Hydrogen Production with *Escherichia Coli* Isolated from Municipal Sewage Sludge

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Abstract

We isolated hydrogen producing microbial strains from municipal sewage sludge in Thailand. The experiments were performed in 0.7 liter of Reinforced Clostridial Media (RCM) at pH 6.8 and 30±1°C. Hydrogen producing isolates were identified as *Escherichia coli* and *Enterobacter* sp. by 16S ribosomal DNA sequence analysis. *E. coli* S3 demonstrated a maximum hydrogen production rate of 16.07±0.53 ml-H_2/l/h after 24 hours of incubation. The maximal cumulative hydrogen yield of 269.92±8.87 ml (calculated at STP) and the hydrogen yield of 0.84±0.06 mol-H_2/mol-glucose were obtained at an initial glucose concentration of 0.5% (w/v), pH 6.8 and 30±1°C.

Key words: bio-hydrogen, *E. coli*, sewage sludge

1. Introduction

Hydrogen is a clean and environmentally friendly fuel. It not only has a high energy content of 122 kJ/g, which is about 2.27 times greater than hydrocarbon fuels [1], but also produces only H_2O when burnt in air. Thus it is a viable alternative to fossil fuel, which is a major source of carbon dioxide. In addition, it can easily be used in fuel cells to generate electricity. There are several well-known methods for hydrogen production such as electrolysis of water [2], steam reforming of hydrocarbons [3] and auto-thermal processes [4], but they are not cost-effective due to high energy requirements. Furthermore, these methods still use electricity derived from fossil fuel combustion.

Biological hydrogen production is a practical alternative to the aforementioned methods. The major biological processes for bio-hydrogen production are bio-photolysis of water by algae [5], photo-fermentation of organic materials, and dark fermentation by bacteria [6]. The fermentative process is more attractive than the others because it is technically simple and can use organic waste materials as substrates [7-8]. Previous studies have been shown that hydrogen gas can be produced by using pure cultures of anaerobic bacteria such as *Clostridium acetobutyricum* [9]. However, there are few reports using pure strains of *Clostridium* sp. to produce hydrogen because of the difficulty in cultivating strictly anaerobic strains [10]. Recently, many researchers have focused on the hydrogen producing ability of microbial strains isolated from waste materials, for instance, cow dung compost [11], leachate [12] and sewage sludge [13-14]. This is because effective hydrogen producing strains isolated from waste materials can produce high levels of hydrogen when organic waste materials are used as substrates.

The objective of this study is to isolate and identify microbial strains capable of producing high hydrogen yield from sewage sludge.

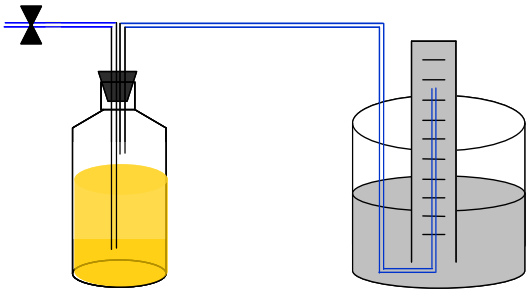
2. Materials and methods

2.1 Screening for hydrogen-producing bacteria

Hydrogen producing bacterial strains were isolated from activated sewage sludge obtained from a sedimentation tank of the Din Daeng Municipal Wastewater Treatment Plant (Bangkok, Thailand). The sewage sludge was pretreated at 100 °C for 20 mins to inhibit the bioactivity of hydrogen consumers as described in Zhu and Beland [15]. The pretreated sludge was serially diluted (10^{-1}-10^{-4}) with Reinforced Clostridial Media (RCM) (MERCK, Germany). Diluted (0.1 ml) sample of each was spread onto a petri dish containing Reinforced Clostridium Agar (RCA) (Merck, Germany) using the method described in APHA [16]. Subsequently, the petri dishes were incubated in anaerobic jars at 35°C for 24-48 hrs. Pure bacterial strains were obtained after successive transfer of individual colonies on RCA plates and incubated under the same conditions mentioned above. To examine their gas producing abilities, a loopful of organisms from each plate of pure cultures was inoculated in 6 ml of RCM in a sealed-test tube containing an inverted Durham tube and incubated at 35 °C for 24-48 hrs.

2.2 Hydrogen production in batch fermentors

A batch experiment was carried out in a 1-L Duran bottle fitted with a silicone stopper and a working volume of RCM 700 ml. A schematic diagram of the fermentor and biogas collector is shown in Figure 1. For inoculum preparation, the pure culture (obtained from section 1.1) was grown in RCM to reach optical density (OD) of 0.8 at a wavelength of 600 nm measured by using a UV-visible spectrophotometer model 1601 (Shimadzu, Japan). Ten percent (v/v) of the inoculum (70 ml) was inoculated into the fermentor containing approximately 630 ml of RCM with initial glucose concentration of 5 g/l and initial pH of 6.8. After inoculation, the fermentor was purged with nitrogen gas (99.99% purity) (Praxair (Thailand) Co., Ltd.) for 5 mins to remove air and then placed in a water-bath with a controlled temperature of 30±1 °C. No attempt was made to control the pH during the experiment. Biogas evolved was allowed to pass through 10% (w/v) NaOH solution (Carlo Erba, Italy) for selective absorption of CO_2. The filtered gas was collected by water displacement in an inverted measuring cylinder. The gas volumes were measured by reading from the cylinder scale at ambient pressure and temperature and then corrected to STP conditions (0 °C and 1 atm). A fermentor containing 700 ml of RCM without inoculum was used as an experimental control. The experiments were carried out in triplicate.



Hydrogen fermentor

Hydrogen gas collector containing 10% NaOH (w/v) to remove CO_2 gas

Figure 1 A schematic diagram of a hydrogen fermentor and a gas collector

2.3 Analytical methods

The composition of a biogas sample was identified and quantified by a gas

chromatograph (GC) equipped with a thermal conductivity detector **(**TCD) **(**CP–3800 model, Varian Analytical Instruments, USA). The carrier gas used was helium (99.999% purity) (Praxair (Thailand) Co., Ltd.) at a flow rate of 5 ml/min. A Plot Fused Silica coated Molecular sieve 5A (10 m x 0.53 mm i.d. x 50 µm film thickness) column (Varian Analytical Instruments, USA) was used as the GC column. The GC temperature program was initially 50°C, held at 50°C for 5 mins, then increased to 100°C at a rate of 4°C/min, and held at 100°C for 2 mins. The temperatures at injection and detector were 100°C and 150°C, respectively. Three percent hydrogen in helium gas (BOC Scientific Co., Ltd., Thailand) was used as hydrogen standard.

Cell densities, pH and glucose concentrations were examined every 3 hours. The cell densities in liquid medium samples were monitored by measuring turbidity at 600 nm using a UV-visible spectrophotometer. Total plate counts were performed by using the spread-plate technique. The glucose concentration of the medium was measured by using the Somogyi Nelson method [17-18]. pH was determined using a pH meter model Cyberscan 2000 (Eutech Cybernetics, Singapore).

2.4 Identification of hydrogen producing isolate

Organisms were gram stained and examined under a light microscope. Microbial strains producing high hydrogen yields were further identified by 16S rDNA sequence analysis. Total genomic DNA of the organisms was isolated by DNA purification kit (Epicentre, USA). A 1,500 bp segment of the 16S rDNA gene was amplified by PCR with a pair of primers designed from conserved sequences of γ-proteobacteria. One (FDNA, 5'-AGAGTTTGATCCTGGCTCAG-3') targeted the beginning and another (RDNA, 5'- GGTTACCTTGTTACGACTT -3') targeted the end of the 16S rDNA gene [19]. PCR of genomic DNA from isolated strains was carried out in a DNA thermal cycler (Perkin Elmer 480, USA). Reaction mixtures of 100 µl contained 100 ng of genomic DNA, 2.5 units of *Taq* DNA polymerase (Promega, USA), 20 pmole of each deoxynucleoside triphosphate (Promega, USA), 1.5 µM $MgCl_2$ (Promega, USA), and 25 pmole of each primer. The PCR protocol consisted of a denaturing step of 95 °C for 5 mins, followed by 30 cycles of denaturation for 30 secs at 95 °C, primer annealing for 30 secs at 55 °C and a minute primer extension at 72 °C. A final extension at 72 °C for 10 mins was then performed. PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Germany) and cloned into plasmids using the PCR cloning kit (pDrive) (Qiagen, Germany). The recombinant plasmid was sequenced in both strands with the Big-Dye^{TM} Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, USA) by using the ABI PRISM^{R} 3700 DNA analyzer. Sequences were compared to those in GenBank by using the BLAST function [20] of the National Center for Biotechnology Information Server, National Institutes of Health, USA.

3. Results and discussion

3.1 Characterization of the hydrogen-producing isolates

Only six organisms (S1-S6) isolated from sewage sludge produced gas in the inverted Durham tubes. All six isolates were gram-negative rods and their H_2 producing capacities were tested.

3.2 Hydrogen production in batch culture

Figures 2 and 3 demonstrate the profiles of cumulative hydrogen production and cell growth of the S1-S6 isolates during batch fermentation, respectively. The production of hydrogen appeared to start

after the mid-exponential growth phase (3 hrs). Maximal hydrogen production rates of all microbial strains occurred after 6 hours when cell growth had entered the early stationary phase. This seems to imply that hydrogen gas was produced rapidly after carbon substrate was consumed to gain biomass. This may be due to a predominant metabolic electron flow towards bio-synthesis, decreasing the availability of electrons for hydrogenase to produce hydrogen gas [13]. Cell growth profiles of S1-S6 isolates using total plate counts and OD_{600} measuring techniques are shown in Figure 3. The cell growth profiles from lag phase to the early stage of stationary phase of both techniques were similar. However, the former technique could clearly differentiate cell growth and cell death. Under the present conditions (initial pH 6.8, 30 °C, 12 hours of incubation and dark fermentation), the S3 isolate produced the maximum yield of hydrogen gas followed by the S2, S6 and S5 isolates. After 24 hours of incubation, the cumulative hydrogen gas production of the S3 isolate was 269.92±8.87 ml at STP (Figure 2 and Table 1), hydrogen production rate was 16.07±0.53 ml-H_2/l/h and hydrogen yield, calculated from peak area of hydrogen standard, was 0.84±0.06 mol-H_2/mol-glucose (table 1). The hydrogen producing rates of S1-S6 strains (Figure 2) are related to their growth profiles at logarithmic growth phase (Figure 3).

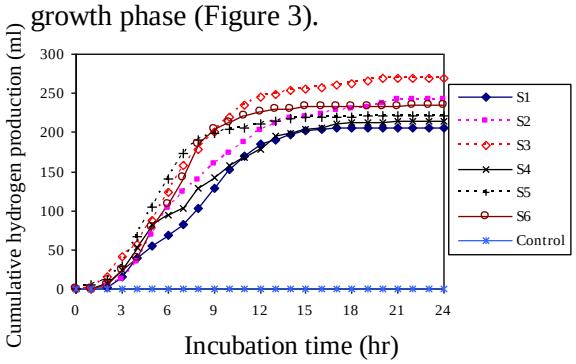


Figure 2 Time-course profiles of cumulative hydrogen production by S1-S6 strains

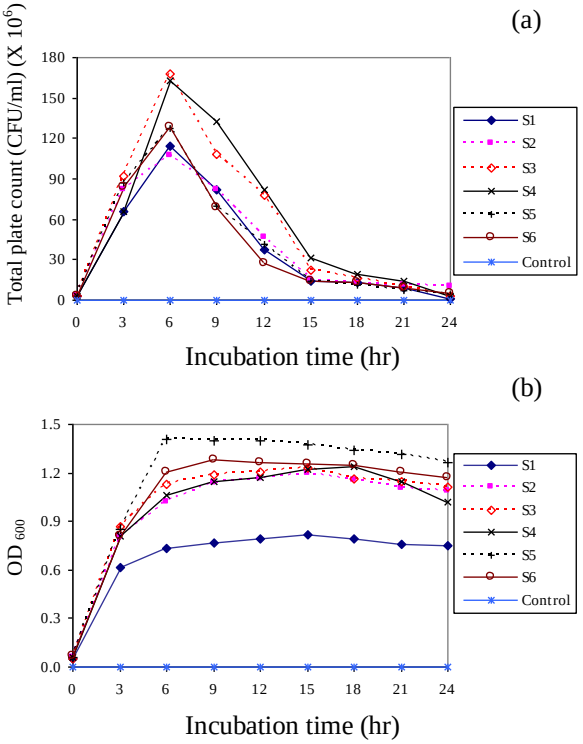


Figure 3 Time-course profiles of cell growth of S1-S6 strains measured by (a) total plate count and (b) OD_{600} using spectrophotometer

pH profiles of the S1-S4 isolates decreased from 6.8 to 5, while that of the S5-S6 isolates decreased from 6.8 to 6 (figure 4). During log phase and early stage of stationary phase, the decrease of pH corresponded with the increase in hydrogen production. This is because microorganisms consumed glucose (figure 5) which was converted to organic acids with production of hydrogen gas [11, 13]. However, if pH decreased lower than the optimum pH range of microorganisms, the growth of microorganism and its hydrogen production rate would decrease significantly [11]. Thus, pH control at an appropriate level may be required to improve the efficiency of hydrogen production. Of the 6 isolates, three with high H_2 producing abilities (S3, S2, S6) were further identified using 16S rDNA sequence analysis.

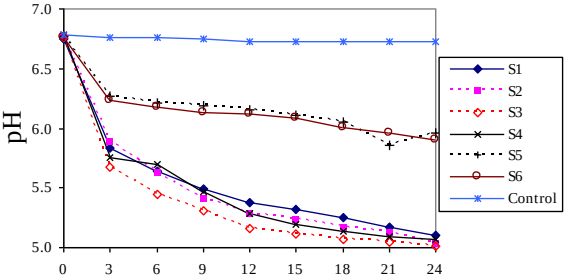


Figure 4 pH reduction during cell growth and H_2 production

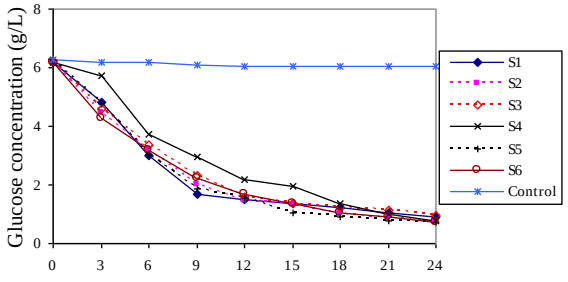


Figure 5 Changes in glucose concentration during cell growth and H_2 production

Table 1 Hydrogen production abilities of microorganisms isolated from sewage sludge after 24 hours of incubation in RCM under anaerobic conditions at 30±1 °C.

Microorganism	Cumulative hydrogen production (ml)	Maximum hydrogen production rate (ml H_2/l/h)	Hydrogen productivity (mol-H_2/mol-glucose consumed)
S1	205.63±10.36	12.24±0.62	0.23±0.09
S2	241.96±3.37	14.40±0.20	0.55±0.08
S3	269.92±8.87	16.07±0.53	0.84±0.06
S4	214.71±3.17	12.78±0.19	0.39±0.06
S5	220.39±6.79	13.12±0.40	0.41±0.08
S6	234.63±7.47	13.97±0.44	0.49±0.07
Control	0	0	0

3.3 Identification of hydrogen producing microbial strains

Using 16S rDNA sequence analysis, S2 and S3 isolates were identified belonging to the genus *Escherichia* while S6 isolate was identified as *Enterobacter* sp. The S2 and S3 isolates showed 100% homology with *E. coli* UTJ89 (CP000243), *E. coli* ATCC8739 (CP000946) and *E. coli* E2437A (CP000800), therefore they were designated as *E. coli* S2 and S3, respectively. In this study, only facultative bacteria were isolated from sewage sludge. This may be due to limitation of our isolation technique as well as the difficulty in cultivating strict anaerobes. The major facultative bacteria used for hydrogen production reported so far include *E. coli* [21-22] and *Enterobacter* sp. [23-24]. In this study, *E. coli* S2 and S3 and *Enterobacter* S6 isolated from the sewage sludge in Thailand also showed high hydrogen production. The *E. coli* S3 isolate, which produced maximum yield of hydrogen gas, will be used to investigate hydrogen production using sewage sludge as substrate in future work.

4. Conclusions

Hydrogen producing microbial strains isolated from municipal sewage sludge collected from a wastewater treatment plant in Thailand were *E. coli* and *Enterobacter sp*. In this study, *E. coli* S3 produced the maximum yield of hydrogen gas after 24 hours of incubation with cumulative hydrogen gas production of 269.92±8.87 ml at STP, hydrogen gas

production rate of 16.07±0.53 ml-H_2/l/h and hydrogen gas production yield of 0.84 ±0.06 mol-H_2/mol-glucose.

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6. References

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