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Immobilization of the Hyperthermophilic Archaeon *Thermococcus onnurineus* Using Amine-coated Silica Material for H_2 Production

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Previously we reported that the hyperthermophilic archaeon, *Thermococcus onnurineus* NA1 is capable of producing hydrogen (H_2) from formate, CO or starch. In this study, we describe the immobilization of *T. onnurineus* NA1 as an alternative means of H_2 production. Amine-coated silica particles were effective in immobilizing *T. onnurineus* NA1 by electrostatic interaction, showing a maximum cell adsorption capacity of 71.7 mg-dried cells per g of particle. In three cycles of repeated-batch cultivation using sodium formate as the sole energy source, immobilized cells showed reproducible H_2 production with a considerable increase in the initial production rate from 2.3 to 4.0 mmol l^{-1} h^{-1}, mainly due to the increase in the immobilized cell concentration as the batch culture was repeated. Thus, the immobilized-cell system of *T. onnurineus* NA1 was demonstrated to be feasible for H_2 production. This study is the first example of immobilized cells of hyperthermophilic archaea being used for the production of H_2.

Keywords: Amine-coated silica particle, cell immobilization, formate, H_2 production, *Thermococcus onnurineus* NA1

Introduction

Cell immobilization has been attracting widespread attention with regard to fermentation processes because it offers many advantages, such as enhanced volumetric productivity via high-cell-density cultivation, feasibility of continuous processing, and easy recovery and reuse of microorganisms [2, 13].

Different techniques have been employed for cell immobilization. In general, these can be divided into the five categories of adsorption on a solid support, covalent attachment to a solid, entrapment in polymeric gels, cell flocculation (aggregation or cross-linking), and encapsulation [12–14]. Among these techniques, cell adsorption on a solid support is extensively used for cell immobilization, as it provides many advantages, such as a simple immobilization process, the continuous replacement of dead cells with active ones, and a high mass transfer condition in comparison with entrapment [5, 8].

Recently, hyperthermophilic archaea such as *Pyrococcus* and *Thermococcus* have been widely studied as a potential microbial platform for the production of biochemical and energy, such as 3-hydroxypropionate and hydrogen [9, 11, 16]. However, in contrast with other mesophilic microorganisms, the cell immobilization of hyperthermophilic archaea has not been well studied thus far. In this study, we investigate the cell immobilization of the hyperthermo-

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philic archaeon *T. onnurineus* NA1, which is known to produce H_2 from various substrates, such as formate, carbon monoxide and starch [4], via physical adsorption between the cell and the porous silica particles coated with positively-charged amine groups. The feasibility, stability and reproducibility of immobilized cells for the production of H_2 from formate were also investigated in repeated-batch cultivation.

Materials and Methods

Strain, supporting materials and cell immobilization

T. onnurineus NA1 (KCTC 10859) was routinely cultured in modified medium 1 (MM1) [4]. Amine-coated porous silica particles with a pore size of 10 or 16 μm (Kromasil-Amino-NH_2), purchased from AkzoNobel (Netherlands), were used as support material for cell immobilization. Diatomite (HayanCell #200, Haedong M-cell Co., Ltd, South Korea) was used as a control because it is also porous and mainly composed of silica [6] but has no positively-charged group such as amine. The particle size was 20-38 μm for diatomite and 10 or 16 μm for Kromasil. After pretreatment through a sequence of washing five times with distilled water, autoclaving, and then drying at 80°C, all particles were stored in an anaerobic chamber (Coy Laboratory Products, USA) filled with an anoxic gas mixture (N_2:H_2:CO_2, 90:5:5). For cell immobilization which was carried out in the anaerobic chamber, 10 ml of the *T. onnurineus* NA1 cell suspension (OD_{600} = 2.74) was added in 25 ml serum bottle containing 10% (w/v) of particles with an adjustment of the initial pH to 6.5 (at room temperature) using 2 N HCl in the case of Kromasil, after which the bottles were sealed with butyl rubber stoppers and aluminium crimp caps. In contrast to the diatomite, the pH of the cell suspension increased to 9.0 (at room temperature) as soon as it was mixed with Kromasil particles. After mixing cells and particles in a shaker for 1 h at 80°C, the serum bottles were set at room temperature for 30 min and the OD_{600} of supernatants was then measured. The adsorptive capacity of Kromasil was tested in the same manner in 1.5 ml tube containing 0.1 g of particles and 1 ml of the cell suspension with an initial OD_{600} concentration of 0.125 to 35.9.

Repeated-batch operation using immobilized-cells

Cell immobilization was conducted as above in a 50 ml serum bottle containing 20 ml of the cell suspension (OD_{600} = 2.5) and 2 g of 10 μm Kromasil. At the end of the immobilization process, the particles were washed at least seven times with MM1 medium such that the OD_{600} of the final washed supernatant was nearly zero (<0.005). The immobilized *T. onnurineus* NA1 cells were then cultured in MM1 medium containing 10 g/l of sodium formate at 80°C for 4 h. The cultivation was repeated two additional times after the removal of free cells from the bottle through an identical washing process. As a control, the final washed suspensions obtained from the washing step before each batch cultivation were also cultured under the same condition additionally to confirm that no free-cells existed in the reaction bottle with the observation of no production of H_2.

Analytical and other methods

The optical density was measured at 600 nm (OD_{600}) with a UV/Vis spectrophotometer (Biophotometer plus, Eppendorf, Germany). The biomass concentration was determined by measuring the amount of cellular proteins of the cell lysates using a DC protein assay kit (Bio-Rad, USA). One unit value of OD_{600} corresponded to 0.453 g-dcw/l based on the assumption that protein comprises approximately 50% of dry cell weight (dcw) [10]. The amount of cells immobilized onto particles was determined from the decreased biomass concentration of free cells during immobilization. The H_2 and formate concentrations were measured by GC and HPLC, respectively, as in a previous report [15]. The H_2 production rate (HPR) was calculated according to the amount of H_2 produced per unit volume of culture broth as a function of time. H_2 productivity was defined as the value of the total amount of H_2 produced per unit volume of culture broth divided by the overall culturing time.

Results and Discussion

Immobilization of *T. onnurineus* NA1 onto amine-coated silica particles

In this study, the immobilized-cell system of *T. onnurineus* NA1 feasible for formate-driven H_2 production was developed. Considering that most bacteria have a net negative surface charge [7] and that *T. onnurineus* NA1 has been found to grow well under relatively extreme conditions such as a high temperature (80°C) and high salinity (3.5% NaCl) in a medium [3, 4], Kromasil-Amino-NH_2, which is a porous

silica particle coated with amine groups on its surface and which has good properties such as high mechanical strength, perfectly spherical shape and exceptional chemical stability, was considered to be an appropriate support material.

As shown in Table 1, it was observed that approximately 91% and 42% of the initial free cells were immobilized onto 10 μm and 16 μm Kromasil, respectively, indicating that a size of 10 μm was more efficient for immobilizing *T. onnurineus* NA1. On the other hand, diatomite, which is also a porous silica particle but has no positively charged groups, failed to immobilize the cells, and no change was observed in the free-cell concentration. Since the cell envelope of *Thermococcales* including *T. onnurineus* NA1 is comprised of a protein surface layer with a negative surface charge, known as an S-layer [1], it was thought that *T. onnurineus* NA1 could be immobilized onto positively charged amine-containing particles by electrostatic interaction, with different efficiency levels with the particle size.

We also determined the adsorptive capacity of the 10 μm Kromasil. Fig. 1 shows that the amount of cells immobilized onto a unit amount of the support was linearly correlated with the initial free-cell concentration up to 10.9 g-dcw/l, after which it leveled off. The maximum amount of immobilized cells to a unit g of Kromasil was estimated be ca. 71.7 mg-dcw.

Table 1 Change of free-cell concentration during immobilization of *T. onnurineus* NA1 cells on silica particles.

Support matrix	Free-cell concentration		Immobilization efficiency (%)[a]
	Initial OD_{600}	Final OD_{600}	
Diatomite (20–38 μm)	2.740	2.740	0.0
Kromasil-Amino-NH_2 (16 μm)	2.740	1.585	42.2
Kromasil-Amino-NH_2 (10 μm)	2.740	0.245	91.1

All samples were mixed in a shaker at 80ºC for 1 h
All data are average value from duplicated experiments
[a]Immobilization efficiency (%) was calculated as follows:
[(Initial OD_{600} − Final OD_{600}) / Initial OD_{600}] × 100

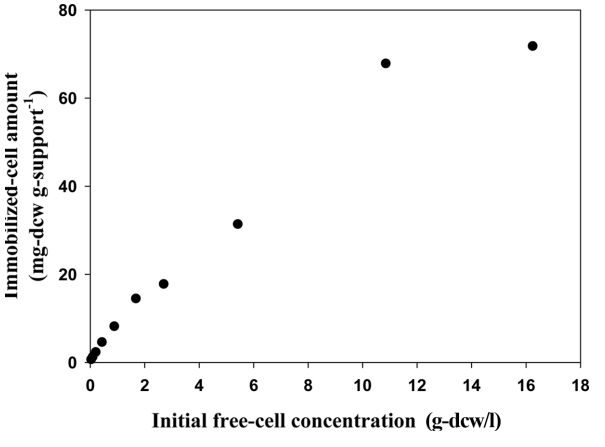

Fig. 1 Adsorption capacity of 10 μm Kromasil-Amino-NH_2 particles with various initial free-cell concentration of *T. onnurineus* NA1. Ten percent (w/v) of Kromasil particles were used and cell immobilization was conducted at 80°C for 1 h.

Formate-driven H_2 production by immobilized-cells of *T. onnurineus* NA1

To investigate the feasibility of immobilized cells of *T. onnurineus* NA1 for formate-driven H_2 production, the immobilized cells were applied in a repeated-batch operation using sodium formate with an initial concentration of 10 g/l as a sole energy source. The cells were immobilized

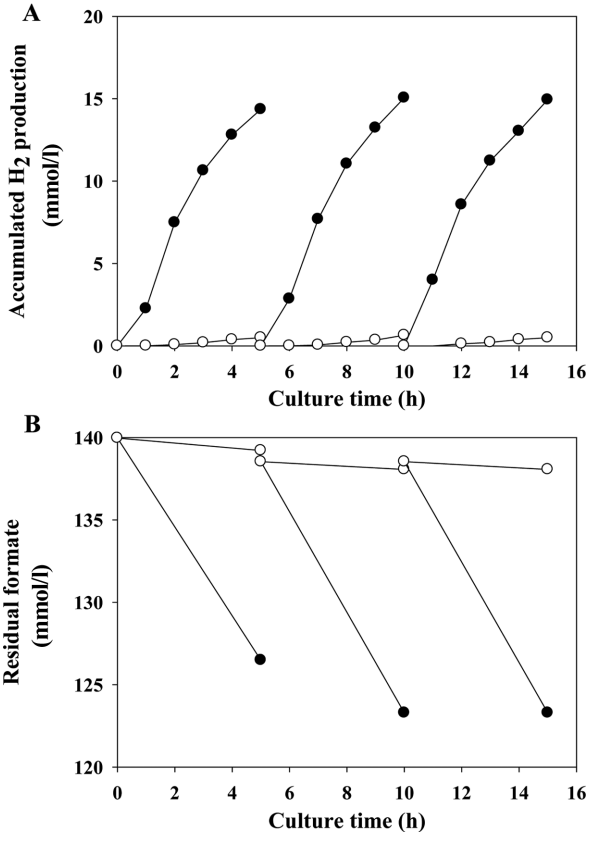

Fig. 2. H_2 production (A) and sodium formate concentration (B) in repeated-batch operation using immobilized-cells of *T. onnurineus* NA1 to 10 μm Kromasil-Amino-NH_2 particles. Symbols in the figure indicate immobilized cells (●) and final washed suspension as control (○).

onto the surfaces of 10 μm Kromasil-Amino-NH_2 particles at an efficiency level of 10.2 mg-dcw per g of support. Using the immobilized cells, three consecutive batches were conducted in a serum bottle. Before each batch culture, free cells were completely removed by thorough washing to ensure that H_2 was not produced at all in the bottle containing the final washed suspension (Fig. 2A, open circle). In all of the batch cycles, the immobilized cells actively produced H_2 from formate with a considerable increase in the initial H_2 production rate (HPR) for 1 h from 2.3 to 4.0 mmol l^{-1} h^{-1} as the batch culture was repeated (Fig. 2A, closed circle), showing a reproducible H_2 production pattern. It was considered that the increased HPR stemmed mainly from the increased immobilized-cell concentration because the initial amount of immobilized cells per unit g of Kromasil, 10.2 mg-dcw, was much lower than the maximum adsorption capacity of 71.7 mg-dcw per g of support. Thus, the free cells generated during cultivation could also be immobilized onto the support. On the other hand, after 2 h in all batches, the HPR suddenly started to decrease despite the presence of enough amount of substrate (Fig. 2B), resulting in a similar final H_2 production, namely, the same H_2 productivity of 3.6–3.75 mmol l^{-1} h^{-1} irrespective of the number of batch cycles (Fig. 2A, closed circle). The decrease of the HPR was likely caused mainly by the increase of the pH during the cultivation, as has been reported during the course of formate decomposition to H_2 [3, 17, 18]. The final pH in each batch culture was about 7.04 (at room temperature), much higher than the initial value. Therefore, it will be desirable to undertake immobilized cell culturing with a controlling pH to enhance H_2 productivity, which is expected to increase as the batches are repeated until the amount of immobilized cells reaches 71 mg-dcw per g of support, the maximum adsorption capacity.

In conclusion, we experimentally demonstrated that *T. onnurineus* NA1 could be immobilized onto amine-coated silica particles via surface charge interaction between the cells and a support material, and the immobilized-cell system of *T. onnurineus* NA1 was shown to be feasible for formate-driven H_2 production. This is the first example of H_2 production by immobilized cells of hyperthermophilic archaea.

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국문초록

아민기가 코팅된 규조토 담체를 이용한 초고온성 고세균 *Thermococcus onnurineus*의 세포 고정화 및 수소생산 연구

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초고온성 고세균 *Thermococcus onnurineus* NA1은 개미산, 일산화탄소, 또는 전분 등을 이용해서 수소를 생산하는 것으로 알려져 있다. 본 연구에서는 *T. onnurineus* NA1의 고정화 세포를 이용한 수소생산을 고찰하였다. 고정화 실험결과, *T. onnurineus* NA1은 표면에 아민기가 코팅된 규조토 담체에 정전기적 인력에 의해 효과적으로 고정화되었고, 1 g의 담체에 고정화 될 수 있는 최대 세포의 양은 71.7 mg-dcw로 확인되었다. 고정화 세포를 이용한 세 번의 반복회분식 배양을 통해 개미산으로부터 수소생산 특성을 고찰하였고, 그 결과 배양이 반복됨에 따라 고정화 세포 농도의 증가에 기인하여 초기수소생산속도가 2.3 에서 4.0 mmol l^{-1} h^{-1}로 상당량 증가됨이 관찰되었다. 따라서, *T. onnurineus* NA1의 고정화세포 시스템은 수소생산을 위한 좋은 대안이 될 수 있을 것으로 사료된다. 본 연구는 초고온성 고세균의 고정화세포를 수소생산에 적용한 첫 번째 사례이다.

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