and manganese were determined by atomic absorption spectrometry, as reported by Rice *et al*. (2012) at the Environmental Research Institute, Chulalongkorn University, Bangkok, Thailand.

RESULTS AND DISCUSSION

Oil Production by *Cy. subsufficiens* NG8.2 in a High C/N Medium and Its Fatty Acid Composition

The *Cy. subsufficiens* NG8.2 had high oil yield (1.26 g/L), oil content (20.12 wt% of DCW), and cell biomass (6.31 g/L) when grown in the high C/N OPM. To the authors' knowledge, this is the first report that *Cy. subsufficiens* is oleaginous. Previous reports have included *Cy. subsufficiens* C6.1 isolated from coconut producing fruity aromatic compounds in non-alcoholic beer (Bellut *et al*. 2019) and *Cy. subsufficiens* DMKU-YNB42-1, a high-ethanol-producing yeast (Jaiboon *et al*. 2016).

Analysis of the *Cy. subsufficiens* NG8.2 oil produced in the high C/N OPM revealed that the dominant fatty acids were oleic (C18:1), palmitic (C16:0), palmitoleic (C16:1), and linoleic (C18:2) acids at 34.64 wt%, 26.32 wt%, 22.25 wt%, and 12.21 wt%, respectively. Other fatty acids found in lesser amounts (0.52 wt% to1.89 wt%) were myristic (C14:0), steric (C18:0), and linolenic (C18:3) acids (Table 2). The major fatty acids normally found in oleaginous yeast oils are oleic, palmitic, stearic, and linoleic acids (Sitepu *et al*. 2014). Only a few oleaginous yeasts produce oil high in palmitoleic acid. Schulze *et al*. (2014) reported that *Pichia segobiensis* SSOH12 oil contained 16 wt% of palmitoleic acid, while the oils from *Candida krusei* DBM 2163 and *Yarrowia lipolytica* CCY 29-26-36 contained 16.0 wt% and 16.4 wt% of palmitoleic acid, respectively, when grown in an OPM containing 30 g/L of glucose with a C/N molar ratio of 30 and a C/P molar ratio of 6 (Kolouchová *et al*. 2015).

Table 2. Fatty Acid Profile of *Cy. subsufficiens* NG8.2 Oil Produced in a High C/N

OPM

Fatty Acid	Percentage (wt%)
Myristic acid (C14:0)	0.52 ± 0.00
Palmitic acid (C16:0)	26.32 ± 0.02
Palmitoleic acid (C16:1)	22.25 ± 0.14
Steric acid (C18:0)	0.69 ± 0.06
Oleic acid (C18:1 n9)	34.64 ± 0.06
Linoleic acid (C18:2 n6)	12.21 ± 0.02
Linolenic acid (C18:3 n3)	1.89 ± 0.00
Others	1.48
Data are shown as the mean ± SD, derived from three independent repeats.	

Oil Production by *Cy. subsufficiens* NG8.2 in 12%-SLH

The *Cy*. *subsufficiens* NG8.2 gave the highest oil yield of 0.99 g/L, which was lower than in the high C/N OPM after 4 d of incubation at 30 °C and 200 rpm in the SLH OPM, while the oil content and cell biomass were 9.82 wt% of DCW and 10.04 g/L, respectively. The SLH had a slightly lower carbon level but substantially greater nitrogen and phosphate levels than the high C/N OPM (Table 3). This might be a reason why the *Cy*. *subsufficiens* NG8.2 produced lower oil yield in the SLH OPM than the high C/N OPM.

Table 3. Comparison of the Compositions of the High C/N Medium, SLH, and SLH-P Oil Production Media

Composition	High C: N[a]	SLH	SLH-P
g/L			
Glucose	50	16.6 ± 0.08	15.43 ± 0.1
Xylose	-	15.87 ± 0.05	14.9 ± 0.00
Galactose	-	0.36	0.36
Arabinose	-	3.63	3.73
Cellobiose	-	ND	ND
Total sugar	50	36.46	34.42
mg/L			
Magnesium (Mg)	49.375	2.5	1.9
Potassium (K)	0.0319	1260	1170
Calcium (Ca)	27.203	362	536
Iron (Fe)	0.0055	1.3	0.79
Manganese (Mn)	-	7.5	0.62
Copper (Cu)	-	<0.1	<0.01
Zinc (Zn)	-	1.8	0.85
Phosphate (PO_4)	3.27	75.7	0.014
g/L			
Total C	20.0	14.58	13.77
Total Nitrogen	0.32	0.6	0.5
C/N molar ratio	626.97	24.31	27.54
C/P molar ratio	18,744.61	194.20	21,182.07
[a] Calculated data, ND (not detectable)			

Maximization of *Cy. subsufficiens* NG8.2 Oil Production in 12%-SLH

As shown in Table 3, the phosphate concentration in the SLH (75.7 mg/L) was decreased by 99.98% to 14 µg/L in the SLH-P, while the total sugar concentration in the SLH (36.46 g/L) was also decreased by 5.6% to 34.42 g/L in the SLH-P. After removal of phosphate, the C/P molar ratio of the SLH increased from 194 to 21,182 in the SLH-P. The total nitrogen concentration of the SLH (0.6 g/L) was also decreased, to 0.5 g/L in the SLH-P, leading to an increased C/N molar ratio from 24.31 in the SLH to 27.54 in the SLH-P. This result is in accord with previous studies, where the sugar and nitrogen concentrations of $Ca(OH)_2$-treated Jerusalem artichoke hydrolysate were slightly lower than in the untreated hydrolysate, and the C/N molar ratio of the untreated hydrolysate (29) increased to 35 in the $Ca(OH)_2$-treated hydrolysate (Bao *et al*. 2018).

When grown in the SLH-P at 30 °C and 200 rpm for 4 d, the *Cy*. *subsufficiens* NG8.2 gave an oil yield of 1.38 g/L, which was 39% higher than in the SLH, with an oil content and cell biomass of 13.20 wt% of DCW and 10.45 g/L, respectively (Fig. 2).

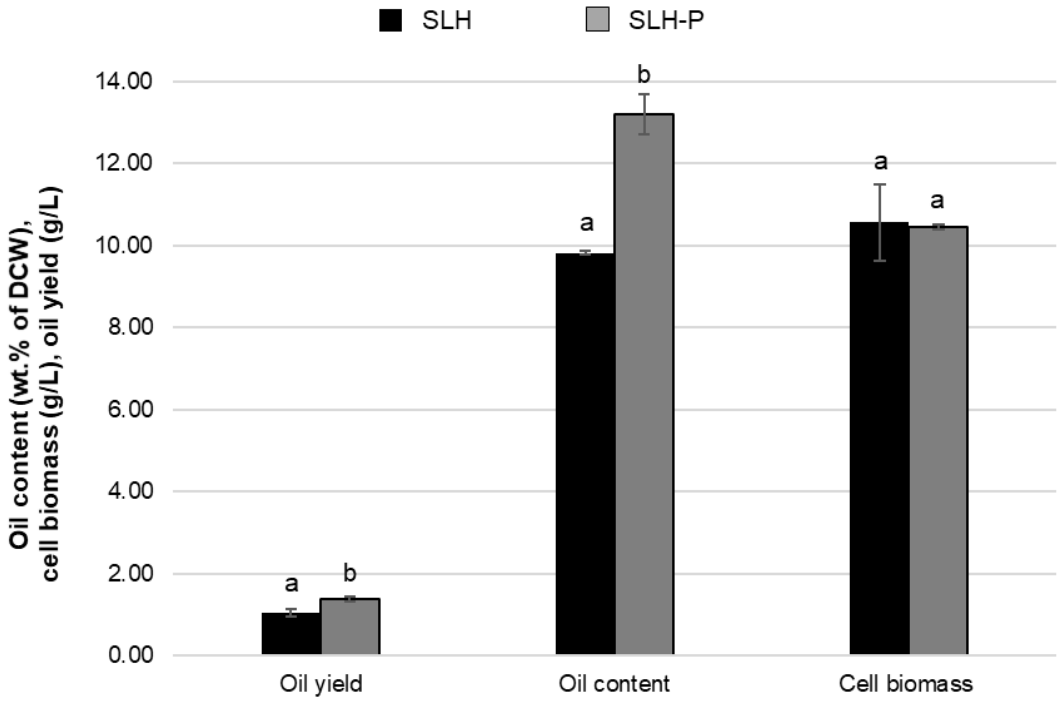


Fig. 2 Comparison of the oil yields, oil contents, and cell biomasses of *Cy. subsufficiens* NG8.2 grown in SLH or SLH-P. Data are shown as the mean ± SD, derived from three separate cultures. Means with a different letter are significantly different ($p < 0.05$, one-way analysis of variance (ANOVA)).

These results broadly agree with other studies, where the oil yield and content of *Cutaneotrichosporon oleaginosum* ATCC 20509 grown in $Ca(OH)_2$-treated water hyacinth hydrolysate supplemented with acetate increased by 4.2 and 4.6 times, respectively, compared to those in untreated hydrolysate (Zhou *et al.* 2019). The oil yield and content of *Cryptococcus curvatus* MUCL 29819 were increased from 0.64 to 0.92 g/L and from 18.3 wt% of DCW to 30.3 wt% of DCW, respectively, when the C/P molar ratio of the OPM was increased from 18 to 1,482 (Huang *et al*. 2018). When grown in *Laminaria* residue hydrolysate treated with $Ca(OH)_2$ (C/P molar ratio of 1,530), oil yield of *Rhodosporidium toruloides* Y4 increased by 3.43 times compared to untreated hydrolysate (Zhang *et al.*

2016). Likewise, the oil production of *Rhodosporidium* (formerly known as *Rhodotorula*) *toruloides* Y4 was directly linked to the C/P molar ratio of the OPM. When *Rhodosporidium toruloides* Y4 was grown in an OPM containing 70 g/L of glucose (C/N molar ratio of 22.3) with C/P molar ratios ranging from 72 to 9,552, the highest oil yield (2.1 g/L) and oil content (62.1 wt% of DCW) were found when the C/P molar ratio was 9,552 (Wu *et al.* 2010). Oil production of *Trichosporon fermantans* CICC 1368 in rice straw hydrolysate was significantly improved when the rice straw hydrolysate was treated with $Ca(OH)_2$, concentrated then adsorbed onto Amberlite XAD-4. The oil content and oil yield increased from 11.6 wt% of DCW to 40.1 wt% of DCW and from 1.7 g/L to 11.5 g/L, respectively (Huang *et al.* 2009). The SLH-P was selected as the OPM in next experiment.

The C/P molar ratio of the SLH-P (no added glucose) was further increased from 21,182 to 24,616 and 27,693 by adding glucose at 5 g/L and 10 g/L, respectively. The *Cy. subsufficiens* NG8.2 had the highest oil yield (1.33 g/L) and cell biomass (9.86 g/L) in the SLH-P with a C/P molar ratio of 21,182 and an oil content of 13.32 wt% of DCW. When grown in SLH-P with a C/P molar ratio of 24,616 (with 5 g/L of glucose), lower biomass (8.53 g/L) and oil yield values but a higher oil content (13.47 wt% of DCW) were obtained compared to in the SLH-P with a C/P of 21,182 (Fig. 3). These results agreed well with a previous report (Papanikolaou and Aggelis 2011), indicating that excess carbon determined the level of accumulated oil, while a limiting nutrient determined the cell biomass formation. *Cutaneotrichosporon oleaginosum* ATCC 20509 gave maximum cell biomass 8.2 g/L when grown in medium (C/N molar ratio of 12.3) with C/P molar ratio of 1360. Increase of the C/P molar ratio of the medium led to a decrease of cell biomass (Zhou et al. 2019).

Almost all of the glucose was consumed within 2 days in the SLH-P with all C/P molar ratios examined. Meanwhile, higher of the C/P molar ratio resulted in lower of the xylose consumption. This might be an effect of the higher osmotic pressure of the increased C/P molar ratio-SLH-P by adding glucose. The highest xylose uptake was observed in SLH-P with C/P molar ratio of 21,182 (Fig. 4). The oil yield and content of *Trichosporon fermentans* CICC 1368 grown in lipid production medium containing 70 g/L of fructose (C/N molar ratio of 23) increased when the C/P molar ratio was increased from 88 to 31,709, while the cell biomass and fructose consumption decreased, and the optimal C/P molar ratio was 6,342 (Bao *et al*. 2018). The SLH-P with a C/P molar ratio of 21,182 (no added glucose) was used in the next experiment.

The SLH-P contained 1.9 mg/L or 78 μM of Mg^{2+}, which was significantly lower than in the high C/N OPM (49 mg/L) (Table 3). In this experiment, $MgSO_4 \cdot 7H_2O$ was added at 0.5 g/L, 0.75 g/L, and 1.0 g/L into the SLH-P to increase the Mg^{2+} concentration to 2.11 mM, 3.12 mM, and 4.13 mM, respectively, and it was then used as the OPM. The *Cy. subsufficiens* NG8.2 exhibited the highest oil yield (1.58 g/L) and content 14.81 wt% of DCW) when the SLH-P contained 3.12 mM of Mg^{2+}, but all added $MgSO_4 \cdot 7H_2O$ concentrations had no significant effects on the cell biomass formation (Fig. 5).

The Mg^{2+} concentration influenced the growth and oil production of *Trichosporon cutaneum* CH002 grown in corncob hydrolysate, where the highest oil content and cell biomass were obtained with 0.3 g/L and 0.4 g/L of $MgSO_4 \cdot 7H_2O$, respectively (Chen *et al.* 2013). Oil production by *Yarrowia lipolytica* ACA-DC 50109 under the double limitation of Mg^{2+} and nitrogen deficiencies was higher than under each individual limitation.

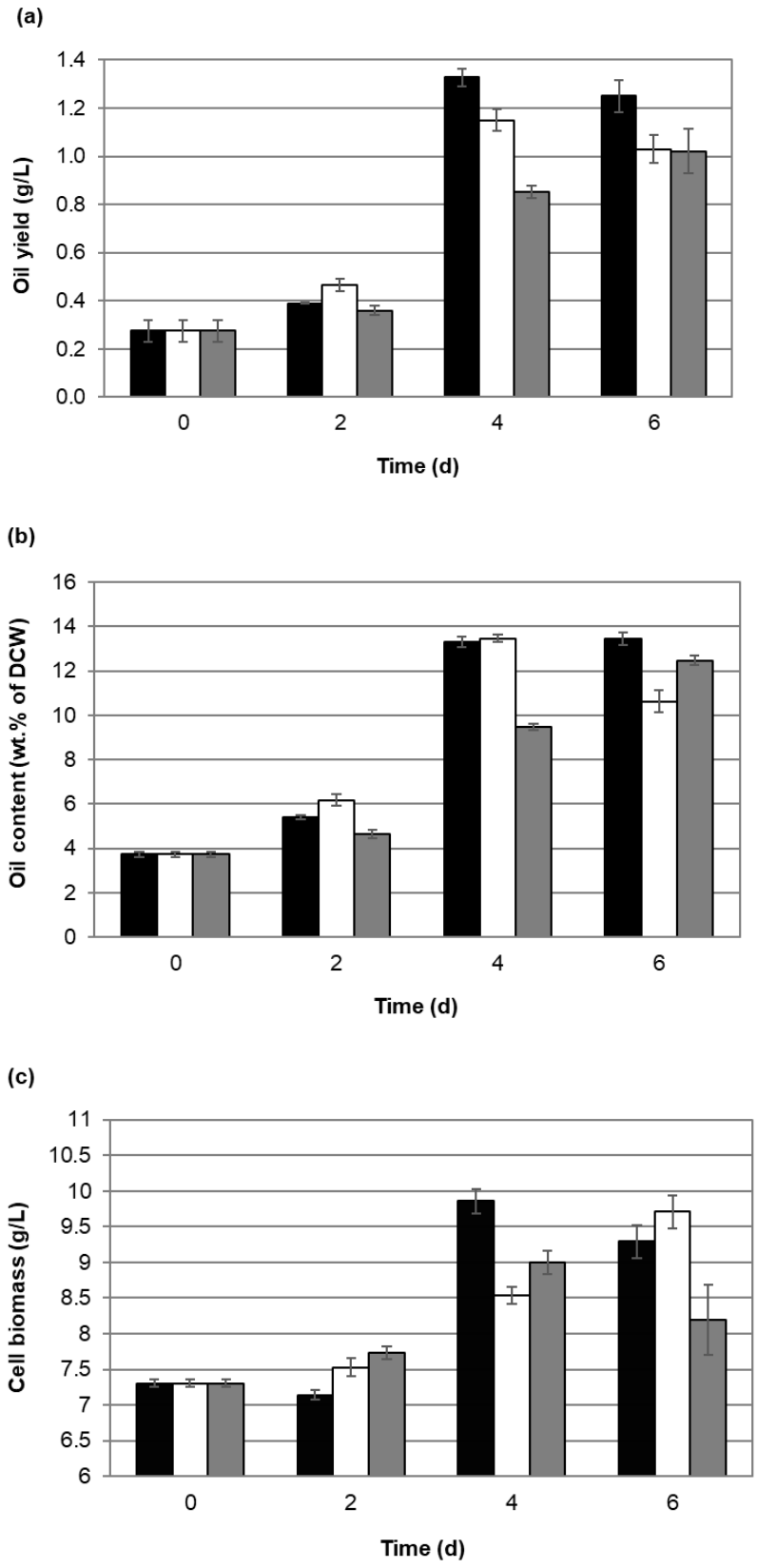


Fig. 3. Comparison of the (a) oil yields, (b) oil contents, and (c) cell biomasses of *Cy. subsufficiens* NG8.2 grown in SLH-P with C/P molar ratios of 21,182 (■), 24,616 (□), and 27,693 (■). Data are shown as the mean ± SD, derived from three separate cultures for each parameter.

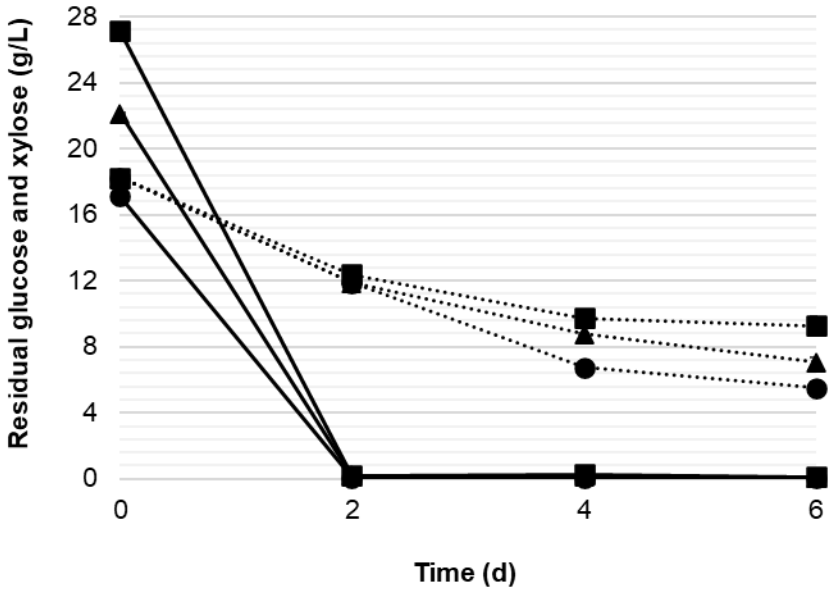
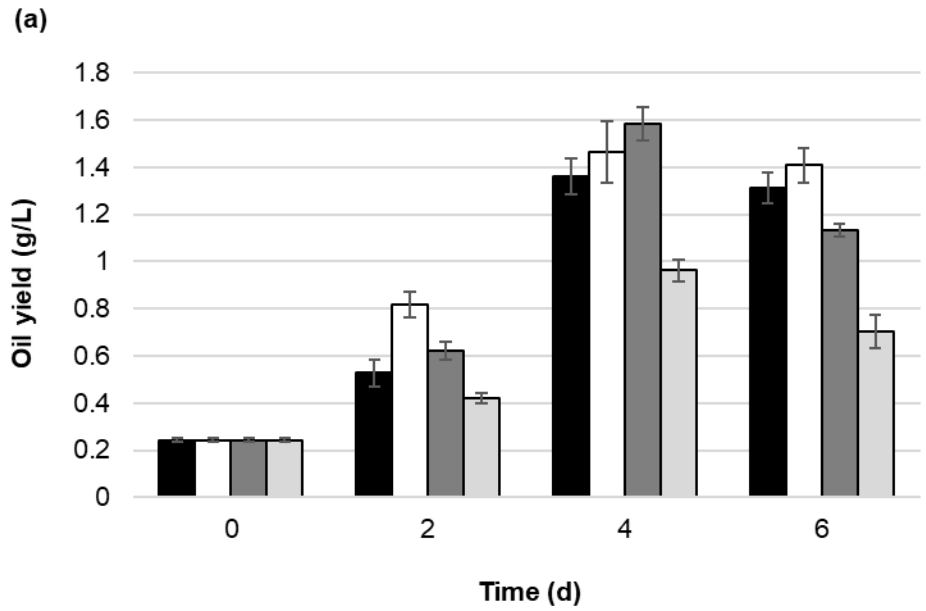


Fig. 4. Glucose (solid line) and xylose (dotted line) consumption profiles of *Cy. subsufficiens* NG8.2 grown in SLH-P with C/P molar ratios of 21,182 (●), 24,616 (▲), and 27,693 (■). Data are shown as the mean ± SD, derived from three separate cultures for each parameter.

Under the double limitation, the activities of both ACL and ME were lower than under each individual limitation condition, although there were no significant differences in the transcription levels of ACL or ME (Bellou *et al*. 2016). The SLH-P containing 3.12 mM of Mg^{2+}, by supplementation with 75 mg/L $MgSO_4 \cdot 7H_2O$, was used as the OPM in the next experiment.



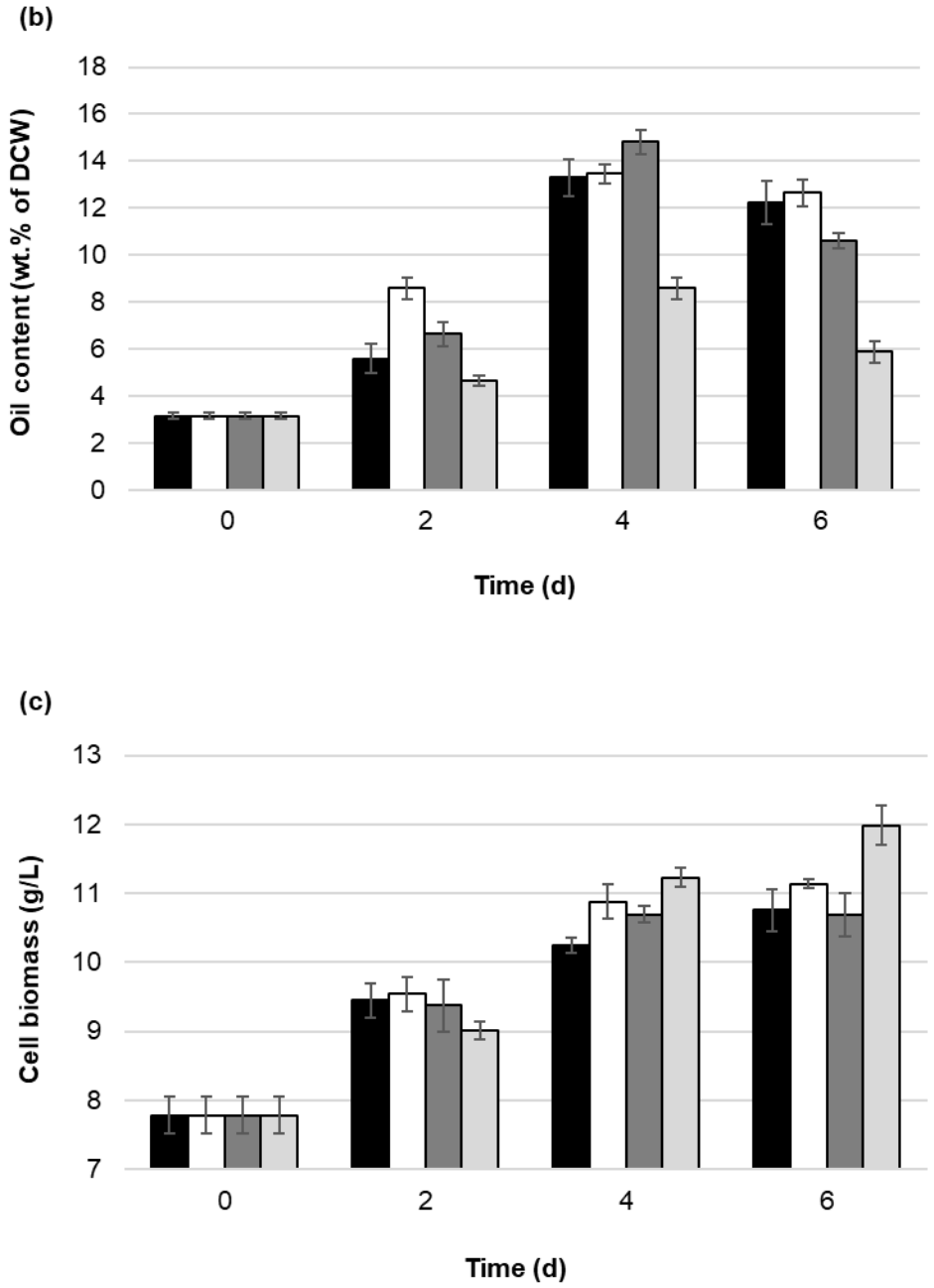


Fig. 5. Comparison of the (a) oil yields, (b) oil contents, (c) and cell biomasses of *Cy. subsufficiens* NG8.2 grown in SLH-P containing 0.078 mM of Mg^{2+} (●), 2.11 mM of Mg^{2+} (▲), 3.12 mM of Mg^{2+} (■), and 4.13 mM of Mg^{2+} (◆). Data are shown as the mean ± SD, derived from three separate cultures for each parameter.

The *Cy. subsufficiens* NG8.2 had the highest oil yield (1.59 g/L) and oil content (14.86 wt% of DCW) in the OPM at a pH of 5.5 at 4 d. The cell biomass was the lowest (10.69 g/L) at a pH of 5.5 but highest (11.69 g/L) at a pH of 6 (Fig. 6). In accordance, a lower pH was previously found to support oil accumulation in oleaginous yeasts (Patel *et al*. 2016). Reduced citric acid efflux at acidic pH's might be the reason why lipid production increased (Zhang *et al*. 2019).

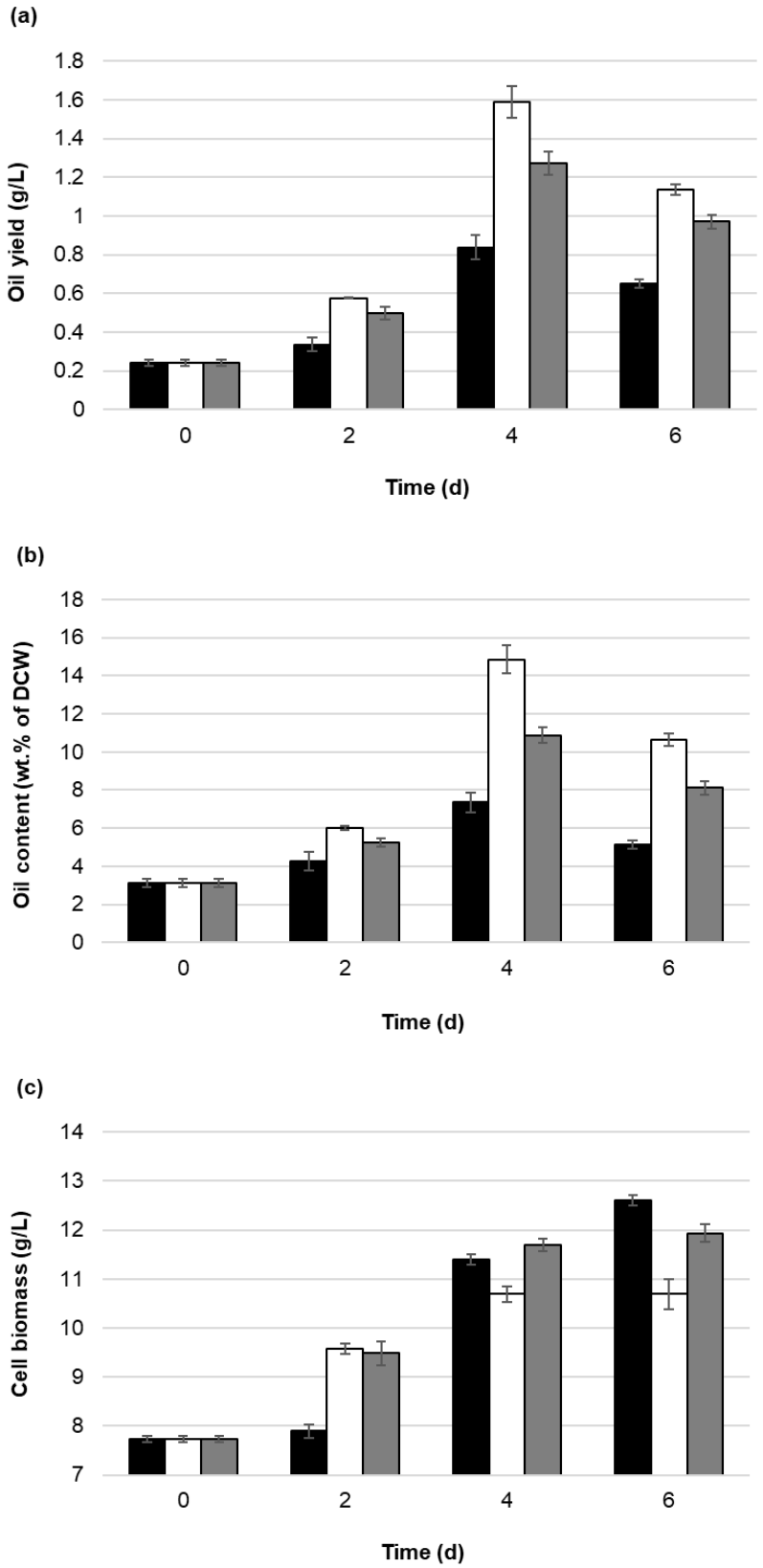


Fig. 6. Comparison of the (a) oil yields, (b) oil contents, and (c) cell biomasses of *Cy. subsufficiens* NG8.2 grown in SLH-P containing 3.12 mM of Mg^{2+} at pH 4.5 (●), 5.5 (▲), and 6 (■). Data are shown as the mean ± SD, derived from three separate cultures for each parameter.

The optimum pH for oil production by *Rhodosporidium toruloides* DMKU3-TK16 in nitrogen-limited OPM containing 30 g/L of glucose is 5.5 (Kraisintu *et al*. 2010). When grown in beet molasses in a pH range of 4 to 7, *Cutaneotrichosporon* (formerly *Cryptococcus*) *curvatus* had the highest oil yield (1.7 g/L) at a pH of 5.5 (El-Fadaly *et al*. 2009). *Rhodotorula kratochvilovae* SY89 had the highest oil content (56.06 wt% of DCW) in an optimized nitrogen-limited OPM containing 50 g/L of glucose at a pH of 5.5 (Jiru *et al*. 2017). Having an optimal pH for oil production in the acidic range is an advantage for industrial yeast oil production due to the reduction in bacterial contamination (Sitepu *et al*. 2014).

Fatty Acid Composition of *Cy. subsufficiens* NG8.2 Oil Produced in SLH

The amount of accumulated oil and the fatty acid profile are highly dependent on the carbon source (*i.e*., glucose and xylose), nutrient limitation (*i.e*., N or P), cultivation conditions (temperature and pH), cultivation time, and setup conditions (batch or fed-batch cultivation) (Kolouchová *et al*. 2016). Therefore, the fatty acid profiles of *Cy. subsufficiens* NG8.2 oils produced in SLH, SLH-P, and SLH-P containing 3.12 mM of Mg^{2+} (pH 5.5) for 4 d were compared. The results revealed that the major fatty acids in the oil produced in the SLH were oleic, palmitic, and palmitoleic acids, while those produced in the SLH-P and SLH-P containing 3.12 mM of Mg^{2+} were palmitic, oleic, and palmitoleic acids. The palmitoleic acid content in the oil decreased from 18.73 wt% (in SLH) to 12.45 wt% (in SLH-P) when the C/P molar ratio increased from 194 in the SLH to 21,182 in the SLH-P (Table 4).

In accordance with these results, the palmitoleic acid contents in oils of *Yarrowia lipolytica* CCY 29-26-36 and *Candida krusei* DBM 2163 produced in two oil production media, which had the same C/N molar ratio (30) but different C/P molar ratios (6 and 1043), decreased from 16.4 wt% to 14.9 wt% and from 16.0 wt% to 14.4 wt%, respectively, when the C/P molar ratio of the OPM increased (Kolouchová *et al*. 2015). The palmitoleic acid content in the oil from *Cutaneotrichosporon* (formerly, *Cryptococcus*) *curvatus* MUCL 29819 decreased from 16.77 wt% to 12.64 wt% when the C/P molar ratio of the OPM was increased from 18 to 1482 (Huang *et al*. 2018).

In this study, the addition of 0.75 g/L of $MgSO_4 \cdot 7H_2O$ into the SLH-P increased the proportions of palmitic and palmitoleic acids but decreased the oleic acid content in the *Cy. subsufficiens* NG8.2 oil. Palmitic acid is catalyzed by fatty acyl CoA synthetase to palmitoyl-CoA, and the palmitoyl-CoA is then converted to palmitoleic acid by delta-9 desaturase. These fatty acyl CoA synthetase require Mg^{2+} for activity (Cook and McMaster 1996). The palmitoleic acid content was greatest (15.80 wt%) in the oil produced in the SLH-P containing 3.12 mM of Mg^{2+} (Table 4).

Several factors in combination, including the oil content, cell biomass, and palmitoleic acid content of the oil produced, determined the palmitoleic acid yield. The SLH-P containing 3.12 mM of Mg^{2+} had the highest palmitoleic acid yield (2.09 mg/g DW sugarcane leaves), while those of the SLH and SLH-P were lower (1.54 mg/g DW sugarcane leaves and 1.43 mg/g DW sugarcane leaves, respectively). The C/N molar ratio of OPM was the main factor affected palmitoleic acid yield of oleaginous yeasts. Palmitoleic acid yields of *Candida krusei* DBM 2163, *Yarrowia lipolytica* CCY 29-26-36, and *Trichosporon cutaneum* CCY 30-5-10 were highest at 430, 260, and 80 mg/g DCW, respectively when C/N molar ratio of OPM was increased from 3 to 30 (Kolouchová *et al*. 2015). *Trichosporon cutaneum* CCY 30-5-10 gave maximum palmitoleic acid yield (over

285 mg/g DCW) in OPM (C/N molar ratio of 70) having ammonium sulfate as nitrogen source (Kolouchová *et al*. 2016).

Table 4. Fatty Acid Profiles of *Cy. subsufficiens* NG8.2 Oil Produced in SLH, SLH-P, or SLH-P Containing 3.12 mM of Mg^{2+} (pH 5.5) for 4 d

Fatty Acid	Percentage (wt%)		
	12%-SLH	12%-SLH-P	12%-SLH-P with 3.12 mM of Mg^{2+}
Myristic acid (C14:0)	1.00 ± 0.01	1.37 ± 0.05	1.79 ± 0.11
Palmitic acid (C16:0)	28.10 ± 2.31	42.71 ± 0.43	46.59 ± 1.35
Palmitoleic acid (C16:1)	18.73 ± 1.88	12.45 ± 0.70	15.80 ± 0.43
Steric acid (C18:0)	2.46 ± 0.07	2.37 ± 0.70	3.31 ± 1.00
Oleic acid(C18:1)	31.69 ± 1.10	31.62 ± 0.37	24.42 ± 0.24
Linoleic acid (C18:2)	14.52 ± 0.35	7.08 ± 0.09	4.57 ± 0.14
Others	3.51	2.42	3.51
Data are shown as the mean ± SD, derived from three independent repeats.			

CONCLUSIONS

1. Sugarcane leaves hydrolysate (SLH) had potential as raw material for palmitoleic acid production using *Cyberlindnera* (*Cy.*) *subsufficiens* NG8.2.
2. Treatment of the SLH with $Ca(OH_2)$ increased the *Cy. subsufficiens* NG8.2 oil production but decreased palmitoleic acid content of the oil.
3. $Ca(OH_2)$-treated SLH (SLH-P) supplemented with Mg^{2+} increased the *Cy. subsufficiens* NG8.2 oil production and palmitoleic acid content of the oil.
4. *Cy. subsufficiens* NG8.2 grown in the SLH-P supplemented with 3.12 mM of Mg^{2+} at pH 5.5 and 30 °C, with 200-rpm aeration, for 4 d gave maximum palmitoleic acid yield at 2.09 mg/g DW sugarcane leaves.

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