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Bioconversion of Glycerol from Biodiesel Production to Ethanol by *Enterobacter aerogenes* TISTR 1468

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The objective of this study is to examine the potential applications of glycerol, a by-product derived from *jatropha* biodiesel production, via bioconversion into ethanol by *Enterobacter aerogenes* TISTR 1468. The purity of crude glycerol sample can be enhanced using sulfuric acid in varying concentrations. It was found that the addition by weight of 5% sulfuric acid could increase the glycerol purity from 27.68% to 94.43% with 65.42% of glycerol yield. Once purified, the glycerol sample was applied at 30 g/L to form the carbon substrate required in the anaerobic fermentation process by which ethanol can be obtained through the use of *E. aerogenes* for 120 hours at a constant 37 °C. Under these conditions, purified glycerol yielded 8.39 g/L ethanol while crude glycerol yielded 6.54 g/L ethanol. The ethanol produced via this approach was shown to be highly efficient in comparison with commercial glycerol with purity at 99.5%, which produced 7.46 g/L ethanol under the same condition.

Keywords: Biodiesel production, Crude glycerol, *Enterobacter aerogenes,* Ethanol, *Jatropha curcas L.*

Dwindling oil reserves allied to environmental concerns have resulted in increasing interest in alternative energy sources such as biodiesel or bioethanol. These sources are considered sustainable and less harmful to the environment, with biodiesel at the forefront of the current surge in global interest[1,2,3,4,5]. The transesterification of vegetable oils or animals fats is the principal means of producing biodiesel, and offers the benefit of using renewable materials in contrast with regular diesel. Furthermore, the biofuel is sulfur-free, non-toxic, and generates lower levels of carcinogenic pollution[6].

Biodiesel production in Thailand is dependent upon two plant species: *Elaeis guineensis* and *Jatropha curcas* Linn. It has been proposed that *jatropha* oil biodiesel might serve as a substitute for at least a part of the diesel fuel currently used in Thailand for transport purposes[7]. This biodiesel production process leads to the creation of around 10% (w/w) glycerol as the primary by-product, although a number of impurities are present, such as soap, fatty acids, methanol, salts, water and other non-glycerol substances, depending on the catalysts and feedstock used in the procedure[8,9]. These impurities lower the value of the crude glycerol obtained, and even safe disposal requires pre-treatment. As the demand for biodiesel has risen, the quantities of low-grade glycerol as a by-product have also increased, so there are undoubtedly advantages to be found in any method which allows its conversion to a higher value product, both in economic terms and also with regard to the environmental benefits.

Glycerol, 1,2,3-propanetriol, is a trihydric alcohol which has a wide range of uses across

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various industries such as food and drinks, drugs, pharmaceuticals, cosmetics, toothpaste, toiletries, plastics, tobacco, pulp and paper, paint, leather, textile, lubricants and automotive[10,11]. However, to use it in these applications is necessary a degree of glycerol purity higher than 95%[2,12].

It is thus necessary to develop a means of using the by-product glycerol generated from biodiesel production, by removing the impurities in order to meet the requirements of industry[8]. The idea of converting waste glycerol into a more useful product has become very interesting, and the method of using microbes to perform the conversion has been suggested as an effective and sustainable option, which helpfully avoids the use of chemicals. There is also no need for high energy input or complex pre-treatment, so the approach can be considered highly advantageous[13,14,15,16]. It has been noted that a number of microbes are able to convert waste glycerol into more useful products, including ethanol[10,17] methane[18], 1,3-propanediol[19], citric acid[20], succinic acid [21] and hydrogen [22,23]. The bioconversion of glycerol into ethanol is of particular interest due to it is widely used in many industries as solvents and also can be used as an alternative fuel for vehicles[3,6]. To obtain ethanol as the principal product from glycerol, *Enterobacter aerogenes*, which is a gram-negative species and member of the Enterobacteriaceae family is the ideal choice[3,12,24].

Given the information thus far, the aim of this research was to examine bio-ethanol production using anaerobic fermentation techniques with *E. aerogenes* TISTR 1468 and glycerol derived as a by-product of the *jatropha* biodiesel production.

MATERIALS AND METHODS

Glycerol sample

The sample of crude glycerol was derived from biodiesel production involving the transesterification of 50 kg *jatropha* oil and 10 L of methanol, using potassium hydroxide as the catalyst. The chemical composition of the isolated crude glycerol was then examined. The commercial glycerol used in the study was obtained from Ajax Finechem (Australia) and had a purity of 99.5%.

Purification of crude glycerol

Having acquired the crude glycerol, it was subsequently treated with sulfuric acid in order to raise the level of purity. This process commenced by lowering the viscosity by dissolving the glycerol in methanol. Sulfuric acid (96%, Carlo Erba, Italy) was then added in five differing concentrations: 3%, 4%, 5%, 6%, and 7% (w/w). The resulting mixture was then stirred for 30 minutes at 400 rpm. Separation of the mixture was then performed using a separatory funnel, and the product was permitted to stand for several hours. This allowed the mixture to form three separate layers, comprising salts at the bottom, glycerol in the middle, and methyl ester on the top. Since the glycerol was the layer of interest, it was then separated out of the mixture and analyzed in order to determine the ideal concentration of sulfuric acid to achieve the best purification outcome.

The purified crude glycerol sample which was deemed to represent the best outcome in terms of purification was then selected as the carbon substrate in the ethanol fermentation process.

Microorganism and culture conditions

Microorganism: A strain of bacterium *Enterobacter aerogenes* TISTR 1468 was obtained from the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

Medium: The preculture medium comprised commercial glycerol 15 g, tryptone 5 g, K_2HPO_4 14 g, KH_2PO_4 6 g, $(NH_4)_2SO_4$ 2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g per litre of distilled water.

Cultivation conditions: E. aerogenes TISTR 1468 from the agar slant was inoculated into 100 ml of the preculture medium in a 250 ml flask and cultivated on a rotary shaker at 150 rpm and 37 °C for 12 hours.

Ethanol production via fermentation

The process requires the starter culture (2% v/v) to be introduced to the fermentation medium (50 ml) in 125 ml serum bottles. The fermentation medium had the same composition as the preculture medium except that 30 g of crude and purified glycerol (from the best purification condition) were used instead of commercial glycerol. The assessment of the optimal glycerol concentration to use as the carbon sources in the fermentation process, the concentrations of commercial glycerol were varied at 10, 20, 30, 40, and 50 g/L. The serum bottles were tightly sealed with rubber stoppers and were flushed with N_2 gas to create anaerobic conditions. Each bottle was

subsequently incubated using a water bath shaker at 37 °C for 120 hours at an agitation speed of 60 rpm. Sampling for analysis of the concentrations of ethanol and glycerol in fermentation broth were performed at every 24 hours interval. All treatments were conducted in triplicates.

Analysis methods

Glycerol sample characterization

The sample of crude glycerol was characterized by its contents of glycerol, methanol, ash, water and matter organic non-glycerol (MONG). Further analysis was carried out with regard to pH and the infrared spectrum, as described below:

Glycerol and methanol

High Performance Liquid Chromatography (HPLC, Shimadzu, Japan) was used to examine the glycerol and methanol contents in the samples. Analysis was performed with a refractive index (RI) detector using an Aminex HPX-87H ion exclusion column (300 x 7.8 mm, Bio-Rad, USA) at a constant 60 °C. For the mobile phase, 5 mM H_2SO_4 was used with a flow rate of 0.6 ml/min.

Ash

Analysis of the ash content was accomplished by burning 1 g glycerol sample in a furnace at 750±10 °C for 10 minutes (ISO 2098-1972).

Water

Volumetric Karl Fischer titration was used to evaluate the water content (ISO 2097-1972).

Matter Organic Non-Glycerol (MONG)

To determine MONG, the percentages of glycerol, methanol, ash and water are subtracted from 100%.

pH

The pH values were determined by measuring a solution comprising 20 g glycerol sample in 100 ml distilled water.

Fourier transforms infrared (FT-IR) analysis

An Attenuated Total Reflection Fourier Transform Infrared Spectrometer (ATR-FTIR, diamond ATR) (Nicolet IR200, USA) was used to determine the infrared spectrum of crude glycerol, commercial glycerol, and purified glycerol.

Analysis of ethanol and glycerol contents in fermentation broth

HLPC was used to analyze the concentrations of ethanol and glycerol in samples of the fermentation broth, under conditions identical to those used in the analysis of the glycerol and methanol contents in the crude glycerol sample.

RESULTS AND DISCUSSION

Characterization of glycerol sample

The typical appearance of crude glycerol produced as a by-product of biodiesel obtained through the transesterification of *jatropha* oil using potassium hydroxide as the alkali catalyst was dark brown, while the pH values were high (11.8). The chemical compositions of the crude glycerol sample are shown in Table 1. HPLC and FT-IR were employed to determine the purity and the compositions of glycerol sample as shown in Fig. 1 and Fig. 2. The chemical composition analysis showed that the crude glycerol sample contained 27.68% (w/w) glycerol. Methanol, ash, water and MONG were present as impurities in the sample.

The infrared spectrum of the crude glycerol was recorded and compared to that from commercial glycerol [Fig. 2 (a), (b)]. Crude glycerol was revealed to contain a number of impurities which could be observed at the absorption

Table 1. Chemical composition of the crude glycerol sample

Component	Percentage (w/w)*
Glycerol	27.68
Methanol	12.53
Ash	5.30
Water	2.00
Matter organic non-glycerol (MONG)	52.49

* based on the weight of the glycerol sample

Table 2. Percentage purities of the glycerol samples after purification at different conditions

Sulfuric acid concentration (% w/w) *	Purity (%)
3	62.08
4	87.72
5	94.43
6	91.09
7	90.28

* based on the weight of the glycerol samples

frequency at 1580 cm^{-1} (COO- functionality) and at 1750 cm^{-1} (C=O functionality). These indications correlate respectively with soap and free fatty acids. In the commercial glycerol sample, these impurities could not be observed. The absorption frequency at 1500-1200 cm^{-1} confirmed the glycerolic moiety of the pure glycerol, and can be explained by the manner in which the C-H in-planes and the O-H bending overlap within the glycerol molecules. OH stretching was noted at 3500-3000 cm^{-1} and 1600 cm^{-1}, confirming the existence in every sample of the OH group[25].

Purification of crude glycerol

The crude glycerol was purified using sulfuric acid at five different concentrations. This allowed the most effective concentration to be determined. The results of HPLC analysis revealed that 5% sulfuric acid by weight gave the best outcome, raising the purity level to 94.43% [Fig. 1 (b), Table 2]. Figure 2 (c) showed the infrared spectrum of the glycerol after purification. The results appear similar to those for commercial glycerol.

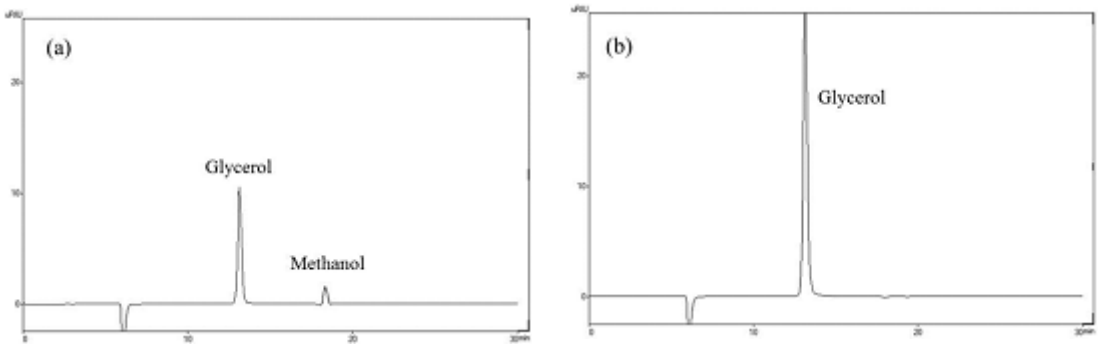

Fig. 1. HPLC chromatograms of (a) crude glycerol and (b) purified glycerol

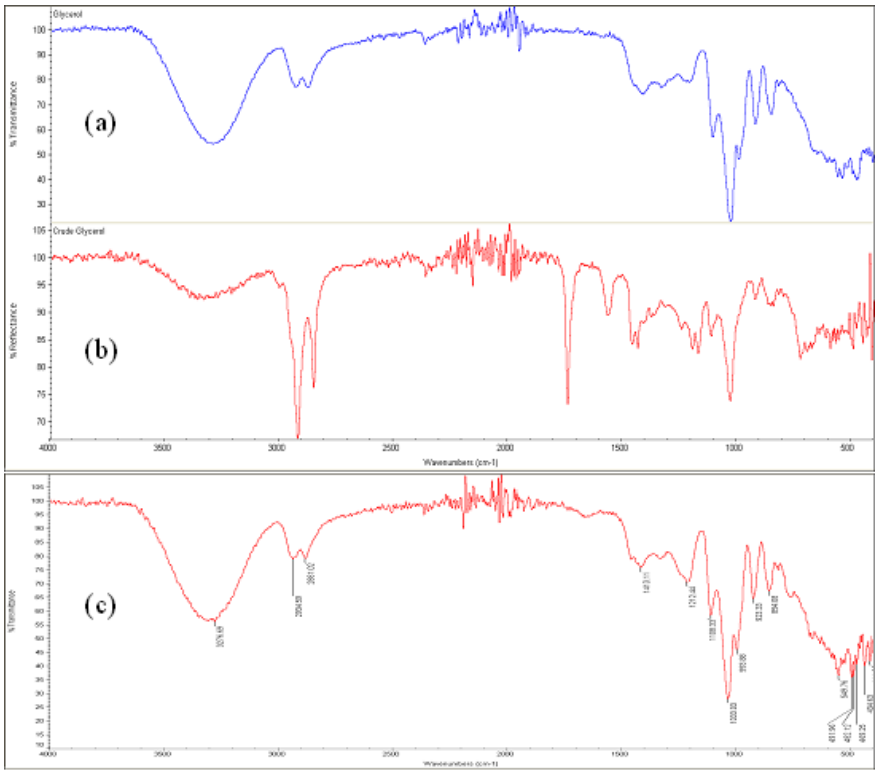

Fig. 2. FT-IR spectra of (a) commercial glycerol (b) crude glycerol and (c) purified glycerol

Fermentation for ethanol production

The optimal glycerol concentration for fermentation

It was necessary to record the ideal initial glycerol concentration to be used in the fermentation process involving *E. aerogenes* TISTR 1468. Therefore, the fermentation process used five different concentrations of commercial glycerol (10-50 g/L) as the carbon source in order to carry out the evaluation. The fermentation was undertaken with serum bottles (125 ml) which contained the cultivation medium (50 ml). These were placed in a water bath shaker at an agitation speed of 60 rpm and maintained at 37 ºC for 120 hours. Figure 3 showed how the initial glycerol concentration affects glycerol consumption and the production of ethanol during the cultivation of *E. aerogenes*. During fermentation, it also produced H_2 (g) and some acid products at the same time[24]. The outcome confirmed that the most suitable concentration for glycerol in order to create the greatest ethanol concentration was 30 g/L at 120 hours, which resulted in a concentration level of 7.46 g/ L.

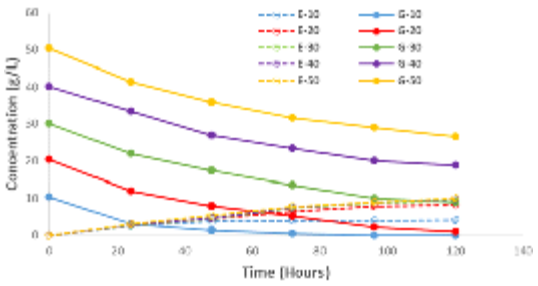

Fig. 3. The effects on glycerol consumption and ethanol production of the initial glycerol concentration during the fermentation of *E. aerogenes* at 37 °C for 120 hours

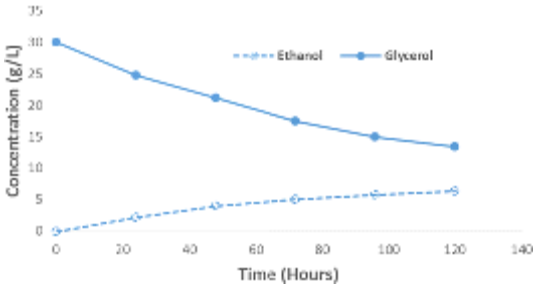

Fig. 4. Concentrations of ethanol and glycerol in fermentation broths during the fermentation using 30 g/ L crude glycerol at 37 °C for 120 hours

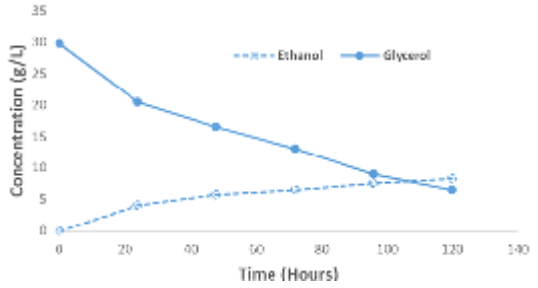

Fig. 5. Concentrations of ethanol and glycerol in fermentation broths during the fermentation using 30 g/ L purifed glycerol at 37 °C for 120 hours

Fermentation of crude and purified glycerol for ethanol production

Ethanol production levels of 6.54 and 8.39 g/L were recorded for crude and purified glycerol at 30 g/L respectively when these were used as carbon substrates in the fermentation process. Figures 4 and 5 showed the results.

CONCLUSION

Crude glycerol, with its high pH of 11.8 and low glycerol content of 27.68% is the principal by-product when biodiesel is produced via the transesterification of *jatropha* oil using a potassium hydroxide catalyst. To increase the purity, sulfuric acid is used, and the optimal concentration for this step was shown to be 5% (w/w), since it resulted in the highest glycerol sample purity level of 94.43%. The 30 g/L samples of crude and purified glycerol were then used in the anaerobic fermentation process at 37 °C for 120 hours to produce ethanol using *E. aerogenes*. The crude and purified glycerol served as a carbon source to produce 6.54 and 8.39 g/L respectively of ethanol. The ethanol which was produced using this approach involving by-product glycerol was shown to be highly efficient in comparison with the ethanol produced using commercial glycerol, which would yield 7.46 g/L under similar conditions.

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