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Hydrogen and Ethanol Production from Glycerol-Containing Wastes Discharged after Biodiesel Manufacturing Process

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H_2 and ethanol production from glycerol-containing wastes discharged after a manufacturing process for biodiesel fuel (biodiesel wastes) using *Enterobacter aerogenes* HU-101 was evaluated. The biodiesel wastes should be diluted with a synthetic medium to increase the rate of glycerol utilization and the addition of yeast extract and tryptone to the synthetic medium accelerated the production of H_2 and ethanol. The yields of H_2 and ethanol decreased with an increase in the concentrations of biodiesel wastes and commercially available glycerol (pure glycerol). Furthermore, the rates of H_2 and ethanol production from biodiesel wastes were much lower than those at the same concentration of pure glycerol, partially due to a high salt content in the wastes. In continuous culture with a packed-bed reactor using self-immobilized cells, the maximum rate of H_2 production from pure glycerol was 80 mmol//h yielding ethanol at 0.8 mol/mol-glycerol, while that from biodiesel wastes was only 30 mmol//h. However, using porous ceramics as a support material to fix cells in the reactor, the maximum H_2 production rate from biodiesel wastes reached 63 mmol//h obtaining an ethanol yield of 0.85 mol/mol-glycerol.

[**Key words:** hydrogen, ethanol, *Enterobacter aerogenes*, glycerol, biodiesel]

Biodiesel fuels are defined as fatty acid methyl or ethyl esters from vegetable oils or animal fats and they are used as fuel in diesel engines and heating systems (1, 2). Since biodiesel fuels have various advantages such as an alternative to petroleum-based fuel, renewable fuel, a favorable energy balance, lower harmful emissions and nontoxic fuel, they have drawn much attention recently. Although biodiesel fuels are produced chemically and enzymatically, glycerol is essentially generated as the by-product (3, 4). Glycerol generated is presently applied, for example, as a ingredient of cosmetics, but a further increase in the production of biodiesel fuels would raise the problem of efficiently treating wastes containing glycerol.

The microbial conversion of glycerol to various compounds has been investigated recently with particular focus on the production of 1,3-propanediol, which can be applied as a basic ingredient of polyesters (5–7). The fermentation of glycerol to 1,3-propanediol has been studied using microorganisms such as *Klebsiella pneumoniae* (8–10), *Citrobacter freundii* (11, 12), *Clostridium butyricum* (13, 14) and *Enterobacter agglomerans* (15). However, the biological production of H_2 and ethanol from glycerol is also attractive because H_2 is expected to be a future clean energy source and ethanol can be used as a raw material and a supplement to gasoline.

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Enterobacter aerogenes HU-101, isolated as a high-rate H_2 producer from methanogenic sludge (16, 17), can convert various carbohydrates, such as sugars and sugar alcohols, to H_2, ethanol, 2,3-butanediol, lactate and acetate. H_2 can be biologically produced either by photosynthetic microorganisms (18, 19) or fermentative anaerobes (20). Among the latter, *Clostridium* species have received much attention for their ability to produce either solvents (butanol and acetone) or acids (butyrate and acetate) as well as H_2 (21). We have studied H_2 production using *E. aerogenes* (22, 23) because *E. aerogenes*, unlike clostridia, exhibits uninhibited growth in an atmosphere of 100% H_2. During the course of these studies, we found that *E. aerogenes* HU-101 mainly produces H_2 and ethanol with a minimal production of other by-products when glycerol was used as the substrate. Thus, the microorganism can be utilized for the high-yield production of H_2 and ethanol from biodiesel wastes containing glycerol.

In this study, we evaluated the culture conditions of *E. aerogenes* HU-101 for the efficient production of H_2 and ethanol from biodiesel wastes. We also demonstrated the continuous production in a packed-bed reactor with and without a support material.

MATERIALS AND METHODS

Microorganism and culture conditions The microorganism used in this study was *E. aerogenes* HU-101 isolated from a methanogenic sludge developed in our laboratory (24). Cultures were

maintained at −80°C with 15% glycerol. A synthetic medium used in this study contained (per liter) 7.0 g of K_2HPO_4, 5.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.021 g of $CaCl_2 \cdot 2H_2O$, 0.12 g of $Na_2MoO_4 \cdot 2H_2O$, 2.0 mg of nicotinic acid, 0.172 mg of Na_2SeO_3, 0.02 mg of $NiCl_2$ and 10 ml of trace element solution containing 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of H_3BO_4, 0.01 g of $AlK(SO_4)_2 \cdot H_2O$, 0.001 g of $CuCl_2 \cdot 2H_2O$ and 0.5 g of Na_2EDTA per liter. A complex medium was prepared by adding the desired concentrations of yeast extract and tryptone to the synthetic medium. The biodiesel wastes containing glycerol were supplied from a biodiesel manufacturing factory in Hiroshima prefecture, Japan. The biodiesel fuel was chemically produced with potassium hydroxide as the alkali catalyst. The wastes contained 41% (w/w) glycerol. The amount of total organic carbon (TOC) in the wastes was 540 g/*l*, of which 524 g was soluble. The impurities were mainly composed of ash (8%, w/v) and methanol (25%, w/w). Although 0.04% (w/w) diacylglycerol and 0.01% (w/w) monoacylglycerol were contained in the wastes, triacylglycerol was not detected.

A modified Hungate technique in combination with the serum bottle technique (25) was used to culture the bacterium anaerobically. The medium without glycerol and phosphate buffer was boiled for 20 min, cooled on ice with continuous bubbling of N_2 gas, dispensed into serum bottles sealed with black butyl rubber stoppers, and then sterilized (18 min, 121°C). Concentrated aqueous solutions of glycerol and phosphate buffer autoclaved separately were then injected into the medium using a hypodermic syringe. After the inoculation of 2 ml of seed culture into serum bottles (approximately 125 ml bottles containing 50 ml of the culture medium) and adjustment of the pH to 6.8, the bottles were incubated at 37°C with agitation (120 rpm) (16).

Packed-bed reactor A cylindrical glass column reactor ($\phi 2.7 \times 17$ cm height) with a working volume of 60 ml was used for the continuous culture. Fresh medium was supplied from the bottom by a peristaltic pump (Decarf N-10; Taiyo, Tokyo) and evolved gas and effluent liquid were discharged from the top of the reactor (22). Two ml of the seed culture was transferred into the reactor. After 12 h of incubation in the batch mode, continuous cultivation was initiated by feeding the sterilized medium at a dilution rate of 0.1 h^{-1} with the peristaltic pump. The cells were cultivated anaerobically at 37°C without controlling pH. After the accumulation of cell flocs was observed at the bottom of the reactor, the volume and the content of gas produced were measured periodically. A quasi-steady state was confirmed, except for the cell mass in the reactor, on the basis of a constant H_2 evolution rate, remaining glycerol concentration and pH of the effluent. These values were measured at least twice per day. Dilution rate was increased stepwisely.

Nagao Porcell (diatomaceous clay; particle size, 4 to 10 mm [diameter]; apparent density, 0.38 g/ml; true density, 2.17 g/ml; porosity, 81%; average pore diameter, 128 μm; Nagao & Co., Okayama) was used as a support material to increase the number of cells retained in the reactor for continuous culture with biodiesel wastes (26).

Analyses Gas production was measured periodically by the displacement of saturated aqueous sodium chloride in a graduate cylinder. The concentrations of CO_2 and H_2 were determined by gas chromatography (GC 8A; Shimadzu, Kyoto) with a thermal conductivity detector (27). Lactate, acetate, ethanol, and 1,3-propanediol were measured using an HPLC system as previously described (28). Glycerol and formate were determined by enzymatic analysis using F-kit glycerol and F-kit formate (Roche Diagnostics K. K., Tokyo), respectively. The cell concentration was not measured because the medium containing biodiesel wastes was turbid.

RESULTS AND DISCUSSION

Medium composition for treatment of biodiesel wastes To ferment biodiesel wastes to H_2 and ethanol using *E. aerogenes*, it would be desirable not to add any supplements that support cell growth to reduce the cost of fermentation and wastewater treatment after fermentation. Therefore, batch fermentation was first carried out with biodiesel wastes diluted with deionized water. When biodiesel wastes were diluted to 80 mM glycerol with deionized water, glycerol was not completely consumed even after 48 h and no growth was observed after 48 h. This indicated that some nutrients should be added to ferment glycerol in biodiesel wastes. Therefore, the synthetic medium was used for dilution of biodiesel wastes. The rate of glycerol utilization further increased using the synthetic medium. When biodiesel wastes were diluted to 80 mM glycerol with the synthetic medium, glycerol was completely utilized after 24 h, yielding H_2 at 0.89 mol/mol-glycerol and ethanol at 1.0 mol/mol-glycerol (data not shown), respectively. The addition of both yeast extract and tryptone to the synthetic medium was effective in increasing the rates of H_2 and ethanol production (Fig. 1). Even in the medium containing 0.5 g/*l* yeast extract and 0.5 g/*l* tryptone, ethanol and H_2 production levels markedly increased after 12 h compared with those of the synthetic medium. The addition of 5 g/*l* yeast extract or tryptone was effective in increasing the rate of glycerol consumption as in the case of adding both (data not shown), suggesting that

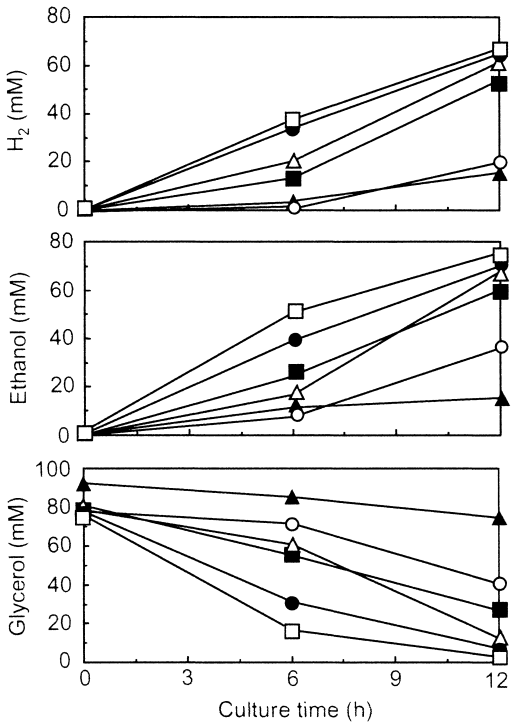

FIG. 1. Typical time courses of H_2 and ethanol production in batch culture using biodiesel wastes diluted with deionized water (closed triangles), synthetic medium (open circles) and complex medium containing 0.5 (closed squares), 1 (open triangles), 2.5 (closed circles), and 5 g/*l* (open squares) each of yeast extract and tryptone. Culture conditions: initial pH, 6.8; glycerol, 80 mM. Experimental values represent averages of at least duplicate cultures.

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