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Dark bio-hydrogen fermentation by an immobilized mixed culture of *Bacillus cereus* and *Brevumdimonas naejangsanensis*

Zhihong Ma, Chan Li, Haijia Su*

Beijing Key Laboratory of Bioprocess, Beijing Advanced Innovation Center for Soft Matter Science and Engineering, Beijing University of Chemical Technology, Beijing 100029, People's Republic of China

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Based on the immobilized mixed culture technology, both the hydrogen production and synergy mechanism between *Bacillus cereus* A1 and *Brevumdimonas naejangsanensis* B1 were investigated. Different immobilization carriers were chosen. In terms of hydrogen yield and the multi-cycle use of the carriers in batch fermentation, corn stalk as carrier was found to be a better candidate than fiber material (polyester fiber) and activated carbon (AC). The obtained cumulative hydrogen production was 2205 mL/L within 180 h, significantly higher than that of the suspended fermentation. The average cumulative hydrogen production and hydrogen yield were 1845 mL/L and 1.50 mol H_2/mol glucose for ten cycles of repeated fermentation batches respectively, which was 62.5% higher than that of suspended fermentation. The experimental results also showed that the system could use starch as direct substrate and the tolerance of the immobilized system to the substrate loading was improved. The activities of amylase and hexokinase were respectively 2 to 3 and 2 times higher than in the suspended fermentation due to the synergistic effect of co-immobilization compared with the suspended fermentation.

1. Introduction

In the past decades, the increasing demand of energy and rapid economic development has stimulated the development of alternative energy sources. Hydrogen energy, as one of the most promising energy candidates, attracts increasing attention due to its environmentally-benign and highly-efficient characteristics [1,2]. Many methods to produce hydrogen were examined and applied, but most of them are energy intensive and fossil-fuel based, which makes hydrogen production more costly and unsustainable [3]. On the contrary, biohydrogen production is energy-saving, efficient and pollution-free [4]. Current research of biohydrogen production mainly concentrates on suspended-cell systems. However, the disadvantages such as easy washout of cells lead to a low efficiency of hydrogen production. Therefore the investigation of immobilized-cell systems has become a hot topic in the hydrogen production [5].

Since pure bacteria are prone to be contaminated along with the fermentation, sterilization is hence again required, increasing the workload. Besides, pure bacteria cannot be recycled and the use of pure bacteria in biohydrogen production becomes difficult to be industrially applied. Bao et al. did research about bio-hydrogen fermentation by mixed culture of *Bacillus* sp. and *Brevumdimonas* sp. The results showed that the hydrogen yield was doubled in mixed culture compared with pure culture [6,7].

Researches moreover showed that the synergistic effect of mixed cultures could broaden the type of substrate, improve the adaptability of the system to the environment, and enhance the efficiency of fermentation [8]. The substrate is one of the major costs in biohydrogen production, [9]. Previous studies used simple sugars, such as glucose, maltose, and xylose, as substrates for hydrogen production. A yield of 2.31 mol H_2/mol xylose was reported by An et al. using *Clostridium beijerinckii* YA001 [10]. Haroun et al. used glucose as substrate, and the hydrogen yield was 2.27 mol H_2/mol glucose [11]. Alvarez et al. used the G088 strain to produce hydrogen with glucose as substrate, and the hydrogen yield was 1.70 mol H_2/mol glucose [12]. But, the cost of using simple sugar as substrates is still high, which increases the total production cost of hydrogen. Since cheap polysaccharoses such as starch and cellulose are unable to be degraded directly owing to the lack of hydrolytic enzymes, it is important (i) to look for cheap and suitable substrates that can considerably decrease the fermentation cost, and (ii) to find functional microorganisms that can directly

* Corresponding author.
E-mail address: suhj@mail.buct.edu.cn (H. Su).

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hydrolyze the starch or cellulose into fermentable reducing sugars [13].

Immobilized-cell systems can be operated continuously at low retention time without suffering from washout of cells. Compared with suspended cells, immobilized cells offer additional advantages such as being more tolerant to environmental perturbation, being more stable in operation, being reusable and promoting a higher biological activity due to the higher cell density [14–16].

Considering the effect of different immobilization methods on the hydrogen production, adsorption and embedding are mostly used [17–19]. Embedding either spreads cells into the porous carrying materials, or embeds cells in a gel-forming polymer, thus yielding immobilized cells. Anjana et al. reported that the hydrogen production rate by immobilized *cyanobacterium Lyngbya perelegans* in alginate was raised approximately two times compared to the suspended-cell system [20]. The mass transfer resistance using the embedding is however too high, so that the biochemical reaction rate is low. Many researchers hence choose adsorption, which is based on electrostatic fixation of the enzyme or microorganism cells upon a carrier to attain the goal of immobilized cells. It is the simplest method with mild preparation conditions and higher cell activity. The adsorption carriers must be internally porous, have a large specific surface area and high mechanical strength. It is reported that the hydrogen production using a porous glass carrier was two times higher than that of suspended fermentation [21]. Kirli et al. reported that the hydrogen yield with immobilization in plastic scouring sponge pads covered by metal mesh increased to 2.1 mol/mol glucose [22].

There are some common carriers used in dark bio-hydrogen fermentation, such as sodium alginate [20], porous glass [21], sponge [22], agar [23], ceramic [24] etc. In this study, we consider that agricultural waste is biodegradable, renewable, biocompatible and non-toxic, which makes it attractive as adsorption material. At present, applicable materials are corn stalk, sunflower stalk and bagasse. Currently, most of the stalks are burned, which leads to low efficiency and environment pollution. Using agriculture stalk as an immobilized carrier, not only achieves agricultural stalk resource recovery, but also provides a highly efficient, low-cost carrier for immobilized microorganism. Therefore, corn stalk was investigated as carrier in this study, and compared with other carrier materials.

In order to comprehend the synergistic mechanism of two bacteria using starch substrate, the immobilized mixed culture system of the two hydrogen production bacteria was investigated. The optimal immobilization carrier was obtained by comparing the hydrogen production rate and reusability of different carriers. The influences of the immobilized mixed strains on pH, on the tolerance to the substrate, towards the substrate utilization rate, and the enzyme activity were studied, and the mechanism of biohydrogen production in the system of anaerobic fermentation was explored.

2. Materials and methods

2.1. Microorganisms

In this study, the mixed culture of A1 and B1 was used. They belong to *Bacillus cereus* A1 (CP015727) and *Brevumdimonas naejangsanensis* B1 (CP015614), respectively [25,26]. They were screened and separated from a sludge in an anaerobic digestion reactor, detailed in previous literature [6].

2.2. Immobilization methods

As the carrier, polyester fiber material, activated carbon (AC) and corn stalk were studied. A certain quantity of carriers was added in the seed medium before it was sterilized. Then, the seed medium was inoculated with the mixed strains (A1/B1 = 1/1), at a ratio determined by previous literature [6]. The microorganisms were grown in the seed medium for 72 h at 37 °C to achieve the immobilization. Then the carriers were removed and transferred to the fermentation medium for hydrogen production.

The composition of the seed and the fermentation medium were established previously [6].

2.3. Experimental setup and procedure

The experimental setup was same as that of Bao et al. [6]. Each reactor was filled with 0.9 L fermentation medium, and 9 g of carrier that had absorbed the microorganisms (in the immobilized fermentations) or 100 mL seed medium with the mixed strains (in the suspended fermentations). Other procedure consult the previous literature [6].

After one batch fermentation, the carriers were removed and added into a new fermentation medium for the next batch experiment without any additional inoculation.

2.4. Analytical methods

In this study, some main parameters such as pH, total sugar concentration, hydrogen production, volatile fatty acids (VFAs), were investigated according to standard analytical procedure [6,7].

3. Results and discussion

3.1. Comparison of immobilized and suspended fermentation process

In this work, fiber material, activated carbon (AC) and corn stalk adsorption materials were chosen as immobilizing carriers, and they are all cheap, accessible, and porous materials.

The cumulative H_2 production are presented in Fig. 1. The cumulative amount of hydrogen increased with various degrees for three immobilized fermentations compared with suspended fermentation. A cumulative amount of hydrogen of 1011 mL/L and a hydrogen yield of 1.04 mol H_2/mol glucose was obtained with the suspended fermentation. Compared with the suspended fermentation mode, the yield was increased by 34.4%, 49.4% and 118.1% for fiber, AC and corn stalk respectively. The cause of this difference is that the adhesion strength between cells and carrier is different for different carriers, and the toxicity to microorganism is also different [25].

In the fiber immobilized fermentation system, the substrate utilization ratio was lower and the same as in the suspended fermentation, being around 60% in all cases, as shown in Fig. 2 b. The variation of the pH (Fig. 2 a) in the fiber tests was the highest of all, and the final pH was the lowest, leading to the lowest cumulative amount of hydrogen. On the contrary, the pH of corn stalk was the most stable and the substrate utilization rate in the corn stalk immobilized fermentation was around 90%. Although the substrate utilization rate in the activated carbon immobilized fermentation also reached 90%, the amount of hydrogen was much lower than that in the corn stalk case, tentatively explained by the fact that the starch was decomposed in the activated carbon immobilized fermentation, but had not been used to produce hydrogen completely, while some sugars were decomposed into some intermediate metabolites.

The main end products in the liquid phase of different experiments, were acetic acid and butyric acid (Fig. 3). The acetic acid was the highest in suspended fermentations, being 1.08 g/L or 37.8% of the total acid content. In fiber and activated carbon immobilized fermentation, the change of butyric acid content was not obvious,