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Isolation of Bacteria in Clarifier Effluent of Sour Pork Industry Able to Produce 1,3-Propanediol from Crude Glycerol

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Abstract. The objective of this research was to isolate bacteria that were capable of utilizing glycerol as carbon source and study the optimal condition of 1,3-propanediol production from crude glycerol. The bacteria isolates B-2, B-4 and B-7 was grow and produce 1,3-propanediol in basal medium formula 1, 2 and 3 that containing glycerol as a sole carbon sources. The bacteria B-2 produced highest 1,3-propanediol in basal medium formula 2. In addition, the various factors that influenced to crude glycerol fermentation in basal medium formula 2 by the bacteria B-2 were investigated. It was found that the optimal conditions of 1,3-propanediol production were 10% bacterial inoculums and crude glycerol about 50 g/L were added. pH of basal medium was adjusted around 7 and the bacterial culture was incubated at 30^{o}C with shaking at 100 rpm for 3 days. The highest yield of 1,3-propanediol was obtained about 0.4167 g/L.

Introduction

Crude glycerol about 5-10% is produced from transesterification of oil for biodiesel production [1]. The global biodiesel production was over 15 billion litres in 2009 and it is still increasing. The forecast for the worldwide production is over 45 billion litres in 2020 [2]. The increased biodiesel production has a created a surplus of crude glycerol, resulting in lower glycerol price. Furthermore, crude glycerol could be considered as a hazardous waste because of its low flash point [3]. The conversion of crude glycerol to higher-value products could be a way to decrease the cost of biodiesel production and to avoid accumulation of crude glycerol. Among potential products from glycerol, 1,3-propanediol (1,3-PDO) has been of increasing interest for many industry. The main application of 1,3-PDO was used as a substrate in polymerization of a new type of polyester, polytrimethylene terephthalate (PTT), which is a type of polyester used in engineering of thermoplastics and in the production of carpets, textile and fibers industries [4]. Moreover, it has been in the production of foods, lubricants and medicines [5]. It also used to improve the properties of solvents, adhesives, laminates, resins, detergents and cosmetics [4]. Over 105 tons of 1,3-PDO are produced annually, primarily through chemical synthesis [6]. However, production of 1,3-PDO from glycerol using chemical method has several disadvantages such as low selectivity, requirement of high pressure and temperature, the use of toxic organic solvents, low yields (5-15% w/w), production of toxic intermediates [7], and the consequently high price hinders the utilization of 1,3-PDO in polymer industries. Therefore, there is much interest in developing an improved and more environmentally favorable process for 1,3-PDO production. One potential method for improved 1,3-PDO production is via the microbial fermentation of glycerol [8]. The use of natural organisms to produce 1,3-propanediol is well studied in bacteria. The common mechanism of glycerol fermentation involves a reductive pathway and an oxidative pathway [4]. In the reductive pathway, a vitamin B_{12}-dependent glycerol dehydratase catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde, and this compound is further reduced to 1,3-PDO by 1,3-propanediol oxidoreductase. In the oxidative pathway, glycerol is dehydrogenated to dihydroxyacetone by a NAD^{+}-linked glycerol dehydrogenase, which is then converted to dihydroxyacetone phosphate by an ATP-dependent dihydroxyacetone kinase. Dihydroxyacetone phosphate is an intermediate of the glycolysis that can be further converted to acetate and to other products, depending on the type of

bacterium [9]. The natural producers of 1,3-PDO from glycerol are of genera *Klebsiella, Clostridium, Citrobacter, Enterobacter* and *Lactobacilli* [10,11,12,13,14,15]. Among these organisms, *C. butyricum* and *K. pneumonia,* have been reported as the best natural producers because of their substrate tolerance, facultative anaerobes, yield and productivity [16,17]. Unfortunately, a major problem is that the best 1,3-propanediol producers are pathogenic [17] and completely anaerobic conditions through the process of fermentation is operated [18]. Therefore, this research aims to find new bacterial strains from clarifier effluent of sour pork that was able to produce 1,3-PDO in micro-aerobic conditions.

Material and Methods

First, the wastewater was collected from clarifier effluent of sour pork in Uttaradit Province (Thailand). Sample (1 ml) of waste water was added to 90 ml of PB solution and then 10-fold serially diluted was performed. Each serial dilution was spread onto R2A agar media. Cultures were incubated at 35^{o}C for 48 hr. Colonies that grown under R2A agar were subcultured for pure culture isolation onto yeast extract mineral medium agar containing 20% glycerol. Subcultures were incubated for 3 days at 30^{o}C. After that, the pre-culture was prepared by a full loop of bacterial cell code B-2, B-4 and B-7 were inoculated into a 100 mL liquid medium which consisted of 10g/L peptone, 10g/L yeast extract, 5g/L beef extract, 5g/L ammonium sulfate and 2g/L glycerol and then were cultivated at 30^{o}C in shaker bath at 200 rpm for 3 days. Then, 10% pre-culture were inoculated into three formula of production medium. 1st formula medium consisted of (per liter) 68 g glycerol, 0.26 g K_2HPO_4, 0.02 g KH_2PO_4, 1.23 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.2H_2O$, 0.01 g $FeCl_2.7H_2O$ and 2.0 g yeast extract. 2nd formula medium contained (per liter) 80 g glycerol, 3.4 g K_2HPO_4, 1.3 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.005 g $FeSO_4.7H_2O$ and 2.0 ml trace element. 3rd formula medium composed of (per liter) 100 g glycerol, 0.69 g K_2HPO_4, 0.25 g KH_2PO_4, 6.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$ and 1.0 ml trace element. The composition of the trace elements (per liter) was 0.07 g $ZnCl_2$, 0.1 g $MnCl_2.4H_2O$, 0.06 g H_3BO_3, 0.2 g $CoCl_2.6H_2O$, 0.02 g $CuCl_2.2H_2O$, 0.025 g $NiCl_2.6H_2O$ and 0.035 g $Na_2MoO_4.2H_2O$. The culture medium was incubated at 30^{o}C in an orbital shaker at 100 rpm for 72 hr. Samples were taken from the flask cultures every 6 hr for analysis of cell dry weight and 1,3-PDO. All experimental trials were performed in triplicate.

Second, production of 1,3-propanediol from crude glycerol was investigated. A full loop of bacterial cell code B-2 was inoculated into 100 ml pre-culture medium. The culture was incubated in an orbital shaker (200 rpm) at 30^{o}C for 60 hrs. After that 10% pre-culture medium was added into 500 ml 2nd formula production medium and incubated at 30^{o}C in orbital shaker at 100 rpm for 72 hrs. The factors that effect on 1,3-PDO synthesis of bacterial cell code B-2were investigated such as bacterial inoculums concentration, crude glycerol concentration, initial pH and incubation temperature. All experimental trials were carried out in triplicate.

The cell dry weight was determined gravimetrically after collection of 4 mL culture broth for 20 min at 4^{o}C and 4500 rpm, including a washing step with distilled water, and drying of the obtained pellet at 105^{o}C until constant weight.

The 1,3-PDO concentration in cultivation supernatant was analyzed by HPLC Agilent 1200 equipped with Aminex HPX-87H (300mm x 7.8mm) column and a refractive index detector. The column temperature was 65^{o}C, 0.5 M sulfuric acid was used as mobile phase at a flow rate of 0.6 ml/min, and an injection volume was 20 μL [18].

Results and Discussion

Isolation of 1,3-PDO producing bacteria from wastewater. Three strains of bacteria isolated from wastewater that collected from clarifier effluent of sour pork could produce 1,3-PDO. They were coding as B-2, B-4 and B-7, respectively. The colony was circular shape, white, and undulates. Bacteria code B-2 was grown in medium containing pure glycerol and produced maximum concentration of 1,3-PDO (Table 1). Therefore, bacteria B-2 was used in the next experiment.

Table 1 Bacterial isolates and ability to produce 1,3-PDO from glycerol

Microorganism	Cell dry weight* (mg/L)	1,3-PDO concentration* (mg/L)
B-2	835.30±41.76	417.60±20.88
B-4	532.20±26.66	266.60±13.33
B-7	545.40±27.27	88.90±0.44

*Average±standard deviation error from triplicate

Production of 1,3-PDO from Crude Glycerol. Crude glycerol that collected from transesterificarion of waste oil catalyzed by KOH for biodiesel production was partially purified. Some properties of purified glycerol such as pH, glycerol content, ash, water content, density and viscosity were 7.11±0.35, 64.58±3.22%, 1.90±0.09%, 2.80±0.14%, 1.205±0.06 g/cm^3 and 108.40±5.42 cSt, respectively.

The result of glycerol fermentation by bacteria B-2 was initially investigated. Bacteria B-2 was able to grow in basal medium containing crude glycerol, 80 g/L, which 10% bacterial inoculums were added and performed at 30^{O}C in shaking incubator at 100 rpm for 72 hrs. Cell dry weight and 1,3-PDO content were 751.40±37.50 mg/L and 375.70±18.70 mg/L, respectively. Then, the influence of initial inoculums concentration on growth and production of 1,3-PDO in batch fermentation was examined. The effect of bacterial inoculums concentration was shown in Table 2. The cell dry weight after bacterial cell growth (751.41±37.57 mg/L) and the 1,3-PDO content (375.70±18.78 mg/L) were highest at 10% inoculums. These result indicated that the inoculum at 10% was suitable volume for fermentation that was similar to crude glycerol fermentation of *Klebsiella pneumonia* SU6 [19].

Table 2 Effect of bacterial inoculums concentration on cell growth and 1,3-PDO production

Inoculum concentration (%)	Cell dry weight* (mg/L)	1,3-PDO concentration* (mg/L)
5	417.38±20.86	208.69±10.43
10	751.41±37.57	375.70±18.78
15	393.48±19.67	196.74±9.83
20	462.74±23.13	231.37±11.56

*Average±standard deviation error from triplicate

Glycerol is a carbon and energy source for 1,3-PDO production and its availability has an influence on the product formation [20]. Therefore, the effect of crude glycerol that partially purified on growth and production of 1,3-PDO of bacteria B-2 was investigated. The effect of the crude glycerol concentrations on cell growth and 1,3-PDO production in bath fermentation with 10% inoculums at 30^{o}C and shaking at 100 rpm for three days was shown in Table 3. The highest cell dry weight of bacterial cell growth (822.51±41.12 mg/L) and highest 1,3-PDO concentration (411.25±20.56 mg/L) were found at 50 g/L crude glycerol. When the concentration of glycerol increased, both of cell dry weight and 1,3-PDO production were decreased. This finding indicated that at high amount of glycerol went to be the lethal 3-hydroxypropionaldehyde accumulation which was toxic to microbial cell [17]. Besides, the high concentration of crude glycerol might contain impurities that could inhibit bacterial growth [19].

Table 3 Effect of crude glycerol concentration on bacterial cell growth and 1,3-PDO production

Crude glycerol concentration (g/L)	Cell dry weight* (mg/L)	1,3-PDO* (mg/L)
50	822.51±41.12	411.25±20.56
60	789.41±39.47	394.70±19.73
70	718.93±35.94	359.46±17.97
80	751.40±37.57	375.70±18.78

*Average±standard deviation error from triplicate

The pH is one of the main factors influencing cell growth and 1,3-propanediol production of bacteria since the catalytic activity of the enzyme and the metabolic activity of the microorganisms depend on the extracellular pH [21]. The effect of initial pH in the range of 3-9 on cell growth and

production of 1,3-PDO production were studied using 10% bacterial inoculums and the optimal glycerol concentration at 50 g/L. The results were shown in Table 4. At pH 7.0, the cell growth and 1,3-PDO production were the highest about 833.54±41.67 mg/L and 416.77±20.83 mg/L, respectively at 72 hrs. This corresponded to continuous glycerol fermentation by *Clostridium butyricum* [22]. At higher pH such as 9, cell dry weight and 1,3-PDO production were decreased. These results are consistent with Garg and Jain [23] who reported that alkaline condition favored the formation of other products and decrease in the production of 1,3-PDO.

Table 4 Effect of pH on bacterial cell growth and 1,3-PDO production

pH	Cell dry weight* (mg/L)	1,3-PDO* (mg/L)
3	393.48±19.67	196.74±9.83
5	478.67±23.93	239.33±11.96
7	833.54±41.67	416.77±20.83
9	524.03±26.20	262.01±13.10

*Average±standard deviation error from triplicate

Since, the temperature that needed for cell growth and 1,3-PDO production were different, the effect of temperature (10, 20, 30, 40 and 50^{o}C) was studied. The results were shown in Table 5. Bacterial cell dry weight increased in the temperature range of 10-30^{o}C. However, the temperature from 30^{o}C to 50^{o}C caused the decrease in bacterial growth. The maximum cell dry weight and 1,3-PDO concentration were obtained about 833.54±41.67 mg/L and 416.77±20.83 mg/L, respectively, at 30^{o}C. This phenomenon can be explained that the high temperature might inactivate the enzyme activity in glycolysis and Krebs' cycles, then decreased the carbon metabolism [24]. Therefore, bacterial cell growth was inhibited at high temperature.

Table 5 Effect of temperature on bacterial cell growth and 1,3-PDO production

Temperature (^{o}C)	Cell dry weight* (mg/L)	1,3-PDO* (mg/L)
10	393.48±19.67	196.74±9.83
20	478.67±23.93	239.33±11.96
30	833.54±41.67	416.77±20.83
40	524.03±26.20	262.01±13.10
50	197.35±9.86	98.67±4.93

*Average±standard deviation error from triplicate

Summary

Bacterial strain was isolated from wastewater that collected from clarifier effluent of sour pork was able to synthesize 1,3-PDO from crude glycerol.

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