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Contributed Paper

Potential of L-phenylalanine Production from Raw Glycerol of Palm Biodiesel Process by a Recombinant *Escherichia coli*

Penjit Srinophakun [a, c], Supachai Reakasame [a], Methee Khamduang [a], Kanoktip Packdibamrung [b], Anusith Thanapimmetha*[a, c]

[a] Department of Chemical Engineering, Faculty of Engineering, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.

[b] Department of Biochemistry, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand.

[c] National Center of Excellence for Petroleum, Petrochemicals and Advanced Materials, S&T Postgraduate Education and Research Development Office (PERDO), Bangkok 10330, Thailand.

*Author for correspondence; e-mail: fengjrc@ku.ac.th

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ABSTRACT

The possibility of using raw glycerol from biodiesel process for the bacteria growth and L-phenylalanine production using recombinant *Escherichia coli* BL21(DE3) was investigated. Raw glycerol with 75.50% (w/w) glycerol content was pretreated with concentrated sulfuric acid to reduce the impurities. Type A and type B glycerol containing 91.28% and 83.93% glycerol content were obtained after the acid pretreatment. Raw glycerol, type A and B glycerol and commercial grade glycerol (99.5%) at various concentrations (5, 10, 30 and 50 g/L) were used as a carbon source. The cell dry weight (CDW) and L-phenylalanine concentration obtained after 36 h of cultivation from raw glycerol were much higher than that from other types of glycerol. The highest CDW (3.47 g/L) and L-phenylalanine (55.2 mg/L) were obtained from the medium containing 30 g/L of raw glycerol. The results indicated that raw glycerol from biodiesel process was an interesting carbon source for L-phenylalanine production.

1. INTRODUCTION

Biodiesel which can be used as a fuel in diesel engines and heating systems is monoalkyl esters of long-chain fatty acids derived from vegetable oils or animal fats via transesterification process [1]. There are many advantages of biodiesel over petroleum-based fuel such as its renewability, biodegradability, and lower sulfur and aromatic content [2]. Due to the scarcity of petroleum reserves and the increase in environmental concern, biodiesel becomes more attractive to be produced and used as

an alternative energy. In the biodiesel production process, glycerol is generated as a major by product. For every 10 kg of biodiesel produced, about 1 kg of glycerol is generated [3]. Although high purity glycerol is used as an important feed stock in food, pharmaceutical, cosmetic, tobacco, and many other industries [4], raw glycerol derived from biodiesel production cannot be applied directly in those industries because it contains considerable amount of impurities such as

excess methanol, catalyst residual, and soap [5]. Recently, the increasing worldwide biodiesel production leads to the accumulation of surplus raw glycerol [6]. Therefore, finding alternative ways to utilize raw glycerol would provide the solution for the economic viability of biodiesel production.

Glycerol can be used as a sole carbon source in microbial fermentations to produce various products such as 1, 3-propanediol [7], mannosylerythritol [8] and citric acid [9]. Despite lower price and more availability of raw glycerol compared with pure glycerol, the studies on the use of raw glycerol in fermentation processes have been reported in limited numbers [9-14]. Some was reported that impurities such as soap and salt presenting in raw glycerol had inhibitory effects on the growth of many microorganisms [12-14]. However, some strains of *Clostridium butyricum* and *Klebsiella pneumoniae* have been reported to be able to grow on raw glycerol [11, 13, 15].

Escherichia coli is one of microorganisms frequently used as a host to produce recombinant product in various industrial applications [16]. In addition, *E. coli* is capable of utilizing a variety of carbon sources that are constituents of readily available renewable feedstock. These include carbohydrates such as glucose and xylose, as well as non-carbohydrate carbon sources such as glycerol and fatty acids [17]. Anaerobic fermentation of glycerol by *E. coli* generates various chemicals such as ethanol, succinate, acetate, lactate, and hydrogen [2]. L-phenylalanine production from glycerol by recombinant *E. coli* BL21(DE3) was also reported [18]. Various methods had been shown to achieve the production of L-phenylalanine, and using phenylpyruvate as substrate was characterized as one of them, of which utilized the activation of L-phenylalanine dehydrogenase gene [19]. This genetically modified bacterium was

proven to show higher level of L-phenylalanine dehydrogenase, comparing to a non-engineered form [20]. L-phenylalanine is produced commercially mostly by fermentation involving recombinant *E. coli* and sucrose [21]. L-phenylalanine is an essential amino acid which has been used as a raw material in the production of low-caloric sweetener aspartame [22]. Due to the increasing demand for soft drinks and low-caloric food, the commercial value of L-phenylalanine has increased greatly over the past few years. Therefore, the production of this amino acid has been intensively studied [23]. In previous studies, only pure glycerol was conducted in *E. coli* cultivation [2, 18]. Hence, the purpose of this work is to investigate the potential of using raw glycerol from biodiesel production process for the microbial growth and L-phenylalanine production by recombinant *E. coli* BL21(DE3). The raw glycerol derived from methanolysis of refined bleached deodorized (RBD) palm oil was used without prior purification and its result was compared with commercial grade glycerol (99.5% w/w) and sulfuric acid-pretreated glycerol.

2. MATERIAL AND METHOD

2.1 Microorganism

Escherichia coli BL21(DE3) (genotype: $F^- ompT hsdS_B (r_B^- m_B^-) gal dcm$ (DE3)) (Invitrogen Corporation, Carlsbad, CA, USA) was the host strain used to express the phenylalanine dehydrogenase gene of *Acinetobacter lwoffii*. The phenylalanine dehydrogenase gene was cloned using pET-17b (Novagen; Merck KGaA, Darmstadt, Germany) as an expression vector. This recombinant strain had been constructed at the Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand [20]. To prepare inocula, the cells were cultured in 250-mL Erlenmeyer flask containing 50 mL of LB medium with an addition of 0.05 g/L

of Ampicillin and incubated in an orbital shaker at 200 rpm and 37°C for 24 h. The LB medium contained (per liter) 5 g of yeast extract, 10 g of NaCl and 10 g of tryptone. The pH of the medium was adjusted to 7.4 by 3 N NaOH.

2.2 Raw Glycerol

Raw glycerol used in this study was kindly supplied by Patum Vegetable Oil Co., Ltd (Patumthani province, Thailand). This refinery produces biodiesel using alkali catalyzed (NaOH) transesterification process of RBD palm oil with methanol. Methanol in raw glycerol was prior recovered and reused in the biodiesel process. Raw glycerol was dark brown and semi solid with pH 12 containing 75.50% (w/w) glycerol, 13.30% (w/w) soap, 3.90% (w/w) ash and low methanol content (about 1-2% w/w).

2.3 Raw Glycerol Pretreatment

To increase the purity of raw glycerol, it was evaporated at about 80°C for 1 h to remove the methanol residual. After the temperature of preheated raw glycerol reduced to about 40°C, it was then pretreated by adding concentrated sulfuric acid (97% w/v) until the pH of raw glycerol decreased from 12 to 5 and 3, respectively. The acid addition into raw glycerol led to the formation of sulfate salt and separation of free fatty acids. Each acid-pretreated glycerol (pH 3 and pH 5) was then centrifuged at 2,300 g for 15 min to separate glycerol from sulfate salt (bottom phase). The separated glycerol phases of acid-pretreated glycerol with pH of 3 and 5 were labeled as type A and type B glycerol.

2.4 Fermentation Experiment

The medium employed in *E. coli* BL21(DE3) cultivation contained (per liter) 50 g of $(\text{NH}_4)_2\text{SO}_4$, 0.81 g of MgCl_2 , 2.43 g of KH_2PO_4 , 2.43 g of K_2HPO_4 , 0.85 g of

yeast extract, 0.085 g of thiamine-HCl, 0.002 g of FeSO_4 , 0.002 g of MnSO_4 , 0.05 g of CaCl_2 and 0.01 g of ZnSO_4 [18]. Various types of glycerol (commercial grade glycerol, raw glycerol, and type A and B acid-pretreated glycerol) at 5, 10, 30 and 50 g/L were used as a sole carbon source. The pH of the medium was adjusted to 7.4 using 3 N NaOH. When preparing this medium, thiamine-HCl, MgCl_2 , and the remaining nutrient were sterilized separately and mixed together at the time of inoculation. Thiamine-HCl was sterilized by filtering through a filter with a pore size of 2 mm. MgCl_2 and the remaining nutrients were autoclaved at 121°C (15 psi) for 15 min. The fermentation experiments were carried out in 250 mL Erlenmeyer flasks with a working volume of 50 mL. The medium was inoculated with the inoculum size of 5% (v/v) of the total liquid volume. The cultures were incubated at 37°C and 200 rpm in an orbital shaker. Samples were withdrawn from each flask after 36 h of cultivation to determine the cell dry weight and L-phenylalanine concentration.

2.5 Analytical Methods

Raw glycerol and the recovered acid-pretreated glycerol were characterized by their contents of glycerol, soap, and ash. The parameters were analyzed by the methods below:

- Glycerol was measured using spectrophotometric method of Bondioli et al. [24].
- Soap was determined by colorimetric titration using 0.1 N HCl as a titrant and 0.4% bromophenolblue as an indicator [12].
- Ash contents were analyzed according to standard method ISO 2098-1972.

Cell dry weight of the culture was determined by centrifuging the fermentation broth of the culture at 1,800 g for 10 min, followed by washing with distilled water and drying the cell pellet at 105°C to constant

weight. The supernatant of the fermentation broth was further analyzed for the L-phenylalanine concentration.

L-phenylalanine concentration was analyzed by dabsyl-Cl derivatization coupled with HPLC resolution and UV (436 nm) detection. Dabsyl-Cl derivative of L-phenylalanine contained in the supernatant was prepared according to the method of Khamduang [18] with slight modification. The 0.2 mL aliquot of supernatant was placed in an eppendroff and mixed well with 0.1 mL of 1.5 M NaHCO₃ (pH 9.0) and 0.2 mL of dabsyl-Cl solution (2 mg/mL in acetone), respectively. The mixture was then heated at 70°C for 10 min and dried under vacuum. The residual was then dissolved in 0.5 mL of 70% ethanol. The resulting solution was centrifuged at 14,000 g for 2 min, filtered through a syringe filter (0.2 µm pore size), and analyzed by HPLC (ACE, C18 column, 150×4.6 mm, 5 µm particle) at room temperature. The mobile phase consisted of a 50:50 v/v mixture of a phase A (10 mM potassium dihydrogen phosphate, pH 6.55) and a phase B (acetonitrile and 2-propanol, 75:25 v/v). The flow rate of mobile phase was 1.0 mL/min.

All experiments were performed in triplicate and the results were expressed as mean of the triplicated experiments.

3. RESULTS AND DISCUSSION

3.1 Raw Glycerol Pretreatment

The raw glycerol contained 75.50% (w/w)

glycerol, 13.30% (w/w) soap, 3.90% (w/w) ash, and low methanol content (about 1-2% w/w). It was subjected to an acid pretreatment process to increase glycerol purity by getting rid of impurities presenting in it. The high level of impurities, particularly soap and sodium ions, have been reported to be toxic to many microorganisms [12, 14, 15]. The composition and pH of various types of glycerol are summarized in Table 1. As shown in Table 1, lower pH of type A glycerol was the results of adding more acid in. It was obvious that higher percentage of glycerol in type A contains fewer impurities of soap and ash. The result of adding sulfuric acid was to split the soluble soap in raw glycerol into insoluble free fatty acid and sulfate salt [25]. In this experiment, the insoluble free fatty acid and sulfate salt were separated from the raw glycerol solution by centrifugation as mentioned in material and methods. Consequently, glycerol content in raw glycerol increased from 75.50% (w/w) to 83.93% (w/w) and 91.28% (w/w) in type B and type A, respectively as shown in Table 1. In addition to splitting soap containing in raw glycerol, sulfuric acid also neutralized sodium hydroxide existing in raw glycerol. Both soap splitting and sodium hydroxide neutralization led to the formation of sodium sulfate. As sodium sulfate has a low solubility in glycerol, consequently, it partially crystallized out from raw glycerol. Hence, ash content, a representative of sodium ions in raw glycerol, tended to decrease when pH of acid-pretreated glycerol decreased.

Table 1. Composition and pH of various types of glycerol.

Glycerol	pH	Glycerol % (w/w)	Soap % (w/w)	Ash % (w/w)
Raw glycerol	12	75.50 ± 0.37	13.30 ± 0.06	3.90 ± 0.04
Type B	5	83.93 ± 0.25	0.50 ± 0.00	2.77 ± 0.05
Type A	3	91.28 ± 0.34	n.d.*	2.59 ± 0.02

Note: * Not detected

This result was consistent with the study of Ooi et al. [26]

3.2 Effects of Glycerol on Cell Growth of Recombinant *E. coli* BL21(DE3)

Raw glycerol from biodiesel industry, two types of acid-pretreated glycerol (type A and type B) and commercial grade glycerol (99.5%) were used as a sole carbon source in culturing recombinant *E. coli* BL21(DE3) at various concentrations. Cell dry weight of *E. coli* BL21(DE3) obtained from different medium after 36 h of cultivation are shown in Figure 1. As can be seen in Figure 1, cell dry weight obtained from commercial grade glycerol and type A and B acid-pretreated glycerol were very similar. The maximum cell dry weight of these three glycerol (1.97 g/L of commercial grade glycerol, 2.06 g/L of type A and 2.11 g/L of type B) were obtained

at 10 g/L of glycerol. The obtained cell dry weight slightly decreased when the concentration of glycerol was increased from 10 to 30 and 50 g/L suggesting that the cell being inhibited when the concentration of glycerol exceed 10 g/L. These results are consistent with the previous report for this bacterial cultivation on pure glycerol [27]. Comparing at the same glycerol concentration, the cell dry weight obtained from type B glycerol was slightly higher than that from type A and commercial grade glycerol, respectively. However, an appreciably higher cell dry weight of *E. coli* BL21(DE3) was obtained when raw glycerol was used. The highest cell dry weight (3.47 g/L) was obtained at 30 g/L raw glycerol. An inhibition of raw glycerol on the cell growth was observed at 50 g/L concentration.

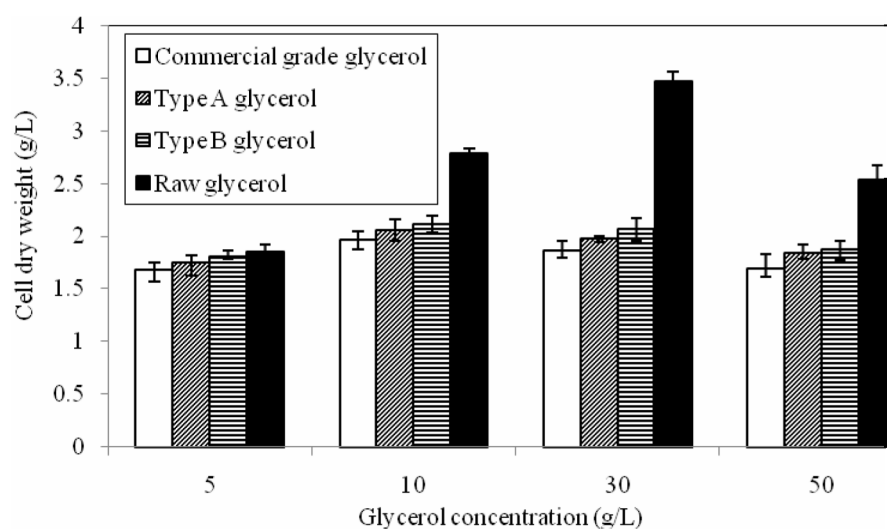


Figure 1. Effects of glycerol concentration and types on cell growth of a recombinant *Escherichia coli* BL21(DE3).

3.3 Effects of Glycerol on L-phenylalanine Production by a Recombinant *E. coli* BL21(DE3)

The effects of various concentrations of different glycerol types on L-phenylalanine

production by a recombinant *E. coli* BL21 (DE3) were investigated by analyzing the supernatant of the culture medium at the end of the fermentation (36 h) as described earlier. The concentrations of L-phenylalanine

obtained from different medium are shown in Figure 2. The results showed that the concentration of L-phenylalanine obtained from the cultures has a similar trend as that of cell dry weight. The L-phenylalanine concentration obtained from raw glycerol was the highest at 55.2 mg/L. For type A and type B acid-pretreated and commercial grade glycerol, the maximum L-phenylalanine concentration was obtained at 10 g/L rather than 30 g/L of raw glycerol. It was observed that L-phenylalanine was growth-associated. Although the yield of L-phenylalanine obtained in this study was accounted to be smaller than a previous report by three orders of magnitude [28], this novel idea of using raw glycerol from refinery biodiesel plant could still be worth exploring.

As the cell dry weight and L-phenylalanine concentration of raw glycerol were much higher than that of commercial grade glycerol, it suggested that a recombinant *E. coli* BL21 (DE3) could utilize raw glycerol more efficiently for its growth and L-phenylalanine production. These results are very interesting. In the background literature, the inhibition effects of raw glycerol on microbial growth

have been always observed [7, 12, 29]. In this study, Figure 1, raw glycerol seems to have positive effects on *E. coli* BL21(DE3) growth. One evidence that supported the ration of raw glycerol in an organismal growth was from Chi et al., 2007. The author found a good correlation between the microalga, *Schizochytrium limacinum*, growth, docosahexaenoic acid (DHA) production, and raw glycerol [14]. In fact, the microbial fermentation of raw glycerol from biodiesel production process has been investigated previously with particular focus on the 1, 3-propanediol production [11-13, 15]. 1, 3-propanediol production by *Clostridium butyricum* VPI3266 [15] and by *Klebsiella pneumoniae* [11]. However, it was reported that raw glycerol from biodiesel production process had higher inhibition effects on microbial cell growth as compared to more purity glycerol. Therefore, 1, 3-propanediol productivity obtained from raw glycerol was lower than that from more purity glycerol. Asad-ur-Rehman et al., 2008 reported that soap was one of important inhibitors of *Clostridium butyricum* DSM5431 [12].

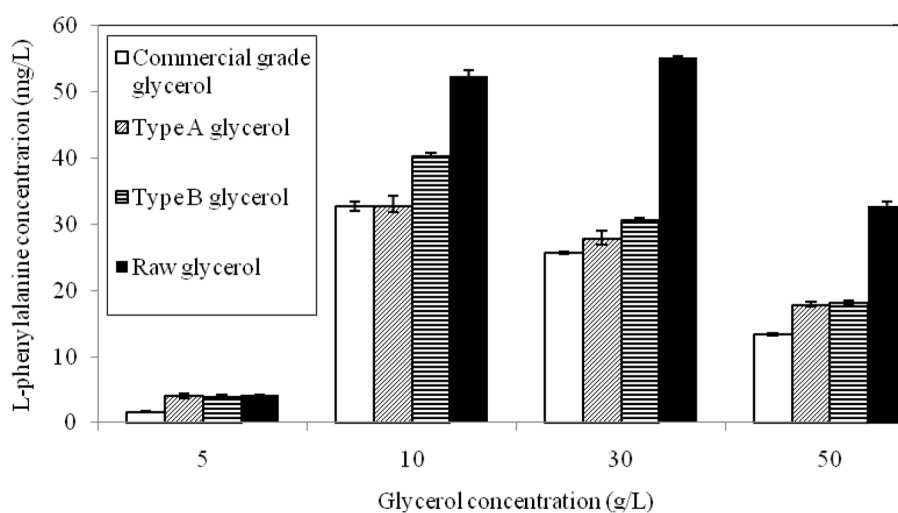


Figure 2. Effects of glycerol concentration and types on L-phenylalanine production by a recombinant *Escherichia coli* BL21(DE3).

3.4 Effects of Impurities on Cell Growth

As shown in Figure 1., the cell dry weight in the medium of raw glycerol was much higher than that in the medium with higher purity glycerol. The raw glycerol used in this study contained considerable amount of impurities (13.30% (w/w) soap and 3.90% (w/w) ash). During medium preparation process, it was observed that when raw glycerol was mixed with other nutrients in distilled water, the white liquid layer presumed to be insoluble soap was formed on the top of the mixture. However, such layer disappeared after autoclaving. In order to test the effect of this white layer, which was hypothesized to be soap, on *E. coli* BL21(DE3) growth, the cells were cultured in soap-contained medium (medium with 10 g/L raw glycerol as in the previous session) compared with soap-separated medium. To prepare the soap-separated medium, the mixture of raw glycerol and other nutrients was left for 30 min in the separating funnel and removed the upper phase (insoluble soap). The lower phase, theoretical soap-separated phase, was

then autoclaved, mixed with sterilized $MgCl_2$ and thiamine-HCl and then used for the cell cultivation. The results were shown in Figure 3. When soap was separated from the medium before autoclaving, 30.6% reduction in the obtained cell dry weight (in comparison to that in soap-contained medium) was observed suggesting that the significantly higher cell dry weight obtained from raw glycerol was mainly attributed to the soap presenting in the medium. Instead of being detrimental to bacterial cell, the soap in raw glycerol has beneficial effects on *E. coli* BL21(DE3) growth. Free fatty acid from soap dissolving in medium (> 90% water) after autoclaving might be used as a carbon source for bacteria [17]. Therefore, cells could grow better.

Since it was hypothesized that a growth of *E. coli* BL21(DE3) was affected by some impurities resided in raw glycerol, the following experiment was conducted to study an effect of sodium salt. The notion that 3.90% (w/w) ash was considered as sodium salt, therefore, was tested for its validity.

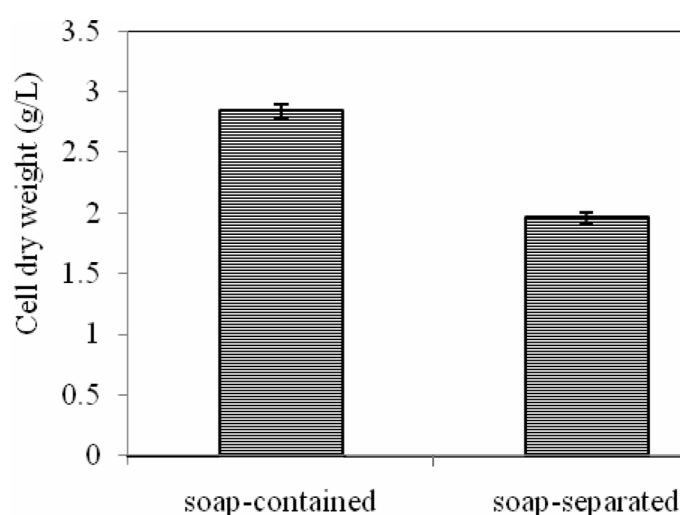


Figure 3. Effects of soap in medium on cell growth of a recombinant *Escherichia coli* BL21(DE3).

Different levels of Na_2SO_4 (0 g/L, 0.5 g/L, 1 g/L and 5 g/L) were added to the medium containing 10 g/L commercial grade glycerol. The dry cell weight obtained from these cultures was determined. As shown in Figure 4, the obtained cell dry weight was not significantly different within the investigated sodium sulfate concentration range. It could

be summarized that the concentration of up to 5 g/L of Na_2SO_4 could not substantially change the cell dry weight of *E. coli* BL21 (DE3). Consequently, the sodium ion (soap form) in raw glycerol appeared to show the non-effect on the growth of this recombinant *E. coli*.

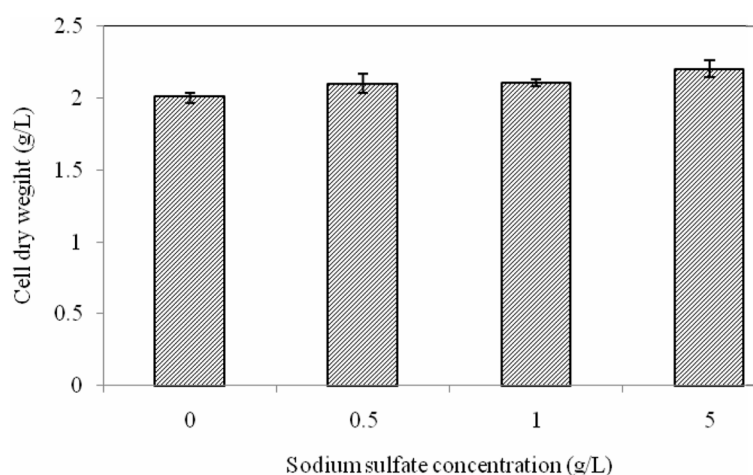


Figure 4. Effects of sodium sulfate concentration on cell growth of a recombinant *Escherichia coli* BL21(DE3).

4. CONCLUSIONS

In this work, the possibility of using raw glycerol from RBD palm oil biodiesel for bacterial growth and L-phenylalanine production by a recombinant *E. coli* BL21 (DE3) was investigated. The raw glycerol from biodiesel production process had low purity (75.50% (w/w) glycerol content). In order to reduce the amount of presence impurities (soap and ash), concentrated sulfuric acid was added into raw glycerol until the pH decreased from 12 to 5 and 3, respectively. Acid-pretreated glycerol of pH 5 and 3 (type B and A glycerol) contained 83.93% (w/w) and 91.28% (w/w) glycerol. The amount of soap decreased from 13.30% (w/w) in raw glycerol to 0.50% (w/w) and non-detectable level in type B and A glycerol. Raw glycerol, type A and B glycerol and commercial grade

glycerol (99.5%) at various concentrations (5, 10, 30 and 50 g/L) were used as a carbon source in culturing a recombinant *E. coli* BL21 (DE3). The medium with 30 g/L of raw glycerol gave the highest cell dry weight (3.47 g/L) and L-phenylalanine concentration (55.2 mg/L). In addition, the impurities contained in the medium of raw glycerol had positive impact to the growth of *E. coli* BL21(DE3). However, the further verification of these substances still needed to be investigated.

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Petrochemicals and Advanced Materials, S&T Postgraduate Education and Research Development Office (PERDO), Bangkok, Thailand.

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