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Biohydrogen Production Using Immobilized Cells of Hyperthermophilic Eubacterium *Thermotoga neapolitana* on Porous Glass Beads

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**Abstract:** Biohydrogen fermentation using immobilized cells of *Thermotoga neapolitana* on porous glass beads was successfully performed in a continuously stirring anaerobic bioreactor (CSABR) system operated under the conditions of temperature 75 °C, pH 7.0 and 5.0 g/L pentose (xylose) and/or hexose (glucose). The results showed that both batch and fed-batch cultivations of the immobilized cells were effective for high-rate and high-yield H\(_2\) production compared with those from the free cells. In the batch cultivation, the H\(_2\) production rate and H\(_2\) production yield of the immobilized cells, respectively achieved the highest values of 5.64 ± 0.19 mmol-H\(_2\) L\(^{-1}\) h\(^{-1}\) and 1.84 ± 0.1 mol H\(_2\)/mol xylose, which were almost 1.7-fold and 1.3-fold higher than those with free cells. The maximum H\(_2\) production rate (6.91 mmol L\(^{-1}\) h\(^{-1}\)) in this proposed method was 1.5-fold higher than that of free cells in the fed-batch cultivation.

**Keywords:** *Thermotoga neapolitana*, Biohydrogen, Immobilized cells, Porous glass beads, CSABR, Fed-batch culture.

1. **INTRODUCTION**

Nowadays, many research efforts have been devoted to the sustainable and alternative energy, which is of critical importance with the ever-growing energy demands and climate change concerns, together with the fossil fuel depletion [1, 2]. Biohydrogen is an ideal, clean and friendly energy source that does not produce environmental pollution as carbon dioxide [3]. Furthermore, it can be produced from various renewable resources, which might be derived from pure sugars (glucose and xylose) [4, 5], agricultural residues, woody biomass [6-8] and biodiesel industry (glycerol waste) [9, 10], via biological conversion. Therefore, fermentative biohydrogen production technology has developed rapidly and considered as a promising treatment technology for organic wastes with efficient clean bioenergy production [11, 12].

The hyperthermophilic bacteria *Thermotoga* have garnered increasing interest for potential biohydrogen generation because of its high yields from a wide range of carbohydrates, such as glucose, sucrose, xylose, glycerol [5, 9, 13-21], and even rice straw [22]. Moreover, the optimum growth temperature of *Thermotoga* at an ambient 75°C makes its H\(_2\) fermentation less sensitive to contamination from methanogenic archaea, with a higher rate of hydrolysis and H\(_2\) yield [23-26].

Many researchers have examined various methods for efficient biohydrogen production with *T. neapolitana*. The optimization of cultivation conditions such as temperature, pH, initial concentration of substrate (carbon and nitrogen source), and ratio of medium volume were studied in small batch cultivation for efficient H\(_2\) production of *T. neapolitana* [23]. N\(_2\) sparging was a useful technique to enhance the H\(_2\) yield of strictly anaerobically fermentative bacterium *T. neapolitana* [4, 9]. In recent years, the immobilized cell systems have become popular alternatives over free-cell systems because they are capable of maintaining higher biomass concentrations and operating at higher dilution rates without biomass washout [27]. However, the H\(_2\) production based on immobilized-cell systems has mainly focused on using mesophilic bacteria such as porous glass beads-immobilized growing cells of *Clostridium butyricum* [28, 29a], agar gels-immobilized cells of *Rhodospirillum rubrum* [30], agar gels/porous glass beads-immobilized cells of aciduric *Enterobacter aerogenes* HO-39 [29b], lignocellulosic materials-supported *Enterobacter cloacae* IIT-BT08 [27], immobilized sewage sludge [31, 32], polydimethylsiloxane-immobilized microbial consortia [33], and so on. To the best of our knowledge, until now, there has been no research on enhanced H\(_2\) production using immobilized cells of *T. neapolitana* on porous glass bead. Even though, the H\(_2\) production using immobilized cells of *T. neapolitana* on acrylic hydrogel with pH-buffering properties was investigated on single report [36]. However, the H\(_2\) production rate and H\(_2\) production yield in batch culture were less than those with free cells [36].
In this study, immobilized cell systems and suspended cell systems using T. neapolitana were studied and compared based on H₂ production. This is the first report on the performance of immobilized cells T. neapolitana on porous glass beads for high-rate H₂ production. Hydrogen production rate, hydrogen content in biogas, soluble metabolites compositions and hydrogen yield were examined. The Fed-batch cultivation at constant pH was conducted for preventing substrate-associated growth inhibition by controlling the nutrient supply.

2. MATERIALS AND METHODS

2.1. Strain and Cultivation Medium

Thermotoga neapolitana strain DSM 4359 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany). The cultures was grown in modified Thermotoga maritima basal culture medium (TMB) at 75 °C and pH 7.5, with 10 % (v/v) inoculation [23]. The medium used for H₂ fermentation consisted of (amounts are in grams per liter of deionised water): 1.5 g KH₂PO₄; 4.2 g Na₂HPO₄·12H₂O (22 mM PO₄³⁻); 0.5 g NH₄Cl; 0.2 g MgCl₂·6H₂O (1.0 mM); 20.0 g NaCl; 2.0 g yeast extract; 5.0 g carbon source (glucose, xylose); 15.0 mL of the trace element solution (DSM-TES, see DSMZ medium 141); and 1.0 mg resazurin, which was used as a redox indicator. The anaerobic conditions for growth were created by adding 1.1 g cysteine hydrochloride as a reducing agent and flushing the headspace of the serum bottles with pure N₂ within 5 min.

2.2. Small Batch Cultivation by Immobilized Cells on Porous Glass Beads

Porous glass beads, vitralPOR (about 4 mm in diameter, 60-300 μm in pore size) were purchased from ROBU Glassfilter-Geräte GmbH Co., Schützenstrasse, Hattert, Germany. 25 mL of porous glass beads, 4 mL of precultured broth of the bacterium and 50 mL basal culture medium were put in a 120 mL serum bottle. Gas phase in upper layer was replaced with nitrogen gas and anaerobic batch cultivation was done at 75 °C and pH 7.5.

2.3. Hydrogen Production from the CSABR System

During immobilizing T. neapolitana cells on porous glass bead, a batch cultivation using a 3 L bioreactor (Biotron, Korea) and charged with 300 mL of porous glass beads, 900 mL of fresh medium and a 100 mL inoculum of T. neapolitana, was performed under constant temperature, pH and agitation at 75 °C, 7.0 and 300 rpm, respectively, using a Biotron controller system [5, 34]. The pH was kept constant by addition of 2.0 N NaOH. The temperature was kept at 75 °C using a heating coil wrapped around the bioreactor. The gas headspace was sparged with a continuous and pure N₂ gas flow; the gas outlet from the reactor was connected to condenser. The flow and partial pressure of the gas headspace in the outlet gas were monitored by a gas meter. After the batch culture, a fed batch culture was started by feeding a fresh medium (100 mL). The feed medium was prepared in a stock solution of the substrate with 50 g L⁻¹ xylose, 20 g L⁻¹ yeast extract, cysteine-HCl, salts, and the trace element solution in the concentration stated in session of strain and cultivation medium and adjusted to pH 7.0 using 2.0 N NaOH, which was added in four doses at a feed rate of 12 mL per min. The growth at pH 7.0 was controlled during the fed-batch culture using 2.0 N NaOH. The complete setup is illustrated on Scheme 1 [5].

2.4. Sampling and Analyses

Scanning electron microscopy of the cells immobilized on porous glass beads was conducted using SEM with a JEOL JSM-7401F cold cathode field emission scanning electron microscope.

The methods for sampling, analyses as well as calculation of H₂ production were described in our previous works [5, 34]. Biomass concentration in free systems was monitored by dry cell weight (DCW). The H₂ gas in the headspace was determined by a gas chromatograph (GC, Hewlett Packard 5890 Series II, USA) employing a thermal conductivity detector (TCD). Substrates (Glucose and xylose) as well as organic acids (acetic and lactic acids) were quantified using an HPLC system equipped with a reflective index detector (Agilent 1100, USA).

3. RESULTS AND DISCUSSION

3.1. Hydrogen Production Using the Immobilized Cells in Small Batch Cultivation

Hexose (glucose) and pentose (xylose) are the hydrolyzed products of lignocellulosic materials and they respectively account for 55-65 % and 35-45 % in the total mass [32]. These substrates were used for H₂ production by T. neapolitana [4, 5, 9, 15, 23, 34]. In this study, preliminary experiments showed that H₂ production using immobilized cells of T. neapolitana on porous glass beads with glucose and xylose substrates
was much higher than those free cell systems (Figure 1). It could be explained that the immobilized cell systems are capable of maintaining higher biomass concentrations and operating at higher dilution rates without biomass washout [27]. At the end of cultivation, the respective \( \text{H}_2 \) production from glucose and xylose were 37 ± 1.4 (904 ± 20 mL\(^{-1}\)) and 38.6 ± 1.3 mmol.L\(^{-1}\) (944 ± 32 mL.L\(^{-1}\)), that were nearly 1.2-fold greater for glucose and 1.3-fold greater for xylose than those of suspended cells with a maximum \( \text{H}_2 \) production (30.1 ± 0.7 mmol.L\(^{-1}\) for glucose and 30.1 ± 0.4 mmol.L\(^{-1}\) for xylose). Compared with the results from the highest previous report in the suspended cells system showed that a maximum \( \text{H}_2 \) production (806 mL. L\(^{-1}\) for glucose and 625 mL. L\(^{-1}\) for xylose [4]) was less than those with immobilized cells in this study. This result indicated efficiency of immobilized cells in \( \text{H}_2 \) production from \textit{T. neapolitana}. The substrate utilization and other products such as acetic acid and lactic acid, which were generated simultaneously with \( \text{H}_2 \) production [4, 13, 15], were also determined and compared between suspended cells and immobilized cells of \textit{T. neapolitana}. Similar to the \( \text{H}_2 \) production result, the levels of acetic acid and lactic acid from fermentation with immobilized cells were higher than those of the suspended cells for both glucose and xylose. The
substrate utilization was observed to be faster in the immobilized cells compared to the suspended cells (Figure 1). A similar result was obtained from previous report [27-38]. Yokoi et al. (1997) found that H₂ production and substrate consumption with immobilized cells of Clostridium butyricum on porous glass beads were higher than the corresponding values with free cells.

The scanning electron microscopy of immobilized cells on porous glass bead (Figure 2) showed that the bacterial cells were closely adsorbed to the bead pores. This allowed the substrates to be supplied readily to the bacteria and hydrogen and other gasses dissolved in culture broth would exit easily from the pores [29b].

3.2. Hydrogen Production Using the Immobilized Cells in the 3 L CSABR

The growth and H₂ production of T. neapolitana in batch cultivation have been reported limited by a rapid decrease in pH and affective from high hydrogen partial pressure [4, 9]. To overcome this problem, both the immobilized cells and suspended cells were conducted in pH-controlled batch bioreactors using a continuously stirring anaerobic bioreactor system at a constant pH of 7.0 (which was optimized in previous report [5]) and xylose as the main substrate. Results in Figure 3 and Table 1 showed that the best H₂ production rate, H₂ yield and acetic acid production in pH-controlled cultivation with the immobilized cells were much higher than those suspended cells. In the immobilized cells system, the H₂ production rate and accumulated H₂ production were respectively achieved the highest values of 5.64 ± 0.19 mmol-H₂.L⁻¹.h⁻¹ (138 ± 5 ml-H₂.L⁻¹.h⁻¹) and 52.9 ± 2.6 mmol. L⁻¹. Respectively, these values were almost 1.7-fold and 1.4-fold higher than those suspended cells (H₂ production rate of 3.3 ± 0.1 mmol-H₂.L⁻¹.h⁻¹ and accumulated H₂ production of 37.1 ± 1.8 mmol. L⁻¹) (Figure 3B and Table 1). This value also indicated a higher (3.5-fold) value than that of the immobilized cells on an acrylic hydrogel with pH-buffering properties (39.8 ± 2.1 ml-H₂.L⁻¹.h⁻¹) [36]. An optimum H₂ production was achieved with acetic acid as the main fermentative end-product [35]. In the present study, the acetic acid generation was measured in both the immobilized cells and free cells systems. Figure 3 showed that the amount of acetic acid production (53 ± 2.7 mmol. L⁻¹) with the immobilized cells at the end of cultivation was higher by approximately 80% comparing with the suspended cells (29.5 ± 1.5 mmol. L⁻¹). The xylose consumption ratio versus cultivation time was shown in Figure 3 to compare the hydrogen-producing properties of the suspended cells and the immobilized cells in the CSABR. The xylose in the medium was completely consumed in the immobilized cells after 18 h of cultivation, while the suspended cells did not completely use xylose (80 % of xylose consumption). In case of glucose as main substrate, Basile et al., 2012

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Figure 2: Scanning electron micrograph of the T. neapolitana immobilized on porous glass beads.
reported that glucose consumption was 50 % and 75 % for the suspended cells and the immobilized cells of *T. neapolitana* on an acrylic hydrogel after 21 h of cultivation, respectively [36]. These results strongly confirmed that the immobilized cells of *T. neapolitana* on porous glass beads are effective for high rate and yield H₂ production compared to the system of the free cells or immobilized cells of *T. neapolitana* on an acrylic hydrogel.

### 3.3. Hydrogen Production Using the Immobilized Cells in Fed-Batch Cultivation

To develop a large-scale H₂ production system as well as preventing substrate-associated growth inhibition when use the immobilized cells of *T. neapolitana*, pH-controlled fed-batch cultivation with xylose substrate doses was carried out by controlling the nutrient supply. To compare the hydrogen-producing properties between the suspended cells and the immobilized cells, fed–batch cultivation in a 3L-CSABR was conducted at the same condition with a constant pH of 7.0 and a concentrated fresh medium containing 5.0 g L⁻¹ xylose.

The feeding time was determined according to the final xylose concentration. The initial xylose concentration was 5.0 g L⁻¹. Figure 4B showed the changes in xylose concentration after the concentrated fresh medium for H₂ production was added into the system. Identical to the suspended cells system (data not shown here), H₂ production occurred after a lag

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**Table 1: Performance and Metabolites in Hydrogen Fermentation of *T. neapolitana* in Batch Mode System**

| Parameters                        | Working volume of 1 L in a 3L CSABR\(^*\) system |
|----------------------------------|--------------------------------------------------|
|                                  | Free cells                                        | Immobilized cells                          |
| Maximum H₂ content (%)           | 33.4 ± 1.6                                        | 43.1 ± 1.98                                 |
| Maximum H₂ production rate (mmol·H₂·h⁻¹) | 3.3 ± 0.1                                       | 5.64 ± 0.19                                |
| H₂ yield\(***\)              | 1.42 ± 0.09                                       | 1.84 ± 0.1                                 |
| Final acetic acid (g L⁻¹)       | 1.77 ± 0.1                                        | 3.19 ± 0.15                                |
| Final lactic acid (g L⁻¹)       | nd\(**\)                                          | nd                                         |
| Xylose consumption (%)          | 80.1 ± 4.5                                        | 99.9 ± 1.1                                 |
| Final pH                        | 7.0                                                | 7.0                                        |

Each measurement was repeated three times and averaged. The cultivation was at 75 °C, pH 7.0 with 5.0 g/L of initial concentration of substrate (xylose).

\(\text{CSABR} = \) continuously stirred anaerobic bioreactor.

\(\text{nd} = \) not detected

\(\text{H₂ yield} = (\text{H₂ formed}, \text{mol})/(\text{substrate consumed}, \text{mol})\).

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**Figure 3:** Growth of *T. neapolitana* on xylose substrate in the batch cultivation using a 3 L-CSABR: (A) Free cells, (B) immobilized cells.
phase of approximately 6 h. After 15 h fermentation, H₂ production quickly reached to a maximum value with a maximum H₂ gas content and the H₂ production rate were near 43% and 5.64 ± 0.2 mmol-H₂ L⁻¹ h⁻¹, respectively (Figure 4A). After 18 h, the H₂ production rate dropped slowly to 5.2 ± 0.26 mmol-H₂ L⁻¹ h⁻¹ with xylose being completely utilized (Figure 4B). The H₂ production rate and substrate utilization in the immobilized cells were much higher comparing with the suspended cells (3.02 ± 0.15 mmol-H₂ L⁻¹ h⁻¹, 87.8 % xylose utilization) [5]. Along with determination of H₂ production and substrate consumption, the acetic acid and lactic acid concentrations were measured and showed in Figure 4B. The acetic acid concentration increased according to the fermentation time and reached 3.2 ± 0.16 g. L⁻¹ after 18 h cultivation. This value was higher than that of the free cells (approximately 1.5 g. L⁻¹) [5]. No lactic acid concentration was determined after 18 h fermentation.

After 18 h, the first fed-batch process started with concentrated fresh medium being immediately added to the system. The final respective xylose and biomass concentrations in the medium were 5.2 ± 0.2 g. L⁻¹ and 3.4 ± 0.17 g DCW L⁻¹ at 18 and 36 h. Similar to the fed-batch cultivation of the free cells [5], the biomass concentration increased rapidly to a constant value of 4.9 ± 0.2 to 5.1 ± 0.2 g L⁻¹ during 27-35 h with the substrate being almost entirely consumed (Figure 4B). In this phase, the maximum H₂ production rate and H₂ content respectively reached 6.9 ± 0.3 mmol-H₂ L⁻¹ h⁻¹ and approximately 53 % at 24 h (Figure 4B). These values were almost 1.5-fold and 1.1 fold higher than those of the free cells system (4.62 ± 0.23 mmol-H₂ L⁻¹ h⁻¹ and 47 %, respectively) [5]. Lactic acid was determined at 21 h. At the end of this phase, acetic acid and lactic acid concentrations were 6.0 ± 0.3 g. L⁻¹ and 1.0 ± 0.02 g. L⁻¹, respectively.

After 36 h, the second fed-batch process began with adding concentrated fresh medium and giving a final substrate concentration of 5.2 ± 0.2 g. L⁻¹ (Figure 4B). The H₂ production rate and H₂ content reached maximum values of 6.2 ± 0.3 mmol-H₂ L⁻¹ h⁻¹ and approximately 47 % (Figure 4B), respectively. These values were much higher than those of the free cells system (4.1 ± 0.2 mmol-H₂ L⁻¹ h⁻¹ and 42 %, respectively) [5]. The H₂ production rate then gradually decreased when the substrate was almost fully utilized. The acetic acid and lactic acid concentrations were 8.1 ± 0.4 g L⁻¹ and 1.9 ± 0.1 g L⁻¹, respectively (Figure 4B).

At 49 h, the concentrated fresh medium was added again with a final substrate concentration of 5.24 ± 0.25 g L⁻¹ (Figure 4B). The highest H₂ content and H₂ production rate were 5.8 ± 0.3 mmol-H₂ L⁻¹ h⁻¹ and 44 % (Figure 4B), respectively at 58 h. These results were higher in comparison with a free cells system (3.9 ± 0.2 mmol-H₂ L⁻¹ h⁻¹ and 40 %, respectively) [5]. At 62 h, the xylose concentration was almost completely utilized. The respective with acetic acid and lactic acid concentrations of 9.5 ± 0.44 g L⁻¹ and 2.8 ± 0.12 g L⁻¹.

After 62 h, fresh medium for hydrogen production was once again added with final xylose concentration of 5.0 ± 0.2 g. L⁻¹ (Figure 4B). A maximum H₂ production rate was achieved at 71 h (approximately 5.8 ± 0.3 mmol-H₂ L⁻¹ h⁻¹) (Figure 4B). At the end of the fed-batch fermentation, the residual xylose, acetic acid,
and lactic acid concentrations were 0.7 ± 0.07 g L⁻¹, 10.7 ± 0.5 g L⁻¹, and 3.6 ± 0.18 g L⁻¹, respectively.

The immobilized cells demonstrated a better hydrogen production in comparison to the free cells. The absorption capacity in the micro-porous structure of porous glass beads might play a role in improving hydrogen production rate and substrate utilization. Constant substrates supplied to bacteria cells which well adsorbed on beads allowed gas diffusion from pore. Therefore, *T. neapolitana* immobilized on porous glass beads in CSABR was effective for high-rate and high-yield H₂ production comparing with those of the free cells. Similar results were obtained with immobilized *C. butyricum* or *E. Aerogenes* HO-39 on porous glass beads (Yokoi et al., 1997), which gave high H₂ production rate and yield. Aruna and Munawar 2012 reported an enhancement of photo H₂ production rate and yield. Aruna and Munawar 2012 reported an enhancement of photo H₂ production rate and yield. Aruna and Munawar 2012 reported an enhancement of photo H₂ production rate and yield. Aruna and Munawar 2012 reported an enhancement of photo H₂ production rate and yield.

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4. CONCLUSIONS

Biohydrogen fermentation using the immobilized cells of *T. neapolitana* on porous glass beads was performed successfully in a continuous stirring anaerobic bioreactor system. The hydrogen production in both the pH-controlled batch and fed-batch cultivation of the immobilized cells was higher than that of free cells system. The H₂ production rate and accumulated H₂ production of the immobilized cells reached highest values of 5.64 ± 0.19 mmol h⁻¹ and 52.9 ± 2.6 mmol L⁻¹, respectively, which were almost 1.7-fold and 1.4-fold higher than those of free cells.

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