Naganishia cerealis IN1S2.5 Oil Production from the Hydrolysate of NaOH-Impregnated & Catalyst Steam Explosion Pretreated Oil Palm Empty Fruit Bunch

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NaOH-impregnation with catalyst steam explosion was found to be an efficient pretreatment method for oil palm empty fruit bunch (OPEFB) as a substrate for oil production by *Naganishia cerealis* IN1S2.5. Cellulase hydrolysis of the pretreated OPEFB yielded glucose at 0.364 g/g. Investigation of *N. cerealis* IN1S2.5 oil production in the OPEFB hydrolysate revealed a maximum oil yield (2.46 g/L) when the C/P molar ratio of the OPEFB hydrolysate was adjusted to 25.71, supplemented with Ca^{2+} and Zn^{2+}, and set to pH 4. The *N. cerealis* IN1S2.5 oil was comprised of oleic (37.6%), palmitic (36.2%), and steric (17.9%) acids, all (w/w), as the major fatty acids. Predicted properties of the produced biodiesel indicated the potential of *N. cerealis* IN1S2.5 oil as a biodiesel feedstock.

Keywords: Oil palm empty fruit bunch; Oleaginous yeast; Steam explosion; Biodiesel

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INTRODUCTION

Some yeasts accumulate a high level of intracellular lipid as oil droplets when grown under an excess of carbon but limited essential nutrient (typically nitrogen) conditions (Jiru *et al*. 2017). Yeasts that accumulate more than 20% (w/w dry cell weight, DCW) intracellular oil are defined as an oleaginous yeast (Ratledge 1989). The oil from oleaginous yeasts is mostly in the form of triacylglycerol with a fatty acid composition that is similar to vegetable oils (Llamas *et al.* 2020). Oleaginous yeast oil is, therefore, a promising renewable non-food oil precursor for conversion to bio-based oleochemicals, such as fuel, soap, plastics, paints, detergents, textiles, rubber, surfactants, lubricants, food, and cosmetic additives, *etc*., which are currently produced from vegetable oils (Probst *et al*. 2016; Vasconcelos *et al.* 2019).

Oil palm production in Thailand was 16.4 million tons in 2019 (Office of Agricultural Economics 2019), which generated approximately 3.3 million tons of oil palm empty fruit bunch (OPEFB) waste (Chang 2014), which is under-utilized.

The OPEFB is a lignocellulosic biomass left over at the palm oil mill after removal of the steam-sterilized oil palm fruit. The steam sterilization of oil palm fruit bunches in the production line renders the OPEFB saturated with water. Incineration of the OPEFB as fuel for steam generation is discouraged due to the large amount of white smoke emission. Although the white smoke is not detrimental to health, it has a strong impact by causing an unpleasant environment. At present, the OPEFB is used as a substrate for mushroom

cultivation and returned to the field as organic fertilizer. The OPEFB is typically composed of 23.7 to 65.0% (w/w) cellulose, 20.6 to 33.5% (w/w) hemicellulose, and 14.1 to 30.4% (w/w) lignin (Chang 2014), and so it has the potential to serve as low-cost source of fermentable sugars, mainly glucose and xylose. Ultimate analysis of the OPEFB revealed a high carbon content of 43.8 to 54.76% (w/w) but a low nitrogen content of 0.25 to 1.21% (w/w) (Chang 2014). The majority of oleaginous yeasts can convert pentose sugars, such as xylose and arabinose, into lipids (Probst *et al*. 2016), making OPEFB a potential suitable feedstock for the cultivation of oleaginous yeasts. However, pretreatment of the OPEFB to break the lignin seal and disrupt the crystalline cellulose structure before enzymatic saccharification is required.

Steam explosion is a physical lignocellulosic pretreatment method that has been shown to be effective in the treatment of OPEFB (Medina *et al*. 2016). In the steam explosion process, the chipped lignocellulosic feedstock is treated with saturated steam at a high temperature (160 to 260 °C) and pressure for a short time period, followed by explosive decomposition of the feedstock due to the suddenly relieved pressure. The high-pressured steam expands the plant cell wall of polysaccharide fibers, which increases the subsequent enzyme accessibility to the cellulose via exposing the internal cellulose surface (capillary tube structure of cellulose) and hydrolyzed acetyl groups of hemicellulose to acetic acid. The explosive decomposition also causes mechanical disruption of the lignocellulosic fibers (Kim 2018; Akhlisah *et al* 2021). Steam explosion is economically practical at an industrial scale due to the lower energy requirement, around a 70% lower energy consumption than conventional mechanical process to obtain the same particle size (Kim 2018).

Addition of an impregnation agent before the pretreatment can further improve the effectiveness of the steam explosion (Siramon *et al*. 2018). Sodium hydroxide (NaOH) was shown to be better than nitric and sulfuric (H_2SO_4) acids at enhancing the enzymatic saccharification of OPEFB, because the alkali removed the lignin more efficiently than the acids (Rashid *et al.* 2011). The presence of lignin interferes with enzymatic hydrolysis by blocking the access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. The NaOH pretreatment also caused swelling, leading to an increased internal surface area and a decreased polymerization and crystallinity of the lignocellulose (Sudiyani *et al.* 2010; Sampora *et al* 2020).

The total amount of oil accumulated in yeast varies considerably among different strains of the same yeast species, but the fatty acid profile of the accumulated oil remains quite consistent within a species if grown under the same condition. Rather, a difference in the fatty acid profile occurs when the composition of the oil production medium (OPM) and incubation time are varied (Sitepu *et al*. 2013). This research aimed to (i) elucidate the efficacy of the NaOH-impregnated and catalyst steam explosion pretreatment method on the enzymatic-saccharification performance of the pretreated OPEFB, and (ii) to maximize oil production by *Naganishia cerealis* IN1S2.5 from the OPEFB hydrolysate (as the OPM) and subsequently characterize the produced oil for its potential applications.

EXPERIMENTAL

OPEFB

Shredded OPEFB with a 7% (w/w) moisture content was obtained from the Thai Tallow and Oil Co. Ltd. (Surajthani province, Thailand). It was milled and sieved to obtain

a 2 to 10 mm particle size. The OPEFB particles were then dried at 65 °C and kept at 4 °C until used.

Microorganism

Naganishia cerealis strain IN1S2.5, a newly isolated oleaginous yeast, was isolated from fertile soil at Doi Inthanon National Park in Chiang Mai province, Thailand. It was grown on yeast-malt (YM) agar slant (3% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 2% agar, all in (w/v), pH 5.5) and kept at 4 °C.

Preparation of the OPEFB Hydrolysate-based OPM

The dried OPEFB particles were soaked at 10% (w/v) in 2 M NaOH for 16 h and then washed with tap water. The NaOH-impregnated OPEFB was then suspended at 3% (w/v) in various concentrations [0.8 to 3.2% (w/v)] of NaOH and subjected to steam explosion at 200 °C, 200 psi for 5 min in a high-pressure reactor (Parr Instrument Company, model 4523, USA). The resultant NaOH-catalyst steam explosion OPEFB was washed with distilled water until the pH reached neutral and then dried overnight at 65 ºC. The dried pretreated OPEFB was then suspended in 100 mM sodium citrate buffer pH 4.5 at 5% (w/v) and hydrolyzed by cellulase (Accellerase™ 1500, Genencor, Finland) at 750 CMC (carboxymethyl cellulase) units/g dry weight (DW) and 183.6 pNPG (p-nitrophenyl-glucoside) units/g DW at 50 °C, 100 rpm for 6 h. After centrifugation (9,803 x g, 4 °C for 15 min), the supernatant (OPEFB hydrolysate) was harvested and analyzed for glucose and xylose concentrations using a Multichannel Biochemistry Analyzer (YSI 7100 MBS, USA), and for total nitrogen concentration using the Kjeldahl method.

The concentration of metal ions was analyzed by the inductive coupled plasma method for zinc, copper, iron, and manganese; and by atomic absorption spectrometry (AAS) for calcium, magnesium, and potassium. The total phosphate content was measured by the ascorbic acid method. The 2.4% (w/v) NaOH-catalyst steam explosion OPEFB was analyzed for alpha-, beta- and gamma-celluloses by method of the Technical Association of the Pulp and Paper Industry (TAPPI)-T203 cm-09 method (TAPPI, 1999) and acid-insoluble lignin by the TAPPI-T222 om-15 method (TAPPI, 2015).

Production of *N. cerealis* IN1S2.5 Oil

The *N. cerealis* IN1S2.5 grown in YM broth at 30 °C, 200 rpm, for 24 h as the inoculum was then transferred at 10% (v/v) into YM broth (50 mL) and incubated at the same condition for 48 h. After centrifugation (9,803 x g, 4 °C, 15 min), the resultant cells were washed twice with sterile distilled water, suspended in a synthetic high carbon/nitrogen (C/N) molar ratio medium [5% glucose, 0.1% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.005% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.001% NaCl, and 0.001% $CaCl_2 \cdot 2H_2O$, all in (w/v), pH 5] (Galafassi *et al*. 2012) or OPEFB hydrolysate as the respective OPM and incubated at 30 °C, 200 rpm. Cultures were centrifuged as above, and the obtained cell precipitate was washed with sterile distilled water and freeze dried by lyophilization. The level of accumulated intracellular oil of the lyophilized cells was analyzed as previously reported (Pranimit *et al.* 2019). Briefly, the lyophilized cells (0.5 g) were suspended in 10 mL of 2:1 (v/v) chloroform: methanol, sonicated at 37 KHz for 15 min, and centrifuged (5,416 x g, 4 °C, 10 min). The supernatant was harvested, mixed with 2 mL of 0.73% (w/v) NaCl, and recentrifuged at 774 x g, 4 °C for 10 min. The lower oil phase was collected, dried by evaporation at room temperature (32 °C), and weighed. The intracellular oil accumulated in 100 g cells (DCW) was expressed as the oil content, as %

(g/g DCW), and the oil yield was calculated from the oil content in % (g/g, DCW) x cell biomass (g DCW/L)/ 100.

Maximization of the *N. cerealis* IN1S2.5 Oil Production in OPEFB Hydrolysate

Oil production of *N. cerealis* IN1S2.5 was maximized in the OPEFB hydrolysate-glucose (OPEFB hydrolysate supplemented with a final glucose concentration of 50 g/L) by addition of various concentrations of KH_2PO_4 [0.1%, 0.2%, 0.4%, 0.6%, and 0.8% (w/v)], and various kinds of metal ions [$FeCl_3 \cdot 7H_2O$ 0.001%, $CaCl_2$ 0.005%, $MnSO_4 \cdot H_2O$ 0.0002%, $ZnSO_4 \cdot 7H_2O$ 0.002%, and $MgSO_4 \cdot 7H_2O$ 0.005%, all (w/v)], varying one factor at a time (univariate analysis). The pH of the optimally supplemented OPEFB hydrolysate was then varied (pH 4, 4.5, 5, 5.5, 6, and 6.5). The condition that gave the highest oil yield was then selected for the subsequent experiments.

Fatty Acid Analysis

The extracted oil was transesterified and analyzed as previously described (Pranimit *et al.* 2019). In brief, wet cells (1 g) were suspended in 8 mL of 10% (w/v) potassium hydroxide (KOH) in methanol, incubated at 80 °C for 2 h, and then cooled down to room temperature. Petroleum ether (1 mL) was added and centrifuged to separate the unsaponified materials. The resultant aqueous phase was harvested, mixed with 3 mL of 6 N hydrochloric acid, and then extracted with diethyl ether, harvesting the diethyl ether phase. Fatty acids were recovered by evaporation at room temperature under nitrogen (N_2) gas and then methyl-esterified with boron fluoride. The obtained fatty acid methyl esters (FAMEs) were extracted by hexane and analyzed by gas chromatography (GC; Agilent 6890N, Delaware, USA) using a capillary column (30 m x 0.32 mm, 0.25 µm thickness) and flame ionization detector. The injector and detector were set at 150 and 250 °C, respectively. Helium was used as the carrier gas at a 2.3 mL/min flow rate. The temperature profile started at 150 °C and was increased to 180 °C at 10 °C/min, to 200 °C at 5 °C/min, to 205 °C at 0.5 °C/min and held at 205 °C for 2 min, then increased to 250 °C at 5 °C/min and held at this temperature for 5 min (total running time of 33 min).

Predicted Biodiesel Properties

The fatty acid composition data of *N. cerealis* IN1S2.5 oil was used to determine the properties of the produced biodiesel using Eqs. 1 to 6 (Hoekman *et al*. 2012; Tanimura *et al*. 2014),

$$\text{Viscosity} = -0.6316AU + 5.2065 \quad (1)$$

$$\text{Specific gravity} = 0.0055AU + 0.8726 \quad (2)$$

$$\text{Cloud point} = -13.356AU + 19.994 \quad (3)$$

$$\text{Cetane number} = -6.6684AU + 62.876 \quad (4)$$

$$\text{Iodine number} = 74.373AU + 12.71 \quad (5)$$

$$\text{Higher heating value} = 1.7601AU + 38.534 \quad (6)$$

where AU is the average unsaturation and is calculated from Eq. 7.

$$AU = \sum N \times C_i \quad (7)$$

In Eq. 7, N is the number of carbon-carbon double bonds of the unsaturated fatty acid, and C_i is the mass fraction of the component.

Analytical Procedure

Statistical analysis was performed using the IBM SPSS® Statistics for Windows software version 22 (SPSS Inc. Chicago, USA). Significant difference between means was evaluated using Duncan's multiple range test (DMRT), accepting significance at the $P < 0.05$ level.

RESULTS AND DISCUSSION

The OPEFB Hydrolysate

After 6 h of cellulase hydrolysis, the NaOH-catalyst steam explosion OPEFB prepared by suspending the NaOH-impregnated OPEFB in 2.4% (w/v) NaOH during steam explosion liberated the highest level of glucose (18.23 g/L or 0.364 g/g) (Fig. 1) and contained 87.8% more cellulose and 95.2% less lignin than the untreated OPEFB (Table 1). The OPEFB hydrolysate prepared by the same method except for suspending the NaOH-impregnated OPEFB in distilled water during steam explosion previously gave only 13.23 g/L reducing sugar (Weeraphan *et al*. 2016). The presence of NaOH during the steam explosion might prevent the lignin from re-adsorbing the cellulose in the treated OPEFB fibers (Zheng *et al*. 2013), and so reduced the level of unproductive binding of cellulase to lignin (Voxeur *et al*. 2015).

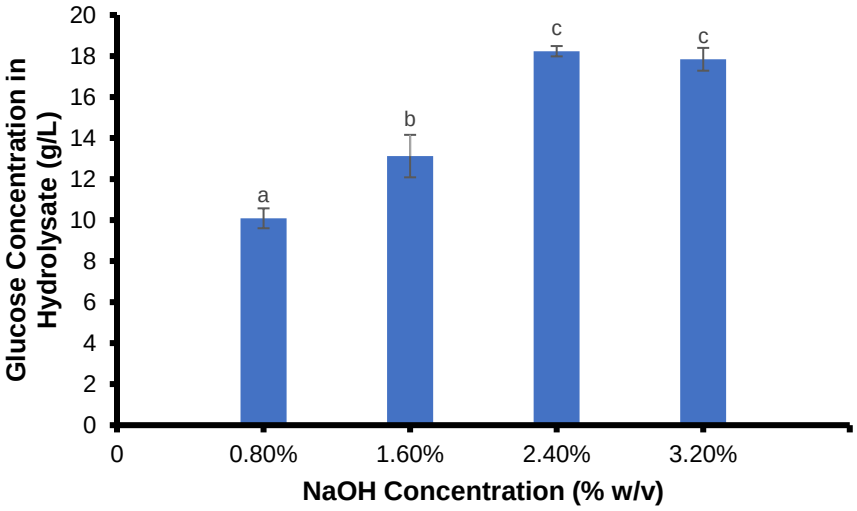

Fig. 1. Effect of the NaOH concentration used in the NaOH-impregnated OPEFB during steam explosion on the subsequent glucose liberation by cellulase hydrolysis. Data are shown as the means ± 1SD, derived from three independent repeats. Means with a different letter are significantly different ($p < 0.05$; DMRT).

The OPEFB impregnated in 0.14 M H_2SO_4 overnight and subjected to steam explosion at 203 °C for 2 min contained 16.5% more cellulose and 74.8% less lignin than the untreated OPEFB. Hydrolysis of the dilute H_2SO_4 impregnated & steam explosion OPEFB by 15 FPU/g cellulase for 12 h yielded glucose at 0.17 g/g (Siramon *et al*. 2018). Steam explosion OPEFB (195 °C, 6 min) contained 19.8% and 49.5% more cellulose and acid insoluble lignin, respectively, than the untreated OPEFB, and liberated glucose at 4.1 g/L when it was hydrolyzed by 10 FPU/g cellulase (Medina *et al*. 2016). Lignin (34.9%)

and hemicellulose (30.8%) were removed from OPEFB pretreated with steam explosion (180 °C, 20 min). Glucose (97.3% based on initial cellulose) was liberated from the pretreated OPEFB hydrolysed by 40 FPU/g cellulase for 72 h (Sari *et al.* 2021).

From the above results, the enzymatic hydrolysate of the 2.4% (w/v) NaOH-catalyst steam explosion OPEFB (OPEFB hydrolysate) was selected for use in subsequent experiments. The chemical composition and some trace elements of the OPEFB hydrolysate are summarized in Table 2.

Table 1. Comparison of the Lignocellulosic Components of the 2.4% (w/v) NaOH-catalyst Steam Explosion OPEFB and the Untreated OPEFB

Component	Untreated OPEFB (% w/w)	2.4% (w/v) NaOH-catalyst steam explosion OPEFB (% w/w)
Cellulose	48.4	90.9
Hemicellulose	14.3	6.8
Lignin	16.8	0.8

Table 2. Chemical Composition of the 2.4% (w/v) NaOH-catalyst Steam Explosion OPEFB Hydrolysate

Chemical composition:	*(g/L)*
Glucose	16.67
Xylose	3.47
Total nitrogen	0.01
Total phosphate	<0.05
Trace elements:	*(mg/L)*
Zinc	2.85
Copper	0.19
Iron	7.95
Manganese	0.89
Calcium	50.42

Oil Production by *N. cerealis* IN1S2.5

When grown in the synthetic high C/N molar ratio OPM for 6 d, *N. cerealis* IN1S2.5 gave an oil yield of 3.67 g/L and an oil content of 24.8% (g/g DW). To our knowledge, this is the first report that *N. cerealis* is oleaginous. Meanwhile, in the OPEFB hydrolysate, the *N. cerealis* IN1S2.5 gave the highest oil yield of 0.41 g/L at 1 d, with an oil content and cell biomass of 4.07% (g/g DCW) and 10.13 (g/L), respectively, which is markedly lower than in the high C/N molar ratio OPM.

Maximization of the *N. cerealis* IN1S2.5 Oil Production in OPEFB Hydrolysate

Yeast oil accumulation is dependent on the C/N molar ratio of the OPM (Sathiyamoorthi *et al*. 2019). A higher sugar concentration in the OPM gives a higher C/N molar ratio and subsequently a higher oil accumulation by oleaginous microorganisms (Ahmad *et al.* 2017). The sugar concentration in the OPEFB hydrolysate-based OPM was increased by adding glucose to a final concentration of 50 g/L (OPEFB hydrolysate-glucose), whereupon the OPEFB hydrolysate-glucose gave the highest oil yield of *N. cerealis* IN1S2.5 at 0.8 g/L after 7 d with an increased oil content to 12.45% (g/g DCW), but a decreased cell biomass to 6.4 g/L (Fig. 2).

In the yeast oil accumulation process, the concentration of the limiting nutrient (typically nitrogen) frequently determines the quantity of cell biomass, whilst an excess carbon source concentration largely determines the amount of oil accumulated (Papanikolaou and Aggelis 2011). However, some nitrogen sources were preferred over others for inducing optimal yeast oil accumulation, and the preferred nitrogen source must be supplied (Probst *et al.* 2016). When the C/N molar ratio of corn stover hydrolysate containing 0.5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ was increased to 30.6 or 96.7 by adding 30.1 or 127.1 g/L glucose, respectively, both the oil content and cell biomass of *Trichosporon cutaneum* were increased from 10.5 to 28.1% (g/g DCW) and 13.25 to 16.06 (g/L), respectively (Huang *et al.* 2011). *Trichosporon coremiiforme* grown in detoxified corncob-acid hydrolysate that contained 45.7 g/L sugar and 0.2 g/L nitrogen had the highest oil content and cell biomass at 37.8% (g/g DCW) and (20.4 g/L), respectively, at 8 d. The addition of more than 40 g/L glucose into the detoxified corncob-acid hydrolysate increased the highest oil content and cell biomass of the *T. coremiiforme* at 8 d to 39.2% (g/g DCW) and 24.5 (g/L), respectively (Huang *et al.* 2013).

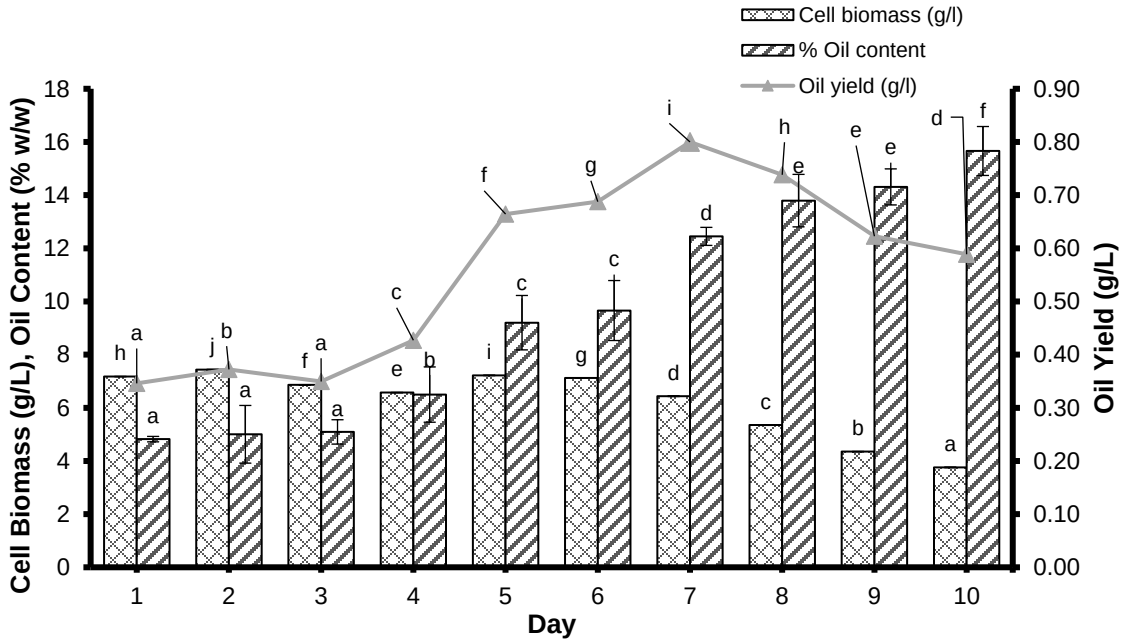

Fig. 2. Cell biomass, oil content, and oil yield of *N. cerealis* IN1S2.5 grown in OPEFB hydrolysate-glucose. Data are shown as the means ± 1SD, derived from three independent repeats. Means with a different letter are significantly different ($p < 0.05$; DMRT).

The optimal C/N molar ratio not only needs to be high but also strongly depends on the OPM composition (Jiru *et al.* 2017). Phosphorus is important for yeast cell growth and metabolism.

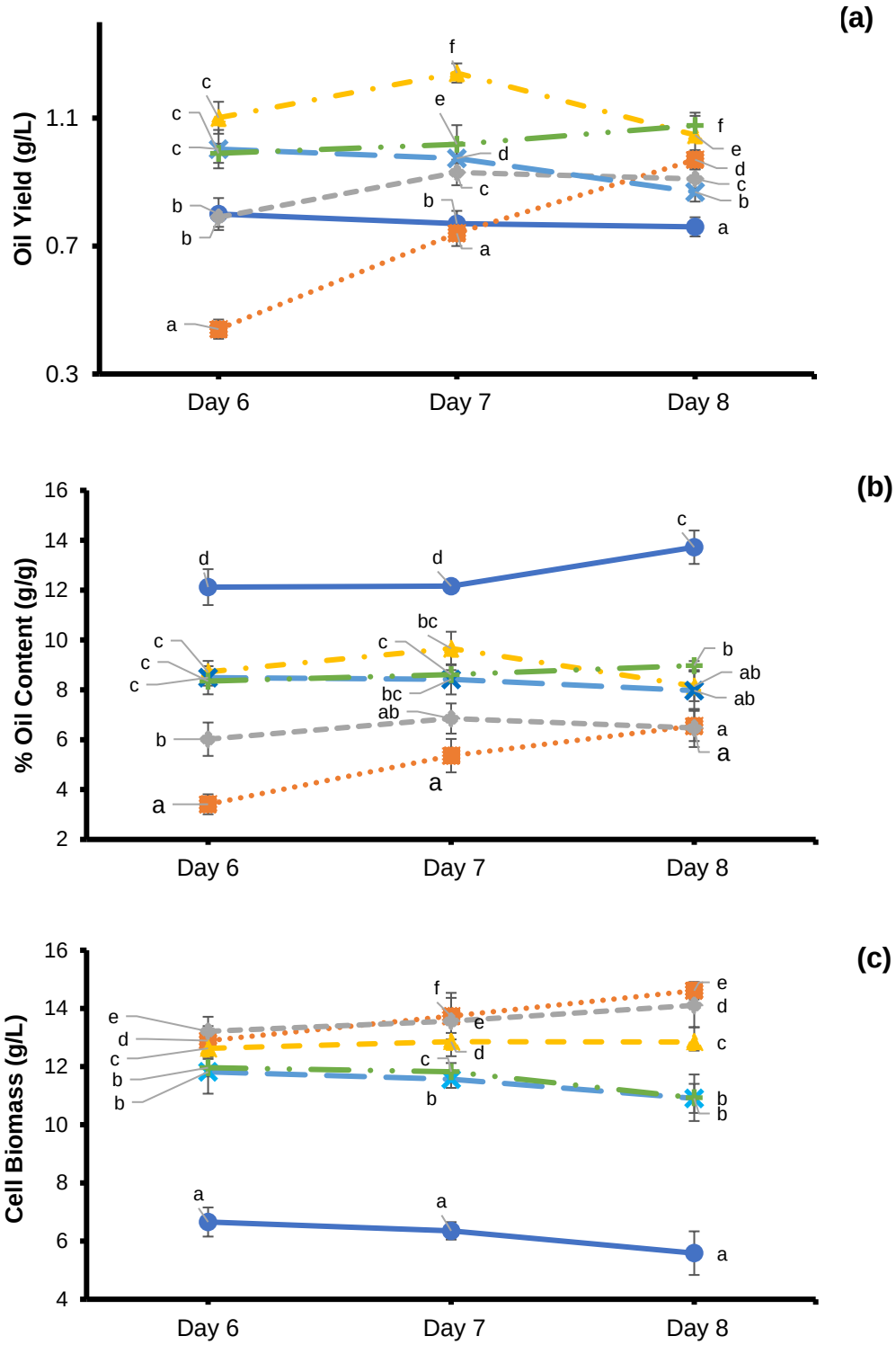

Fig. 3. Effect of the KH_2PO_4 concentration supplemented in the OPEFB hydrolysate-glucose on the oil yield (a), oil content (b), and cell biomass (c) of *N. cerealis* IN1S2.5. KH_2PO_4 concentrations (% w/v): 0 (●), 0.1 (■), 0.2 (♦), 0.4 (▲), 0.6 (**x**), and 0.8 (**+**). Data are shown as the means ± 1SD, derived from three independent repeats.

Yeast oil is accumulated in an oil droplet within a special organelle covered by a phospholipid monolayer (Qin *et al.* 2017), which requires metabolizable phosphorus for the phospholipid synthesis. Thus, increasing the phosphate concentration in the OPEFB hydrolysate-glucose to evaluate the effect on *N. cerealis* IN1S2.5 oil production was initially performed by adding various concentrations of KH_2PO_4. The highest oil yield of the *N. cerealis* IN1S2.5 increased to 1.24 g/L (oil productivity 0.18 g/L/d) when 0.4% (w/v) KH_2PO_4 was added, with an increased cell biomass to 12.86 g/L (Fig. 3). Yeast oil accumulation has been directly linked to the C/P molar ratio of the culture medium (Wu *et al.* 2010). The oil yield of *Candida* sp. NG17 cultured in sugarcane leaves hydrolysate was increased from 5.07 g/L to 6.67 g/L when increasing the C/P molar ratio to 57.5 with 0.1% (w/v) KH_2PO_4 supplementation (Pranimit *et al.* 2019). Addition of 0.1 g/L KH_2PO_4 into OPM containing 15.0 g/L glucose, 15.0 g/L xylose, 4.0 g/L $(NH_4)_2SO_4$, 2.0 g/L peptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L EDTA, and 1% (v/v) trace element solution increased oil yield of *Cutaneotrichosporon oleaginosum* from 3.3 g/L to 3.6 g/L (Zhou *et al*. 2019).

Several metal ions are known to play an important role as a cofactor in fatty acid synthesis (Muhid *et al*. 2008). In this experiment, the OPEFB-glucose plus 0.4% (w/v) KH_2PO_4 was further supplemented with various metal ions known to have positive effects on yeast oil production (Wang *et al.* 2012) [all (w/v)]; 0.001% $FeCl_3 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.0002% $MnSO_4 \cdot H_2O$, 0.002% $ZnSO_4 \cdot 7H_2O$, and 0.005% $MgSO_4 \cdot 7H_2O$. After 7 d of incubation, the addition of $CaCl_2$ was found to result in the highest oil yield at 1.26 g/L, while the addition of $ZnSO_4 \cdot 7H_2O$ gave a higher oil content than the control (Fig. 4). Therefore, the effect of $CaCl_2$ and $ZnSO_4 \cdot 7H_2O$ additions on the oil production of *N. cerealis* IN1S2.5 were further examined.

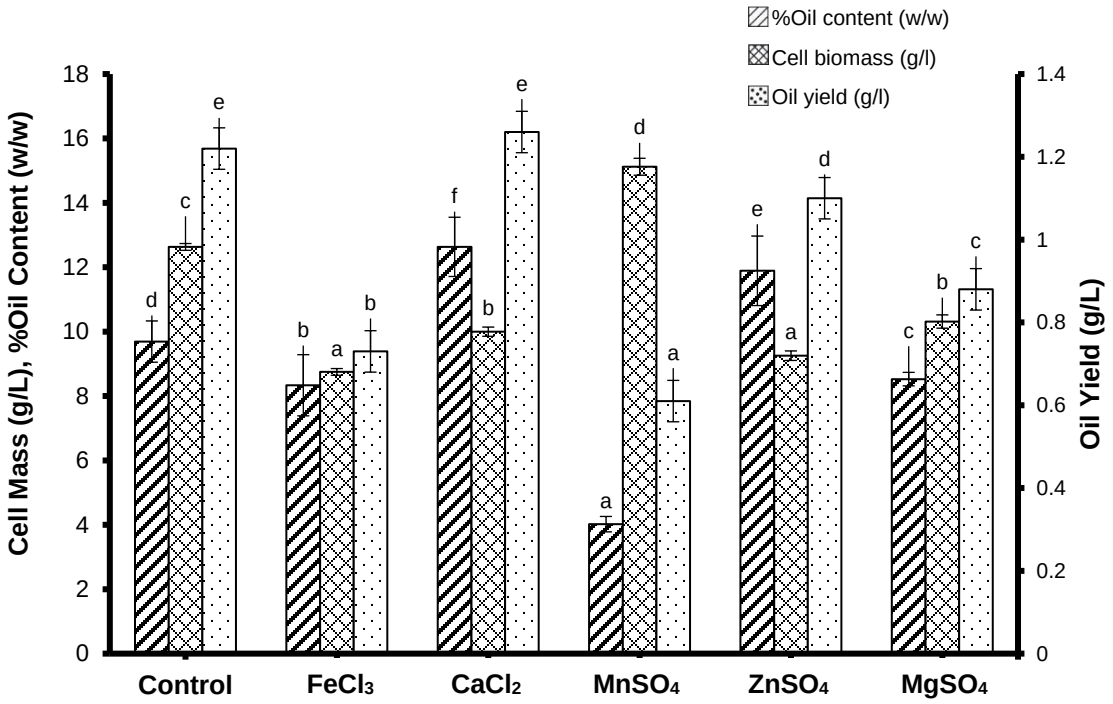

Fig. 4. Effect of various metal ions on the oil yield, oil content, and cell biomass of *N. cerealis* IN1S2.5 when grown in OPEFB-hydrolysate-glucose-KH_2PO_4 for 7 d. Metal ion supplement concentrations were [all (w/v)]:$FeCl_3 \cdot 7H_2O$ 0.001%, $CaCl_2$ 0.005%, $MnSO_4 \cdot H_2O$ 0.0002%, $ZnSO_4 \cdot 7H_2O$ 0.002%, and $MgSO_4 \cdot 7H_2O$ 0.005%. Data are shown as the means ± 1SD, derived from three independent repeats. Means with a different letter are significantly different ($p < 0.05$; DMRT).

Simultaneous addition of 0.005% (w/v) $CaCl_2$ and 0.002% (w/v) $ZnSO_4 \cdot 7H_2O$ increased the oil yield further by 9.68% to 1.34 g/L (Table 3). This is consistent with previous studies. For example, the presence of Ca^{2+} ions was shown to give a beneficial effect on *Lipomyces starkeyi* lipid production (Naganuma *et al*. 1985), while *Rhodosporidium toruloides* oil production was significantly improved in optimized medium containing 1.5 mM/L $CaCl_2$ and 1.91 mM/L $ZnSO_4$ (Li *et al*. 2006). Likewise, the presence of Ca^{2+} and Zn^{2+} ions showed a positive effect on the oil production of *R. toruloides* grown in a cassava starch hydrolysate containing OPM (Wang *et al*. 2012). The oil production of *Candida albidus* was increased from 5.83 to 11.81 g/L in an optimized OPM containing 0.15 g/L $CaCl_2$ and 0.02 g/L $ZnSO_4.7H_2O$ (Enshaeieh *et al*. 2013).

Table 3. Effect of $CaCl_2$ and $ZnSO_4 \cdot 7H_2O$ Supplementation in the OPEFB Hydrolysate-glucose-KH_2PO_4 on the Oil Production by *N. cerealis* IN1S2.5 at 7 d of Incubation

Run	$CaCl_2$	$ZnSO_4 \cdot 7H_2O$	Cell biomass	Oil content	Oil yield
	(% w/v)	(% w/v)	(g/L)	(% g/g)	(g/L)
1	0	0	12.57 ± 0.27^{f}	9.48 ± 0.47^{e}	1.19 ± 0.38^{f}
2	0.005	0	10.82 ± 0.13^{c}	11.46 ± 0.69^{g}	1.24 ± 0.24^{g}
3	0.01	0	11.27 ± 0.23^{d}	9.38 ± 0.34^{e}	1.06 ± 0.31^{e}
4	0	0.002	9.09 ± 0.28^{a}	10.14 ± 0.71^{f}	0.92 ± 0.63^{c}
5	0.005	0.002	9.58 ± 0.22^{b}	13.94 ± 0.65^{h}	1.34 ± 0.42^{h}
6	0.01	0.002	9.213 ± 0.59^{a}	8.03 ± 0.65^{c}	0.74 ± 0.71^{b}
7	0	0.004	12.78 ± 0.91^{g}	7.86 ± 0.94^{b}	1.01 ± 0.93^{d}
8	0.005	0.004	12.08 ± 0.25^{e}	8.33 ± 0.86^{d}	1.01 ± 0.46^{d}
9	0.01	0.004	12.985 ± 0.53^{h}	5.33 ± 0.32^{a}	0.69 ± 0.41^{a}

Data are shown as the mean ± 1SD, derived from three independent repeats. Means within a column followed by a different letter are significantly different ($p < 0.05$; DMRT).

When *N. cerealis* IN1S2.5 was cultured in OPEFB-glucose supplemented with KH_2PO_4, $CaCl_2$ and $ZnSO_4 \cdot 7H_2O$ (supplemented OPEFB) at various pH values (4, 4.5, 5, 5.5, 6, and 6.5) for 7 d, the highest oil yield (2.32 g/L) was obtained at pH 4 (Fig. 5). This is consistent with previous reports, where a lower pH supported lipid accumulation in oleaginous microorganisms (Patel *et al.* 2016). *Rhodotorula glutinis* IIP-30 had an optimal pH for oil production at pH 4 (Johnson *et al.* 1992). A study on the pH-dependent lipid production in *Yarrowia lipolytica* W29 indicated that there was neither any significant alteration in the key enzymes involved in lipid biosynthesis, such as ATP-citrate lyase (ACL), acetyl-CoA carboxylase, and diacylglycerol acyltransferase, nor in the expression level of genes in the tricarboxylic acid cycle at different media pH values. Rather, the transporting activity of the mitochondrial membrane was increased at a higher pH and this stimulated the efflux of citrate from the mitochondria to the cytoplasm, where citrate can be further exported from cell. In contrast, the transcription levels of mitochondrial membrane transporters were decreased at lower pH values, and this reduced the efflux of the citrate. The citrate accumulated in cell, which in turn resulted in the citrate being converted to acetyl-CoA by ACL for subsequent production of lipid (Zhang *et al*. 2019).

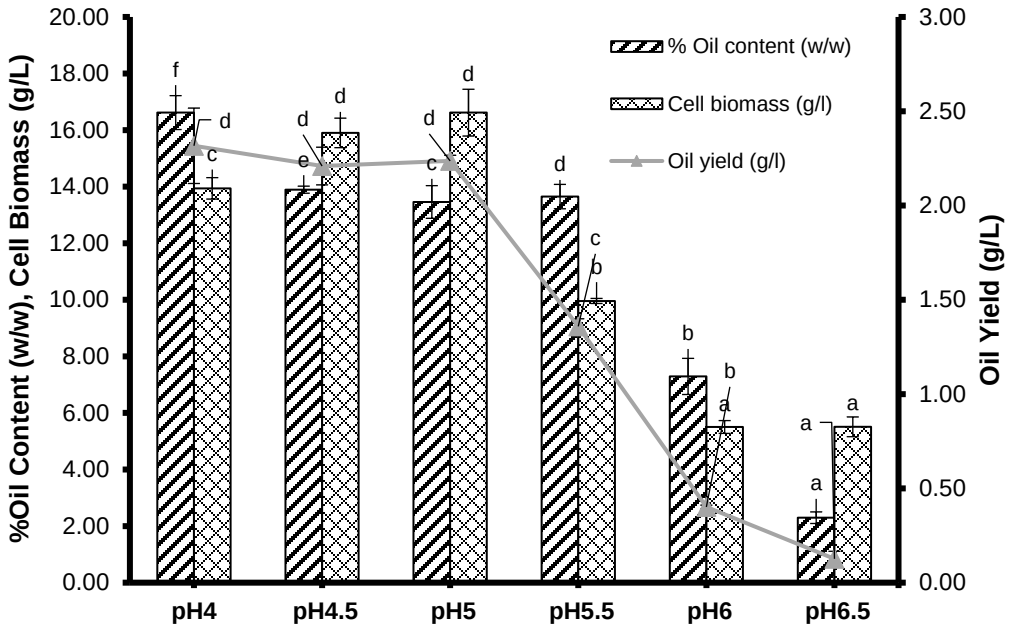

Fig. 5. Effect of the media pH on the oil yield, oil content, and cell biomass of *N. cerealis* IN1S2.5 when grown in supplemented OPEFB for 7 d. Data are shown as the means ± 1SD, derived from three independent repeats. Means with a different letter are significantly different ($p < 0.05$; DMRT).

In this study, when the final concentration of glucose in the supplemented OPEFB at pH 4 was varied at 50, 75, 100, and 125 g/L, the maximum oil yield of *N. cerealis* IN1S2.5 was increased to 2.46 g/L at 7 d when the final glucose concentration was 75 g/L (C/P molar ratio of 25.71) (Fig. 6).

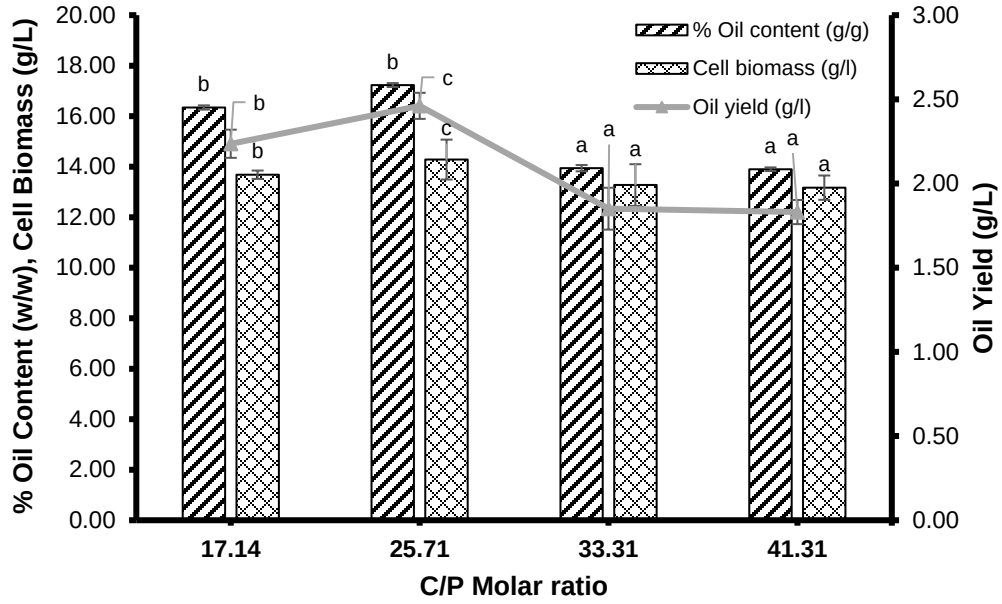

Fig. 6. Effect of the C/P molar ratio of the supplemented OPEFB on the oil yield, oil content, and cell biomass of *N. cerealis* IN1S2.5 at 7 d. Data are shown as the means ± 1SD, derived from 3 independent repeats. Means with a different letter are significantly different ($p < 0.05$; DMRT).

Fatty Acid Composition of *N. cerealis* IN1S2.5 Oil

To realize the potential application of the *N. cerealis* IN1S2.5 oil, its fatty acid composition was determined. When grown in the optimized OPEFB hydrolysate based OPM, the oil was composed of 36.2% (w/w) palmitic acid, 37.6% (w/w) oleic acid, and 17.9% (w/w) steric acid as the dominant fatty acids (Fig. 7). This was different from the *N. cerealis* IN1S2.5 oil produced in the synthetic high C/N molar ratio OPM (Galafassi *et al*. 2012), which contained a much higher oleic acid and lower palmitic acid and steric acid content at 61.7%, 22.6%, and 8.12% (w/w), respectively. The dominant fatty acids of *Rhodosporidium kratochvilovae* HIMPA1 oil grown in aqueous extract of *Cassia fistula* L. fruit pulp were palmitic, steric, and oleic acids at 43.1%, 28.7%, and 17.3% (w/w), respectively (Patel *et al*. 2016). But when the *Rh. kratochvilovae* HIMPA1 was grown in pulp and paper industry effluent, the dominant fatty acids of the produced oil was changed to be oleic, palmitic, and linoleic acids at 45.4%, 21.9%, and 15.9% (w/w), respectively, (Patel *et al*. 2017). Indeed, the feedstock composition has a significant influence on the fatty acid profile of oleaginous oil (Brar *et al*. 2017).

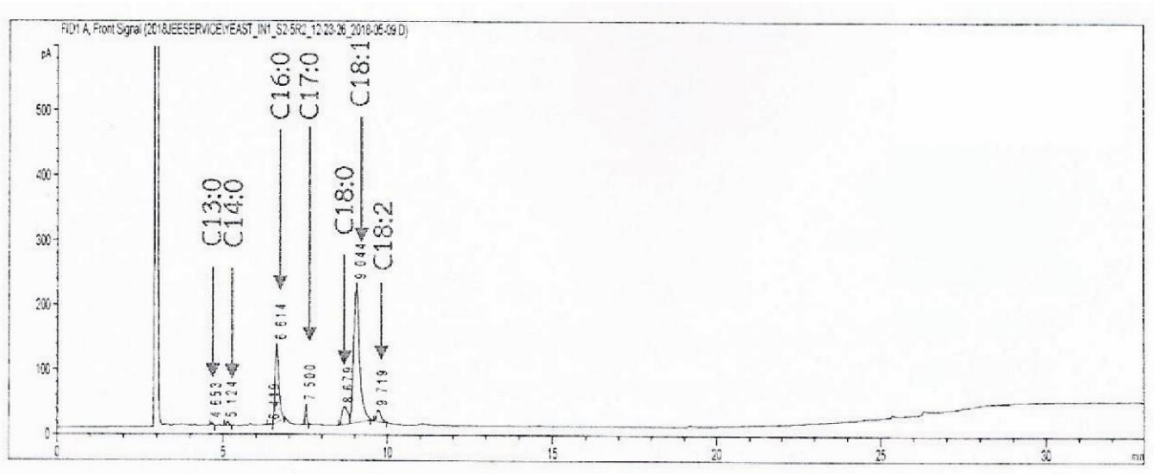

Fig. 7. Chromatogram of fatty acid composition in *N. cerealis* IN1S2.5 oil produced in the OPEFB hydrolysate based OPM

Predicted Biodiesel Properties

Biodiesel properties influenced by the fatty acid composition of the feedstock oil were determined, and the results are summarized in Table 4. The viscosity, specific gravity, cetane number, iodine number, and oxidative stability values of the biodiesel produced from *N. cerealis* IN1S2.5 oil were all within the EU standard (EN14214) and US standard (ASTM D6751).

When the OPEFB hydrolysate containing 115 g/L reducing sugar prepared from alkaline-pretreated OPEFB (3.75 M NaOH, 121 ºC, 1 h) was used as the OPM (C/N molar ratio of 99.6), the cell biomass and oil content of *L. starkeyi* were 14.9 g/L and 6% (g/g DCW), respectively, at 192 h. But when the OPEFB hydrolysate was diluted to 60 g/L reducing sugar (C/N molar ratio reduced to 71.7), the *L. starkeyi* cell biomass decreased to 13.1 g/L, while the oil content increased to 40.3% (g/g DCW) at 108 h. This improvement in the *L. starkeyi* oil production in the diluted OPEFB hydrolysate was explained by the reduced concentration of inhibitors (Thanapimmetha *et al*. 2019).

Table 4. Comparison of the Biodiesel Properties of the Oil from *N. cerealis* IN1S2.5 Grown in OPEFB Medium with Those of the EU and US Standard

	N. cerealis IN1S2.5 oil	EU standard	US standard
Viscosity (mm/s^2)	4.93	3.5-5.0	1.9-6.0
Specific gravity	0.8750	0.86-0.9	-
Cloud point (°C)	14.21	Country specific [a]	Country specific [a]
Cetane number	59.99	Lower limit = 51	47
Iodine number	44.93	Upper limit = 120	-
Higher heating value	39.30	-	-

[a] Agreement upon fuel supplier and purchaser
- Not reported

The OPEFB was pretreated by 0.4% (w/v) H_2SO_4 at 170 °C for15 min and filtered to separate the liquid fraction from the residual solid. The liquid fraction was then detoxified by overliming, and it was defined as the OPEFB liquid hydrolysate (OPEFB-LH). The residual solid was washed, cellulase hydrolyzed, and filtered, with the resultant filtrate being defined as the OPEFB enzymatic hydrolysate (OPEFB-EH). The detoxified OPEFB-LH contained 0.56 g/L furfural (FUR) and 0.08 g/L 5-hydroxymethylfurfural (HMF). Oil production by three oleaginous microorganisms (*Rhodotorula mucilaginosa*, *Aspergillus oryzae*, and *Mucor plumbeus*) in the filter-sterilized hydrolysates supplemented with nutrients at 28 °C with shaking for 7 d was then compared. The highest oil yield was obtained from OPEFB-EH and OPEFB-LH by *M. plumbeus* (4.7 and 1.9 g/L) followed by *A. oryzae* (4.47 and 1.4 g/L), respectively. The higher C/N molar ratio of OPEFB-EH than OPEFB-LH was proposed as a principal reason. The dominant fatty acids of *M. plumbeus* oil produced from OPEFB-EH and OPEFB-LH were oleic [33.8 and 20.4% (w/w)] and palmitic [18.2 and 23.0% (w/w)] acids, while those of *A. oryzae* oil produced from OPEFB-EH and OPEFB-LH were oleic acid at 34.1 and 29.7% (w/w) and palmitic acid at 9.3 and 21.1% (w/w), respectively, (Ahmad *et al*. 2016).

Delignified OPEFB (DOPEFB), in which the toxic by-products of lignin were reduced by alkaline pretreatment [10% (w/v) NaOH at 10% solid loading, 100 ºC, 30 min] was used to prepare hemicellulose and cellulose hydrolysates by a two-step hydrolysis. In the first step, the DOPEFB was hydrolyzed by 0.5% (w/v) H_2SO_4 at 120 ºC for 60 min, and the obtained liquid fraction was defined as the hemicellulose hydrolysate. In the second step, the residual solid was hydrolyzed by 2.5% (w/v) H_2SO_4 at 120 ºC for 60 min and the liquid fraction of this step was defined as the cellulose hydrolysate. Holocellulose hydrolysate was prepared by the one-step hydrolysis of DOPEFB with 2.5% (w/v) H_2SO_4 at 120 ºC for 60 min. The holocellulose hydrolysate contained the highest amount of sugar (38.3 g/L) together with the highest concentration of FUR (0.57 g/L) and HMF (0.44 g/L). The hydrolysates were diluted to contain 20 g/L sugar and used as a carbon source in the hydrolysate-based OPM. The HMF concentration in this OPM was in range of 0.1-0.4 g/L, which was lower than toxic level (1.8-3.0 g/L), while the FUR concentration in the diluted hemicellulose, cellulose, and holocellulose hydrolysates was 0.22. 0.41, and 0.3 g/L, respectively.

Three oleaginous yeasts, *Rhodotorula mucilaginosa* G43, *Kluyveromyces marxianus* X32, and *Candida tropicalis* X37, grew and produced lipid in the diluted

hydrolysates only after they were detoxified by activated carbon or overliming. Of these, *C. tropicalis* X37 gave the highest lipid yield (2.73 g/L) in the cellulose hydrolysate detoxified by overliming, although this medium also had the highest glucose concentration, which might be the reason for the highest lipid production by *C. tropicalis* X37 (Tampitak *et al*. 2015).

In this study, combined strategy of NaOH-impregnation prior to steam explosion and steam explosion in the presence of NaOH significantly enhanced the enzymatic saccharification of OPEFB. Supplementation of phosphate, Ca^{2+}, and Zn^{2+} in the OPEFB hydrolysate-glucose resulted in maximum oil yield of *N. cerealis* IN1S2.5. This result indicates that low-cost carbon source which contains high concentration of phosphate, Ca^{2+} and Zn^{2} is suitable to be used to raise the C/N molar ratio of the OPEFB hydrolysate.

CONCLUSIONS

1. Cellulase hydrolysis of NaOH-impregnated oil palm empty fruit bunch (OPEFB) suspended in 2.4% (w/v) NaOH during steam explosion yielded the highest glucose level at 0.364 (g/g).
2. The maximum oil yield of *N. cerealis* IN1S2.5 was 2.46 g/L when grown in the hydrolysate of the NaOH-impregnated & catalyst steam explosion OPEFB, where the C/P molar ratio was adjusted to 25.71, and the mixture was supplemented with Ca^{2+} and Zn^{2+} at pH 4.
3. Predicted biodiesel properties indicated that this *N. cerealis* IN1S2.5 oil had potential to be feedstock for biodiesel production.

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