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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

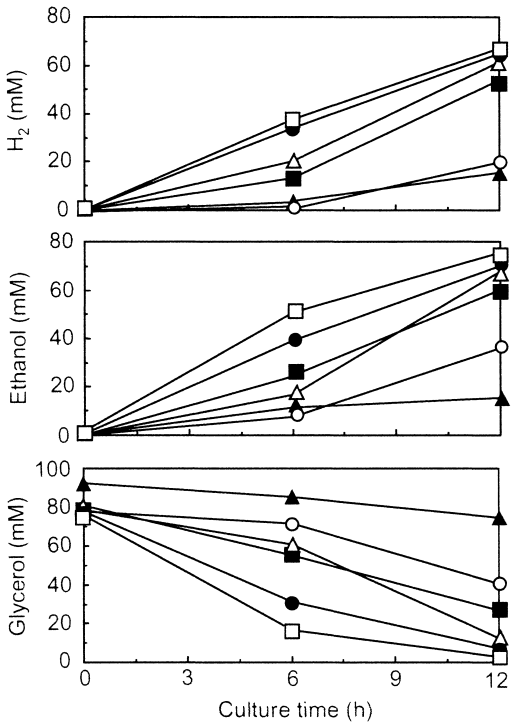


FIG. 1. Typical time courses of $H_2$ and ethanol production in batch culture using biodiesel wastes diluted with deionized water (closed triangles), synthetic medium (open circles) and complex medium containing 0.5 (closed squares), 1 (open triangles), 2.5 (closed circles), and 5 g/*l* (open squares)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

TABLE 1. Yields of end products for glycerol in biodiesel wastes diluted with complex medium

| Parameter | Glycerol concentration (g/*l*) | | | |
|---|---|---|---|---|
| | 1.7 | 3.3 | 10 | 25 |
| Time required for complete glycerol consumption (h) | 4 | 4 | 12 | >48[a] |
| Yield (mol/mol-glycerol) | | | | |
| $H_2$ | 1.12 | 0.90 | 0.71 | 0.71 |
| Ethanol | 0.96 | 0.83 | 0.67 | 0.56 |
| Lactate | ND | 0.05 | 0.11 | 0.17 |
| Acetate | 0.2 | 0.1 | 0.09 | 0.06 |
| 1,3-Propanediol | 0.2 | 0.22 | 0.12 | 0.17 |
| Formate | 0.14 | 0.2 | 0.19 | ND |

Batch cultures were carried out using the complex medium containing various concentrations of biodiesel wastes and 80 mM (1.7–3.3 g/*l* glycerol) or 160 mM (10–25 g/*l* glycerol) phosphate buffer at an initial pH of 6.8. Experimental values represent averages of at least duplicate cultures. ND, Not detected.

[a] Glycerol was not completely consumed within the time shown in the table.

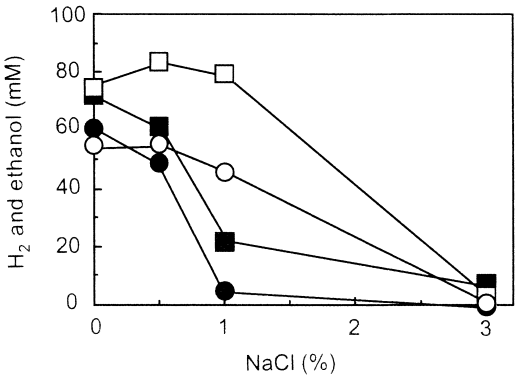


FIG. 2. Effect of sodium chloride concentration on $H_2$ (circles) and ethanol (squares) production by *E. aerogenes* HU-101 from biodiesel wastes (closed symbols) or pure glycerol (open symbols) in synthetic medium. Culture conditions: culture time, 24 h; initial pH, 6.8; glycerol, 10 g/*l*. Experimental values represent averages of at least duplicate cultures.

TABLE 2. Yields of end products for commercially available glycerol

| Parameter | Glycerol concentration (g/*l*) | | |
|---|---|---|---|
| | 5.0 | 10 | 25[a] |
| Time required for complete glycerol consumption (h) | 4 | 6 | 12 |
| Yield (mol/mol-glycerol) | | | |
| $H_2$ | 1.05 | 0.89 | 0.82 |
| Ethanol | 1.00 | 0.86 | 0.80 |
| Lactate | 0.06 | 0.14 | 0.12 |
| Acetate | 0.07 | 0.09 | 0.02 |
| 1,3-Propanediol | 0.06 | 0.16 | 0.14 |
| Formate | 0.10 | 0.12 | 0.01 |

Culture conditions: initial pH, 6.8; pure glycerol; complex medium; phosphate buffer, 80 mM. Experimental values represent averages of at least duplicate cultures.

[a] When glycerol concentration was 25 g/*l*, phosphate buffer was used at 160 mM.

some nutrients such as specific amino acids and vitamins that are still unknown are needed for the better growth of *E. aerogenes*.

**Effect of concentration of biodiesel wastes on fermentation** To minimize the reactor size and running cost, it is desirable that the concentration of biodiesel wastes is as high as possible. Therefore, batch fermentation was carried out with biodiesel wastes diluted with the complex medium, which consisted of the synthetic medium containing 5 g/*l* yeast extract and 5 g/*l* tryptone to 1.7, 3.3, 10 and 25 g/*l* as glycerol concentrations. The culture times needed for the complete utilization of glycerol and yields of end-products at the indicated times are shown in Table 1. The yield of $H_2$ from glycerol (1.12 mol/mol) exceeded the theoretical maximum yield of $H_2$ (1.0 mol/mol) with 1.7 g/*l* glycerol. This suggested that unknown carbon sources or electron sources in the wastes contributed to $H_2$ production. The yield of ethanol was almost the same as the theoretical maximum yield from glycerol and small amounts of acetate and 1,3-propanediol were detected in the case of using 1.7 g/*l* glycerol. However, the yields of $H_2$, ethanol and acetate decreased whereas the yield of lactate increased with the increase in the concentration of biodiesel wastes. Furthermore, when the glycerol concentration was 25 g/*l*, glycerol was not completely consumed even after 48 h and a decreased $H_2$ production was observed.

To determine the sole effect of glycerol concentration on substrate utilization and product formation, the complex medium supplemented with commercially available glycerol (pure glycerol) was applied to batch cultures. The microorganism completely consumed 5 g/*l* or 10 g/*l* pure glycerol within 6 h and 25 g/*l* after 12 h (Table 2). Although the yields of $H_2$ and ethanol were 1 mol/mol-glycerol using 5 g/*l* glycerol, they decreased with the increase in glycerol concentration, as observed in biodiesel wastes. The result indicated that a higher concentration of glycerol decreased the yields of $H_2$ and ethanol.

**Effect of impurities on $H_2$ and ethanol production by *E. aerogenes*** As shown in Tables 1 and 2, the rates of glycerol utilization in the medium with biodiesel wastes supplemented to provide 10 and 25 g/*l* glycerol were much lower than that in the medium with pure glycerol. Biodiesel fuel is currently produced using an alkali-catalyzed technology. The most commonly used alkali catalysts are sodium hydroxide, sodium methoxide and potassium hydroxide (3). Since an alkali is neutralized with an acid after esterification, biodiesel wastes may contain high concentrations of salts such as sodium chloride, which might inhibit cell growth. Figure 2 shows the effect of the concentration of added sodium chloride on the rate of glycerol utilization. Both $H_2$ and ethanol productions at 1% sodium chloride were almost the same as those without sodium chloride in the medium with pure glycerol. On the other hand, when sodium chloride was added to the medium with biodiesel wastes, $H_2$ and ethanol productions significantly decreased even at 1% sodium chloride. Indeed, when biodiesel wastes were diluted to 10 or 25 g/*l* glycerol, the solution should contain about 0.2% or 0.5% ash, respectively (see Materials and Methods). If the resulting ash is considered to be mostly sodium chloride, the decrease in the rate of product formation caused by the presence of the ash in biodiesel wastes seemed to be excessive compared with that caused by the

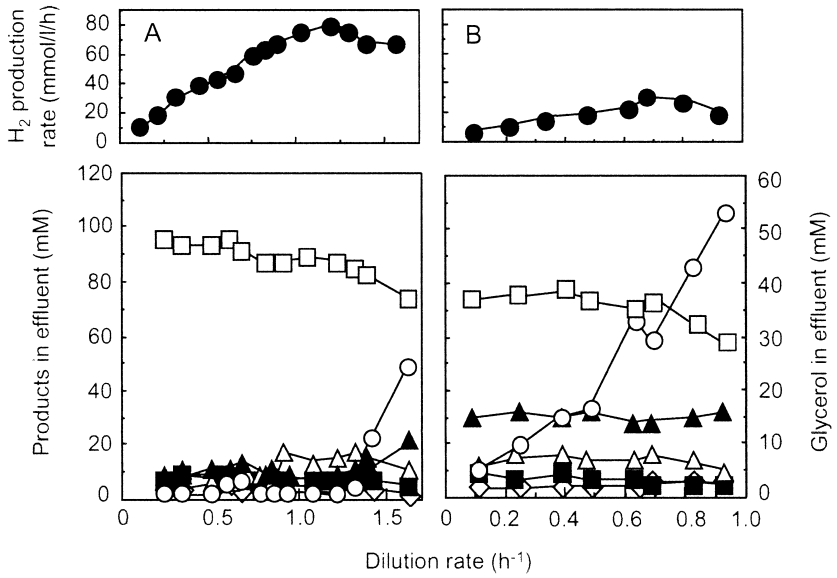


FIG. 3. $H_2$ production rates and end production concentrations during continuous culture by self-immobilization. (A) Cultivation on pure glycerol. (B) Cultivation on glycerol waste discharged from biodiesel process. Symbols: closed circles, $H_2$; open circles, glycerol; open squares, ethanol; closed triangles, formate; open triangles, 1,3-propanediol; closed squares, lactate; open diamonds, acetate. Culture conditions: complex medium (yeast extract, 5 g/*l*; tryptone, 5 g/*l*; glycerol, 110 mM) at 37°C.

presence of sodium chloride in the medium with pure glycerol as shown in Fig. 2. A high salinity of the medium with biodiesel wastes would be one of the causal factors for the inhibition of product formation.

Since about 25% (w/w) methanol was also contained in biodiesel wastes used in this study, about 1.5% methanol should be contained in biodiesel wastes diluted to 25 g/*l* glycerol, the concentration at which glycerol consumption significantly decreased. Therefore, the effect of methanol concentration on the growth of *E. aerogenes* was investigated using the complex medium containing 10 g/*l* pure glycerol. However, even when methanol was added up to 3%, no inhibition of the cell growth and glycerol consumption was observed, suggesting that methanol was not an inhibitory factor for this microorganism at the range of dilutions tested.

**Continuous cultures using packed-bed reactor with self-immobilized cells** To elucidate the production rate of $H_2$ and ethanol from biodiesel wastes, we carried out continuous culture using immobilized-cell systems, which have become common alternatives to suspended-cell systems in continuous operations because they are more efficient in solid/liquid separation and can be operated at high dilution rates without encountering washout of cells. Recent studies have clearly demonstrated that immobilized-cell systems using various support matrices are suitable for continuous hydrogen fermentation (29–32). In particular, since *E. aerogenes* flocculates, a continuous culture system using a fixed-bed reactor with self-immobilized cells have been developed as reported previously (22). Therefore, in this study, this continuous culture system was attempted to increase the productivity of $H_2$ and ethanol.

Figure 3 shows the production rates of $H_2$ and the concentrations of ethanol and other metabolites in the effluent from 110 mM pure glycerol and biodiesel wastes in the complex medium (5.0 g/*l* yeast extract, 5.0 g/*l* tryptone) in continuous culture with self-immobilized cells. The volumetric maximum $H_2$ production rate reached 80 mmol/*l*/h and glycerol was consumed completely at dilution rates up to 1.3 $h^{-1}$ (Fig. 3A). This rate of $H_2$ production was 2.6-fold and 1.3-fold higher than that from glucose using the same strain of *E. aerogenes* and the high-$H_2$-producing mutant AY-2 using the same culture system, respectively (22). This result indicates that glycerol is a very suitable substrate for producing $H_2$ as suggested previously (17). The yield of ethanol was maintained at more than 0.9 mol/mol-glycerol during the culture although the production of 1,3-propanediol increased at a higher dilution rate.

On the other hand, when biodiesel wastes were applied to the same culture system, the number of cells remaining in the reactor was much lower than in the reactor with pure glycerol. The flocs formed in biodiesel wastes were very downy and fragile, and easily washed out from the reactor with the increase in dilution rate. Some components in biodiesel wastes such as salts and/or oils that remained after the biodiesel manufacturing process might disturb the formation of rigid flocs. Residual glycerol was observed even at a low dilution rate (Fig. 3B), possibly, due to the low cell density in the reactor. The maximum rate of $H_2$ production with biodiesel wastes was 30 mmol/*l*/h at a dilution rate of 0.8 $h^{-1}$, which was much lower than that with pure glycerol. Indeed, the lower rate of glycerol consumption resulted in the lower rate of $H_2$ production. Furthermore, the accumulation of formate could also explain why $H_2$ production rate decreased in the case of using biodiesel wastes because formate is generally converted to $H_2$ and $CO_2$ as catalyzed by formate hydrogen lyase, although the reason for formate accumulation is still unclarified.

**Continuous culture using packed-bed reactor with support material** The results obtained from the analysis using the packed-bed reactor system with self-immobiliza-

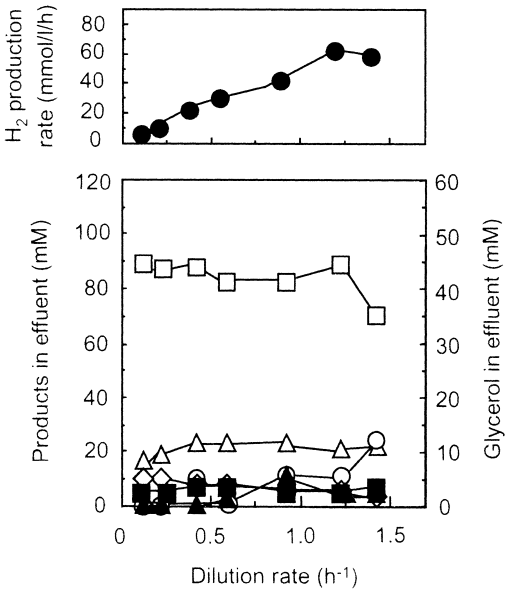


FIG. 4. $H_2$ production rates and end production concentrations during continuous culture using Nagao Porcell. The substrate is glycerol in waste discharged after a biodiesel process. Symbols: closed circles, $H_2$; open circles, glycerol; open squares, ethanol; closed triangles, formate; open triangles, 1,3-propanediol; closed squares, lactate; open diamonds, acetate. Culture conditions: complex medium (yeast extract, 5 g/*l*; tryptone, 5 g/*l*; glycerol, 10 g/*l*) at 37°C.

tion demonstrate that it is difficult to retain a sufficient number of cells at a higher dilution rate when biodiesel wastes are applied. To increase the production of $H_2$ and ethanol more, a packed-bed reactor with a carrier matrix seemed to be an attractive system for preventing the efflux of cell flocs from the reactor. Thus, this system was used and the results of culture using the carrier matrix with the complex medium containing biodiesel wastes are presented in Fig. 4. The cells were successfully retained in the reactor throughout the culture period unlike the culture with self-immobilized cells (data not shown), resulting in an almost complete consumption of glycerol at dilution rates up to 1.2 $h^{-1}$ at which the volumetric maximum $H_2$ production rate increased to 63 mmol/*l*/h with an ethanol yield of 0.85 mol/mol-glycerol. The main by-products were 1,3-propanediol, lactate and formate. This volumetric rate of $H_2$ production from biodiesel wastes by *E. aerogenes* HU-101 was almost the same as that from glucose with the mutant AY-2 of *E. aerogenes* HU-101 (22). This result demonstrates that the use of the carrier matrix was very effective for $H_2$ and ethanol production from biodiesel wastes although the hydrogen production was lower than that in the cultivation using pure glycerol.

In this study, it was shown that $H_2$ and ethanol were produced by *E. aerogenes* HU-101 with a high yield and a high production rate from biodiesel wastes containing glycerol. These findings may lead to a decrease in the use of fossil fuel that may in turn lead to the alleviation of global warming because $H_2$ can be applied to fuel cells to generate electricity and heat without the emission of carbon dioxide, and ethanol can be supplemented to gasoline or used as a resource for biodiesel production instead of methanol which is usually produced from natural gas. Indeed, there are some problems that need to be solved before this technology can have practical applications. For example, it is necessary to increase glycerol concentration used in the production of $H_2$ and ethanol because an excessive dilution of biodiesel wastes using the medium increases the cost for the recovery of ethanol and wastewater treatment. Although $H_2$ and ethanol production from biodiesel wastes was demonstrated using the wild strain of *E. aerogenes* HU-101 in this study, it is necessary to further optimize culture conditions and to breed mutants with a high tolerance to a high concentration of glycerol or salts by conventional breeding methods or genetic engineering. Since 90 g/*l* raw glycerol derived from biodiesel wastes was completely consumed and 47.1 g/*l* 1,3-propanediol was produced by *Clostridium butyricum* F2b after 35 h (33), further explorations of $H_2$ and ethanol-producing microorganisms with such useful properties in nature are also required for this technology to develop.

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