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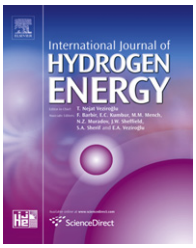

Bioreactor for glycerol conversion into H_2 by bacterium *Enterobacter aerogenes*

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ABSTRACT

Glycerol was used as a substrate for H_2 production by bacterium *Enterobacter aerogenes* in the test tubes and bioreactor. A BioFlo/CelliGen 115 bioreactor (10 L working volume) was utilized to conduct the experiments for conversion of glycerol into H_2 by *E. aerogenes* cells. The highest H_2 production rate was observed under 2% glycerol in the culture medium. The glycerol uptake efficiency by bacteria in the bioreactor was found to be 65% during the 6 day period, matching glycerol uptake efficiency observed in the test tubes experiment (65%).Hydrogen production from glycerol (2% glycerol, v/v) by *E. aerogenes* in the bioreactor and test tubes was measured over the 6 days, showing the maximal H_2 rate at 650 mL g^{-1} dry weight h^{-1}. The yield of H_2 production from glycerol at 0.89 mol/mol in the bioreactor was high, corresponding to the theoretical yield of 1 mol of H_2 per 1 mol of glycerol.

1. Introduction

Glycerol has recently become an abundant commodity due to its generation as a by-product of biodiesel production [1]. World biodiesel production is expected to reach 12 billion liters by the end of 2010 [2], with about one pound of glycerol created for every 10 pounds of produced biodiesel [1]. At present, a surplus of glycerol is destroyed by incineration [2]. However, burning glycerol produces nitrogen oxide as well as carbon dioxide (CO_2), the primary greenhouse gas. The over-production of glycerol considerably affects economic viability of the biodiesel industry [1]. Yet, using the surplus of glycerol for biofuel generation such as biohydrogen (H_2) or bioethanol offers a number of considerable benefits for people and the planet.

Molecular hydrogen is one of the possible energy carriers of the future [3]. The conventional industrial methods for H_2 production are costly and the problem has been so far to find a cheaper way to produce H_2. The biological H_2 production by microbial species has a number of advantages, and it can become a cost effective alternative to the current industrial H_2 production methods [4,5]. There are few bacteria that are able to convert glycerol into H_2 by fermentation under anaerobic conditions. These are the strains of *Klebsiella*, *Citrobacter*, *Clostridium*, *Enterobacter*, and *Escherichia coli* [6,7]. Fermentative metabolic pathways of glycerol utilization were studied intensively using bacterium *Escherichia coli* [1]. Main products of this fermentation are ethanol, formate, and acetate via so-called mixed acid ferementation. *E. coli* can further convert formate into H_2 and CO_2. Strain of *Enterobacter aerogenes*, HU-101, ferments glycerol under anaerobic conditions into H_2 and ethanol, but with a minimal amount of other by-products such as lactate, acetate, 1,3-propanediol, and formate [6]. Exact metabolic pathways of glycerol are not known for of *E. aerogenes*. It is

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well observed, that this bacterium can ferment glucose via 2, 3-butanediol fermentation with main products such as 2, 3-butanediol, H_2 and CO_2, and minimal amounts of ethanol, lactate and acetate [8].

In order to establish industrial production of H_2 by bacteria from glycerol, it is necessary to use bioreactors which provide the most efficient utilization of glycerol, and allow monitoring culture purity and basic process parameters. Bioreactors are types of closed systems that are made of an array of tanks or tubes, in which bacteria can be cultivated under controlled conditions [9]. Here, we studied the conversion of glycerol into H_2 by the facultative aerobic bacterium *E. aerogenes* using a traditional stirred-tank bioreactor (fermenter). Stirred-tank bioreactors are the most common in biotechnology industry. They are relatively simple, easily obtainable, allow growing bacterial cells in high densities, and can promote mass transport of glycerol to bacterial cells [10]. Another advantage of a traditional stirred-tank bioreactor is that it can be operated as a continuous culture over a long period of time [10].

2. Materials and methods

2.1. Bacterial culture

E. aerogenes was obtained from the Carolina Biological Supply Company. Prior to the inoculation of bacterial cells into the bioreactor *E. aerogenes* was grown in test tubes (5.0 mL suspension volume) on the synthetic medium containing inorganic salts and glycerol [6]. Dry weight of cell culture was determined by trapping the bacterial cells on Whatman GF/F filter paper, and drying them at 90 °C to a constant weight.

2.2. Bioreactor

BioFlo/CelliGen 115 benchtop fermentor/bioreactor (New Brunswick Scientific, Edison, NJ, USA) with working volume of 10 L was used in our experiments. The bioreactor was maintained under 37 °C with continuous agitation (50 rpm) and without aeration. The bioreactor was inoculated with bacterial seed culture at cell density of 0.012 g/L. Seed culture was grown in 250 mL flask with 100 mL cell suspension volume for 24 h under 37 °C. The bioreactor was sterilized before inoculation with seed culture.

2.3. Measurement of hydrogen production

Three mL samples of bacterial cell suspension were vacuum degassed (270–300 Torr) in sealed 15 mL vials fitted with rubber stoppers and flashed with N_2 to remove O_2. This procedure induces anaerobiosis after which the samples were incubated for 5 h under 37 °C. Hydrogen production was measured using a Gow-Mac gas chromatograph (Bethlehem, PA) equipped with a molecular sieve 5A column and a thermal conductivity detector. Nitrogen was used as the carrier gas. Each measurement of H_2 production was performed using three samples of bacterial culture and the results were averaged.

2.4. Ethanol and glycerol measurements

Ethanol and glycerol concentrations were determined spectrophotometrically using ethanol or glycerol kits (Bio-Vision, CA) and ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Each measurement of glycerol or ethanol concentrations was performed using three samples of bacterial culture and the results were averaged.

3. Results

3.1. Hydrogen production in test tubes

3.1.1. Calculation of glycerol uptake efficiency

Initially, we tested the conditions for maximal H_2 production by batch bacterial cultures in the test tubes, which represent a much less complex culture system than a bioreactor. Here, we tested an ability of *E. aerogenes* cells to grow on a synthetic medium with glycerol. In these experiments, different amounts of glycerol (v/v) were added to the growth medium. The highest growth and consumption of glycerol by bacterial cells was observed with 2% and 10% glycerol concentrations in the medium. No significant growth was observed on a synthetic medium with 0.1% glycerol. The consumption of glycerol by bacteria is summarized in Table 1.

The efficiency of glycerol uptake (E) by *E. aerogenes* was calculated using the following formula: $E = [(I\text{-}F)/I] \times 100\%$, in which I and F are the initial and final concentrations of glycerol, respectively. The glycerol uptake efficiency was 40% at an original concentration of 1%; 65% at an original concentration of 2%; and 70% at an original concentration of 10% during the six-day period.

3.1.2. Hydrogen production rate

Hydrogen production was measured under different concentrations of glycerol in the synthetic medium. The highest H_2 production rate was observed under 2% glycerol concentration (v/v) in the growth medium (Table 2). In addition, the ethanol production was also measured, and was shown the highest with 2% glycerol in the culture medium.

Table 1 – Glycerol consumption by batch cultures *E. aerogenes* under different initial concentration of glycerol (1%, 2% and 10% in synthetic medium) for a six day period.

Day	Glycerol concentration (initial concentration - 1%)	Glycerol concentration (initial concentration - 2%)	Glycerol concentration (initial concentration - 10%)
0	1.00%	2.00%	10%
1	0.85%	1.00%	7.3%
2	0.75%	0.90%	5.2%
3	0.60%	0.80%	3.3%
6	0.60%	0.70%	3.0%

Table 2 – Hydrogen production by one day-old culture and ethanol production by a 4 day-old culture of *E. aerogenes*, as a function of glycerol concentration in the growth medium.

Glycerol (% v/v in the medium)	H_2 production (mL g^{-1} dry weight h^{-1})	Ethanol production (mM)
0.1	28	0.10
1	184	2.80
2	628	5.00
3	118	3.30
4	42	2.70
5	39	2.30
6	25	1.30
7	23	1.25
8	22	0.70
9	21	0.60
10	20	0.30

3.1.3. *Hydrogen production under optimal glycerol conditions*

After the optimal concentration of glycerol in the synthetic medium for H_2 and ethanol production was determined, H_2 and ethanol generation by bacteria was monitored and measured daily under 2% and 4% glycerol concentration (v/v) in the medium (Figs. 1 and 2). The highest hydrogen production was observed during the first day of running the batch culture, but ethanol production increased gradually and was the highest at the 4th day of culture.

3.2. *Hydrogen production in a bioreactor*

3.2.1. *pH, pO_2 and optical density*

During the six-day operation of the bioreactor, we monitored pH, O_2 concentration and optical density of the bacterial culture. The starting pH of the medium was 7.0; it dropped to 6.45 during the first two days of bioreactor operation, and remained at this level until the end of the experiment. Oxygen was depleted to a zero level during the first 24 h of operation. Optical density of the bacterial culture increased up to 0.23–0.24 during the first two days and remained at this level until the end of the bioreactor operation.

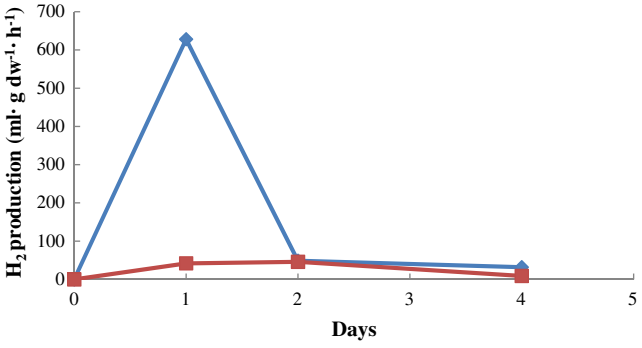

Fig. 1 – Hydrogen production by *E. aerogenes* in batch culture (test tubes) as a function of time. ◆ H_2 production under 2% glycerol (v/v) in the growth medium. ■ H_2 production under 4% glycerol (v/v) in the growth medium.

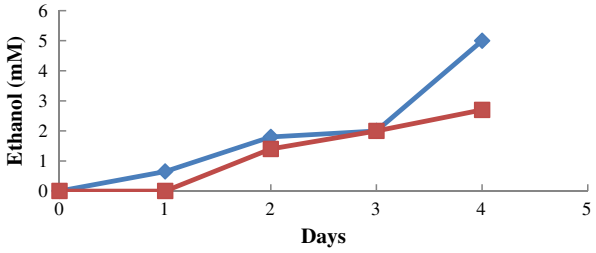

Fig. 2 – Ethanol production by *E. aerogenes* in batch culture (test tubes) as a function of time. ◆ ethanol production under 2% glycerol (v/v) in the growth medium. ■ ethanol production under 4% glycerol (v/v) in the growth medium.

3.2.2. *Calculation of glycerol uptake efficiency and biomass yield*

In the bioreactor, bacterial cells metabolized glycerol gradually during the six days of operation. The glycerol uptake efficiency in the bioreactor was calculated using the same formula as shown above (Section 3.1.1). Demonstrated efficiency of glycerol uptake was 65% which matched the observed glycerol uptake efficiency in the test tubes experiment for the same time period (Table 1). In addition, the biomass yield (g cell dry weight per g of consumed glycerol) was estimated in the bioreactor. The average biomass yield for 6 day period of the bioreactor operation was 2 g of cell dry weight per g of glycerol consumed. The maximal biomass yield on the second day of bioreactor operation was 2.75 g of cell dry weight per g of glycerol consumed compared to the maximum theoretical biomass yield for glycerol of 4.7 [8].

3.2.3. *Hydrogen production*

The assessment of H_2 and ethanol-producing activity by bacteria in bioreactor in the presence of 2% glycerol (v/v) was performed (Fig. 3). Hydrogen production by *E. aerogenes* from glycerol was observed for six days at the maximum rate of 650 mL g^{-1} dry weight h^{-1}. No H_2 production was observed during the first 24 h of bioreactor operation due to the high oxygen levels inside the bioreactor vessel. Bacterial H_2 production occurs only under anaerobic conditions, because the enzyme hydrogenase, which catalyzes it, is highly sensitive to O_2 [3]. Hydrogen in our bioreactor experiment began to form after 24 h, with production rate reaching its maximum

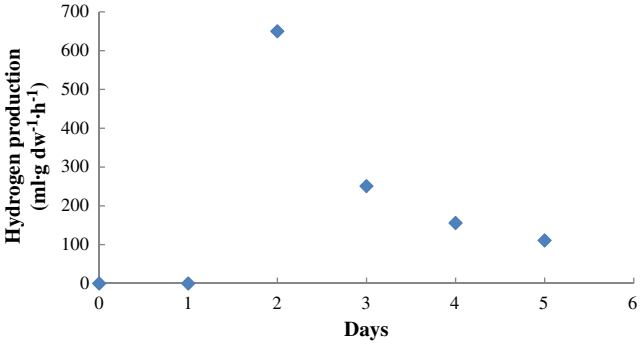

Fig. 3 – Hydrogen production by *E. aerogenes* in the bioreactor.

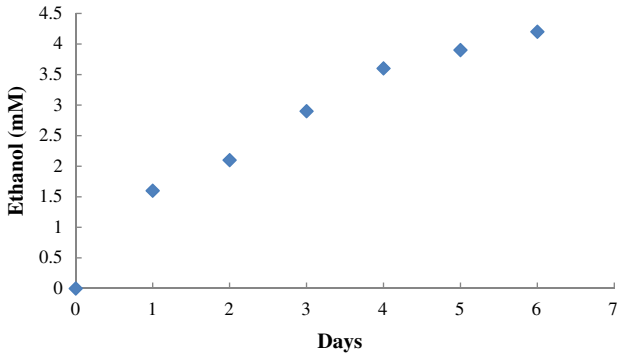

Fig. 4 – Ethanol production by *E. aerogenes* in the bioreactor.

on the second day of operation. After that time, H_2 production started to decline, which corresponded to the stationary phase of bacterial culture with no increase in optical density. After six days the operation of bioreactor was stopped due to the low rates of H_2 production. Still, the observed rates of hydrogen production in the bioreactor were similar to the rates of H_2 production in the test tubes. Together with H_2, the bacterial cells in the bioreactor produced ethanol (Fig. 4). Ethanol production levels by *E. aerogenes* in the bioreactor were similar to the ethanol production in the test tubes.

3.2.4. *Yield of hydrogen from glycerol*

The maximal yield of H_2 from glycerol (2% v/v) on the 2nd day of bacterial growth was relatively high at 0.89 mol of H_2 per mol of glycerol matching the theoretical yield of 1 mol of H_2 produced per 1 mol of glycerol consumed. On the other hand, the ethanol yield was very low (less than 0.001 mol/mol of glycerol) during the same period.

3.2.5. *Energy conversion efficiency of glycerol into hydrogen*

The energy conversion efficiency of glycerol (2% glycerol v/v) into H_2 by *E. aerogenes* was calculated. The heat of H_2O formation, (241 KJ mol^{-1}) was used as the energy content of the H_2 produced. The heat of combustion of glycerol of 1571 KJ mol^{-1} is the energy content of glycerol. The yield of H_2 from glycerol is 0.89 mol/mol of glycerol as shown above. Thus, energy conversion efficiency = [(241 × 0.89)/1571] × 100% = 13.7%

4. Discussion

In this study we used a traditional stirred-tank bioreactor for glycerol conversion into H_2 by bacterium *E. aerogenes*. We exceeded our expectations by obtaining higher H_2 production rates from glycerol (Table 3) and a high H_2 yield that matched the theoretical yield. The observed rates are comparable to H_2 production rates by other microorganisms which generate H_2 by fermentation [11,12]. The glycerol uptake efficiency (65%) by *E. aerogenes* was exactly the same in the bioreactor and test tubes. This indicates the possibility that bioreactor for glycerol conversion into H_2 can be operated without agitation, which may offer a significant saving of energy for industrial applications of this technology. It is likely that consumption of glycerol by bacterial cells in the bioreactor was at first carried out via process of respiration until O_2 was completely depleted during the 24 h period. Hydrogen was not produced at this time. After O_2 removal from bioreactor by bacteria, the conversion of glycerol into H_2 took place under anaerobic conditions via fermentation. Ethanol was another product of fermentation in the bioreactor, although its yield was very low. These findings, as well as the molar ratio of H_2 to ethanol (0.89/0.001), suggest that ethanol was not the main product of glycerol fermentation. We did not measure the accumulation of any additional fermentation products, such as CO_2 or acids. We assume that the bacterial cells ferment glycerol via 2, 3-butanediol fermentation with main products such as 2, 3-butanediol, H_2 and CO_2. An indication to this was provided by a non-significant pH drop in the bioreactor vessel during our experiments. An alternative pathway, such as mixed acid fermentation, would include a substantial change of pH of the growth medium [8].

Table 3 – Rates of microbial H_2 production obtained from literature and from our experiments.

Microorganism	Rate of hydrogen production (mL g^{-1} dry weight · h^{-1})
E. aerogenes, fermentation of glucose [10]	400
Mixture of bacteria, starch fermentation [11]	900
E. aerogenes, fermentation of glycerol (our results)	650

It is important to point out that glycerol conversion into H_2 was observed under anaerobic condition via fermentation. Fermentation is the most widely used and easily maintained bioprocess in microbiological and biotechnological industries [13]. Simplicity and effectiveness of fermentation may help to quickly establish industrial process based on our findings.

5. Conclusions

1. Higher H_2 production rates can be obtained with a simple anaerobic conversion of glycerol by a common bacterium *E. aerogenes* in a traditional stirred-tank bioreactor.
2. The experiments allowed to successfully scale-up bacterial H_2 production from test tubes to pilot scale bioreactor.
3. This work demonstrates possibility to convert glycerol, the by-product of biodiesel production, into the valuable fuel – molecular hydrogen.

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