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Dark Fermentation of Crude Glycerol by Locally Isolated Microorganisms to Hydrogen

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The need for sustainable management of the waste along with the availability of crude glycerol (CG) has generated interest on glycerol-based cultures. To date, CG is used as cheap substrate in fermentation in replace of glucose or other types of substrates. In this study, raw CG is used as a sole substrate for the locally isolated microorganisms from biodiesel sludge to produce bio-hydrogen via dark-fermentation. Prior to the isolation of potential microbes, preliminary test was done to ensure that hydrogen is among the gases produced during the fermentation. This preliminary test was conducted using the wastewater. Mixed-microbes including the potential microbes were expected to be in the wastewater and help in the bioconversion of both pure and crude glycerol to hydrogen. The experiment using pure commercial glycerol was also conducted to compare the performance and conversion of the hydrogen. The gas was collected in a sampling bag. Analysis of the gas was done using GC-TCD with argon as carrier gas. Isolation and screening of potential hydrogen producer has successfully isolated 10 potential microorganisms. Screening was based on their ability to produce gas globules in the agar. Findings on the gas analysis showed that the microorganisms produced only hydrogen and carbon dioxide. No traces of methane was observed from the GC analysis indicating that no methanogenic microbes in the mixed-cultures, and no foam was produced during the dark-fermentation using crude glycerol as carbon source. In contrast, foam is formed in the fermentation of pure commercial glycerol. From the findings, it can be concluded that the isolated microbes has the potential to produce hydrogen from the raw crude glycerol.

1. Introduction

Hydrogen, biologically or chemically processed, is known to be a good alternative renewable fuel in replacing the petroleum-based fuel in future. This is because hydrogen can offer a clean and sustainable environment since its combustion only produce water as by-product (Abdeshahian, et al., 2014). Production of hydrogen by chemical methods involve reforming of hydrocarbons such as steam reforming, (partial oxidation) gasification, autothermal reforming, aqueous-phase reforming (APR), and supercritical water reforming processes (Cardoso et al., 2014) While biologically, hydrogen can be produced using biophotolysis, photo-fermentation, dark fermentation, or combination of these ways (Abo-hashesh, et al., 2011). Both chemical and biological processes have their own advantages and disadvantages, but biological approach is more economical and clean (Abdeshahian et al., 2014) as chemical conversion releases greenhouse gases (Abdeshahian et al., 2014), forming CO_x,NO_x,SO_x,C_xH_y compounds, ash, and other organic compounds that have adverse effect to environment (Azwar, et al., 2014).

Above all the biological methods, dark-fermentation is reported as the best method since the production can occur continuously without the need for the light and is considered as the simplest process (Azwar, Hussain and Abdul-wahab, 2014b). Furthermore, this method offers a high rate production of hydrogen by a variety of available cheap feedstocks and wastes (e.g: crude glycerol, biomass, animal waste, etc) (Azwar et al., 2014b), a variety of fermentative anaerobic bacteria that have high growth rates (Abo-hashesh et al., 2011), low energy requirement, and produce valuable by-product such as volatile fatty

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acids (butyric, lactic and acetic acids) and alcohols (ethanol, methanol) (Lee, et al., 2014). Comparisons of biological process of hydrogen production are listed in Table 1.

Table 1: Comparison of biological process of hydrogen production (Lee et al., 2014)

Biological Process	Advantages	Disadvantages
Direct and indirect photolysis	H_2 can be produced directly from sunlight and water Higher solar conversion energy (10 folds compared to other sources)	Low rates of H_2 production by natural borne-organisms Required carrier gas to collect the evolved gas
Photofermentation	Bacteria in this group can use wide spectrum of light Variety of waste materials can be used	requires high activation energy to drive nitrogenase, which is the enzyme responsible for H_2 production in photosynthetic bacteria Low solar conversion efficiencies, typically not much higher than that for algal biophotolysis systems. In addition, phototrophic H_2 production with Photosynthetic bacteria are extremely doubtful due to ammonia and oxygen contents, making it difficult in practical applications.
Dark fermentation	Simple process with low energy requirements, higher rates of H_2 production, economically feasible or better process economy, and the ability to generate H_2 from a large number of carbohydrates (or other organic materials) can produce H_2 all day long without light It produces valuable metabolites such as butyric, lactic, and acetic acids as by-products It is an anaerobic process, so there is no O_2 limitation problem	Low H_2 yield compared to physicochemical methods. The bacteria are unable to overcome the inherent thermodynamic energy barrier to full substrate decomposition due to which low H_2 yields was observed

Crude glycerol (CG) was once declared as waste because of the abundances that lead to its descending price and high purification cost as well as its adverse effect to environment if disposed without any proper treatment. However, vast development in research has proven that CG can offer many industrial beneficial products. CG can also be used as a cheap substrate to substitute glucose in the fermentation processes. Conversion of CG to hydrogen via dark fermentation also proven to give higher yield of hydrogen compared to other methods. According to Abo-hashesh et al. (2011), in theory, 12 mol of hydrogen can be produced from one mole of glucose. However, the maximum yield of hydrogen reported to so far is only 2 mol/mol of glucose by facultative anaerobic microbes and 4 mole by strict anaerob. While a review by Jyoti et al. (2012) stated that Sabourin-Provost and Hallenbeck (2009) claimed to obtain 6 mol of hydrogen per mol of glycerol. Maru, et al. (2013) claimed to have obtained 0.85 mol H_2/mol glycerol from dark fermentation of glycerol using Enterobacter spH1. Meanwhile, (Chookaew, et al., 2011) produced 0.18 mol H_2/mol glycerol by thermotolerant Klebsiella sp. TR17. The use of CG as feedstock in biohydrogen production is still new because previous works focused more on bioconversion of pure glycerol using pure or genetically modified culture. Thus this paper aim to look into the possibility of obtaining hydrogen producing microorganisms from local biodiesel wastewater plant and further convert it to hydrogen via dark fermentation using crude glycerol as the sole carbon source. It involved two stages: preliminary study on dark fermentation of hydrogen production using indigenous microbes direct from the wastewater, and isolation for the potential hydrogen producer from the biodiesel wastewater.

2. Methodology

Microorganisms used in this study were isolated from biodiesel wastewater obtained from one of the biodiesel plant in Pasir Gudang, Johor, while crude glycerol were obtained from Carotino and pure glycerol were purchased from Sigma.

Prior to the isolation, a pre-liminary dark fermentation study was conducted using crude glycerol and pure glycerol as substrate to learn about the possible production of hydrogen by the indigenous microbes presence in the biodiesel. For cultivation, 30 mL of pre-boiled medium will be added into 50 mL serum bottles and sealed with butyl rubber stoppers, prior to sterilization (15 min, at 121 °C). After cooling down, 3 mL of pre-activated culture will be inoculated into the serum bottles, under a laminar flow cabinet. Subsequently, the medium (containing 10 % v/v inoculum) were flushed with nitrogen for 2 min to create anaerobic environment to support the growth of the microorganism, using a hypodermic sterile syringe with a 0.22 mm filter, before being incubated at 37 °C with continuous stirring (120 rpm).

The minimal medium used in this study was modified from Varrone et al. (2013). The composition of the medium per liter of distilled water are shown in Table 2.

Table 2: Composition of the minimal medium used for the experiment

Compositions	Concentration (g/L)
glycerol (crude or pure)	20.0
$K_2HPO_4.3H_2O$	3.4
KH_2PO_4	1.3
$MgSO_4.7H_2O$	0.2
$(NH_4)_2SO_4$	2.0
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.02
$FeSO_4.7H_20$	0.005

Analysis of hydrogen produced was made using GC-TCD (column used: HP Plot Molsieve 5A, capillary 30 mm x 530 µm x 25 µm nominal) with argon as carrier gas. The liquid sample was analysed using HPLC and GC-FID. During hydrogen production process, aqueous samples were collected at regular interval (24 h). Glycerol concentration of such samples was measured by using a spectrophotometric method of glycerol analysis proposed by Bondioli et al. (2005).

Isolation for the potential hydrogen producer was done using pour plate technique. The fresh sludge (0.1 mL) was dropped onto the petri dish and then the agar medium was poured on it. Once the agar solidifies, the plates were incubated at 37 °C for 72 h. Observation and colony count were made on the microorganisms' growth. All the isolated microorganisms were screened for the potential hydrogen producer. Screening for the potential producer was made based on the gas globules produced inside the agar.

3. Results and Discussion

3.1 Dark Fermentation of Glycerol to Biohydrogen

Prior to the isolation of potential microorganisms for biohydrogen production, a pre-liminary dark fermentation study was carried out using the indigenous mixed-microorganisms in the wastewater. This pre-liminary study was done qualitatively to confirm the presence of biohydrogen producers in the wastewater sample. Gas obtained from the fermentation study was analysed using GC-TCD, and results confirm that some microorganisms presence in the wastewater have the potential to produce biohydrogen. GC analysis conducted on the collected gas (after 72 h) shows that there are only hydrogen and carbon dioxide produced by the mixed-microorganisms (Figure 1). This finding is in accordance with the study done by Reungsang et al. (2013) who also obtained only biohydrogen and CO_2 by UASB granules from glycerol, except that the granules were heat-treated before the experiment. No methane was produced, indicating that there are no methanogenic microorganisms in the mixed-cultures.

Based on area of H_2 of chromatogram, it is found that the hydrogen produced using crude glycerol is more than the pure glycerol. Crude may contain impurities that help in adaptation of the microorganisms. Thus, without the need to adaptation, the microorganisms favour the crude glycerol more than the pure glycerol. While most literature indicates negative effect of CG impurities, Xu, et al., (2012) proved that only methanol inhibit the microbial conversion but other impurities showed positive influences on microbial growth by increasing biomass concentration and lipid production.

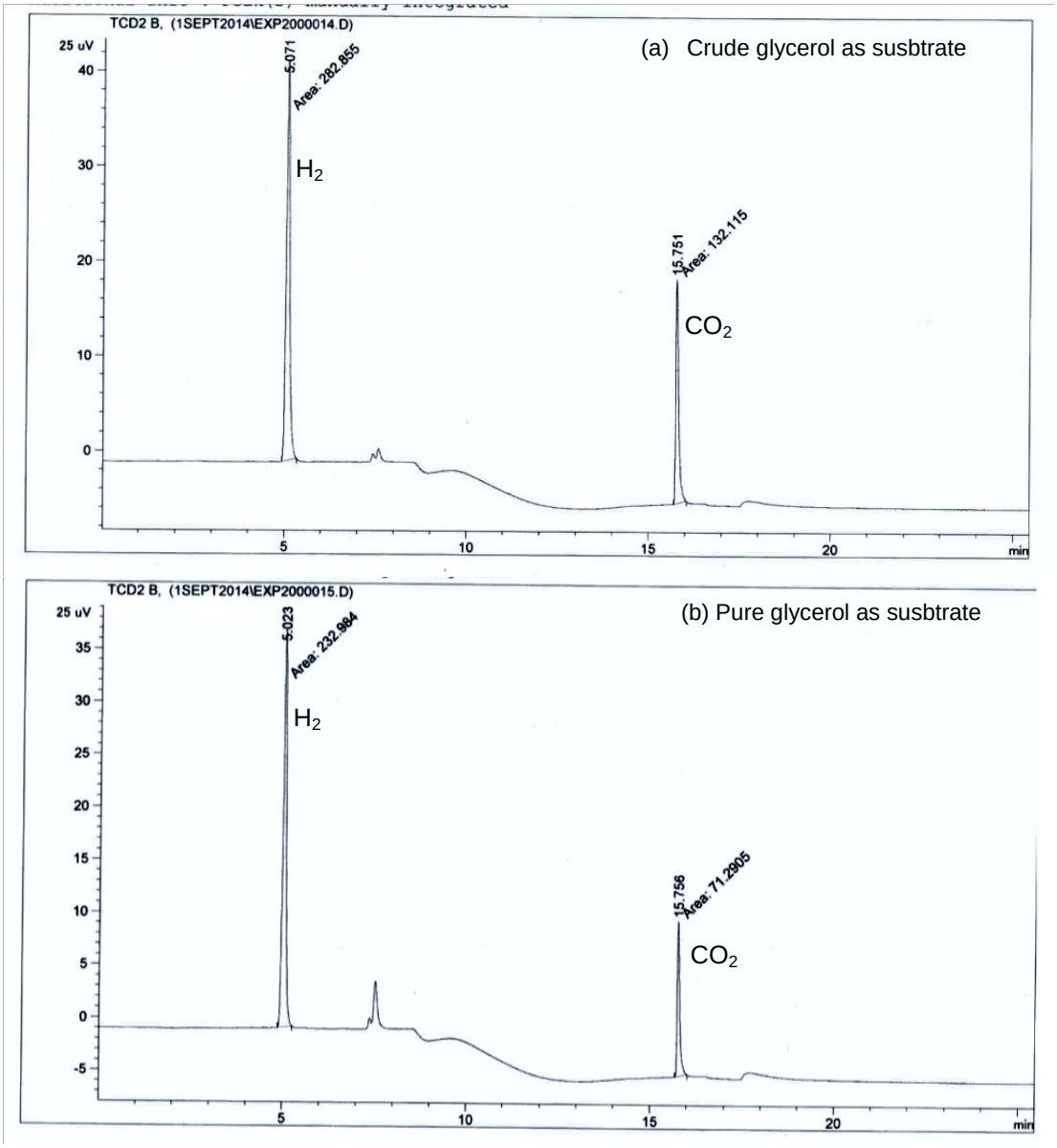

Figure 1: Presence of biohydrogen from dark fermentation of wastewater using (a) crude glycerol and (b) pure glycerol as substrate

3.2 Isolation and Screening for Potential Hydrogen Producer

Literatures reported that several microorganisms have ability to produce biohydrogen. In this study, the source of microorganisms was obtained from wastewater of biodiesel plant (anaerobic sludge pond). Thus, there may be chances to successfully isolate a few microorganisms. Isolation was done aseptically in anaerobic condition. Since there is no proper incubation chamber/anaerobic jar to incubate the isolates in anaerobic condition, a conventional way was employed. The isolation was performed using pour plate method and incubated in closed jar for 72 h at 37 °C.

Observations made after 72 h (Figure 2) show that by using pure glycerol as substrate gave only 54 isolates with most of the isolates are circular, raised, and cream in colour but only two showed the ability to produce gas (shown by the existence of gas globules inside the agar). The sizes also are small with entire edge and smooth texture. As for crude glycerol, the colonies observed are somewhat different from those in pure glycerol. Not just that, there are only 8 isolates grown in the plates, with all eight isolates are cream in colour, translucent, circular, raised, and moderate in size.

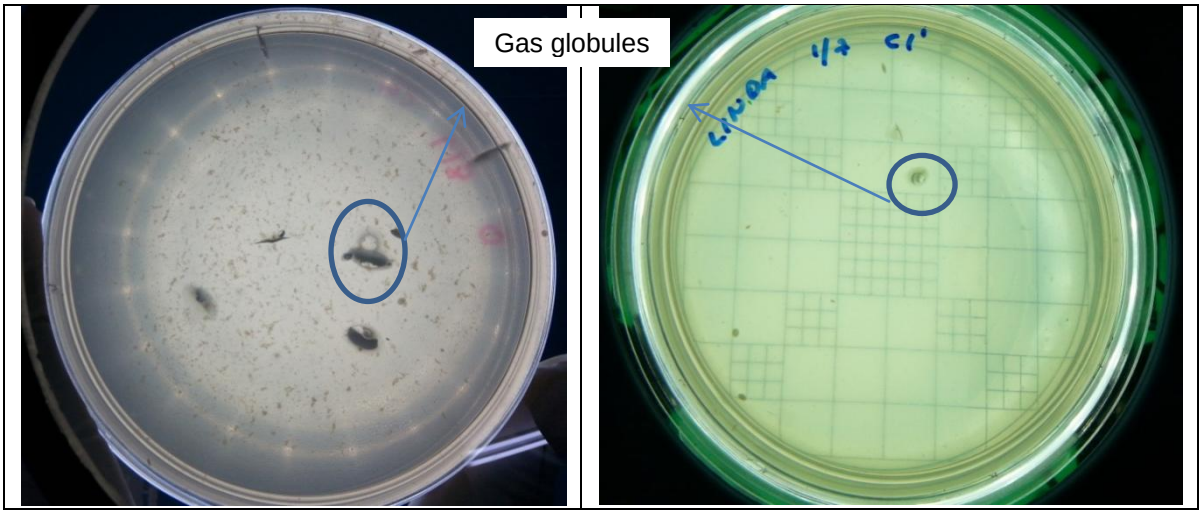

Figure 2: Gas globules showing the gas produced by the colony

4. Conclusions

This study confirms that there are potential hydrogen producers in the biodiesel wastewater with no methanogenic bacteria. Although there may be thousands of bacteria presents in wastewater, only those with the potential of producing gas were able to grow in the minimal medium. Findings showed that the new locally isolated microorganisms have the potential to produce hydrogen. These bacteria also can consume crude glycerol as the substrate and convert it to mainly hydrogen and carbon dioxide. Results also indicate the absence of methanogenic bacteria since no methane was produced. To optimize the yield, further research needs to be carried out on the characterization of the strains and the operating conditions.

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