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Rapid Screening Method for Isolation of Glycerol-consuming Bacteria for Ethanol Production

(Kaedah Saringan Pesat untuk Pengasingan Bakteria Penggunaan-Gliserol untuk Penghasilan Etanol)

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ABSTRACT

Large numbers of glycerol-consuming bacteria are present in nature; hence bioconversion of glycerol into biofuel which is bioethanol is one of the interests. The effective screening procedure is needed to screen and isolate broad ranges of bacteria from environment. The screening method was modified based on enzymatic oxidation of ethanol, which is correlated to reduction of 2,6-dichlorophenol-indophenol dye that resulted in the formation of yellow zone. Approximately 300 colonies were able to grow on minimal media using glycerol as sole carbon. Only about 70 isolates showed positive result when using the modified ethanol production assay after pre-screening stage. The formation of decolourized zone was apparent using modified assay containing 5 mL/L of 0.05M 2,6-dichlorophenol-indophenol, 10 mL of reaction mixture and 500 µl/L of enzyme, respectively. The ethanol production capability of the isolates was further proven by anaerobic fermentation as a quantitative method. This modified method is applicable in screening for ethanol producer from glycerol as carbon source allows rapid and more bacteria can be screened.

Keywords: 2,6-dichlorophenol-indophenol; ethanol production; glycerol-utilization; screening and isolation

ABSTRACT

Bio-penukaran gliserol kepada bioetanol menjadi satu kepentingan kerana sejumlah besar bakteria di dalam alam sekitar didapati berupaya untuk menggunakan gliserol. Oleh yang demikian, kaedah penyaringan yang berkesan diperlukan untuk menyaring dan memencilkan bakteria dalam jumlah yang banyak dari alam sekitar. Kaedah penyaringan telah diubah suai berdasarkan pengoksidaan enzim etanol yang berkait rapat dengan pemudaran pewarna 2,6-diklorofenol-indofenol dan seterusnya menghasilkan zon kuning. Kira-kira 300 koloni bakteria didapati dapat hidup apabila media minimum digunakan dengan gliserol sebagai sumber karbon utama. Hanya 70 daripada koloni tersebut menunjukkan keputusan positif daripada kaedah analisis etanol yang diubah suai pada peringkat pra-saringan. Pembentukan zon warna yang pudar dapat dilihat dengan jelas apabila menggunakan komposisi yang telah diubah suai iaitu 5 mL/L 0.05M 2,6-diklorofenol-indofenol, 10 mL campuran tindak balas dan 500 µl/L enzim. Keupayaan bakteria pencilan tempatan tersebut dibuktikan dengan fermentasi anerobik sebagai kaedah kuantitatif. Kaedah yang diubah suai ini boleh diguna pakai untuk menyaring lebih banyak bakteria yang berpotensi sebagai pengeluar etanol daripada gliserol sebagai sumber karbon dengan lebih cepat.

Kata kunci: Menyaring dan memencilkan; penggunaan gliserol; penghasilan etanol; 2,6-diklorofenol-indofenol

INTRODUCTION

Glycerol is produced as by-product during the production of biodiesel. The glycerol production is expected to increase in parallel to the increasing demand of biodiesel. Various valuable chemicals that have been produced from glycerol using biological conversion include 1,3-propanediol (Biebl et al. 1992; Hao et al. 2008; Papanikolaou et al. 2000), 1,2-propanediol (Jung et al. 2011), dihydroxyacetone (Bauer et al. 2005; Bories et al. 1991; Claret et al. 1999), ethanol (Dharmadi et al. 2006; Ito et al. 2005; Jarvis et al. 1997; Nakas et al. 1983), succinate (Lee et al. 2004, 2001), propionic acid (Barbirato et al. 1997; Himmi et al. 2000), citric acid (Imandi et al. 2007; Papanikolaou et al. 2002), lactic acid (Hong et al. 2009), polyhydroxylalcanoate or polyhydroxybutyrate (Ashby et al. 2004; Ibrahim & Steinbuchel 2009; Lee et al. 2004) and biosurfactants (Zhang et al. 2005). Bioethanol is one of renewable and environmental-friendly types of biofuel. In practice, most bioethanol fermentation relies on sugar and starch feedstocks. Currently, ethanol fermentation using lignocellulosic biomass as feedstock has taken place as utilization of food crops, such as corn grain and cane juices, to produce ethanol raises major nutritional and ethical concerns (Bai et al. 2008). However, a typical lignocellulosic ethanol fermentation process usually requires 5 to 7 days (120-170 h) which leads to contamination problem in large scale fermentation (Pimentel 2009). Therefore, developing a new strategy on bioethanol production by using cheap and highly abundant biomass with simpler molecule structure, such as crude glycerol can be an advantage.

The application of yeasts in bioethanol fermentation is most well reported in literatures in which sugar-based materials were used as the main feedstock (Willke & Vorlop 2004). *Enterobacter aerogenes* HU101 (Ito et al. 2006), *Klebsiella planticola* (Jarvis et al. 1997) and the species of *E. coli* (Dharmadi et al. 2006) are among the bacteria that have been reported as ethanologen using glycerol as carbon source. The studied candidates are often unable to convert glycerol efficiently into ethanol at higher glycerol concentration, i.e. more than 10 g/L. Hence, isolating potential ethanologens that can utilise glycerol efficiently at higher concentration (more than 10 g/L) using simple screening method may further encourage the application of glycerol in fuel ethanol production. The conventional method of screening ethanologens involves selection by cultivating colonies in YPD (yeast-peptone-dextrose) broth and subsequently analyzing ethanol produced by high performance liquid chromatography, gas chromatography or enzyme assay. This screening and isolation procedure was time consuming and limiting the number of isolates to be screened. A rapid screening method for yeast involved in ethanol fermentation from xylose was reported by Jacobs et al. (1983). This simple screening method involves oxidation/reduction of ethanol and NAD^+ into acetaldehyde and $NADH_2$ in the presence of alcohol dehydrogenase. Reduced form of NAD^+ ($NADH_2$) couples with 2,6-dichlorophenol-indophenol dye (blue compound) to become reduced form of 2,6-dichlorophenol-indophenol (yellow).

This paper describes a modified screening method based on Jacobs et al. (1983) procedure to detect ethanol-producing bacteria which utilized glycerol as carbon source. The isolates were subsequently subjected to simple fermentation process as quantitative test.

Materials and Methods

Screening and Isolation Procedure

The medium used as bacterial isolation containing 1 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 1 g/L yeast extract, 15 g/L NaCl and supplemented with 20 g/L of glycerol as major carbon source. NaCl was supplemented to mimic the characteristic of glycerine wastes. Approximately 1 g of soil sample or 1 mL of wastewater sample was inoculated into 120 mL serum bottle containing 50 mL of medium which was prepared in anaerobic chamber. After overnight incubation at 37°C, a 0.1 mL of fermentation broth was transferred into new serum bottle containing 50 mL fresh media. After 4 series of incubation, a 0.1 mL of broth was diluted and plating on the agar-based medium in anaerobic condition. Further purification was done in order to get single colonies.

Single colonies grown on agar plate were subjected to further screening process which is a modified method from Jacobs et al. (1983). The isolates were first grown onto agar and incubated in anaerobic jar for 24 h. The rapid screening method was done in two different media, minimal media as mentioned previously, and Luria-bertani (LB) media supplemented with glycerol (20 g/L) and 15 g/L NaCl. Then, a 5 mL of reaction mixture consists of (per litre) agar (10 g), sodium phosphate buffer (0.1 M, pH 8), 50 µL 0.05 M 2,6-dichlorophenolindophenol (blue dye), 100 µL yeast alcohol dehydrogenase (diluted 1 in 10) and 100 µL 0.15 M NAD solution, was poured onto plate to allow solidification at room temperature for 30 min. Then, a 3 mL of 0.005 M 5-methyl-phenazinium methyl sulphate was spread on the agar plate and the plate was further incubated at 30°C for 30 min. The assay is based on the enzymatic reaction, where the presence of ethanol was determined by yellow zones formation surrounding colonies against a blue background. In this experiment, several modifications were performed which include the amount of 0.05 M 2,6-dichlorophenol-indophenol solution (blue compound), enzyme (9000 U/mL activity) and 0.15 M NAD solution added into the reaction mixture. Firstly, the amount of 0.05M 2,6-dichlorophenol-indophenol solution (blue compound) used was varied from 50 µL, 500 µL, 5 mL and 10 mL in a liter of reaction mixture. In this case, NADH was used as control. The ratio of enzyme alcohol dehydrogenase and NAD^+ was maintained as 1:1. Then, the amounts of enzyme alcohol dehydrogenase (9000U/mL activity) and 0.15 M NAD^+ ranging from 100 to 1000 µL (per litre) were tested in this study. Finally, appropriate amount of reaction mixture was characterized according to following volume: 3, 5 and 10 mL.

Confirmation Test via Batch Fermentation

To prove this method is applicable in screening for ethanol producer, selected isolates with yellow zones were subjected to anaerobic fermentation using glycerol as carbon source. Positive control used in this experiment was *Enterobacter aerogenes* HU101 as well-known ethanol producer from glycerol (Ito et al. 2005). A 50 mL of media broth (minimal and LB-glycerol) was prepared, sparged with nitrogen gas for 15 min to create anaerobic condition, and transferred into closed serum bottle. A single colony was inoculated into the media under anaerobic condition and then incubated at 37°C, 120 rpm for 24 h. The ethanol produced from the fermentation was measured quantitatively.

Result and Discussion

Screening and Isolation Procedure

The method adopted in this study was according to Jacobs et al. (1983) in which it was initially used to determine ethanol production from yeasts by using xylose as carbon source. This rapid method involved simple procedure and hence large number of isolates can be compared on the agar plate. The ethanol formed by grown colony was detected by enzymatic technique whereby the alcohol dehydrogenase and 2,6-dichlorophenol-indophenol were used as catalyst

and redox mediator, respectively (Smith & Olson 1975). Acetaldehyde and NADH were produced through this ethanol oxidation in the presence of NAD^+. The NADH produced will couple with 2,6-dichlorophenol-indophenol dye, which is blue compound (oxidized) and then turned to colourless compound (reduced) (Smith & Olson 1975). The colourless zone can be visualized by developing yellow zone by using 5-methyl-phenazinium methyl sulphate. The enzymatic oxidation of ethanol is a correlation to the reduction of dye which is directly proportional to the amount of ethanol present in the sample (Fernandez et al. 1987; Smith & Olson 1975). However, difficulty in observing clear zone was encountered when screening method was used for ethanol producing bacteria as shown in Figure 1(a). As shown in Figure 1(b), the clear zone was apparent when 5 mL/L of 0.05 M 2,6-DCP was added in reaction mixture as compared to 50 µl/L, 500 µl/L and 10 mL/L of dye. On the other hand, a 500 µl/L of enzyme alcohol dehydrogenase and NAD^+ was sufficient to convert ethanol into acetaldehyde and NADH (Figure 1(c)). Clear zone could be observed by using 10 mL of reaction mixture per assay plate instead of 3 and 5 mL of reaction mixture as shown in Figure 1(d). The reaction mixture of 3 and 5 mL was not sufficient to cover the surface area of the plate. Dilution of ethanol produced from grown isolates may occur if more than 10 mL of reaction mixture was used. Others parameters such as incubation temperature and incubation time were maintained as described previously, since higher temperature and prolonged reaction time led to unstable colour formation.

Approximately 300 colonies were successfully grown in glycerol-supplemented minimal medium. These colonies were then subjected to modified screening procedure as mentioned earlier to isolate ethanol-producing bacteria. The ethanol produced by the isolates was detected by clear zone formed around the colonies which become yellow in the presence of phenazine methosulphate. The diameter of the clear zone signifies the amount of ethanol produced by the isolates, where it was measured from edge of the colony. About 70 isolates showed positive results with the diameter ranging from 0.02 - 0.50 cm after 24 h of incubation. Table 1 shows the diameter of clear zone produced by the selected isolates including positive control. It is clear that isolate B11 and AM108 are the potential ethanol producing bacteria since the diameter obtained by these two isolates were comparable to positive control, *Enterobacter aerogenes* HU101. These two isolates were identified as Gram negative bacteria which are in agreement with most of the ethanol-producing microorganisms involved in glycerol fermentation (Biebl 2001; Lee et al. 2001).

In addition to minimal medium, the clear zones developed by the selected isolates were compared by culturing the isolates in LB medium. The LB medium containing high nitrogen source can enhance the growth of microorganism and subsequently ethanol production. The diameter of clear zone for all selected isolates grown in LB media increased as shown in Table 1. It was observed that isolate H1 might be a potential ethanol producer since the diameter of clear zone developed on LB agar plate

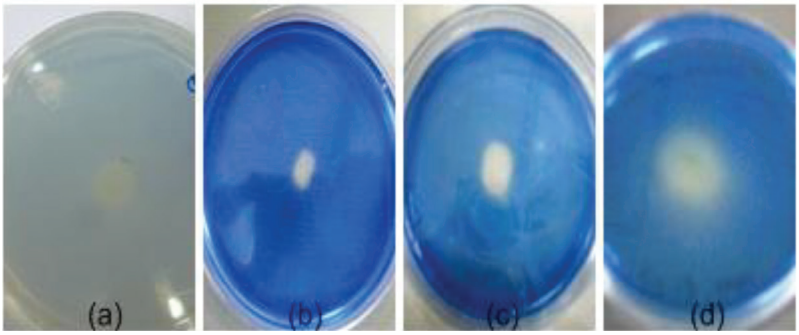

FIGURE 1. Clear zone observed at various conditions. NADH was used as control (a) Negative results following method by Jacobs et al. (1983) (b) Clear zone developed at 5 mL/L of 0.05M 2,6-DCP in reaction mixture (c) Clear zone at 500 µl/L of enzyme and NAD^+ in reaction mixture and (d) Clear zone developed at 10 mL of reaction mixture per assay plate

TABLE 1. Diameter of clear zone of selected strains

Strains	Glycerol-based minimal medium		Glycerol with LB medium	
	[#]24 h	72 h	24 h	72 h
E. aerogenes HU101	[*]0.25 ± 0.07	0.325 ± 0.04	0.45 ± 0.07	0.55 ± 0.07
H1	0.15 ± 0.07	0.2 ± 0	0.35 ± 0.07	0.475 ± 0.18
B11	0.25 ± 0.07	0.3 ± 0	0.4 ± 0	0.45 ± 0.07
AM108	0.25 ± 0.07	0.3 ± 0	0.35 ± 0.07	0.5 ± 0.14

The results are the average of triplicate experiments
*diameter in cm
[#]Colony incubation time

TABLE 2. Ethanol production in minimal media and LB media using glycerol as carbon source

STRAIN	Minimal media ethanol produced (g/L)		LB media ethanol produced (g/L)	
	*24 h	72 h	24 h	72 h
E. aerogenes HU101	0.623 ± 0.01	0.777 ± 0.37	1.804 ± 0.42	1.772 ± 0.51
H	0.159 ± 0.04	0.194 ± 0.04	1.948 ± 0.60	1.934 ± 0.78
B11	0.790 ± 0.31	0.866 ± 0.18	1.926 ± 0.21	1.854 ± 0.20
AM108	0.360 ± 0.10	0.629 ± 0.24	2.640 ± 0.08	2.437 ± 0.44

The results are the average of triplicate experiments
*Colony incubation time

was comparable to that isolates B11 and AM108; even though the diameter of clear zone by H1 on minimal agar plate was smaller. Some additional nutrients are required to favor the ethanol production, especially by enhancing the biomass growth, which is supplemented by organic nitrogen source such as yeast extract and peptone presence in LB medium. The increase of diameter could also be observed when the incubation time was lengthened. Several advantages are observed by modifying the screening method recommended by Jacobs et al. (1983) which include suitable for screening ethanol producing bacteria using glycerol as carbon source, clearer zone obtained and lesser incubation time which is within 24 h.

CONFIRMATION TEST VIA BATCH FERMENTATION

Further quantitative ethanol measurement was undertaken in order to prove the reliability of the modified screening and isolation procedure by performing batch anaerobic fermentation. Glycerol fermentation was compared in 120 mL serum bottle using minimal and LB media, respectively. Table 2 shows the ethanol production by isolates H1, B11, AM108 and *Enterobacter aerogenes* HU101 (positive control). It seems that all isolates produced ethanol in both minimal and LB media. This finding supports the observation of modified screening method. In glycerol-based minimal medium, isolate B11 produced higher amount of ethanol than *Enterobacter aerogenes* HU101 and the other two isolates. The result obtained in this experiment is in agreement with the diameter of clear zone observed on agar plate. Moreover, LB medium supplemented with glycerol enhanced the growth and subsequently ethanol production of all isolates as higher amount of ethanol was produced in comparison to minimal medium. However, isolate AM108 produced 2.64 g/L of ethanol at 24 h of incubation, which was the highest among all the tested strains. It seems that nutrients present in LB media may play an important role in enhancing the ethanol production. The isolates obtained in this study have shown ethanol production capability and therefore further investigation is recommended.

CONCLUSION

A modified screening method was successfully developed which allows more number of isolates to be screened within 24 h for ethanol detection. This qualitative assay is able to screen ethanol producing bacteria using glycerol as carbon source. The amount of 0.05 M 2,6-dichlorophenol-indophenol solution, reaction mixture per assay plate and enzyme alcohol dehydrogenase with NAD^+ solution, were characterized to obtain significant color changes in the presence of ethanol. The reliability of this modified screening method was further supported by quantitative ethanol measurement from batch fermentation.

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