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Research article

Production of oil with increasing palmitoleic acid content by *Cyberlindnera subsufficiens* NG8.2 from oil palm empty fruit bunch via co-fermentation with cassava starch

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Abstract

Palmitoleic acid is a monounsaturated fatty acid that has been shown to have many important health benefits, with broad applications in medicines and cosmetics. Intracellular oils accumulated in yeast cells are mostly in the form of triacylglycerols, of which some are contain palmitoleic acid. This work investigated oil palm empty fruit bunch (OPEFB) as a potential feedstock for the production of oils with a high palmitoleic acid content using *Cyberlindnera subsufficiens* NG8.2. The low sugar concentration of the OPEFB hydrolysate (OPEFBH) was overcome by the co-utilization of cassava starch hydrolysate (CSH). The maximum oil titer of *Cy. subsufficiens* NG8.2 at 30°C was almost the same in a mixture of either OPEFBH plus CSH (OPEFBH-CSH) or OPEFBH plus glucose (OPEFBH-G) at the same final glucose concentration (50 g/L). However, the oil produced using OPEFBH-CSH contained a higher palmitoleic acid content (18.51% weight per weight, w/w) than from OPEFBH-G (10.41%, w/w). Supplementation of OPEFBH-CSH with 1 g/L KH_2PO_4, 0.05 g/L $MgSO_4 \cdot 7H_2O$ and 0.01 g/L $CaCl_2 \cdot 2H_2O$ (pH 5.0) increased the oil titer from 1.17 g/L to 1.96 g/L. Reducing the incubation temperature from 30°C to 25°C increased the palmitoleic acid content from 18.03% (w/w) to 21.49% (w/w) without any significant effects on the oil titer and oil composition.

Introduction

Palmitoleic acid, or omega-7, is a monounsaturated fatty acid that can be obtained in small quantities from animal fat products, vegetables and marine oils (Kolouchová et al., 2015). It has several beneficial effects on human health, such as: 1) reducing the blood-circulating low density lipoprotein cholesterol, which is a cardiovascular disease

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risk factor (Griel et al., 2008); 2) improvement of cytokine, laminin, and type IV collagen levels in liver cirrhotic patients (Gao et al., 2003); 3) prevention of β-cell apoptosis leading to type-2 diabetes (Morgan and Dhayal, 2010); and 4) inhibition of Gram-positive bacterial growth (Wille and Kydonieus, 2003). Oils from sea buckthorn (*Hippophae rhamnoides*) and macadamia nut (*Macadamia integrifolia*) are known to have high amounts of palmitoleic acid, with concentrations of about 40% (weight per weight, w/w) and 12–22% (w/w), respectively, (Kolouchová et al., 2015). However, both sea buckthorn and macadamia nut have limited availability and the quality of their oils varies depending upon the cultivar, locality and seasonality (Orsavova et al., 2015).

Yeasts containing ATP citrate lyase (ACL) accumulate oil, mostly as triacylglycerols (TAG), in their cells for energy storage when cultured using surplus carbon but restricted essential nutrients (most often nitrogen) conditions (Madani et al., 2017). Yeasts that accumulated more oil than 20% (w/w) dry cell weight (DCW) are defined as oleaginous yeasts (Ageitos et al., 2011). In some oleaginous yeasts, such as *Kluyveromyces polysporus* DBM 2171 (Kolouchová et al., 2016), *Candida krusei* DBM 2163, *Yarrowia lipolytica* CCY 29-26-36 (Kolouchová et al., 2015), and *Pichia segobiensis* SSOH12 (Schulze et al., 2014), the oils have been reported to contain high concentrations of palmitoleic acid. The use of yeasts to produce oil has several advantages over plants, as the oil quality is independent of seasonality and locality, there is no requirement for a large land area for plantation, the yeast can utilize various kinds of carbon sources, including sugars derived from lignocellulose (Probst et al., 2015) and the fatty acid composition of the oil can be managed (Kolouchová et al., 2015).

In 2018, there were 16.4 million tonne of oil palm fruit bunch produced in Thailand (Office of Agricultural Economics (2019). After the oil palm fruit bunches have been removed in the process of palm oil production, the residual oil palm empty fruit bunch (OPEFB) is left as lignocellulosic waste and accounts for 20% by weight of the oil palm fruit bunch (Chang, 2014), with more than 3 million tonnes of OPEFB being generated annually. Currently, the OPEFB is used for landfill and a small amount is used as substrate for mushroom cultivation. The OPEFB is composed of 48.40% (w/w) cellulose, 14.30% (w/w) hemicellulose and 16.80% (w/w) lignin (Weeraphan et al., 2021). Impregnation with sodium hydroxide (NaOH) and subsequent NaOH-catalyst steam explosion has been shown to be an effective pretreatment method for increasing the subsequent cellulose hydrolysis of the OPEFB to simple sugars. OPEFB hydrolysate obtained after enzymatic hydrolysis of the pretreated OPEFB contained 16.67 g/L of glucose, 0.01 g/L of nitrogen and less than 0.05 g/L phosphate (Weeraphan et al., 2021).

Recently, the production has been reported of oil of *Cy. subsufficiens* NG8.2, an oleaginous yeast, from a synthetic high C/N medium containing 50 g/L glucose, 0.1 g/L yeast extract, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $(NH_4)_2SO_4$, 0.1 g/L NaCl, and 0.1 g/L $CaCl_2 \cdot 2H_2O$ (modified from Galafassi et al. (2012)), without phosphate supplementation) composed of 22.25% (w/w) palmitoleic acid (Hoondee et al., 2021). The high carbon/nitrogen (C/N) and carbon/phosphorus (C/P) ratios of the OPEFB hydrolysate (OPEFBH) make it an interesting potential feedstock for *Cy. subsufficiens* NG8.2 oil production.

Increasing the C/N ratio of the oil production medium (OPM) by the addition of sugar resulted in increased oil accumulation of oleaginous microorganisms (Ahmad et al., 2017). Cassava starch, which contains 72–85% (w/w) of starch and low amounts of fat and protein (Ek et al., 2020), was reported to be a good carbon source for yeast oil production (Wang et al., 2012). With almost 30 million t of cassava being generated each year in Thailand (Office of Agricultural Economics, 2020), it is a plentiful product.

In the current study, OPEFBH was used as feedstock for *Cy. subsufficiens* NG8.2 oil production. Cassava starch hydrolysate (CSH) was evaluated as a sugar source compared to glucose to increase the sugar concentration of OPEFBH and its effect was determined on the palmitoleic acid content of the *Cy. subsufficiens* NG8.2 oil produced.

Materials and Methods

Oil palm empty fruit bunch

Shredded OPEFB collected from the Thai Tallow and Oil Co. Ltd., Surat Thani province, Thailand was hammer-milled and sieved. The resultant OPEFB with a particle size of 2–10 mm was dried overnight at 65°C and stored at room temperature.

Microorganism

Cy. subsufficiens NG8.2 deposited under accession number MSCU1058 in the culture collection at the Department of Microbiology, Faculty of Science, Chulalongkorn University was used in this study. It was grown on yeast malt extract (YM) agar (30 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract,

5 g/L peptone, 2 g/L agar, pH 5.5) at 30°C for 48 hr and stored in the refrigerator for short term storage.

Preparation of oil palm empty fruit bunch and cassava starch hydrolysate

OPEFBH (the hydrolysate of NaOH-impregnated OPEFB using the NaOH as a catalyst for pretreatment steam explosion of OPEFB), was prepared as described previously (Weeraphan et al., 2021).

For the formation of CSH, cassava starch was suspended in distilled water at 20% (weight per volume) and then liquefied with α-amylase (158.64 U/g dry weight, DW) at 85°C and pH 5.8 for 4 hr, and then saccharified with glucoamylase (98.7 U/g DW) at 60°C and pH 4.5 for 2 hr. After centrifugation at 9803×*g* for 20 min, the resultant clear syrup was filtered through Whatman No. 1 filter paper (Cytiva LLC; Marlborough, MA, USA). The filtrate, referred to as CSH, was analyzed for its glucose concentration. The α-amylase and glucoamylase used were from Siam Victory Chemicals, Co. Ltd., Thailand.

Production of Cy. subsufficiens NG8.2 oil

The *Cy. subsufficiens* NG8.2 oil was produced using a two-stage method because the nutrient requirement for each stage was different. In the first (cell mass production) stage, the *Cy. subsufficiens* NG8.2 was grown on YM agar (pH 5.5) at 30°C for 24 hr and suspended in YM broth (50 mL) at an initial optical density at 660 nm of 0.8. After incubation at 30°C and 200 revolutions per minute (rpm) for 24 hr, the resultant culture (5 mL) was transferred into fresh YM broth (50 mL) and incubated using the same conditions for 48 hr. Cells obtained after centrifugation (9,803×*g*, 4°C, 15 min) were washed twice with sterile distilled water.

For the second (oil production) stage, the washed cells were inoculated into OPM and incubated at 30°C and 200 rpm for 7 d. Synthetic high C/N medium (50 g/L glucose, 1 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L KH_2PO_4, 0.01 g/L NaCl, 0.01 g/L $CaCl_2 \cdot 2H_2O$, pH 5.5; Galafassi et al., 2012), OPEFBH supplemented with glucose (OPEFBH-G) to a final glucose concentration of 50 g/L, or OPEFBH supplemented with CSH (OPEFBH-CSH) to a final glucose concentration of 50 g/L (glucose concentration measurement based on Multichannel Biochemistry Analyzer (YSI 2700 Select; YSI Incorporated; Yellow Springs, OH, USA) was used as the OPM as indicated. The culture was harvested every 24 hr and centrifuged (9,803×*g*, 4°C, 15 min). The cell precipitate washed with sterile distilled water was lyophilized and weighed. The weight of the lyophilized cells was defined as the resultant cell mass (in grams dry cell weight, DCW). The intracellular oil accumulated in the lyophilized cells was extracted and quantified as reported by Pranimit et al. (2019). The intracellular oil content of 100 g of cells (DCW) was expressed as the oil content (percentage weight per weight DCW), and the oil titer was calculated from the oil content (percentage weight per weight DCW) × cell mass (grams DCW per liter) / 100.

Fatty acid analysis

The extracted *Cy. subsufficiens* NG8.2 oil was transesterified and subsequently analyzed using gas chromatography as described by Weeraphan et al. (2021).

Statistical analysis

Data were analyzed using analysis of variance for independent measures, followed by Duncan's multiple range test or independent sample t test comparisons (SPSS version 22; IBM; Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$. The full factorial experiment for investigated effect of Mg^{2+} and Ca^{2+} as minerals co-supplementation was designed using Minitab® version 19.1 in triplicate experiment (Minitab Inc.; State College, PA, USA).

Results and Discussion

Comparison of oil and palmitoleic acid production in synthetic high C/N medium, oil palm empty fruit bunch hydrolysate-glucose and oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate

The oil production amounts by *Cy. subsufficiens* NG8.2 in synthetic high C/N medium, OPEFBH-G and OPEFBH-CSH were compared. The maximum oil titer (1.17 g/L) was obtained in OPEFBH-CSH at day 5, with an oil content and cell mass of 8.95% (w/w, DCW) and 13.11 g/L, respectively. The oil titer, oil content and the cell mass obtained in the OPEFBH-G were 1.14 g/L, 8.43% (w/w, DCW), and 13.49 g/L, respectively. The lowest level of oil titer (0.71 g/L), oil content (3.57% w/w, DCW), and cell mass (10.71 g/L) were in the synthetic high C/N medium (Fig. 1). The higher oil content of *Cy. subsufficiens* NG8.2 in the OPEFBH-CSH than in the OPEFBH-G or synthetic high C/N media might

have been a result of the complex composition of the OPEFBH and CSH. The level of oleaginous yeast oil production is known to be strongly dependent on the OPM composition, such as the availability and concentration of both the carbon and nitrogen sources and the C/N ratio, including concentrations of trace elements and inorganic salts (Li et al., 2008).

The oil produced in the OPEFBH-CSH, OPEFBH-G and synthetic high C/N OPM contained 18.51%, 10.41% and 9.50% (w/w) palmitoleic acid, respectively, (Table 1), which was equivalent to palmitoleic acid production of 188 mg/g oil dry weight (DW), 105 mg/g oil DW and 98.59 mg/g oil DW, respectively. Feedstock composition, including the C/N ratio, is known to have a significant influence on the fatty acid composition of oleaginous oils (Brar et al., 2017; Gientka et al., 2017). The dominant fatty acid of *Rhodosporidium kratochvilovae* HIMPA1 oil produced in an aqueous extract of *Cassia fistula* fruit pulp was palmitic acid at 43.06% (w/w), according to Patel et al. (2015), while those acids produced in the effluent from a pulp and paper industry factory were changed to oleic acid at 45.43% (w/w), according to Patel et al. (2017). Due to the highest palmitoleic acid content in oils produced in the OPEFBH-CSH, maximization of the *Cy. subsufficiens* NG8.2 oil production was performed in the OPEFBH-CSH.

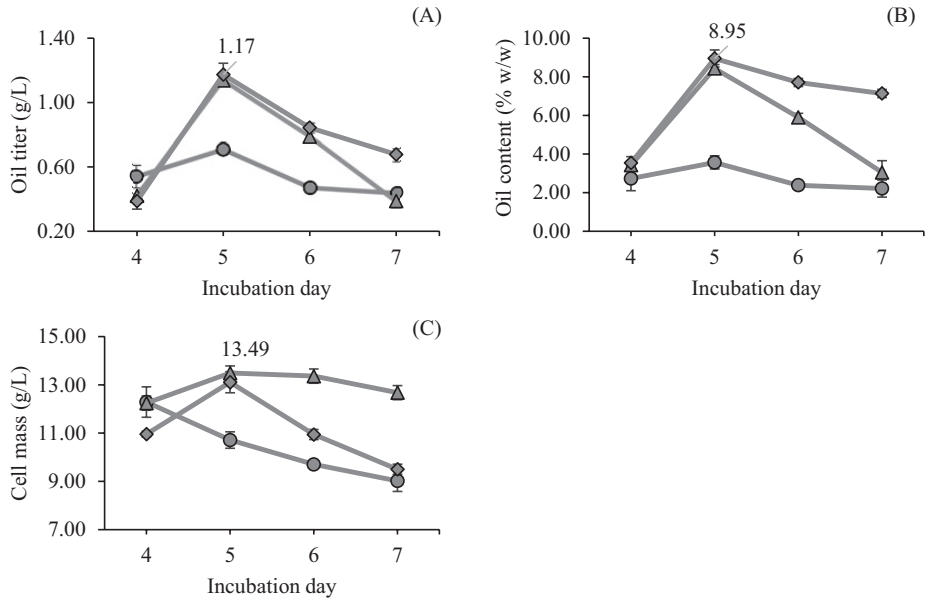

Fig. 1 (A) oil titer; (B) oil content; (C) cell mass of *Cyberlindnera subsufficiens* NG8.2 grown in oil production media consisting of synthetic high C/N (●), oil palm empty fruit bunch hydrolysate-glucose (▲) and oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate (◆), where error bars indicate ± SD and w/w = weight per weight

Table 1 Fatty acid concentration of *Cyberlindnera subsufficiens* NG8.2 oil produced in various kinds of oil production media when incubated for 5 days

Fatty acids	% (weight per weight)		
	Synthetic high C/N medium	OPEFBH-G medium	OPEFBH-CSH medium
Lauric acid (C12:0)	3.83±0.02^{a}	0^{b}	0^{b}
Myristic acid (C14:0)	1.30±0.01^{b}	1.99±0.02^{a}	1.23±0.00^{c}
Palmitic acid (C16:0)	38.99±0.07^{b}	40.31±0.19^{a}	23.92±0.02^{c}
Palmitoleic acid (C16:1)	9.50±0.02^{c}	10.41±0.03^{b}	18.51±0.03^{a}
Steric acid (C18:0)	17.16±0.21^{a}	15.62±0.14^{b}	1.47±0.13^{c}
Oleic acid (C18:1)	22.31±0.27^{b}	22.13±0.23^{b}	44.13±0.01^{a}
Linoleic acid (C18:2)	5.49±0.01^{b}	5.27±0.04^{c}	10.32±0.06^{a}
Others	1.42±0.00^{b}	4.27±0.00^{a}	0.42±0.00^{c}

OPEFBH-G = oil palm empty fruit bunch hydrolysate-glucose; OPEFBH-CSH = oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate
Data are shown as the mean ± SD, derived from three independent experiments. Means in a row superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Maximization of Cy. subsufficiens NG8.2 oil production in oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate

The *Cy. subsufficiens* NG8.2 oil production in the OPEFBH-CSH was maximized based on univariation of nutritional and cultivation factors at 30°C (pH5.5) as follows: 1) the final glucose concentration of the OPEFBH-CSH was varied to 50, 60, 70 or 80 g/L by varying the volume of the CSH supplemented; 2) the level was determined of oil production in the OPEFBH-CSH supplemented with yeast extract (0, 1, 2 or 4 g/L) or $(NH_4)_2SO_4$ (0, 1, 2 or 4 g/L); 3) oil production was determined of the *Cy. subsufficiens* NG8.2 in OPEFBH-CSH containing various concentrations of KH_2PO_4 (0, 1, 2 or and 4 g/L).

The conditions that provided the highest oil titer and oil productivity were selected as partially optimized OPEFBH-CSH for further studies as: 4) the *Cy. subsufficiens* NG8.2 oil was produced in the partially optimized OPEFBH-CSH, (from steps 1–3 above), which was further supplemented with $MgSO_4 \cdot 7H_2O$ (0, 0.5 or 1 g/L) and $CaCl_2 \cdot 2H_2O$ (0, 0.1 or 0.2 g/L) using a two-factor three level full factorial design; 5) then, the *Cy. subsufficiens* NG8.2 oil was produced in the optimized OPEFBH-CSH at various initial medium pH values (4, 4.5, 5, 5.5, 6 or 6.5) and incubated at 30°C or 25°C. All experiments were conducted in triplicate replication. The set of conditions that produced the highest oil titer in the above experiments was selected for further studies.

Effect of glucose concentration

A higher sugar concentration in the OPM was reported to increase in the C/N ratio and subsequently increase in the oil production of oleaginous microorganisms (Ahmad et al., 2017). When grown in OPEFBH-CSH with various final glucose concentrations (50, 60, 70 or 80 g/L), the *Cy. subsufficiens* NG8.2 produced a maximum oil titer (1.17 g/L) at day 5 in OPEFBH-CSH containing an initial total glucose concentration of 50 g/L. In OPEFBH-CSH having an initial total glucose concentration of 60, 70 or 80 g/L, the oil titers were 1.13 g/L, 1.09 g/L and 1.06 g/L, respectively. The high osmotic pressure of the OPEFBH-CSH with glucose concentrations of 60, 70 or 80 g/L might have been the reason for the lower oil titer obtained. Thus, the OPEFBH-CSH containing 50 g/L glucose was selected for further studies. In accord with this result, the optimal glucose concentration for *Rhodotorula kratochvilovae* SY89 oil production in nitrogen-limited media was 50 g/L, producing a maximum oil titer of 6.95 g/L (Jiru et al., 2017).

Effects of nitrogen source and C/N versus C/P ratios

Different oleaginous yeasts favor different nitrogen sources for oil production (Probst et al., 2015). The level was examined of the *Cy. subsufficiens* NG8.2 oil produced in OPEFBH-CSH (50 g/L glucose) supplemented with yeast extract or $(NH_4)_2SO_4$ at 0, 1, 2 or 4 g/L. The addition of yeast extract at 4 g/L produced a maximum oil titer of 1.23 g/L (oil productivity of 0.21 g/L/d), whereas 1.17 g/L, 1.17 g/L or 1.14 g/L of oil titer were obtained from 0 g/L, 1 g/L and 2 g/L of yeast extract supplemented. In comparison, the addition of 1 g/L $(NH_4)_2SO_4$ produced a maximum oil titer of 1.21 g/L (oil productivity of 0.20 g/L/d), while the addition of 0 g/L, 2 g/L or 4 g/L $(NH_4)_2SO_4$ produced oil tiers of 1.17 g/L, 1.14 g/L and 1.09 g/L, respectively. At these optimal concentrations of yeast extract and $(NH_4)_2SO_4$, the oil contents were 9.14% and 8.85%, respectively, (w/w DCW), while the cell masses were 13.52 g/L and 13.63 g/L, respectively.

Suitable nitrogen sources either in combination or alone are necessary for oil production in oleaginous microorganisms. For example, *Rhodotorula kratochvilovae* SY89 provided a maximum oil titer (7.27 g/L) in nitrogen-limited media containing 50 g/L glucose when both $(NH_4)_2SO_4$ and yeast extract were used as the nitrogen sources in combination (Jiru et al., 2017). In addition, yeast extract was reported to increase the oil production of *Rhodosporidium toruloides* (Saran et al., 2017), while *Pichia guilliermondii* produced the highest oil titer (4.50 g/L) in OPM (C/N ratio 30) with NH_4Cl as the nitrogen source compared to only 0.20 g/L with $NaNO_3$ as the nitrogen source. The oil titer of the *P. guilliermondii* obtained in the OPM with NH_4Cl as the nitrogen source was almost the same as with $(NH_4)_2SO_4$ (Chopra and Sen, 2018).

When the *Cy. subsufficiens* NG8.2 was grown in the OPEFBH-CSH supplemented with various concentrations of KH_2PO_4 (0, 1, 2 or 4 g/L), the maximum oil titer (1.40 g/L) and oil productivity (0.23 g/L/d) were obtained in OPEFBH-CSH supplemented with 1 g/L KH_2PO_4, with an oil content of 10.80% (w/w, DCW) and a cell mass of 12.93 g/L at day 6. Supplementation of OPEFBH-CSH with 0, 2 or 4 g/L of KH_2PO_4 yielded oil titers of 1.17 g/L, 1.26 g/L and 1.25 g/L, respectively (Fig. 2). Intracellular yeast oil accumulation occurs in an oil droplet encased with a phospholipid monolayer, and so requires phosphate (Qin et al., 2017). The optimal C/P ratio of the OPM is important for maximization of yeast oil production (Papanikolaou and Aggelis, 2011). Addition of 1 g/L KH_2PO_4 into OPEFBH-CSH reduced the C/P ratio from 2.70 to 2.58 but increased the maximum oil titer of the *Cy. subsufficiens* NG8.2 by 19.65%, from 1.17 g/L to 1.40 g/L.

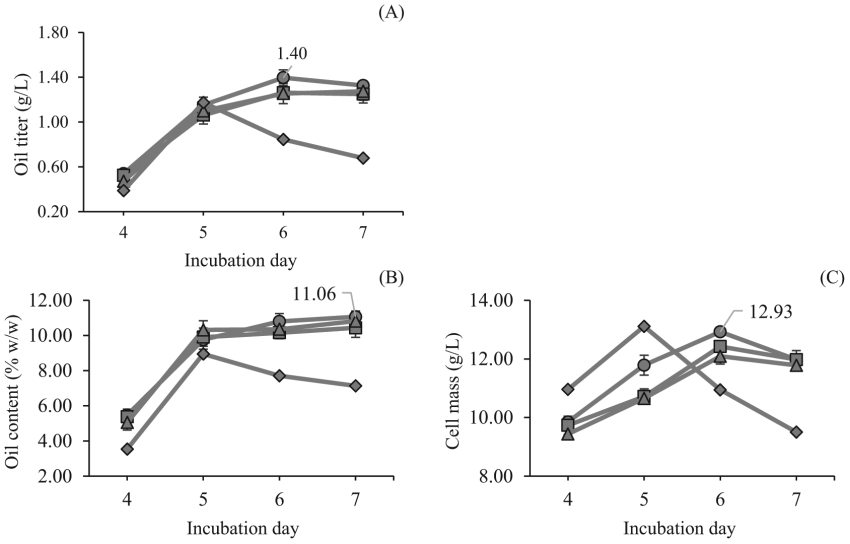

Fig. 2 (A) oil titer; (B) oil content; (C) cell mass of *Cyberlindnera subsufficiens* NG8.2 grown in oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate supplemented with KH_2PO_4 at 0 g/L (◆), 1 g/L (●), 2 g/L (■) or 4 g/L (▲), where error bars indicate ± SD and w/w = weight per weight

In comparison, the highest oil titer of *Candida* sp. NG17 grown in sugarcane leaf hydrolysate (C/P ratio of 17.33) increased 31.56% from 5.07 g/L to 6.67 g/L, when supplemented with 1 g/L KH_2PO_4 (C/P ratio of 10.40), according to Pranimit et al. (2019). The addition of 4 g/L KH_2PO_4 into OPEFBH containing glucose at 50 g/L, which decreased the C/P ratio from 70,000 to 16.97, resulted in a 55% increase in the maximum oil titer for *Naganishia cerealis* IN1S2.5, from 0.80 g/L to 1.24 g/L (Weeraphan et al., 2021). This revealed that the greater the difference in the C/P ratio from its optimal value, the lower the level of oil titer obtained. The oil produced using OPEFBH-CSH supplemented with 1 g/L KH_2PO_4 (C/P ratio of 2.58) contained 18.28% (w/w) palmitoleic acid, while that produced in OPEFBH-CSH supplemented with 4 g/L yeast extract contained 17.54% (w/w) palmitoleic acid. From these results, the OPEFBH-CSH supplemented with 1 g/L KH_2PO_4 was selected for further studies.

Effect of Mg^{2+} and Ca^{2+} co-supplementation on oil production

Optimal concentrations of Mg^{2+} and Ca^{2+} co-supplemented in OPEFBH-CSH (C/P ratio of 2.58) for *Cy. subsufficiens* NG8.2 oil production were determined using a two-factor, three-level full factorial design. The effects of these divalent cations for oil titer were evaluated, since under conditions of an over-abundance of carbon but insufficient essential nutrient (most often nitrogen), oleaginous yeasts express cytosolic ACL activity and divert the carbon flow from energy production via the tricarboxylic acid cycle to lipid biosynthesis (Probst et al., 2015). ACL is known to require Mg^{2+} as a cofactor for activity (Amaretti et al., 2010), while Ca^{2+} signal transduction regulates lipid biosynthesis in response to nitrogen-deficient stress adaptation (Chen et al., 2014). The experimental trials and response are shown in Table 2. The simultaneous addition of $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and $CaCl_2 \cdot 2H_2O$ (0.1 g/L) resulted in the highest oil titer (1.91 g/L) and oil content (14.17% w/w, DCW) at day 6. In OPEFBH-G supplemented with the optimal concentrations of KH_2PO_4, Mg^{2+} and Ca^{2+} (at pH 5.5), the maximum oil titer of *Cy. subsufficiens* NG8.2 was 1.24 g/L (0.25 g/L/d oil productivity).

In comparison, the optimal concentration of $MgSO_4 \cdot 7H_2O$ for oil production by *Trichosporon cutaneum* in corncob hydrolysate was 0.30 g/L (Chen et al., 2013). Increased oil contents of *Scenedesmus obliquus* and *Chlorella vulgaris* were observed under increased concentrations of Mg^{2+} or Ca^{2+} (Gorain et al., 2013). This suggested that different oleaginous yeasts and OPM required different concentrations of Mg^{2+} and Ca^{2+} for oil accumulation. Thus, OPEFBH-CSH (C/P ratio of 2.58) supplemented with 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 g/L $CaCl_2 \cdot 2H_2O$ (optimized OPEFBH-CSH) was selected for further studies.

Effect of pH

When *Cy. subsufficiens* NG8.2 was grown in the optimized OPEFBH-CSH at different initial pH values, the highest oil titer (1.97 g/L), oil productivity (0.33 g/L/d), and oil content [14.50% (w/w, DCW)] were achieved at pH 5.0, whereas the

Table 2 Optimal concentrations of $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ co-supplemented in oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate (C/P ratio of 2.58) on *Cyberlindnera subsufficiens* NG8.2 oil production at day 6

Trial	$MgSO_4 \cdot 7H_2O$ (g/L)	$CaCl_2 \cdot 2H_2O$ (g/L)	Cell mass (g/L)	Oil content (% w/w)	Oil titer (g/L)
1	0	0	12.90±0.09^{d}	10.80±0.22^{b}	1.39±0.04^{c}
2	0	0.1	12.90±0.10^{d}	10.93±0.83^{b}	1.41±0.11^{c}
3	0	0.2	12.92±0.06^{d}	11.25±0.10^{b}	1.45±0.01^{c}
4	0.5	0	13.10±0.06^{c}	13.69±0.23^{a}	1.79±0.04^{b}
5	0.5	0.1	13.48±0.07^{a}	14.17±0.07^{a}	1.91±0.02^{a}
6	0.5	0.2	13.57±0.05^{a}	13.78±0.52^{a}	1.87±0.07^{ab}
7	1	0	13.26±0.03^{b}	13.58±0.40^{a}	1.80±0.05^{b}
8	1	0.1	13.53±0.02^{a}	13.51±0.23^{a}	1.83±0.03^{ab}
9	1	0.2	13.54±0.04^{a}	13.63±0.19^{a}	1.85±0.03^{ab}

w/w = weight per weight

Data are shown as mean ± SD, derived from three separate cultures for each parameter. Means in a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

highest cell mass was obtained at pH 5.5 (13.64 g/L) compared to 13.57g/L at pH 5.0 (Fig. 3). This was in contrast to other reported results, where the optimal pH level for oil production of *Rhodotorula glutinis* (Tao et al., 2008), *Cutaneotrichosporon curvatus* (El-Fadaly et al., 2009), *R. toruloides* DMKU3-TK16 (Kraisintu et al., 2010) and *Rhodosporidium kratochvilovae* SY89 (Jiru et al., 2017) were 5.5, although *Trichosporn cutaneum* produced its highest oil titer at pH 6.0 (Chen et al., 2013). Different optimal pH levels for oil production by different oleaginous yeasts have been reported, with the optimal pH of each oleaginous yeast being influenced by the carbon source available in the OPM (Jiru et al., 2017).

Effect of temperature

The oil content, cell mass and oil titer were compared for *Cy. subsufficiens* NG8.2 grown in the optimized OPEFBH-CSH for 6 days at 25°C and 30°C. The oil content at 25°C was higher than at 30°C [16.93% versus 14.74% (w/w, DCW)], whereas the cell mass at 30°C was higher than at 25°C (13.30 g/L versus 11.90 g/L). However, the higher oil titer obtained at 25°C (2.01 g/L) than at 30°C (1.96 g/L) was not significantly different (Fig. 4). Different optimal temperatures for the oil accumulation of different oleaginous yeasts have been reported. The cell mass of *Trichosporon cutaneum* was reported to increase slowly as the temperature increased from 25°C

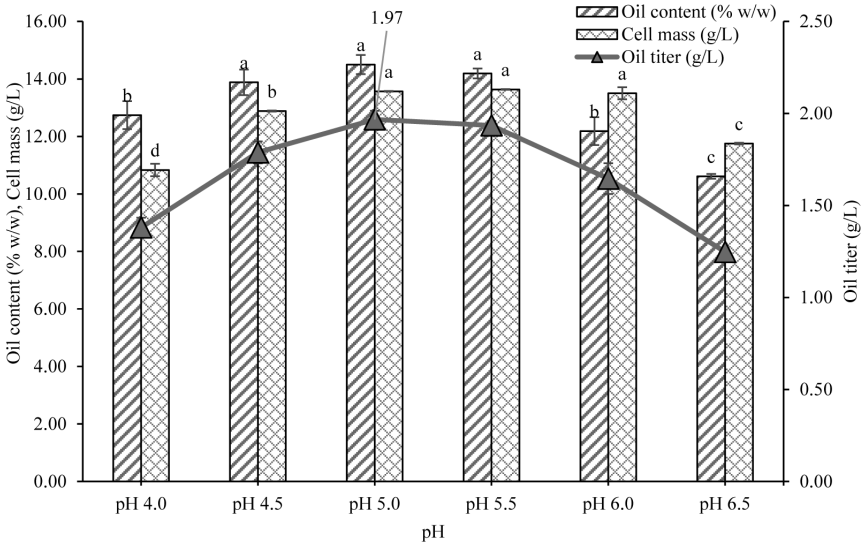

Fig. 3 Oil content, cell mass and oil titer of *Cyberlindnera subsufficiens* NG8.2 grown in optimized oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate for 6 d at different initial pH values, where error bars represent ± SD; different lowercase letters above bars indicate significant ($p < 0.05$) differences among means; w/w = weight per weight

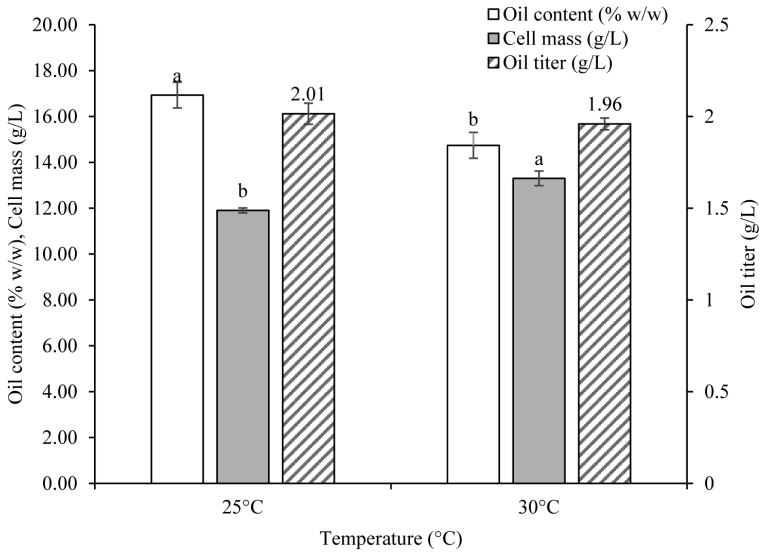

Fig. 4 Histograms showing oil content, cell mass and oil titer of *Cyberlindnera subsufficiens* NG8.2 grown in optimized oil palm empty fruit bunch hydrolysate-cassava starch hydrolysate at 25°C or 30°C, where error bars represent ± SD, different lowercase letters above bars indicate significant ($p < 0.05$) differences among means; w/w = weight per weight

to 32°C, whereas the oil content reached a maximum at 28°C, due to the sub-optimal activity of enzymes involved in lipid biosynthesis at lower or higher temperatures than this (Chen et al., 2013). In contrast, *Rhodotorula kratochvilovae* SY89 produced its maximum oil content, cell mass and oil titer in nitrogen-limited media at 30°C (Jiru et al., 2017). Cultivation temperature critically influences oil accumulation by oleaginous yeasts (Jiru et al., 2017).

Fatty acid composition of Cy. subsufficiens NG8.2 oil produced at different temperatures

As the incubation temperature has a significant effect upon the fatty acid composition of the produced oil (Papanikolaou and Aggelis, 2011), the fatty acid composition was analyzed of the *Cy. subsufficiens* NG8.2 oil produced at 25°C and 30°C. Although, the oil had the same dominant fatty acids (oleic, palmitic, palmitoleic) at each temperature, there was an alteration in the concentration of the dominant fatty acids. The oil produced at 30°C contained 31.00%, 29.39% and 18.03% (w/w) oleic, palmitic and palmitoleic acids, respectively, whereas at 25°C, the proportion of palmitic acid of the oil decreased to 27.01% (w/w), while the oleic and palmitoleic acids increased to 34.22% (w/w) and 21.49% (w/w), respectively (Table 3).

Traditional biotechnological yeast species (*Kluyveromyces polysporus, Torulospora delbrueckii, Saccharomyces cerevisiae*) and oleaginous yeasts (*Candida krusei, Yarrowia lipolytica,*

Table 3 Fatty acid composition of the *Cyberlindnera subsufficiens* NG8.2 oil produced at 25°C or 30°C

Fatty acid (% weight per weight)	Temperature (°C)	
	25	30
Myristic acid (C14:0)	1.60±0.04^{b}	1.91±0.03^{a}
Palmitic acid (C16:0)	27.01±0.20^{b}	29.39±0.07^{a}
Palmitoleic acid (C16:1)	21.49±0.12^{a}	18.03±0.03^{b}
Stearic acid (C18:0)	1.93±0.05^{b}	3.48±0.03^{a}
Oleic acid (C18:1)	34.22±0.16^{a}	31.00±0.12^{b}
Linoleic acid (C18:2)	8.35±0.03^{a}	8.38±0.01^{a}
α-Linolenic acid (C18:3)	0.64±0.03^{a}	2.35±0.02^{a}
Others	4.75^{a}	5.47^{a}

Data are shown as mean ± SD values, derived from three separate cultures for each parameter. Means in a row superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Trichosporon cutaneum) were screened to identify a suitable palmitoleic acid-producer using a defined OPM with glucose as the carbon source. The rResults indicated that *Candida krusei* was the best in terms of palmitoleic acid production and the C/N ratio was the main factor affecting the content of palmitoleic acid. In optimized medium (C/N ratio of 30 and C/P ratio of 6), the palmitoleic acid content in oils of *Candida krusei* DBM 2163 was 16.00% (w/w) and palmitoleic acid production was 430 mg/g oil DW. Using these conditions, *K. polysporus* DBM 2171, *Y. lipolytica* CCY 29-26-36 and *T. cutaneum* CCY 30-5-10 produced palmitoleic acid at 157 mg/g oil, 260 mg/g oil and 80 mg/g oil DW. An increase in the C/N ratio to 70 resulted in decreased palmitoleic acid production by *K. polysporus* DBM 2171 to 30–62 mg/g oil DW (Kolouchová et al., 2015). Palmitoleic acid production by *Cy. subsufficiens* NG8.2 in the optimized OPEFBH-CSH (C/N ratio of 291.47 and C/P ratio of 2.58) at 25°C was 213.93 mg/g oil DW. The high C/N ratio of the optimized OPEFBH-CSH might have been a reason for the low palmitoleic acid production of *Cy. subsufficiens* NG8.2.

The change in the fatty acid profile at the lower temperature (25°C) revealed that *Cy. subsufficiens* NG8.2, as with other microorganisms, had mechanisms to respond to changes in environmental conditions to maintain its membrane fluidity and functionality, known as homeoviscous adaption (Sinensky, 1974). The unsaturated and short chain fatty acids have a lower melting point than saturated and long chain fatty acids and hence, adaption to lower temperature is realized by incorporating a higher proportion of unsaturated and short chain fatty acids (Bajerski et al., 2017). The sum of the degree of polyunsaturation of *Rhodotorula glacialis* DBVPG 4785 oil decreased from 55.40% at 0°C to 40.60% at 20°C (Amaretti et al., 2010).

The use of low-cost substrates for yeast oil production is crucial for reducing the production cost and to make it economically viable. Although OPEFB is a lignocellulosic waste that has a high abundance and availability, the sugar concentration of OPEFBH is insufficient to be of benefit for the industrialization of yeast oil production. In the present study, the co-utilization of OPEFBH with CSH was a simple but successful strategy to both increase the sugar concentration and balance the nutrient requirements in the OPEFBH feedstock for *Cy. subsufficiens* NG8.2 to produce oil with an increasing amount of palmitoleic acid.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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