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## Waste Management

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## Chemical and biological conversion of crude glycerol derived from waste cooking oil to biodiesel

Jiaxin Chen <sup>a,b</sup>, Song Yan <sup>b</sup>, Xiaolei Zhang <sup>a,\*</sup>, Rajeshwar Dayal Tyagi <sup>b</sup>, Rao Y. Surampalli <sup>c</sup>, J.R. Valéro <sup>b</sup>

<sup>a</sup> School of Civil and Environmental Engineering, Harbin Institute of Technology (Shenzhen), Shenzhen, Guangdong 518055, PR China

<sup>b</sup> INRS Eau, Terre et Environnement, 490, rue de la Couronne, Québec G1K 9A9, Canada

<sup>c</sup> Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC, PO Box 886105, Lincoln, NE 68588-6105, USA

## ARTICLE INFO

## Article history:

Received 16 May 2017

Revised 24 October 2017

Accepted 25 October 2017

Available online xxxx

## Keywords:

Crude glycerol

Energy

Lipid accumulation

Biodiesel

## ABSTRACT

In this study, crude, purified, and pure glycerol were used to cultivate *Trichosporon oleaginosus* for lipid production which was then used as feedstock of biodiesel production. The purified glycerol was obtained from crude glycerol by removing soap with addition of  $H_3PO_4$  which converted soap to free fatty acids and then separated from the solution. The results showed that purified glycerol provided similar performance as pure glycerol in lipid accumulation; however, crude glycerol as carbon source had negatively impacted the lipid production of *T. oleaginosus*. Purified glycerol was later used to determine the optimal glycerol concentration for lipid production. The highest lipid yield 0.19 g/g glycerol was obtained at 50 g/L purified glycerol in which the biomass concentration and lipid content were 10.75 g/L and 47% w/w, respectively. An energy gain of 4150.51 MJ could be obtained with 1 tonne of the crude glycerol employed for biodiesel production through the process proposed in this study. The biodiesel production cost estimated was 6.32 US \$/gal. Fatty acid profiles revealed that C16:0 and C18:1 were the major compounds of the biodiesel from the lipid produced by *T. oleaginosus* cultivated with crude and purified glycerol. The study found that purified glycerol was promising carbon source for biodiesel production.

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### 1. Introduction

One of the most urgent issues in the world is to seek renewable, sustainable, and affordable energy source due to the risk of the depletion of petro energy. Biodiesel is gaining increasing attention as it can be produced by renewable and cheap materials. The dramatic increase in demand of biodiesel resulted in its increased production from various types of oils. The common method of biodiesel production is trans-esterification in which oils or fats react with short chain alcohol (generally methanol) with acid or base as catalyst. In the process, crude glycerol is generated as by-product. About 0.10 kg of glycerol is generated per kilogram of biodiesel produced. It is normally called crude glycerol and mainly contains glycerol (20–96% w/w), free fatty acids, soaps, catalyst, salts, methanol etc. (Gao et al., 2016; Hansen et al., 2009; Hu et al., 2012). The composition of crude glycerol varies from one biodiesel production plant to another and is mainly determined by the feedstock oil composition and quality, the oil and methanol molar ratio used in trans-esterification, type of catalyst used, and the detailed procedure

such as with or without methanol recovery (Athalye et al., 2009; Uprety et al., 2017; Yen et al., 2012).

Crude glycerol is a complex material, and the proper utilization to attain its maximum value is desirable for its appropriate handling. Purification of crude glycerol was the most applied method before biodiesel boom. However, due to a substantial decrease in the price of purified glycerol (1.54 US \$/kg before 2000 and 0.66 US \$/kg after 2007), the purification is getting less attractive. Therefore, direct use or partial purification of crude glycerol is becoming promising. Due to the large demand on energy in the current world, use of crude glycerol for energy production has been widely reported (Nartker et al., 2014; Oliveira et al., 2015; Trchounian et al., 2016). Bioconversion of glycerol to biodiesel is an interesting way of utilization of original or partially purified crude glycerol. Oleaginous microorganisms such as *Schizochytrium* sp., *Yarrowia lipolytica*, *Rhodotorula* sp., and *Cryptococcus* sp. are reported capable of assimilating glycerol to produce lipid which is the raw material of biodiesel production (Deeba et al., 2016; Gao et al., 2016; Polburee et al., 2015; Ryu et al., 2013).

Current studies revealed that the composition of crude glycerol had great impact on lipid accumulation in microorganisms (Cerón-García et al., 2013; Polburee et al., 2015). Normally, high

\* Corresponding author.

E-mail address: [xiaolei.zhang2016@foxmail.com](mailto:xiaolei.zhang2016@foxmail.com) (X. Zhang).

glycerol content in crude glycerol tends to lead high lipid accumulation in microorganism. Study showed that the lipid accumulation reached around 60% w/w (lipid g/ biomass g) with pure glycerol as carbon source, which was only 20% w/w (lipid g/ biomass g) when crude glycerol was applied as carbon source (Polburee et al., 2015). The highest biomass and lipid concentration reached 26.7 g/L and 18.5 g/L when utilized crude glycerol with 85% glycerol content, respectively, but they were 18.0 g/L and 13.4 g/L when the crude glycerol with glycerol content of 33%, respectively (Xu et al., 2012). The content of impurities including methanol and soap have showed significant effect on cell growth as well as lipid accumulation (Liang et al., 2010a; Lorenz et al., 2017). It was revealed that biomass productivity of *Cryptococcus curvatus* cultivated with crude glycerol medium was only 67% of that from pure glycerol with the same carbon concentration (Liang et al., 2010a). Lipid accumulation in *Kodamaea ohmeri* with crude glycerol was only 50% of that with pure glycerol (Kitcha and Cheirsilp, 2011). Gao et al. (2016) found that the lipid productivity of *Rhodospiridium toruloides* in crude glycerol cultivation was reduced around 18% compared with that of pure glycerol cultivation (Gao et al., 2016). Overall, crude glycerol with high glycerol content and low impurity content showed advantage on lipid production from oleaginous microorganism.

Crude glycerol composition is highly related to the character of feedstock oil of biodiesel production. The crude glycerol with high glycerol content (>60%) was normally generated in the biodiesel production from plant seed oils which have low free fatty acid (FFA) content. It suggests that FFA content in the feedstock oil has great impact on the final glycerol content in crude glycerol as FFA can be transferred to soap during the transesterification in base catalyzed reaction. As the prices of plant seed oils are increasing, waste cooking oils which have high FFA content are largely employed in biodiesel production, and hence the production of crude glycerol with high soap content is gradually increasing. In order to reduce the influence on lipid production from microorganism, soap can be removed from crude glycerol prior to the utilization. By soap removal, the glycerol content will be increased in the crude glycerol, and thus the purified crude glycerol could enhance the production of biomass and lipid from oleaginous microorganisms. In addition, it would bring extra value to the process if the recovered soap could be also converted to useful product. In fact, soap in crude glycerol can be converted to FFA which can be used to produce biodiesel with acid as catalyst.

In this study, crude glycerol generated in the biodiesel production from waste cooking oil, which has low glycerol content, was used as carbon source for oleaginous yeast cultivation after FFA recovery. The obtained FFA from crude glycerol purification was then transferred to biodiesel through esterification. The study provides a way to maximize the value of crude glycerol with low glycerol content. In addition, the work increases energy efficiency as energy (biodiesel) production by-product (crude glycerol) again converted to energy (biodiesel). It provides a feasible method to manage crude glycerol and create energy. The flow diagram of the study is shown in Fig. 1.

## 2. Materials and methods

### 2.1. Materials

Crude glycerol was kindly provided by a biodiesel production plant, in Quebec, Canada. Oleaginous microorganism *Trichosporon oleaginosus* (ATCC20509) was employed in this study to produce lipid.

### 2.2. Crude glycerol characterization

**Density and pH:** The weight of 2 mL of crude glycerol was measured at room temperature. The density of crude glycerol was determined by dividing the weight with the volume (2mL). To determine the pH, 1.0 g of crude glycerol was dissolved in 50 mL of deionized (DI) water. The pH of the solution was measured by a digital pH meter at room temperature (Hu et al., 2012).

**Glycerol content:** The glycerol content was determined according to the method reported by Bondioli and Della Bella (2005). 3,5-diacetyl-1,4-dihydrolutidine, a yellow complex, was formed in a two-step reaction. In the first step, glycerol reacted with sodium periodate to form formaldehyde, following, acetyl acetone was added to generate the complex of 3,5-diacetyl-1,4-dihydrolutidine. The complex was measured by UV-Vis Spectrophotometer at 410 nm. The glycerol content was calculated according to standard curve ( $=0.05645 \times \text{conc.} - 0.07437$ ;  $R^2 = 0.99534$ ). The method is a well established one for glycerol determination (Lima et al., 2012; Sidnei et al., 2011). It could rapidly and accurately determine glycerol concentration in liquid. To verify the method, glycerol was also determined with High Performance Liquid Chromatography (HPLC), and results showed that Bondioli and Bella method was reliable. Thus, Bondioli and Bella method was used in this study to determine glycerol concentration.

**Soap content:** The soap content was estimated as reported by Liang and co-workers (Liang et al. (2010b)). The pH of 50 g crude glycerol was adjusted to 1.0 with 85%  $\text{H}_3\text{PO}_4$ . After well mixing, the solution was centrifuged at 5000 rpm for 20 min. The top red dark layer (in the centrifuged liquid) which was FFA was collected and weighed. The soap content was calculated according to soap amount =  $304 \times \text{FFA amount}/282$ ; where 304 is average soap molar mass and 282 is the average FFA molar mass.

**Biodiesel content:** Biodiesel content was analyzed with Gas Chromatography coupled with Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500) to control the result quality. Helium was used as the carrier gas. The dimensions of the column used were 30 m  $\times$  0.25 mm, with a phase thickness of 0.2  $\mu\text{m}$ . The calibration curve was prepared by injecting known concentrations of an external standard, mixture comprising 37 FAMES (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1,3-dichlorobenzene was also used as internal standard with a concentration of 50 ppm.

**Ash content:** The 10 g of crude glycerol was heated at 750 °C for 3 h (Manosak et al., 2011). After the sample was cooled down to room temperature, the residue ( $W_3$ ) was weighed and then the ash content was calculated ( $W_3/10 \times 100\%$ ).

**Catalyst content:** Crude glycerol was sampled from a company in which NaOH was used as the catalyst in the transesterification process to produce the biodiesel. To determine the content of NaOH, 10 g of crude glycerol was adjusted to pH 7 with 1 M HCl and the consumed volume of acid 1 M HCl (V) was recorded and used to calculate the NaOH content ( $=40 \times 1 \times V/10$ ; where 40 is NaOH molar mass, 1 is HCl molar concentration, V is the volume of 1 M HCl consumed to bring the pH to 7; and 10 is crude glycerol amount) in crude glycerol.

**Methanol content:** The methanol content was determined with Heidolph Laborota 4011 digital evaporator. The 100 mL (107.3 g) of crude glycerol was subjected to 60 °C for 15 min. The evaporated methanol ( $W_4$ ) was collected and the methanol content in the crude glycerol was calculated as follows:  $W_4/107.3 \times 100\%$ .

**Water content:** The 10 g of crude glycerol was heated at 105 °C until weight constant ( $W_5$ ). The weight loss during the heating was due to the evaporation of water and methanol. The sum of water and methanol content was calculated as follows:  $[(10 - W_5)/10 \times 100\%]$ . After subtracting methanol content, water content was obtained.

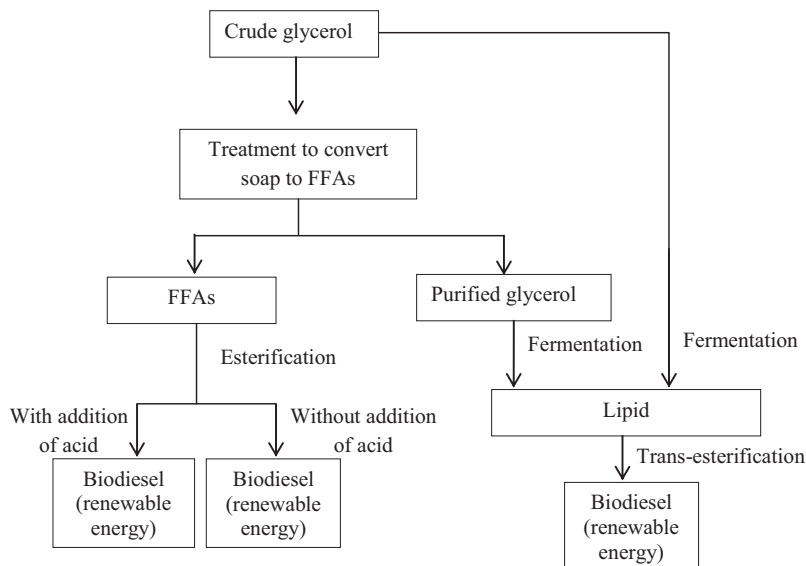


Fig. 1. The flow diagram of the study.

### 2.3. Soap conversion to free fatty acid (FFAs)

According to the information of the catalyst amount added during trans-esterification provided by biodiesel production industry, different volume (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mL) of 85% phosphoric acid was added to 40 mL of crude glycerol, respectively, to determine the optimal acid addition for converting soap to FFAs. After well mixing, the mixtures were allowed to separate into three layers with the FFA as the top layer, the precipitate (salt) in the middle, and purified glycerol in the bottom. The FFA and purified glycerol were collected and stored, respectively, for further utilization.

### 2.4. Lipids production with glycerol

Crude and purified glycerol (obtained by removing soap) was used as carbon source to cultivate oleaginous microorganism *T. oleaginosus* for lipid production. Prior to utilization, they were sterilized at 121 °C for 15 min to remove methanol due to the concern on that methanol could inhibit the growth of *T. oleaginosus*.

The pre-culture was prepared by inoculating a loopful of *T. oleaginosus* in the sterilized media containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. After 24 h incubation, the pre-culture was transferred to the sterilized fermentation medium containing (per liter): 2.7 KH<sub>2</sub>PO<sub>4</sub>, 0.95 Na<sub>2</sub>HPO<sub>4</sub>, 0.404 NH<sub>4</sub>Cl, 0.2 MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g yeast extract, 0.1 EDTA, 0.04 CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.0055 FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.0052 citric acid·H<sub>2</sub>O, 0.001 ZnSO<sub>4</sub>·7H<sub>2</sub>O, and 0.00076 MnSO<sub>4</sub>·H<sub>2</sub>O and 25 g crude, purified, or pure (Certified ACS, Fisher Scientific) glycerol (Meesters et al., 1996; Zheng et al., 2012). The pH of the medium was adjusted to 6.5 and then sterilized at 121 °C for 15 min prior to inoculation. The fermentation was performed in shake flasks under aerobic conditions. The incubation was performed at 28 °C with 170 rpm.

To study glycerol concentration effect on lipid accumulation of *T. oleaginosus*, the purified glycerol was used and the investigated concentration was 25, 50, 75, and 100 g/L. The fermentation was conducted similarly as that with different type of glycerol (crude, purified, and pure). The maximum lipid accumulation of *T. oleaginosus* normally was at 48–96 h, thus the fermentation was conducted for 120 h (Gong et al., 2016; Seo et al., 2013).

### 2.5. Residual glycerol analysis

The residual glycerol after fermentation was analyzed with Bondioli and Bella method as described above.

### 2.6. Lipid extraction from yeast biomass

The standard chloroform and methanol extraction procedure with minor modification was employed to determine the lipid content in the biomass (Folch et al., 1957; Vicente et al., 2009). Biomass was harvested from the fermented broth by centrifugation at 5000 rpm for 15 min followed by 2 times washing with distilled water, and then dried by lyophilisation. The extraction was performed as following: the M (around 200) mg lyophilized biomass was mixed with 4 mL solvent containing chloroform and methanol (2:1 v/v); the solution was subjected to 60 °C for 4 h; the mixture was then centrifuged at 5000 rpm for 15 min and the supernatant (solvent phase) was withdrawn and transferred into a pre-weighed glass vial (W<sub>6</sub>); the procedure was repeated for the second time extraction, and the supernatant was transferred to the same vial (the pre-weighed glass vial). The vial containing the supernatant obtained from the two extractions was put in the oven set at temperature of 60 °C to evaporate the solvents. It was considered that the evaporation was complete when the weight of the vial stopped changing, and the weight (W<sub>7</sub>) was noted down. The lipid amount was calculated by the difference of W<sub>6</sub> and W<sub>7</sub>. The lipid content in the biomass of *T. oleaginosus* was calculated as: (W<sub>7</sub>-W<sub>6</sub>)/M × 100%, where M was the biomass amount used in the extraction (around 200 mg in this study). The obtained lipid was then converted to biodiesel through trans-esterification.

### 2.7. Free fatty acid content in lipids extracted from biomass

The FFA content was determined with titration method (Woyewoda et al., 1976). Samples collected at 48 h fermentation were used to determine FFA content in lipids. The lipid was extracted as described before on Section 2.6. The extracted lipids was dissolved in 5 mL hexane and transferred to a 100 mL conical flask. Hexane was then evaporated at 60 °C. The 10 mL of chloroform: methanol 2:1 v/v mixture was added to the conicals flask with and without (blank) lipids, respectively, and then two drops

of phenolphthalein was added, respectively as well. The 0.01 N KOH filled in 25 mL burette was added to the conical flasks drop by drop with gentle agitation. The titration was ended when a pink colour was observed and persisted at least for 5 s. The volume of KOH used was recorded to calculate the FFA content using Eq. (1).

$$\text{FFA content as oleic acid}(\%) = 28.2 \times N \times (V - B) / W_{\text{lipid}} \times 100\% \quad (1)$$

where V = the volume (mL) of titration solution; B = the volume (mL) of the blank; N = the normality of the titration solution (KOH);  $W_{\text{lipid}}$  = the weight of the oil sample (grams).

### 2.8. Esterification of FFAs and trans-esterification of lipids

The FFA obtained from soap (as described above) were converted to fatty acids methyl esters (FAMES, biodiesel) by reacting with methanol in the absence or presence of acid. The 5 mL of acidic (sulfuric acid 2% v/v in methanol) methanol was added to 0.2 g of FFA. The mixture was then heated to 50 °C for 24 h. The 1,3-dichlorobenzene was used as internal standard. After reaction (24 h), the 5% NaCl solution was added (100 mL per gram of lipids), and then FAMES was extracted by washing two times with hexane (100 mL per gram of lipid), and the upper hexane layer was recovered by phase separation. The FAMES in hexane was washed with 2% sodium bicarbonate (20 mL per gram lipid), and the top layer was dried in oven at 60 °C (Halim et al., 2011).

The lipids obtained by solvent extraction from *T. oleaginosus* in vials was first dissolved in hexane (5 mL); then mixed with methanol. Lipid to methanol molar ratio was 1:6 (0.3 mL methanol for per gram lipid). Sodium hydroxide (0.5% w/w oil) was used as catalyst. The mixture was then subjected to 55 °C in oil bath for 2 h. The 1,3-dichlorobenzene was used as internal standard with a concentration of 50 ppm. The procedure of FAMES recovery was similar as that of FAMES converted from FFA.

The FAMES in hexane were analyzed using a Gas Chromatography coupled with Mass Spectroscopy (GC-MS) (Perkin Elmer, Clarus 500) as described above for biodiesel content determination.

All the experiments were performed in triplicate and average results were reported with standard deviation less than 5%.

## 3. Results and discussion

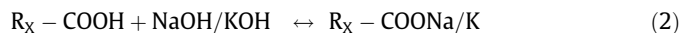
### 3.1. Crude glycerol composition

The composition of crude glycerol was determined and the results were given in Table 1. It was observed that the crude glycerol has low glycerol content ( $31.8 \pm 0.3\%$  w/w) and high soap content ( $21.1 \pm 0.3\%$  w/w). It would be due to the high content of FFA in the feedstock of the alkaline catalytic biodiesel production. Soap could be an inhibitor of cell growth as it can attach on cells and

interfere to the nutrient transportation from fermentation medium to cell bodies. Therefore, soap removal was performed.

### 3.2. Free fatty acid recovery from soap

The high soap content in crude glycerol (Table 1) is due to presence of high concentration of FFA in the feedstock oil. In alkaline condition ( $\text{pH} > 7$ ), FFAs react with base (NaOH or KOH) to form soap (the equilibrium of the reaction of Eq. (2) is shifted to right). On the contrary, FFA will be released due to the dissociation of soap (the equilibrium of the reaction of Eq. (2) is shifted to left) at low pH (FFA recovery process by lowering pH of the crude glycerol).



where  $R_x$  presented  $C_nH_{2n+1}$  for saturated and  $C_nH_{(2(n-m)+1)}$  for unsaturated ( $m = 1, 2, 3, \dots; n > m$ ).

To recover FFA, the pH of the crude glycerol was lowered to less than 7. It was observed that FFA stood in the top, purified glycerol was in the bottom, and the middle layer was the salt precipitates after pH adjustment. This observation was different from few other reports in the literature, which obtained the middle layer as glycerol and bottom layer as salt precipitates (Kelly, 2006; Swearingen, 2006). It could be due to the difference of the composition of the crude glycerol, which led to the variation in density of the precipitates. The results of FFAs recovery was shown in Table 2. There was 8.11 g of FFA in 40 mL crude glycerol (at pH 1). The phase separation of samples 2–10 with addition of 85%  $H_3PO_4$  started after 15 min settling, and completed at around 72 h. With addition of 1 mL of 85%  $H_3PO_4$  (sample 1), layer separation was not observed until 3 h. The FFA amount obtained from samples 2–10 was almost the same (Table 2), and there was only 1.32 g FFAs obtained in sample 1. Compared to other samples, sample 2 (2 mL of 85%  $H_3PO_4$  in 40 mL of crude glycerol) gave comparable FFA recovery efficiency (99.2% w/w) and highest concentration of glycerol (54.96% w/w) with shortest settling time (36 h). Therefore, 2 mL acid addition to 40 mL crude glycerol was considered the optimal acid requirement for FFA recovery when gravity settling was used for phase separation and thus to recover glycerol from its crude form.

### 3.3. Conversion of free fatty acids to biodiesel

As mentioned, FFA can consume alkaline catalyst to form soap; hence, acid catalyst should be used in the conversion of FFA to biodiesel (FAMES). In the process of FFA recovery from soap,  $H_3PO_4$  was added to lower the pH. It suggested that the recovered FFA contained the acid; therefore, the addition of acid in the esterification could be avoided. To investigate if it is still necessary to add the acid in the reaction to produce biodiesel from FFA, esterifications of FFA with and without  $H_2SO_4$  were conducted. The results showed that the FAMES yield (g FAMES/g FFAs) was 90.8% with  $H_2SO_4$  and 32.1% without  $H_2SO_4$ , respectively.

According to calculations, the  $H^+$  present in FFA is 0.49 mmol per 0.2 g FFA. 5 mL methanol was added to react with 0.2 g FFA; therefore, the  $H^+$  concentration in the system was 0.10 mol/L. With addition of acid,  $H^+$  concentration in the system was increased to 0.75 mol/L. Esterification is a reversible reaction and  $H^+$  concentration determines the direction of reaction at equilibrium and thus affecting the rate of reaction or rate of conversion. The low conversion rate of FFAs to biodiesel in the esterification reaction without acid addition would be due to the low concentration of  $H^+$ . In order to determine the ideal reaction time, experiment was prolonged from 24 h to 48 and 72 h, as a result the biodiesel yield (g FAMES/g FFA added) was increased from 32.1% to 38.7% and 39.1%, respectively. This suggested that the reaction reached its

**Table 1**  
Composition of crude glycerol and purified glycerol.

Items	Crude glycerol	Purified glycerol
Glycerol content (% w/w)	$31.8 \pm 0.3$	$55.0 \pm 0.2$
Soap content (% w/w)	$21.1 \pm 0.3$	0.0
Catalyst content (NaOH) (% w/w)	$2.8 \pm 0.2$	0.0
Biodiesel content (% w/w)	$1.2 \pm 0.0$	$1.5 \pm 0.0$
Ash (% w/w)	$2.3 \pm 0.1$	$4.2 \pm 0.2$
Methanol (% w/w)	$15.3 \pm 0.3$	$18.5 \pm 0.6$
Water (% w/w)	$24.4 \pm 0.2$	$20.8 \pm 0.8$
pH	$8.93 \pm 0.04$	$3.93 \pm 0.25$
Density (g/mL)	$1.073 \pm 0.06$	$1.101 \pm 0.03$

**Table 2**  
Free fatty acid recovery from crude glycerol.

Sample	Crude glycerol (mL)	Acid (H <sub>3</sub> PO <sub>4</sub> ) addition amount (mL)	pH	FFA (g)	FFA recovery efficiency (%)	Glycerol content (% w/w)
1	40	1	6.60	1.32	5.5	43.11 ± 0.41
2	40	2	3.93	8.04	99.2	54.96 ± 0.24
3	40	3	3.55	8.04	99.2	53.66 ± 0.38
4	40	4	3.22	8.05	99.3	52.49 ± 0.16
5	40	5	3.16	8.06	99.4	51.34 ± 0.25
6	40	6	3.14	8.08	99.7	50.25 ± 0.22
7	40	7	2.97	8.09	99.8	49.26 ± 0.19
8	40	8	2.76	8.09	99.8	48.33 ± 0.36
9	40	9	2.74	8.09	99.8	47.12 ± 0.22
10	40	10	2.70	8.09	99.8	46.18 ± 0.20

equilibrium in about 48 h under the condition of addition of 5 mL methanol per 0.2 g FFAs, catalyst amount of 0.1 mol H<sup>+</sup>/L, and reaction temperature of 50 °C. Even though, the biodiesel yield increase to 38.7% after 48 h reaction, but it is much lower than that of the esterification with acid addition after only 24 h reaction, which was 90.8%. It indicates that acid addition as catalyst is still required in order to make the process acceptable in practice when considering for utilization of the recovered FFA from crude glycerol for biodiesel production.

The obtained biodiesel contains mainly C18 and C16 with little amount of C14. In the reaction without acid (as catalyst) addition, FAMES consists of 58.3% C18, 37.1% C16, and 2.9% C14, and the saturation fatty acid degree was 56.4%. In case with acid (as catalyst) addition, FAMES contains 62.5% C18, 27.9% C16, and 0.7% C14 with saturation fatty acid degree of 66.2%. FAMES produced from FFA are similar as biodiesel produced from palm oil (55–68% C18 and 32–45% C16 with saturation fatty acid degree of 50–55%) (Ekpa et al., 1994; Verma et al., 2016). It suggested that the FAMES generated are suitable to use as biodiesel.

#### 3.4. Effect of glycerol type on the biomass and lipid production

*T. oleaginosus* could use crude glycerol but high glycerol concentration would inhibit cell growth (Liang et al., 2010a; Meesters et al., 1996). The optimal glycerol concentration for cell growth was between 20 and 40 g/L (Liang et al., 2010a; Meesters et al., 1996). In fact, carbon to nitrogen (C/N) ratio has great impact on lipid accumulation. As reported, the optimal C/N ratio for *T. oleaginosus* growth and lipid production was about 30 (Hassan et al., 1996; Liang et al., 2010a; Ryu et al., 2013). The aim of the study is to produce lipid with glycerol. To investigate glycerol type (crude glycerol, purified glycerol and pure glycerol) effect on cell growth and lipid production, the glycerol concentration of 25 g/L was employed in the work as it is between 20 and 40 g/L as well as made that the C/N ratio of the medium was around 30 (N = 0.3 g/L) (Ryu et al., 2013).

Pure, purified, and crude glycerol were used as carbon source for lipid production from *T. oleaginosus*. A lag period was observed in biomass growth (Fig. 2), which could be due to the fact that the inocula were not grown in the similar composition medium as the pre-culture medium, and thus an adaptation period may be required by the strain when transferred to the new medium (Gong et al., 2016; Xu et al., 2012). In order to minimize the lag period, the inocula should be produced in the similar medium as the production medium.

The fermentations continued 120 h, and samples were taken for determining glycerol, biomass and lipid. The glycerol was completely consumed within 72 h fermentation and the biomass concentration and lipid content rapidly increased during the period for the case of pure and purified glycerol (Fig. 2). The biomass concentration of pure and purified glycerol fermentation reached the

highest at 72 h, which were 10.90 g/L for pure glycerol and 10.32 g/L for purified glycerol. In crude glycerol fermentation, the highest biomass concentration (7.58 g/L) also took place at 72 h. The highest maximum specific growth rate ( $\mu_{max}$ ) occurred in the fermentation with pure glycerol (0.036 h<sup>-1</sup>) as raw material followed by 25 g/L purified glycerol (0.034 h<sup>-1</sup>) and crude glycerol (0.025 h<sup>-1</sup>) (Table 3).

The pure glycerol was slightly better than purified glycerol in terms of biomass production and maximum specific growth rate. Unlike pure glycerol, methanol is present in the purified glycerol. However, it wouldn't be the cause of the slightly low biomass concentration observed in purified glycerol (10.32 g/L) comparing to that in pure glycerol (10.90 g/L) cultivation. The boiling point of methanol is 65 °C and sterilization of the medium by autoclaving at 121 °C for 15 min will eliminate the methanol from the medium (Pyle et al., 2008). In fact, purified glycerol also contains metals and others impurities which are derived from feedstock oil or from the chemicals that are added during the biodiesel production process. It was reported that metals had negative impact on cell growth (Liang et al., 2010a; Pyle et al., 2008). Hence, the impurities in purified glycerol may inhibit biomass growth and lead to lower biomass density than that in pure glycerol (Capone et al., 1983; Polburee et al., 2015).

Compared to the biomass concentration in the fermentation with purified and pure glycerol at 72 h, crude glycerol showed inhibition on cell growth. Similar trend was reported by other researchers (Cerón-García et al., 2013; Liang et al., 2010a; Polburee et al., 2015). In crude glycerol, soap content was around 21% w/w. Both the soap and the cell surface are polar, and thus the soap could easily attach to the cell's surface, which can cause the inhibition of the nutrients transfer (Athalye et al., 2009; Sarma et al., 2014). The purified glycerol was obtained from crude glycerol after soap removal. This would be the reason of the higher biomass density observed in the purified glycerol than that in crude glycerol (Table 3).

During fermentation, lipid was rapidly accumulated in the cells from 0 to 72 h and the maximum lipid content was observed at 72 h, which were 49% for pure glycerol and 44% for purified glycerol, respectively, (Fig. 2). After the glycerol consumed up, biomass concentration and lipid content gradually went down which would be due to that lipid were degraded by cells as energy for maintaining their activities. In the cultivation with the initial crude glycerol concentration of 25 g/L, there was still glycerol left unused at the end of the fermentation (120 h). During fermentation with crude glycerol, the pH of the broth was gradually decreasing from 6.5 to 5.1. As the pH of the fermentation broth went down, soap in the crude glycerol could be converted to FFA. However, the soap and FFA amount in the sample were low (both were less than 0.08 g in 5 mL), and thus they were not detected during analysis. According to other report, *T. oleaginosus* was capable of assimilate FFA (Yang et al., 2015), and thus the strain might start to utilize FFA

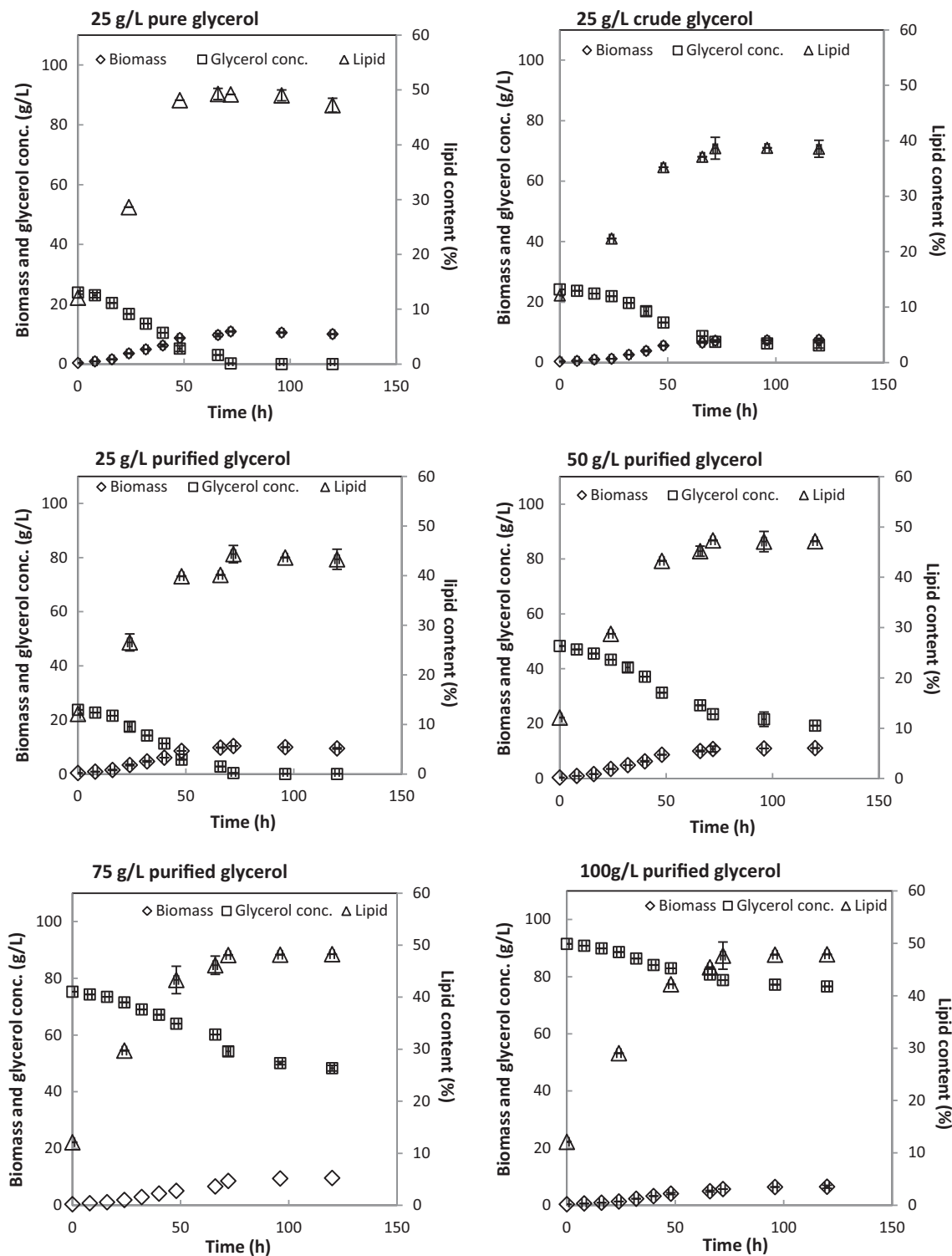


Fig. 2. Biomass and glycerol concentration changing with time for different glycerol concentration; data are means of three replicates with error bars indicating standard deviations.

as carbon source as well, which led to the glycerol consumption became slowly.

From 72 to 120 h, biomass concentration and lipid content declined (in pure and purified glycerol fermentation) or kept stable (in crude glycerol fermentation), which suggested that the fermentation could be stopped at 72 h in order to avoid lipid consumption and waste energy (Fig. 2). The lipid yield and lipid productivity

were 0.22 g lipid/g glycerol and 1.79 g/L/d for pure glycerol, 0.18 g lipid/g glycerol and 1.52 g/L/d for purified glycerol, and 0.15 g lipid/g glycerol and 0.97 g/L/d for crude glycerol, respectively (Table 3). Biomass and lipid yields reveal the carbon utilization efficiency by microbes. The biomass ( $Y_{x/c}$ ) and lipid ( $Y_{l/c}$ ) yields of *T. oleaginosus* while grown in different types of glycerol at 25 g/L concentration displayed the following trend, pure

**Table 3**  
Growth and lipids production parameters for *T. oleaginosus* grown in different types of glycerol.

Glycerol type	Gly <sub>0</sub> (g/L)	C/N ratio	Time (h)	Gly <sub>t</sub> (g/L)	X (g/L)	P <sub>X/N-T</sub> (g/L-d)	L (g/L)	P <sub>L/N-T</sub> (g/L-d)	μ (h)	Y <sub>L/X</sub> (g/g)	Y <sub>X/G</sub> (g/g)	Y <sub>L/G</sub> (g/g)
Pure glycerol	25	90	72	0.17 ± 0.04	10.90 ± 0.07	3.63	5.36 ± 0.03	1.79	0.036	0.49 ± 0.05	0.44 ± 0.01	0.22 ± 0.00
Crude glycerol	25	90	72	5.66 ± 0.91	7.58 ± 0.44	2.53	2.92 ± 0.01	0.97	0.025	0.39 ± 0.01	0.39 ± 0.03	0.15 ± 0.01
Purified glycerol	25	90	72	0.33 ± 0.02	10.32 ± 0.05	3.44	4.57 ± 0.05	1.52	0.034	0.44 ± 0.01	0.42 ± 0.02	0.18 ± 0.00
	50	180	72	22.25 ± 0.05	10.75 ± 0.02	3.69	5.24 ± 0.02	1.74	0.035	0.47 ± 0.02	0.40 ± 0.02	0.19 ± 0.00
	75	270	72	49.31 ± 0.99	9.61 ± 0.5	3.20	4.63 ± 0.00	1.54	0.021	0.48 ± 0.01	0.37 ± 0.02	0.18 ± 0.02
	100	360	72	76.59 ± 0.05	6.48 ± 0.03	2.16	3.10 ± 0.03	1.03	0.019	0.48 ± 0.01	0.28 ± 0.01	0.13 ± 0.00

Representation of biomass (X, g/L), lipid (L, g/L), and initial/final glycerol (Gly<sub>0</sub>/Gly<sub>t</sub>, g/L) concentrations at 72 h fermentation at maximum concentration of lipid was achieved. Y<sub>L/X</sub>, g/g-yield of lipids with respect to dry biomass, P<sub>L/N-T</sub>-lipid productivity, Y<sub>X/G</sub>, g/g -biomass yield with respect to glycerol consumed, P<sub>X/N-T</sub>-biomass productivity, Y<sub>L/G</sub>, g/g -lipid produced per glycerol consumed, μ -specific growth rate, and consumed glycerol values are presented for all trials. Culture conditions: growth on 500 ml flasks at 170 rpm and T = 28 °C with initial pH 6.5 ± 0.1.

glycerol > purified glycerol > crude glycerol (Table 3). Similar trend was also reported by other researchers: Liang et al. (2010a) found that pure glycerol as carbon source was the superior to crude glycerol and purified glycerol in terms of lipid accumulation in *T. oleaginosus*, and Papanikolaou and Aggelis (2002) also reported that pure glycerol was better than the purified and crude glycerol as carbon source for lipid accumulation in *Y. lipolytica* (an oleaginous yeast) (Liang et al., 2010b; Papanikolaou and Aggelis, 2002). As the strain employed in the fermentations were the same, it indicates that pure glycerol as carbon for lipid production was better than the purified one, and the purified one was better than the crude glycerol. However, the lipid productivity showed that the purified glycerol had similar performance as the pure glycerol, and much superior than the crude glycerol. It indicates that the purified glycerol would be promising carbon source for lipid production from *T. oleaginosus*.

### 3.5. Effect of glycerol concentration on the biomass and lipid production

Purified glycerol was superior on biomass and lipid production by *T. oleaginosus* compared to crude glycerol as carbon source (Table 3); therefore, purified glycerol was further used to investigate glycerol concentration effect on the biomass and lipid production from *T. oleaginosus*. With glycerol concentration of 25 g/L, the glycerol was completely consumed with 72 h in purified glycerol fermentation, and biomass concentration and lipid content started to drop after 72 h. In order to enhance biomass and lipid production and prevent the inhibition on cell growth and lipid accumulation that could be caused due to the low carbon concentration, glycerol concentration of 50, 75, and 100 g/L were studied.

The biomass concentration increased with fermentation time proceeding at glycerol concentration of 25, 50, 75, and 100 g/L (Fig. 2). Increase in glycerol concentration from 25 to 50 g/L, the maximum biomass concentration slightly increased, while further increase in glycerol concentration the biomass concentration was significantly decreased (Table 3). Biomass yield decreased with the increase of the purified glycerol concentration, and the highest value was 0.42 g/g glycerol consumed at 25 g/L and the lowest was 0.28 g/g glycerol consumed at 100 g/L glycerol concentration. A decrease in biomass concentration at high glycerol concentration was due to substrate inhibition. Similar results have been reported by other researchers (Meesters et al., 1996). In their study, the growth of *Cryptococcus curvatus* (the previous name of *T. oleaginosus*) was restricted when the concentration of glycerol was higher than 64 g/L. An inhibitory impact of high glycerol concentration (>60 g/L) on growth of *Schizochytrium limacinum* (oleaginous microalgae) was also observed (Liang et al., 2010b). On the other hand, oleaginous yeast *Yarrowia lipolytica* was not influenced

by glycerol concentration in the range from 20 to 164 g/L (Papanikolaou and Aggelis, 2002). For microalgae cultivation, the maximum biomass density (around 14 g/L) was obtained at a wide glycerol concentration range (from 35 to 85 g/L) (Liang et al., 2010a). Glycerol concentration effect on biomass production occurs not only with respect to different types of microorganisms (microalgae or yeast) used to cultivate, but also in the same types of microorganisms. It indicates that each microorganism has their own feature in utilization of glycerol. The maximum biomass density (around 10.75 g/L) was obtained at glycerol concentration of 50 g/L after 72 h fermentation, which was similar as that in the fermentation with 25 g/L pure glycerol (10.90 g/L) or 25 g/L of purified glycerol (10.32 g/L) in 72 h. It indicated that increase in glycerol concentration did not appreciably increase the biomass concentration.

The lipid yield were almost the same in the fermentation with 25 (0.18 g lipid/g glycerol), 50 (0.19 g lipid/g glycerol), and 75 (0.18 g lipid/g glycerol) g/L glycerol concentration, but it was substantially decreased to 0.13 g/g after glycerol concentration increased to 100 g/L (Table 3). The lipid productivity was 1.52 g/L/d for 25 g/L, 1.74 g/L/d for 50 g/L, 1.54 g/L/d for 75 g/L, and 1.03 g/L/d for 100 g/L, respectively. The results were different from reported by other researchers, in which glycerol concentration from 90 to 100 g/L gave the highest lipid yield and productivity (Kitcha and Cheirsilp, 2011; Yen et al., 2012). The difference could be due to the difference of the strains employed and the composition of the glycerol utilized (Polburee et al., 2015; Saenge et al., 2011; Yen et al., 2012). It was expected that the lipid yield would be enhanced with the increase of carbon concentration; however, the results obtained in the study showed that high carbon concentration (75 and 100 g/L glycerol) has inhibited the lipid production. It was observed that the glycerol consumption became slow even though there was still plenty of glycerol left in the medium, which was 23.43 g/L and 54.23 g/L in the fermentation with 50 g/L and 75 g/L glycerol concentration, respectively. This may be due to the fact that inhibitors (such as toxic protein or ethanol) maybe produced along with cell growth (Nevoigt, 2008). In order to eliminate the inhibition problem, fed-batch process approach can be adopted instead of the batch process. The concentrations of inhibitors are diluted during feeding process of a fed-batch culture. Some researchers have reported that very high biomass concentration (more than 100 g/L) were achieved in fed batch fermentation (Meesters et al., 1996).

In this study, it was found that the purified glycerol has great potential for lipid production from microbe. The lipid yield of different strains cultivated in glycerol medium by different researchers was summarized in Table 4. It clearly displayed that the lipid yield obtained in this study was comparable with other studies. Thus, purified glycerol could be utilized as a carbon source for lipid

**Table 4**  
Glycerol concentration effect on lipid accumulation.

Strains	Glycerol type	Optimal glycerol conc. (g/L)	Lipid yield (g/g glycerol)	References
<i>Aspergillus niger</i> LFMB1	Crude glycerol	60	0.20	(André et al., 2009)
<i>Aspergillus niger</i> NRRL 364	Crude glycerol	60	0.21	(André et al., 2009)
<i>Schizochytrium limacinum</i>	Purified glycerol	35	0.26	(Liang et al., 2010b)
<i>Kodamaea ohmeri</i>	Crude glycerol	100	0.20	(Kitcha and Cheirsilp 2011)
<i>Trichosporonoides spathulata</i>	Crude glycerol	100	0.18	(Kitcha and Cheirsilp 2011)
<i>Rhodotorula</i> sp. LFMB 22	Crude glycerol	30	0.10	(Chatzifragkou et al., 2011)
<i>Chlorella protothecoides</i>	Crude glycerol	30	0.33	(O'Grady and Morgan 2011)
<i>Rhodotorula glutinis</i>	Crude glycerol	100	0.10	(Saenge et al., 2011)
<i>Rhodospiridium toruloides</i>	Crude glycerol	50	0.15	(Xu et al., 2012)
<i>Rhodotorula glutinis</i>	Crude glycerol	90	0.13	(Yen et al., 2012)
<i>Trichosporon oleaginosus</i>	Purified glycerol	50	0.19	This study

production by *T. oleaginosus* with the concentration between 25 and 50 g/L.

### 3.6. Fatty acid profile of *T. oleaginosus* biomass extracted lipid

The FFAs content were 0.44%, 1.19%, 0.46%, 0.44%, 0.43%, and 0.44% w/w on lipids derived from *T. oleaginosus* obtained after 72 h cultivation with 25 g/L pure glycerol, 25 g/L crude glycerol, and 25, 50, 75 and 100 g/L purified glycerol, respectively. The FFA content obtained in this study (less than 1.2% w/w lipids) was significantly different from those (9% w/w lipids) observed by other researchers (Thiru et al., 2011). This would be due to the different treatment of biomass. In this study, the wet biomass was dried by lyophilisation, which preserved the nature of the lipids, and then lipids were extracted with solvent. The extracted fresh lipid was directly used to determine FFA content without storage, which prevented the risk of triacylglycerol (TAG) decomposition to FFAs. In the study of Thiru et al. (2011), the fermented broth was homogenized (the risk of degradation) followed by solvent extraction of lipid from wet yeast cells with no indication if fresh lipid was used for FFA content determination. A study has reported that storage of microbial lipid above 0 °C for 24 h could increase the FFA content from less than 0.1–20% w/w lipid and decrease the TAG content from 72 to 51% w/w lipid (Chen et al., 2012). It clearly indicated that TAG was degraded to FFA during storage. Therefore, the high FFA content observation in other study would be due to the storage of the biomass which resulted in TAG degradation to FFA. While in our study, fresh biomass (without storage) was used in the lipid extraction.

Comparing the composition of lipid obtained using different types of glycerol, relatively high FFA content was found when crude glycerol (1.19% w/w total lipid) was used as raw material. This could be due to the presence of FFA in crude glycerol. As discussed above, FFA existed in the crude glycerol medium due to soap dissociation at pH 6.5 (Table 2); therefore, FFA could attach onto the cell surface. During cell harvesting, washing was performed twice with distilled water, yet FFA would not be dissolved in water and would remain stick to the cell surface. Thereafter, the FFA attached on the surface of cells could be extracted along with the lipid during organic solvent extraction process. Thus, the FFA content of the biomass obtained from the cultivation with crude glycerol was higher compared to that with purified and pure glycerol. The FFA content of lipid extracted from biomass grown on pure and purified glycerol was almost the same, and the purified glycerol concentration (25–100 g/L) didn't impact the FFA content (0.46%, 0.44%, 0.43%, and 0.44% w/w total lipid for 25, 50, 75 and 100 g/L purified glycerol, respectively). For all extracted lipids irrespective of glycerol type used to grow the biomass, the FFA content was lower than 2% w/w lipid, hence alkaline NaOH could be used as catalyst in the trans-esterification process.

The fatty acid profile of the lipid extracted from biomass was shown in Table 5. The majority of fatty acids were C16:0 and C18:1, which was similar to *Jatropha* seed oil (currently used in commercial biodiesel production practice in some countries). It suggests that the lipid from *T. oleaginosus* cultivated with glycerol is suitable in usage as biodiesel production feedstock. The saturation degree (the sum of Cn:0) of the lipid was around 30–40% w/w total lipid. It reveals that the biodiesel produced from the lipid derived from purified glycerol had high oxidation stability.

### 3.7. The productivity of the proposed process

In this study, the crude glycerol generated from biodiesel production was used to produce biodiesel. There were two parts on biodiesel production: (1) the FFA recovered from crude glycerol purification step was converted to biodiesel through esterification; (2) the purified crude glycerol was used as carbon for oleaginous yeast cultivation, and the lipid accumulated in the yeast was then transferred to biodiesel. Based on the results (Table 2), 8.04 g of FFA could be recovered from 40 mL of crude glycerol, and the biodiesel yield was 90.8% with acid as catalyst after 24 h esterification of FFA. It suggested that 182.51 g of biodiesel can be generated from FFA recovered from 1 L of crude glycerol. The 1 kg crude glycerol contains 318 g glycerol (Table 1), accordingly, 1 L crude glycerol will contain 341.21 g glycerol as the density of crude glycerol is 1.073 kg/L. The fermentation studies revealed that the lipid yield from glycerol could reach 0.19 g lipid/g glycerol after 72 h (Tables 3 and 4), hence, the biodiesel obtained from fermentation with glycerol as carbon would be around 63.53 g (=0.19 × 341.21 × 98%; 98% is the trans-esterification efficiency according to our study). Overall, in this study, the 1 L crude glycerol could produce 246.04 g biodiesel within 72 h which includes 182.51 g biodiesel from recovered FFA from crude glycerol (24 h) and 63.53 g biodiesel from glycerol fermentation (72 h). The first run of the process would take around 150 h (including crude glycerol purification, fermentation, and trans-esterification). However, it would be only 72 h required in long term operation as fermentation is the limiting step in time demand and other part involving can be simultaneously performed during fermentation.

To the best of our knowledge, so far, the highest lipid yield from glycerol reported was 0.33 g lipid/g glycerol after 72 h fermentation (Table 4). It suggests that 1 L crude glycerol could maximum produce 110.35 g biodiesel from fermentation when considering that each step from fermentation to biodiesel formed all performs under the optimal condition. It is still lower than the biodiesel productivity from crude glycerol reported in this study, which could produce 246.04 g biodiesel from 1 L crude glycerol within 72 h. It clearly indicates that the process in this study significantly enlarged crude glycerol to biodiesel productivity comparing that of using crude glycerol only for fermentation without recovering FFA and converting it to biodiesel.



**Table 5**  
Fatty acid profile of lipid from *T. oleaginosus*.

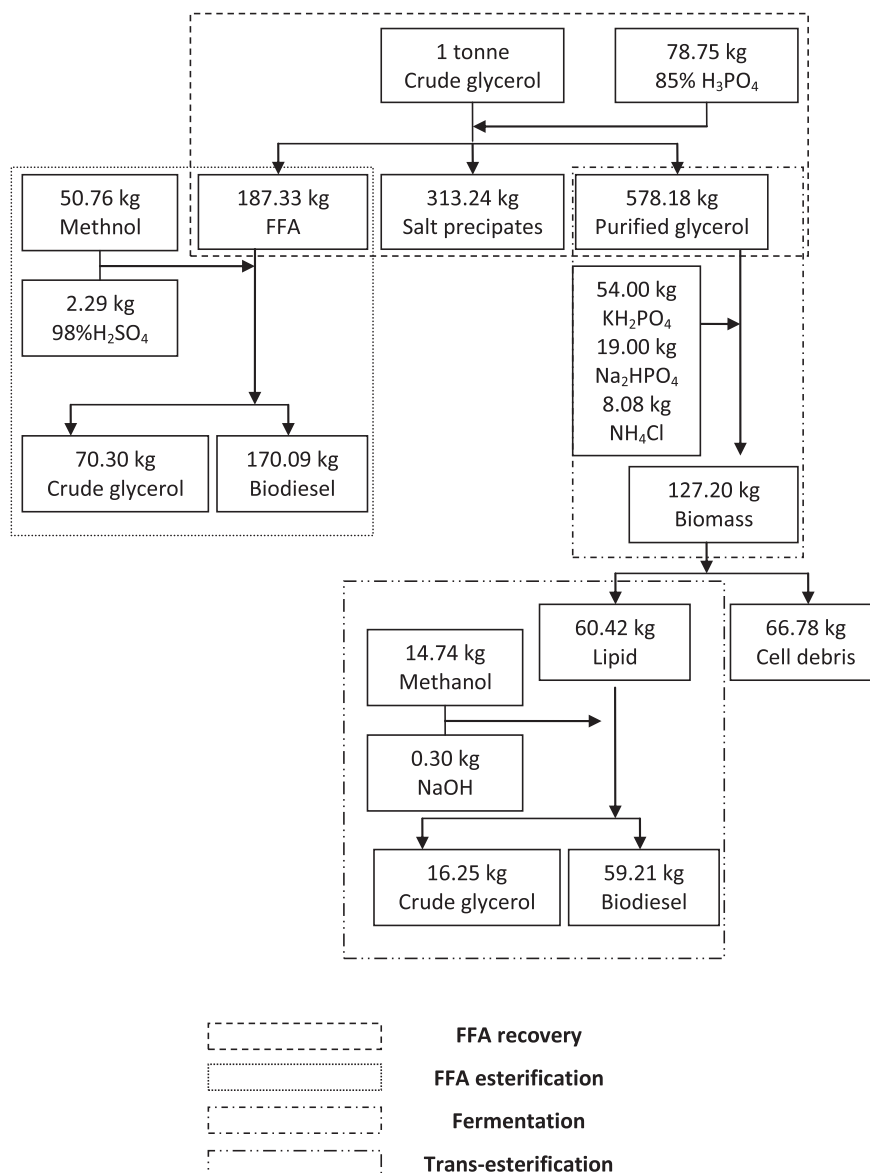
Fatty acid	Relative amount of total fatty acids (% w/w)							<i>Jatropha</i> seed oil <sup>a</sup>
	Lipid from Pure glycerol (25 g/L)	Lipid from crude glycerol (25 g/L)	Lipid from purified glycerol (25 g/L)	Lipid from purified glycerol (50 g/L)	Lipid from purified glycerol (75 g/L)	Lipid from purified glycerol (100 g/L)		
C14:0	0.1	2.2	0.1	0.3	0.1	0.1	0.1	–
C15:0	0.9	0.2	0.6	0.5	0.8	0.7	0.7	–
C16:0	19.3	22.6	20.1	21.0	20.7	20.2	20.2	12–20
C16:1	1.0	0.6	0.8	0.5	0.9	0.7	0.7	–
C18:0	13.4	19.5	15.2	14.9	15.0	14.2	14.2	5.0–10
C18:1	50.6	46.7	49.7	50.2	50.6	50.6	50.6	37–63
C18:2	7.5	5.8	8.6	7.9	7.2	7.8	7.8	10–19
C18:3	0.39	0.42	0.33	0.40	0.51	0.39	0.39	–
C20:0	1.1	0.5	0.8	0.6	0.6	0.8	0.8	–

<sup>a</sup> (Wassner et al., 2016).

### 3.8. Mass and energy balance of the proposed process

Conversion of crude glycerol to biodiesel is aimed to produce extra energy to the biodiesel production. In this study, the bio-

diesel was produced from two parts including esterification of the FFA recovered from crude glycerol and trans-esterification of lipid accumulated in *T. oleaginosus* cultivated with the purified glycerol (after FFA recovery) (Fig. 1). Though, energy (biodiesel)



**Fig. 3.** The mass balance of biodiesel production from crude glycerol.

was produced from the crude glycerol, energy was also consumed as the esterification, trans-esterification, and lipid production from the purified glycerol derived from crude glycerol, were energy consumption processes. The energy balance (=energy produced – energy consumed) of biodiesel production from crude

glycerol is an important indicator to evaluate if the process is practically feasible. The energy balance was calculated based on 1 tonne of crude glycerol used to produce biodiesel. The mass and energy balance was shown in Fig. 3 and Table 6, respectively.

**Table 6**  
Energy balance of biodiesel production from crude glycerol.

Process		Unit energy	Mass amount	Energy required (MJ)
Crude glycerol treatment				
Energy input	H <sub>3</sub> PO <sub>4</sub> (MJ)	5.30 MJ/kg	78.75 kg	417.38
	Crude glycerol	0.00 MJ/kg	1000.00 kg	0.00
Subtotal (MJ)				417.38
FFA to biodiesel				
<i>Esterification</i>				
Energy input	Methanol	20.00 MJ/kg	50.77 kg	1015.30
	H <sub>2</sub> SO <sub>4</sub> (MJ)	7.1 MJ/kg	2.30 kg	16.31
	Mixing (MJ)	0.03 kWh/kg	5.10 kWh	18.37
	Heating (MJ)	0.24 MJ/kg	170.09 kg	40.82
		biodiesel	biodiesel	
Subtotal (MJ)				1090.80
<i>Purification</i>				
Energy input	Distillation	313.5 kJ/kg	170.09 kg	53.32
		biodiesel	biodiesel	
Subtotal (MJ)				53.32
Purified glycerol to biodiesel				
<i>Fermentation</i>				
Energy input (sterilization: 0.11 kg steam/m <sup>3</sup> with 80% energy recovery, energy content of steam 26 MJ/kg steam; the lasting period of agitation and aeration was 72 h)	Total volume (m <sup>3</sup> )		6.36	
	Sterilization	26.00 MJ/kg (80% energy recovery)	0.70 kg	18.19
	KH <sub>2</sub> PO <sub>4</sub>	10.30 MJ/kg	54.00 kg	556.20
	Na <sub>2</sub> HPO <sub>4</sub>	8.21 MJ/kg	19.00 kg	155.99
	NH <sub>4</sub> Cl	8.64 MJ/kg	8.08 kg	69.81
	Water	0.04 MJ/kg	5.78	0.23
	Agitation	7.30 W/m <sup>3</sup>	3.34kwh	12.03
	Aeration, 0.5 vvm	1 kW/m <sup>3</sup>	457.92	1648.51
			kwh	
Subtotal (MJ)				2460.96
Inoculation (5% of total fermentation) (MJ)				123.05
<i>Harvesting</i>				
Energy input	Centrifugation (MJ)	1.00 kWh/m <sup>3</sup>	6.36 kWh	22.90
Subtotal (MJ)				22.90
<i>Lipid extraction</i>				
Energy input (the extraction volume was 2.54 m <sup>3</sup> , and performed at 60 °C for 4 h)	Chloroform	7.63 MJ/kg	0.85 L	17.47
	Methanol	20.00 MJ/kg	0.42 L	6.72
	Heat	2.72 kW/m <sup>3</sup>	27.68 kWh	99.64
	Chloroform and methanol recovery (G)	19.50 kWh/tonne		368.61
Subtotal (MJ)				492.44
<i>Transesterification</i>				
Energy input	Methanol (MJ)	20.00 MJ/kg	14.73 kg	294.73
	NaOH (MJ)	18.50 MJ/kg	0.30 kg	5.59
	Mixing (MJ)	0.03 kWh/kg	1.78 kwh	6.39
	Heating (MJ)	0.24 MJ/kg	59.21 kg	14.21
		biodiesel	biodiesel	
Subtotal (MJ)				344.59
<i>Purification</i>				
Energy input	Distillation	313.5 kJ/kg	59.21 kg	18.56
		biodiesel	biodiesel	
Subtotal (MJ)				18.56
Total input				5024.00
Energy output (MJ)	Biodiesel	37.8 MJ/kg	229.30 kg	8667.65
		biodiesel		
	Crude glycerol	0 MJ/kg	86.54 kg	0.00
	Cell debris	7.59 MJ/kg	66.78 kg	506.86
Total output				9174.51
Energy gain (MJ)				4150.51
Energy conversion efficiency (SUM:energy output/SUM:energy input)				1.83

From the mass balance calculation, it can be seen that 1 tonne of crude glycerol could produce 229.30 kg biodiesel, 86.54 kg crude glycerol, and 66.78 kg cell debris (Fig. 3). The energy balance was calculated based on the mass balance study. In the energy balance study, assumptions were made: (1) the biodiesel production from crude glycerol took place in the biodiesel production industry (where crude glycerol was generated), which suggested that there was no energy input from the transportation of crude glycerol; (2) the energy input from the utilization of crude glycerol was zero and the crude glycerol generated in esterification and trans-esterification was also zero; (3) methanol and H<sub>2</sub>SO<sub>4</sub> were not recovered after esterification and remained in crude glycerol; (4) the fermentation of *T. oleaginosus* for lipid production was conducted under aseptic condition, the power consumption was adopted from our previous study (Zhang et al., 2017); (5) the energy input in biomass harvesting, lipid extraction and trans-esterification was similarly estimated as utilized in our previous study (Zhang et al., 2017); (6) in the trans-esterification of lipid to biodiesel, methanol and NaOH were not recovered after the reaction; (7) the cell debris was assumed to be used as animal feed (Zhang et al., 2016).

The energy input during crude glycerol treatment namely FFA recovery was mainly from the utilization of 85% H<sub>3</sub>PO<sub>4</sub>, which was 417.38 MJ. After FFA was separated from the crude glycerol, it was used to synthesize biodiesel through esterification with the addition of methanol and H<sub>2</sub>SO<sub>4</sub> under heating. The energy input was from the utilization of methanol, H<sub>2</sub>SO<sub>4</sub>, and energy to provide the heating, which was 1090.80 MJ. After biodiesel generation, distillation was performed to purify the product and thus 53.32 MJ of energy was consumed (Table 3).

In this study, the other part of biodiesel was produced from the lipid accumulated in *T. oleaginosus* cultivated with the purified glycerol obtained from crude glycerol after FFA recovery. It included the fermentation of *T. oleaginosus*, biomass harvesting, lipid extraction, trans-esterification, and biodiesel purification (Table 6). During fermentation, chemicals (KH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, NH<sub>4</sub>-Cl) were added as nutrient source. Oxygen was supplied by aeration and agitation was used for mixing. The energy input in the fermentation was 2460.96 MJ plus 123.05 MJ (pre-culture production). After fermentation, centrifugation (22.90 MJ) was performed to collect the biomass followed by extraction (492.44 MJ) with chloroform and methanol at 60 °C. The extracted lipid was then converted to biodiesel through trans-esterification which consumed 344.59 MJ. The produced biodiesel contained water, hence purification was employed, which had an energy input of 18.56 MJ. The total energy input of the biodiesel production from the purified glycerol derived from crude glycerol was 5024.00 MJ. The energy output of the processes was from the produced biodiesel (8667.65 MJ) and the generated cell debris (506.86 MJ). It was observed that the total energy output (9174.51 MJ) was greater than the total energy input (5024.00 MJ). It indicates that utilization of crude glycerol with high soap content to produce biodiesel was an energy gain process, which was 4150.51 MJ. The energy conversion efficiency calculated by dividing the total energy input with the total energy output was 1.83. The energy balance study revealed that converting the crude glycerol with high soap content to biodiesel through the method presented in this study (Fig. 1) was an energy feasible application of crude glycerol.

### 3.9. Cost estimation of the proposed process

The cost was estimated based on the similar assumption as in the energy balance and 1 tonne of crude glycerol used to produce biodiesel. The cost was mainly from the utilization of raw materials and utilities, equipment depreciation, labor, and laboratory/QC/QA. SuperPro Designer was employed to estimate the cost. The plant

life span was set for 10 years, and the equipment depreciation was calculated. The chemical and equipment prices were adopted from the ICIS Pricing and IRON solutions Official Guides, respectively. The prices of electricity and labor were set at 0.10 US \$/kWh and 10 US \$/h. The annual production days were 330 per year.

Based on these assumptions, the biodiesel cost was 6.32 US \$/gal (=1.87 from raw materials US \$ + 2.34 US \$ from equipment depreciation + 0.76 US \$ from utilities + 1.35 US \$ from labor), which was higher than the commercial biodiesel price (3.78 \$/gal). It indicated that utilization of crude glycerol for biodiesel production was not cost acceptable on the basis of 1 tonne crude glycerol utilized with lipid yield of 0.19 g/g glycerol and the fermentation time of 72 h. There are several methods to reduce the biodiesel production cost, which are:

- (1) The raw material cost was from the usage of nutrients and solvents. In fact, nutrients could be replaced by addition of waste such as sludge which has been reported to be potential nitrogen, phosphors, and trace elements (Zhang et al. 2017), which would reduce the cost.
- (2) With the increase of the plant scale, the equipment depreciation and labor cost can be decreased, which can thus reduce the total production cost.
- (3) Increase of lipid yield and reduce the fermentation time, the cost from the equipment depreciation, the labor, and the utilities can be reduced.

Overall, to make the process cost favourable, cheap nutrient source has to be supplied, fermentation has to be optimized, and plant scale should be increased.

## 4. Conclusions

This study has demonstrated that crude glycerol from the biodiesel production industry could be used for producing lipid which could be further converted to biodiesel. Due to the large amount soap content in the crude glycerol, simple purification was conducted to recover FFA from crude glycerol. The recovered FFA was, in fact, also good source for biodiesel production through esterification. The obtained purified glycerol performed similar as pure glycerol in biomass growth and lipid accumulation. The study has provided a method to maximize the productivity of crude glycerol to biodiesel. The energy balance study showed that the process could provide energy gain.

## Acknowledgments

Sincere thanks are due to the Natural Sciences and Engineering Research Council of Canada (Grant A 4984, Strategic Grant- STPGP 412994-11, Canada Research Chair) for their financial support. The views and opinions expressed in this paper are those of the authors.

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