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Fermentative hydrogen production by a new mesophilic bacterium *Clostridium* sp. 6A-5 isolated from the sludge of a sugar mill

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ABSTRACT

The fermentative hydrogen production capability of the newly isolated *Clostridium* sp. 6A-5 bacterium was studied in a batch cultivation experiment. Various culture conditions (temperature, initial pH, and glucose concentration) were evaluated for their effects on cell growth and hydrogen production (including the yield and rate) of *Clostridium* sp. 6A-5. Optimal cell growth was observed at 40 °C, initial pH 7.5–8, and glucose concentration 16–26 g/L. The optimal hydrogen yield was obtained at 43 °C, initial pH 8, and glucose concentration 10–16 g/L. Hydrogen began to evolve when cell growth entered the mid-exponential phase and reached the maximum production rate at the late exponential and stationary phases. The maximum hydrogen yield, and rate were 2727 mL/L, and 269.3 mL H_2/L h, respectively. These results indicate that *Clostridium* sp. 6A-5 is a good candidate for mesophilic fermentative hydrogen production.

1. Introduction

The depletion of fossil fuels and energy-related environmental pollution make it crucial to find alternative or renewable energy sources. Among recognized alternatives to fossil fuels, hydrogen is regarded as a clean energy carrier due to its high energy content and renewable and clean characteristics. Biological methods for producing hydrogen are less expensive and energy-intensive than chemical or electrochemical techniques because they are carried out at ambient temperature and pressure [1].

Biohydrogen processes include microbial photosynthetic, dark fermentation, and microbial electrolysis. Among these, dark fermentation is an efficient approach for biological hydrogen production, and it has advantages for industrial use because it is technically simple, has a high production rate, and can use a variety of organic substrates [2–6]. When combined with the treatment of organic waste and wastewater, dark fermentation has a powerful advantage when applied in practice. There are many species of fermentative hydrogen-producing bacteria. Among them, *Clostridium* sp. is one of the most common and effective hydrogen producers. It is also the dominant species existing in microflora of anaerobic hydrogen fermentation processes, especially in the inoculum sludge that was enriched by heat-pretreatment [7–9]. Many species of *Clostridium* are strong and efficient producers of hydrogen, including *Clostridium butyricum* [4], *Clostridium beijerinckii* RZF-1108 [10], *Clostridium amygdalinum* strain C9 [11], *Clostridium cellulosi* D3 [3], *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) [12], and *Clostridium acetobutylicum* [13]. *Clostridium* sp. can extract energy from carbohydrates using various metabolic pathways that are promoted or inhibited by the culture conditions. Therefore, control culture conditions can enhance hydrogen production.

Fermentative bacteria use glucose to produce hydrogen with a theoretical yield of 4 or 2 mol H_2/mol glucose when acetic or butyric acid, respectively, is produced as the terminal metabolite. In practice hydrogen yields are lower than the theoretical yield, because some carbohydrate is converted to bacterial cells, and end products other than acetic acid are produced. To date, the low yields and production rates are two major barriers for the technologies of biohydrogen production by fermentative bacteria in the laboratory which has inhibited its commercial take-up. It is necessary to improve the level of hydrogen production for practical application. Fermentative hydrogen production is a complex process that is greatly influenced by many factors, including

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temperature, initial pH, and substrate concentration, etc. Thus, optimizing these culture conditions can enhance the hydrogen yield. Wang and Jin [14] found that fermentation parameters, including carbon and nitrogen sources, growth temperature and pH, and inoculum size, greatly influence hydrogen yield and the hydrogen production rate. Zhao et al. [10] investigated the effects of temperature, initial pH, glucose concentration, nitrogen source, and inoculum volume on hydrogen production by a newly isolated hydrogen-producing fermentative bacterium *C. beijerinckii* RZF-1108. Although culture conditions have been investigated widely, the optimal culture conditions for different strains were quite different. Recently, hydrogen fermentation at high temperature has been researched for the purpose of accelerating the rate of hydrogen production. Moreover, high hydrogen yields were accomplished by some of thermophilic bacteria [15]. However, because of the high energy input needed, thermophilic operation is less likely to be the technically and economically favored option. Accordingly, screening of new mesophilic bacteria was carried out.

In this study, a mesophilic hydrogen-producing anaerobe was isolated from the sludge of a sugar mill and designated *Clostridium* sp. 6A-5. Effects of initial pH, temperature, and glucose concentration on hydrogen production were investigated to identify the optimum conditions for hydrogen production by the pure bacterial isolate.

2. Materials and methods

2.1. Isolation of the bacterial strain

The bacterium used in this study, *Clostridium* sp. 6A-5, was isolated from the sludge of Funan sugar mill located in Guangxi Province, China. Heat pretreatment is one of the most common and effective technique for screening of hydrogen producing bacteria [7,8]. It is reported that heat treatment not only reduces the non-spore-forming bacteria, but also activates germination in clostridia spores by altering the germination receptor [7]. Sludge was enriched by heating at 100 °C for 90 min, then regulating it to room temperature. A 10 g heat treated sludge sample was incubated in 100 mL of LM medium at 37 °C.

LM medium was made following Li et al. [16]. LM medium had been used for hydrogen production by freshwater strains. The LM medium composed of (g/L); glucose 20, beef extract 2, tryptone 4, and yeast extract 1, K_2HPO_4 1.5, $MgCl_2$ 0.1, $FeSO_4 \cdot 7H_2O$ 0.1, L-cysteine 0.5; vitamin solution (containing pyridoxine hydrochloride 0.05 g/L, L-ascorbic acid 0.025 g/L, riboflavine 0.025 g/L, citric acid 0.02 g/L, vitamin B1 0.02 g/L, D-biotin 0.01 g/L, and para-aminobenzoic acid 0.01 g/L) 10 mL; trace element solution (containing $CoCl_2 \cdot 6H_2O$ 0.2 g/L, $ZnSO_4 \cdot 7H_2O$ 0.05 g/L, $MnSO_4 \cdot 7H_2O$ 0.01 g/L, H_3BO_3 0.01 g/L, $CaCl_2 \cdot 2H_2O$ 0.01 g/L, Na_2MoO_4 0.01 g/L), 10 mL. Initial pH value of the medium was adjusted to 7.0. Agar medium was prepared by adding 1.5% (w/v) agar to the LM medium.

The serum bottles were flushed with high-pure N_2 (99.999%) to remove oxygen and sealed by rubber plugs to made anaerobic condition. After 2 days incubation, 3 mL of the culture broth was transferred to 100 mL of LM medium for enrichment. These procedures were repeated three times to obtain enriched hydrogen-producing bacterial strains. After the enrichment step, the bacterial strain was isolated using a method described elsewhere [17].

2.2. Strain identification

Morphology of the isolated bacterium 6A-5 was performed using a light microscope (Olympus, Japan) and an electron microscope (Hitachi su1510, Japan). The chromosomal DNA was isolated from strain 6A-5 using the standard method [18], and identification based on 16S rDNA gene sequence analysis was carried out. Forward primer used was 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492 R (5′-GGTTACCTTGTTACGACTT-3′) [19]. Each PCR mixture (total volume, 50 μL) consisted of 5 μL of 10 × PCR buffer, 1 μL of each primer (10 mmol/L), 1 mL of dNTP (10 mmol/L), 1 μL of Taq polymerase (5 U/L), and 1 μL of DNA template and the adequate amount of distilled water added to reach the desired volume. The PCR program was as follows: initial denaturation for 4 min at 96 °C; 10 cycles of 30 s at 94 °C, 45 s at 54 °C, 1 min at 72 °C; 20 cycles of 30 s at 94 °C, 45 s at 52 °C, 1 min at 72 °C; and extension of incomplete products for 10 min at 72 °C. PCR products were examined by electrophoresis on 1% (w/v) agarose gels containing ethidium bromide (0.5 mg/mL). The PCR products were purified using a Wizard PCR Preps DNA purification system (Promega, USA) according to the manufacture's instructions and sequenced by the company (Sangon, China). Best matches for the acquired 16S rDNA sequences were identified by existing DNA sequences in the GenBank database using BLAST. Phylogenetic trees were constructed using the neighbor-joining method with MEGA program. Bootstrap analysis for 1000 replicates was performed to estimate the confidence of the tree topologies [20].

2.3. Hydrogen production experiments

Batch cultivation of *Clostridium* sp. 6A-5 was performed in a 150 mL-serum bottle. The volume of the culture liquid was 100 mL, and the agitation was 120 rpm. 3 mL of the precultured broth was inoculated in 100 mL of LM medium for hydrogen production test. The reactors were flushed with ultra high-pure N_2 (99.999%), then sealed with rubber plugs. To investigate the effect of cultivation temperature on the evolution of hydrogen, the bottles were placed in different incubators (DHZ-032LR refrigerated shaker, Shenergy Biocolor Bioscience and Technology Co., Ltd, Shanghai, China), at following temperatures 25 ± 1, 30 ± 1, 34 ± 1, 37 ± 1, 40 ± 1, 43 ± 1, 46 ± 1, and 50 ± 1 °C, respectively. To investigate the effect of initial pH on the evolution of hydrogen, the following initial pH values 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0 were tested. To investigate the effect of glucose concentration on the evolution of hydrogen, the following glucose concentrations were tested 4, 7, 10, 13, 16, 20, 23, 26 g/L. Cell growth and hydrogen content were determined every 3 h. The final pH value, oxidation-reduction potential (ORP) were determined after gas evolution stopped.

After the one factor tests, the process of hydrogen production at the optimum culture condition was examined. Cell growth, hydrogen content, pH value and oxidation-reduction potential were determined every 3 h.

All experiments were conducted in triplicate. The hydrogen in the biogas produced by the culture was collected using the liquid displacement method. Hydrogen yield was calculated through the volume of biogas and hydrogen content in the biogas.

2.4. Kinetic modeling

The Logistic model (Eq. (1)) was adapted to describe the kinetics of the microbial growth [21]:

$$\frac{dX}{dt} = k_c X\left(1 - \frac{X}{X_{max}}\right) \quad (1)$$

where k_c (h^{-1}) is the apparent specific growth rate; X (g VSS/L) is the microbial concentration; and X_{max} (g/L) is the maximum microbial concentration.

A modified Gompertz equation (Eq. (2)) was employed to model the formation of hydrogen [21]: