Characteristics of hydrogen and bioflocculant production by a transposon-mutagenized strain of *Pantoea agglomerans* BH18

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Abstract: Based on the transposon-mutagenized library of *Pantoea agglomerans* BH18, the mutant strain 2-103 was obtained using mutant screens for high simultaneous production of hydrogen and bioflocculant. The mutant strain 2-103 was able to grow in medium with 50 µg/mL of kanamycin and could be amplified using Tn7-based transposon PCR amplification in cells from several passages, indicating that the mutant strain 2-103 transposon insertion was stable. The mutant strain 2-103 cell culture from batch experiments with varying initial pH values, glucose concentrations and NaCl concentrations was determined. The result indicated that the optimum initial pH for hydrogen production and bioflocculant activity was 7.0 and 8.0, respectively. The optimal glucose concentration for hydrogen and bioflocculant production was 20 g/L. In addition, the mutant strain 2-103 could produce hydrogen and bioflocculant under marine and fresh culture conditions. The mutant strain 2-103 had the highest production of hydrogen and bioflocculant with (1.55 ± 0.07) mol H₂/mol glucose and $(83.7 \pm 5.89)\%$ under marine culture conditions, which increased to (1.78 ± 0.08) mol H₂/mol glucose and $(93.5 \pm 6.24)\%$ under fresh culture conditions. This study found that the mutant strain 2-103 has the ability to produce hydrogen and

biofloculant within a range of initial pH values, glucose and NaCl concentrations, which is beneficial for the improvement of cost-effective bio-treatment processes.

Keywords: Transposon-mutagenized library; Mutant screen; Hydrogen production; Biofloculant activity

1. Introduction

Hydrogen is recognized as one of the most promising energy carriers [1]. Microbial hydrogen production by photosynthetic bacteria or fermentative bacteria has been demonstrated to be an effective process for hydrogen generation. Hydrogen production by fermentative bacteria is technically simpler than production by photosynthetic bacteria. In addition, fermentative hydrogen production can utilize a variety of renewable energy sources such as organic waste and is recognized as a promising approach [2]. Although fermentative hydrogen production has attracted great interest in research recently, this method has not been implemented in large-scale processing due to some major issues that remain unresolved. One of these issues is the production of potentially harmful by-products resulting from the fermentative hydrogen-producing process, such as waste fermenting liquor [3]. Waste fermenting liquor contains high levels of organic substances and requires further treatment before disposal [4]. This issue needs to be resolved before hydrogen production is industrialized using this method.

Biofloculants received practical attention in wastewater treatment, as they are biodegradable and regarded to be environmentally safe [5-6]. However, the high production cost is still the major impediment to the industrial application of biofloculants. To reduce the cost of biofloculant production, several low-cost substrates, such as rice straw, starch wastewater and fermenting waste liquor, were

researched and utilized by bioflocculant-producing microbial strains [7-9]. The procedure of using the fermenting liquor to produce bioflocculants has been recognized as an effective solution for cost-effective bioflocculant production and disposal of fermenting liquor [10].

Studies investigating the utilization of fermenting liquor from hydrogen production found that some strains (*Enterobacter* sp. BY-29 [11], *Ethanoligenens harbinense* [12], *Bacillus* sp. XF-56 [13]) have the ability to produce hydrogen and bioflocculant simultaneously. Applying techniques that utilize hydrogen-producing bacteria that simultaneously produce bioflocculant helps reduce the cost of wastewater treatment.

To date, strains able to produce hydrogen and bioflocculant simultaneously were mainly obtained using wild type screening. In order to expand microbial resources and clarify the mechanism of simultaneous hydrogen and bioflocculant production by hydrogen-producing bacteria, it is necessary to employ molecular biology techniques [14-15]. Classically, transposon mutagenesis has been demonstrated to be an excellent tool for mutating microorganisms in bacterial genetic research [16]. In a previous study, we constructed a transposon-mutagenized library of *Pantoea agglomerans* BH18. This library consisted of a population of insertion mutants with the transposable element at variable positions [17]. To the best of our knowledge, there have been no reports that assess simultaneous hydrogen production and the bioflocculant activity of bacterial strains using molecular biology techniques.

In this study, we screened the transposon-mutagenized strain 2-103, which has the potential for simultaneous hydrogen and bioflocculant production. Batch experiments for hydrogen and bioflocculant production by the mutant strain 2-103 were performed with varying initial pH values, glucose and NaCl concentrations. On the basis of the

transposon-mutagenized *Pantoea agglomerans* BH18 library, this study provides an effective approach to investigate the characteristics of hydrogen and biofloculant production by hydrogen-producing bacteria. These results provide valuable insights into the development of cost-effective approaches for industrializing hydrogen production.

2. Materials and Methods

2.1 Strain and culture growth

Pantoea agglomerans BH18 (NO. EU590682) was grown in LM-H medium as described previously [17]. The medium (g/L) was composed of glucose 10.0, tryptone 4.0, yeast extract 1.0, beef extract 2.0, NaCl 30.0, K₂HPO₄ 1.5, FeSO₄ 0.1, MgCl₂ 0.1 and L-cysteine 0.5. The transposon-mutagenized strains of *Pantoea agglomerans* BH18 were inoculated in the same medium supplemented with 50 µg/mL kanamycin sulfate. *Pantoea agglomerans* BH-18 and the mutant strains were incubated at 37°C with a shaking speed of 120 rpm under anaerobic conditions.

2.2 Screening for mutant strains

In the primary screening, the mutant strains were cultured in the medium, as follows (g/L): glucose 10.0, yeast extract 0.5, urea 0.5, NaCl 30.0, KH₂PO₄ 2.0, K₂HPO₄ 5.0 and MgSO₄ 0.2. After 2 days of growth at 37°C, these transformed cell cultures were spread onto the same medium agar plates with 50 µg/mL kanamycin sulfate. After cultivating for 2 days, single colonies with mucoid and ropy morphology were selected and transferred to the liquid medium for culture. In the secondary screening, hydrogen production and biofloculant activity by the mutant strains were determined. Wild type strain *Pantoea agglomerans* BH18 was used as the control. Finally, the

mutant strain with the highest hydrogen and biofloculant production was isolated.

The stability of the transposon insertion generated by the mutant strain was tested using the following method. Briefly, at the end of fermentative hydrogen production, 1 mL of cell suspension was transferred into 100 mL of medium with 50 µg/mL kanamycin sulfate. After 2 days of anaerobic incubation, 1 mL of culture broth was inoculated for the next cultivation using the same medium under anaerobic conditions. After four subcultures, genomic DNA of the aliquot cells was extracted as a template for polymerase chain reaction to confirm the transposon insertion. The transposon-specific PCR (primer TnL: TTAAGGATTATTTAGGGAAG; TnR: ACAATAAAGTCTTAAAC) was conducted as follows: initial denaturation of DNA for 4 min at 94°C; 30 cycles at 94°C for 30 s, at 49°C for 30 s, at 72°C 30 s and extension of incomplete products at 72°C for 10 min.

2.3. Optimization of batch conditions

The effects of initial pH, glucose concentration and NaCl concentration on cell growth, hydrogen production and biofloculant activity by the mutant strain were determined. To determine the optimum initial pH value for production of hydrogen and biofloculant, the following range of pH values was selected: 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. A range of glucose concentrations (5, 10, 20, 30, 40 and 50 g/L) and NaCl concentrations (0, 4, 30, 40, 50 and 60 g/L) were used in the batch operations. All tests were performed in triplicate.

2.4. Analytical methods

The cell growth of the mutant strain was determined by optical absorbance at 600 nm

using a spectrophotometer (UV-1800, Shimadzu). Hydrogen production was determined using a gas chromatograph (Model 6820, Agilent). In detail, biogas production was collected using the drainage method. The chromatograph was equipped with a thermal conductivity detector and nitrogen at the speed of 30 mL/min was used as the carrier gas. The oven, injector and detector were kept at 40, 200 and 200°C, respectively. The biofloculant activity was measured by kaolin suspension according to methods described previously [18]. The kaolin suspension was prepared by adding 0.5 g kaolin clay to 100 mL deionized water and mixed with 0.5 mL of 10% CaCl₂ solution. Next, 0.5 mL of fermenting liquor was added to the 100 mL of kaolin suspension and the pH value was adjusted to 7.0. The mixture was stirred at 120 rpm for 30 s, and allowed to stand for 10 min. The optical density of the upper solution was measured using a spectrophotometer (UV-1800, Shimadzu) at 550 nm. The mixed solution without biofloculant was used as the blank control. The biofloculant activity was calculated as follows: biofloculant activity = (OD_A-OD_B)/OD_A*100, where OD_A and OD_B represent the optical densities of the control and the sample at 550 nm, respectively.

3. Results and Discussion

3.1. Screening for the mutant strain 2-103

Based on the transposon-insertion library of *Pantoea agglomerans* BH18, we isolated nine mutant strains whose hydrogen production and biofloculant activity exceeded that of the wild type strain by 20%. Of these transposon-mutagenized strains, the mutant strain with the highest hydrogen and biofloculant production was screened and named as strain 2-103. Hydrogen production of the mutant strain 2-103 (1.55 ±

0.07 mol H₂/mol glucose under marine conditions) exceeded that of the wild type strain by 40%. The mutant strain 2-103 produced bioflocculant as a byproduct during fermentative hydrogen production. The bioflocculant activity of the mutant strain 2-103 was $(83.7 \pm 5.89)\%$, which was approximately 2.5-fold greater than that of the wild type strain. To the best of our knowledge, this report describes the first time a transposon-mutagenized strain with simultaneous hydrogen and bioflocculant production was obtained using molecular biology techniques.

The mutant strain 2-103 was replated in the medium with kanamycin to test the stability of the transposition event. Figure 1 shows that the Tn7-based transposon with a 1699 bp transposon fragment was amplified by mutant strain 2-103 in the cells from several passages. These results indicate that the kanamycin resistance was maintained and that the target transposon fragment of the mutant strain 2-103 was stable and effective.

3.2. Bacterial growth

To determine the cell growth of the mutant strain 2-103 during the fermentation, the optical absorbance at 600 nm was monitored at various time intervals. As shown in Figure 2, the mutant strain 2-103 grew logarithmically after 12 h and arrived at a stationary phase at 24 h. At this stationary phase, cell growth of the mutant strain 2-103 reached its maximum. For the wild strain *Pantoea agglomerans* BH18, the time that cells reached the exponential phase and stationary phase was 8 and 16 h, respectively. The mutant strain 2-103 showed slower growth compared with the wild type. In general, cell growth of genetically engineered mutants is usually decreased when compared to the wild type strain [19].

The mutant strain 2-103 began to produce hydrogen in the late exponential phase, with maximum hydrogen production occurring between 24 and 48 h. As shown in Figure 2, hydrogen production and cell growth was not synchronous. This phenomenon is similar to what had been observed in a previous report [20]. The bioflocculant activity of the mutant strain 2-103 was also determined at different time intervals. Figure 2 shows that bioflocculant activity increased with increments of cell growth and had a remarkable improvement during the stationary phase (24–36 h). This result is consistent with prior studies that revealed that bioflocculant production was almost in parallel with cell growth of *Bacillus mojavensis* 32A and *Chryseobacterium daeguense* W6 [15, 21].

3.3. Effects on the production of hydrogen and bioflocculant

3.3.1. Initial pH

The effects of initial medium pH on cell growth and production of hydrogen and bioflocculant were investigated. As shown in Table 1, the mutant strain 2-103 could grow at a wide range of initial pH values (5.0–10.0). The optimum initial pH for cell growth and hydrogen production of the mutant strain 2-103 was at 8.0 and 7.0, respectively, which indicated again that hydrogen generation was not in parallel with cell growth [22]. The maximum hydrogen production yield (1.55 ± 0.07) mol H₂/mol glucose by the mutant strain 2-103 was obtained at the optimum initial pH of 7.0. The initial pH of the medium is known to be an important factor influencing hydrogen production. Disagreements exist regarding the optimum pH for hydrogen-producing strains isolated from different environments. The reported optimum pH values for hydrogen production range from 6.0 to 8.0 [23–25].

The mutant strain 2-103 was isolated and grown under marine conditions. The

optimum initial pH for cell growth and bioflocculant production by the mutant strain 2-103 was 8.0, which is consistent with the pH of seawater. The maximum bioflocculant activity of the mutant strain 2-103 was (87.5 ± 6.38) % at an initial pH of 8.0. The bioflocculant activity and cell growth by the mutant strain 2-103 simultaneously reached their maximum at an initial pH of 8.0. This result is similar to a previous report that demonstrated a close relationship between cell growth and bioflocculant production in *Paenibacillus polymyxa* AM49 [26].

3.3.2. Glucose concentration

Glucose was used as a carbon source during anaerobic cultivation of the mutant strain 2-103. The effect of initial glucose concentration on the production of hydrogen and bioflocculant was examined. Figure 3 shows that the initial glucose concentration had a noticeable effect on cell growth and hydrogen production. With increasing glucose concentrations (from 5 to 50 g/L), cell growth of the mutant strain 2-103 first increased and subsequently decreased. The optimum glucose concentration for cell growth of the mutant strain 2-103 was 20 g/L. Maximum hydrogen production (1.68 ± 0.08 mol H₂/mol glucose) of the mutant strain 2-103 also occurred at a glucose concentration of 20 g/L. Many strains were previously reported to effectively use glucose as a carbon source for hydrogen production with different optimum glucose concentrations ranging from 4 to 40 g/L [27-28].

Glucose concentration is an important parameter for bioflocculant production because low initial concentrations could result in low biomass accumulation, long lag times and a low rate of fermentation [29]. Figure 3 also shows the effect of glucose concentration on bioflocculant activity. Different glucose concentrations led to

changes in bioflocculant activity of the mutant strain 2-103. Bioflocculant activity reached its maximum (91.5 ± 5.82 %) when the initial glucose concentration was 20 g/L. In accordance with our results, some reports also utilized glucose as the optimum carbon source for bioflocculant production with glucose concentrations fixed at 10-20 g/L [30-31].

3.3.3. *NaCl concentration*

Salt is essential for cells and is composed primarily of NaCl. NaCl concentration is one of the most important parameters influencing hydrogen production and bioflocculant activity [32-33]. The properties of hydrogen and bioflocculant by the mutant strain 2-103 under fresh and marine culture conditions were investigated. As seen in Figure 4, differences in hydrogen and bioflocculant production by the mutant strain 2-103 were observed at various NaCl concentrations (0, 4, 30, 40, 50 and 60 g/L). Hydrogen production and bioflocculant activity of the mutant strain 2-103 decreased as NaCl concentrations increased. Hydrogen production and bioflocculant activity was obviously weak at a NaCl concentration of 60 g/L. Under marine conditions (NaCl concentration of 30 g/L), the maximum hydrogen production and bioflocculant activity by the mutant strain 2-103 was (1.55 ± 0.07) mol H₂/mol glucose and (83.7 ± 5.89) % respectively, whereas under the fresh culture condition (NaCl concentration of 4 g/L) it increased to (1.78 ± 0.08) mol H₂/mol glucose and (93.5 ± 6.24) %.

The mutant strain 2-103 was able to grow and produce hydrogen and bioflocculant at all NaCl concentration ranges tested, which indicated that the mutant strain was a salt-tolerant bacterium as it was able to function under either fresh or marine culture

conditions. *Pantoea agglomerans* BH18 was isolated from mangrove sludge. Among various marine environments, mangrove is located at the transition between marine and terrestrial environments with drastic variations in NaCl concentrations. The mutant strain 2-103 basically maintained its salt-tolerance from the wild type strain. These results suggest that the strain 2-103 may have the potential to be used for the production and clarification of biohydrogen under a wide range of water types from fresh to marine conditions.

4. Conclusions

The mutant strain 2-103, which could produce hydrogen and bioflocculant simultaneously, was screened based on the transposon-mutagenized library of *Pantoea agglomerans* BH18. The transposition event of the mutant strain 2-103 was found to be highly stable during the several passages. In batch cultures of the mutant strain 2-103, the optimum condition for hydrogen production and bioflocculant activity was in medium containing an initial pH of 7.0 (8.0), glucose concentration of 20 g/L (as a carbon source) and NaCl concentration of 4 g/L. The highest production of hydrogen and bioflocculant by the mutant strain 2-103 was (1.78 ± 0.08) mol H₂/mol glucose and (93.5 ± 6.24) %, respectively during the 72 h cultivation period under fresh culture conditions. This study revealed that the mutant strain 2-103 could simultaneously produce hydrogen and bioflocculant, which will largely reduce the productive costs of hydrogen and bioflocculants and has the characteristics desired for application in cost-effective wastewater treatments.

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Legend

Fig. 1 The identification of transposon insertion by PCR amplification of the mutant strain 2-103

M: Marker, W: Wild type strain, 1-5: PCR products of transposon Tn7 insertion in the five consecutive passages by the mutant strain 2-103

Fig. 2 Variation of cell growth, hydrogen production and bioflocculant activity by the mutant strain 2-103 during the fermentation (n = 3, Error bars = s.e.m.)

Fig. 3 Effect of glucose concentration on cell growth, hydrogen production and bioflocculant activity by the mutant strain 2-103 ($n = 3$, Error bars = s.e.m.)

Fig. 4 Effect of NaCl concentration on cell growth, hydrogen production and bioflocculant activity by the mutant strain 2-103 ($n = 3$, Error bars = s.e.m.)

Tab.1 Cell growth, hydrogen production and bioflocculant activity by the mutant strain 2-103 in different initial pH using 10 g/L glucose as carbon source