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Basic Studies of Hydrogen Evolution by *Escherichia coli* Containing a Cloned *Citrobacter freundii* Hydrogenase Gene

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ABSTRACT

Citrobacter freundii genes that complemented *Escherichia coli* hyd- (hydrogenase activity) mutation were cloned in plasmids pCBH4 (6.2 kb) and pCBH6(5.7 kb) (*1,2*). Hydrogen evolution by the transformant *E. coli* HK-8(pCBH4 or pCBH6) was investigated. The optimum culture temperature of recombinant *E. coli* cells for hydrogen evolution from glucose was in the neighborhood of 18°C. The recombinant *E. coli* cells cultured at this condition showed a several-fold increase of hydrogen evolution, as compared with that of the wild-type cells. The plasmid-retention stability of this recombinant *E. coli* was extremely high, especially plasmid pCBH4, which was completely retained during 2 wk without any restriction. Hydrogen production by immobilized recombinant *E. coli* was then investigated using cells cultured at 18°C. The hydrogen evolution rate from glucose and Lennox-broth were about twofold higher than that of *E. coli* C600, and this high hydrogen evolution rate was maintained for more than 1 mo.

Index Entries: Hydrogenase gene; *Citrobacter freundii; Escherichia coli*; hydrogenase activity; immobilization; hydrogen evolution from glucose.

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INTRODUCTION

The authors previously reported on success in stabilizing the hydrogen evolution system of *Clostridium butyricum* or a blue-green algae, *Anabaena* N-7363, by immobilizing the bacteria in agar gels, allowing us to construct a continuous hydrogen producing bioreactor system (*3–5*). These bacteria theoretically evolve 4*M* of hydrogen from 1*M* of glucose (*6*). The practical yield of hydrogen from glucose by wild-type bacteria is, however, approximately only 10–20% of this, because of alternate metabolic pathways leading to production of organic acids. We therefore commenced studies on the breeding of bacteria possessing higher hydrogen productivity. In previous papers we reported hydrogen production by *Escherichia coli* containing a cloned hydrogenase gene from *Citrobacter freundii* (*2*). Figure 1 shows the detailed restriction maps of cloned hydrogenase genes. The hydrogen producing activity of the transformant HK-8(pCBH4 or pCBH6) from organic compounds was several-fold higher than that of *E. coli* C600 (wild type). Nevertheless, the hydrogen producing activity from pyruvate or glucose is very low, as compared with that from formate. Therefore, an enhancement in hydrogen evolution from glucose (or molasses) is one of the most important challenges of a practical hydrogen evolution system.

In this paper, we describe the basic study of this recombinant *E. coli* regarding utilization for the hydrogen evolution system. The effect of culture temperature on hydrogen evolution and the stability of hydrogenase gene cloned in *E. coli* were investigated. Finally, recombinant *E. coli* was immobilized in polyacrylamide-hydrazide (PAAH) gel and used for the hydrogen evolution.

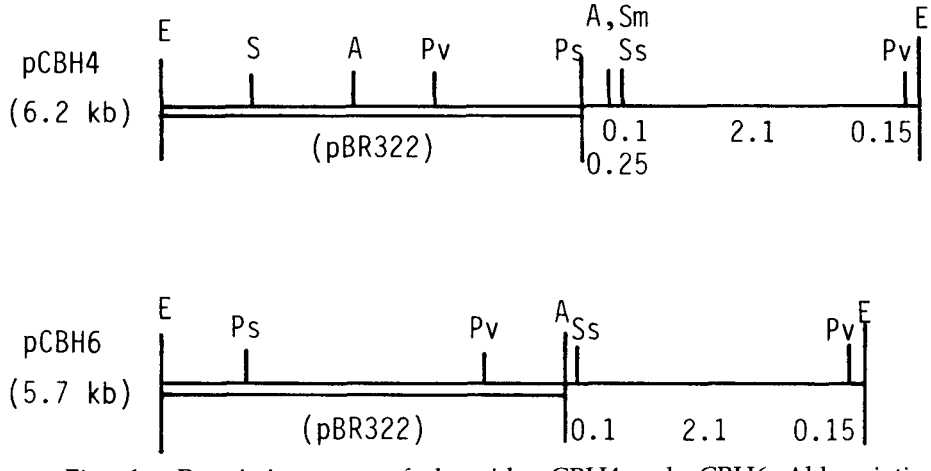

Fig. 1. Restriction maps of plasmids pCBH4 and pCBH6. Abbreviations; A, *Ava*I; E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*II. The numbers indicate the size of the DNA fragment between two restriction sites in kb.

MATERIALS AND METHODS

Strains and Media

Strains and media were the same as described in the previous paper (2). The cells used for the assay were cultured under anaerobic conditions at three different temperatures (18, 27, and 37°C) in Lennox-broth (L-broth; 1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose in distilled water, pH 7.2).

Assay of Hydrogenase Activity

Assay of hydrogenase activity was the same as described in the previous paper (2), except for the following treatment. [1] One milliliter of the precultured medium was inoculated into 200 mL of L-broth and cultured anaerobically at 18°C for about 12 h (for 3–4 h at 27 or 37°C) until it became the late log phase cells. [2] Assay of hydrogenase activity was performed at 30°C.

Assay of Hydrogen-Producing Activity from Organic Compounds

Hydrogen-producing activities from organic compounds (glucose or L-broth) were measured at 30°C as described in the previous paper (2).

Immobilization

The immobilization of microorganisms was carried out by the method of Beetman and Rehm (*7*) with slight modification. Microorganisms were immobilized in PAAH gel prepared according to the procedure of Freeman and Aharonowitz (*8*). Four grams of wet cells cultured at 18°C for 12 h were suspended in saline (0.9%, 4 mL). This suspension was mixed with 20 mL of 5% (w/v) PAAH solution and 4 mL of 3% (w/v) sodium alginate. This mixture was added dropwise to a 0.1*M* calcium chloride solution to solidify the alginate. The gels thus formed were subsequently transferred to a 0.25% (w/v) glyoxal solution to cross-link PAAH. The gels were shrunk, and the final cell concentration in the gels was approximately 14% (w/w).

Measurement of Plasmid Stability

Plasmid retention stability was measured by examining the hydrogenase activity of cells. Immobilized cells were maintained anaerobically in L-broth at 30°C, and cells grown outside of the gels were spread onto Lennox agar plates containing no antibiotics and incubated for over 20 h at 37°C. Hydrogenase activities of colony cells were qualitatively tested by the methyl viologen (MV) filter-paper method (*9*).

Hyd^+ cells reduce MV^{2+} to MV^+, producing a blue-purple color, but Hyd^- cells remain cream colored under hydrogen atmosphere.

Subsequently, it was found that the cells of Hyd^+ colonies could grow on the plates containing antibiotics (tetracycline, 20 μg/mL, or ampicillin, 100 μg/mL, according to the plasmid's antibiotic resistance), but the cells of Hyd^- colonies could not grow on them. The response to the MV filter-paper test, thus indicated positive colonies as containing hybrid plasmids and negative colonies as possessing no plasmids. Therefore, the retention ratios of plasmids were formularized by the equation:

$$\text{Plasmid retention (\%)} = \frac{\text{Sum of } Hyd^+ \text{ colonies}}{\text{Sum of total colonies}} \times 100$$

Hydrogen Evolution by Immobilized Cells

One-half gram of the immobilized cells was suspended in 10 mL of either 0.1% (w/v) glucose solution (0.1*M* phosphate buffer solution, pH 7) or L-broth and transferred to a 50-mL Shrenk flask. This mixture was then incubated anaerobically at 30°C for 3 h and the hydrogen evolution monitored by gas chromatography; this incubation lasted for 24 h. After this measurement, immobilized cells were washed with saline and resuspended in fresh medium. Immobilized cells thus prepared were kept at 25°C until the next use. Hydrogen evolution was measured twice a week for period exceeding 1 mo.

RESULTS AND DISCUSSION

Effect of Culture Temperature on Hydrogen Evolution

The optimum culture temperature for hydrogen evolution determined by the MV assay was found to be different from hydrogen evolution when assayed using glucose. The hydrogenase activity (hydrogen evolution rate from reduced MV) of the transformant HK-8(pCBH4 or pCBH6) cultured at 37°C was higher than when cultured at 18°C (Fig. 2). Hydrogenase activity of the transformant HK-8(pCBH4 or pCBH6) was approximately two times higher than that of *E. coli* C600, when cultured at the same temperature. The hydrogen evolution rate of the transformant HK-8(pCBH4 or pCBH6) from glucose was also higher than that of *E. coli* C600, when cultured at the same temperature (Fig. 3). In contrast with the temperature profile of hydrogenase activity, however, the hydrogen evolution rate of the transformant from glucose was higher when cultured at 18°C than when cultured at 37°C. *Escherichia coli* C600 also exhibited a similar tendency, but the transformant HK-8(pCBH4 or

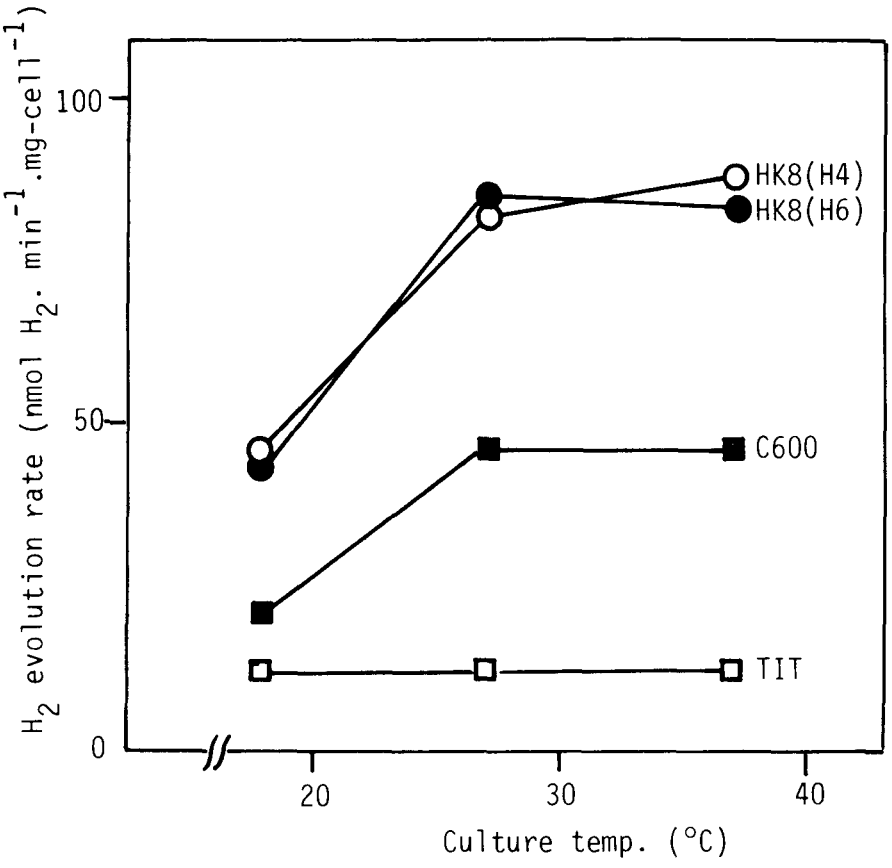

Fig. 2. Hydrogenase activities of various strains. The cells in L-broth were cultured anaerobically at temperatures of 18, 27, and 37°C up to late log-phase. Washed whole cells were inoculated into 10 mL of MV solution (pH 7.0, 30°C), and hydrogen evolved under argon atmosphere was assayed using gas chromatography.

pCBH6) was much more strongly pronounced. That is to say, as the culture temperature of *E. coli* (particularly, the transformants) became lower (37 → 27 → 18°C), hydrogen evolution rates from glucose were tending upward. On the other hand, hydrogen evolution by *C. freundii* TIT0101 remained virtually constant at different temperatures. After all, when the strains were cultured at 18°C, the hydrogen evolution rate of the transformant HK-8(pCBH4 or pCBH6) from glucose was approximately 2.5-fold higher than that of *E. coli* C600 and approximately 2-fold higher than that of *C. freundii* TIT0101. These results suggest that the enzyme(s) functioning in the anaerobic glycolytic pathway, upstream of the hydrogenase system, is (are) a significant bottleneck to hydrogen evolution. It is possible that this (these) bottle-neck enzyme(s) may be more plentifully produced when the strains are cultured at 18°C than when cultured at 37°C. Therefore, we concluded that the cells cultured at 18°C may be efficient for the hydrogen evolution system.

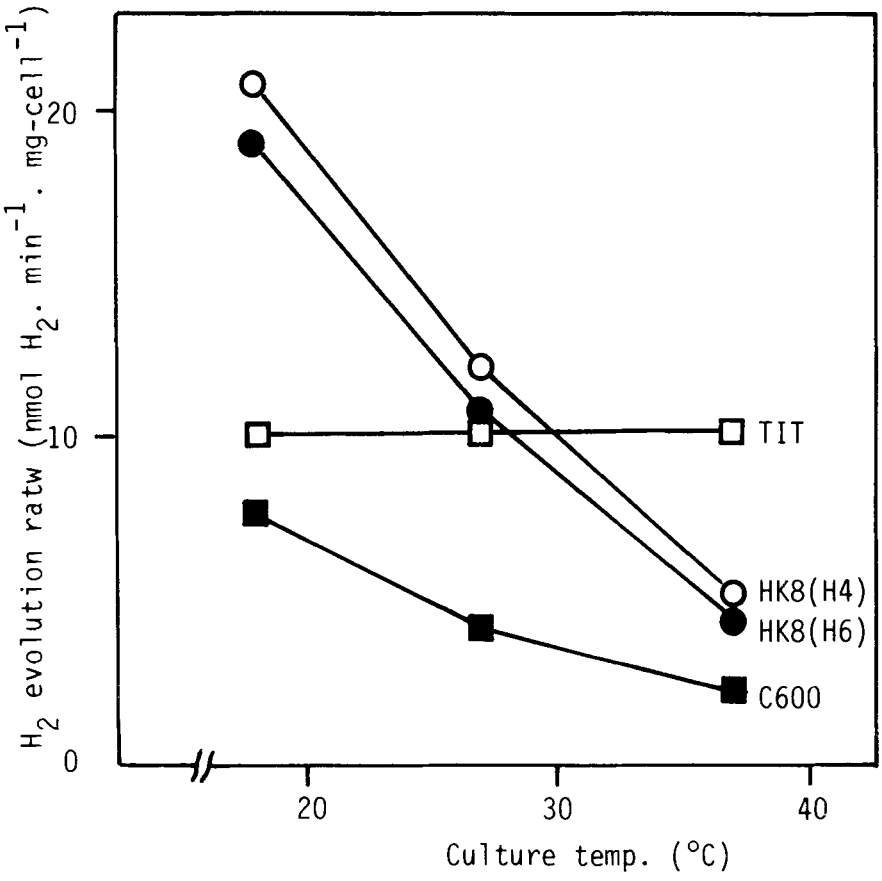

Fig. 3. Hydrogen evolution rates of various strains from glucose. Experimental procedure was the same as that described in Fig. 2, but glucose solution was used instead of MV solution.

Plasmid Retention Stability

Plasmid retention was examined. Figure 4 shows plasmid retention stabilities of the transformants HK-8(pCBH4) and HK-8(pCBH6). These results show that hybrid plasmid pCBH6 (5.7 kb) was rapidly lost (plasmid retention; approximately 39% after 24 d), but pCBH4 (6.2 kb) was almost completely retained even after 37 d. As is commonly known, dose effects of multicopy plasmids are frequently observed during gene cloning. But, in the majority of cases, the effects are temporary, and the hybrid plasmid retention stabilities are insufficient for practical application. The retention stability of pCBH4 is far superior to that of pCBH6, in spite of a similarity in size. It is not clear what the cause of this significant difference is; it may be related to a recognized fact that plasmid pCBH4 was formed by spontaneous deletion (*2*).

Hydrogen Evolution by Immobilized Cells

Hydrogen-evolving activity of *E. coli* free cells from glucose rapidly decreased. Though the cell suspension of the recombinant *E. coli* HK-

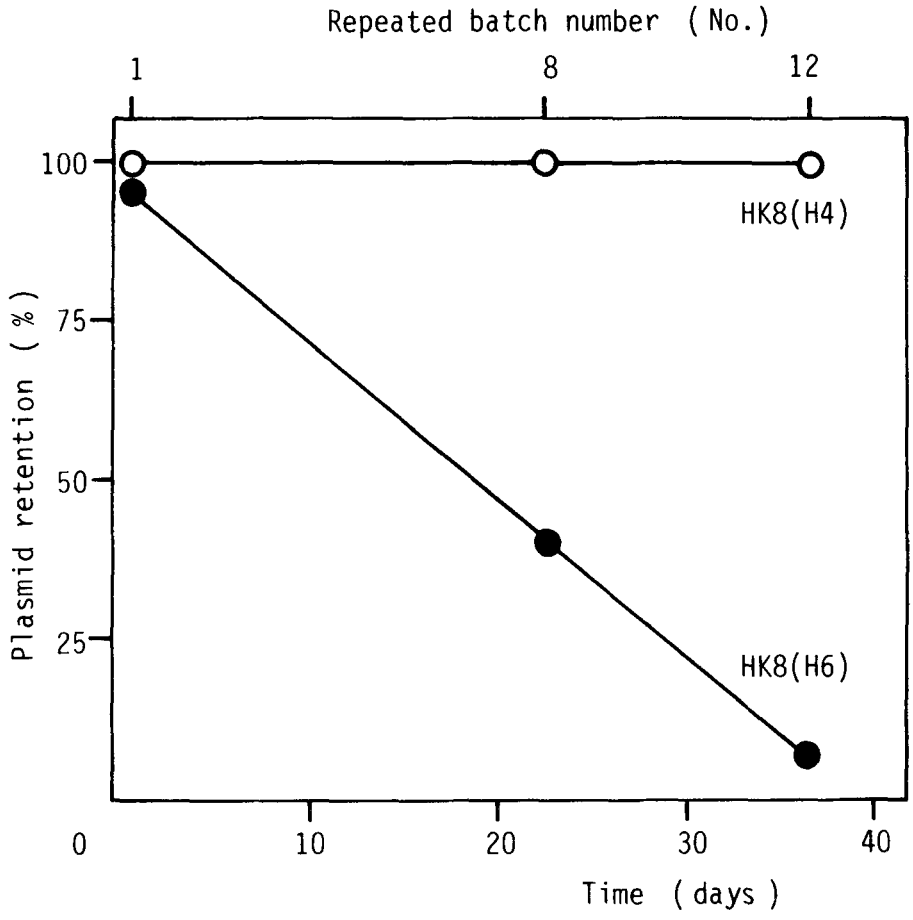

Fig. 4. Plasmid retention stabilities of the transformant cells. Immobilized cells were maintained anaerobically in L-broth at 30°C, and cells grown outside of gels were spread on L-agar plates and incubated for over 20 h at 37°C. Hydrogenase activities of colony cells were tested by the MV filter paper method. The cells of Hyd^+, indicated a blue-purple color, had the hybrid plasmids.

8(pCBH4) was preserved at low temperature (5°C), hydrogen-evolving activity from glucose after 2-d preservation was 1/2–3 times that of the first day (data not shown). Therefore, the whole cells of *E. coli* HK-8(pCBH4) and C600 were immobilized in PAAH gels and used for hydrogen evolution.

Figure 5 shows the time course of hydrogen evolution from glucose. In the early stage, hydrogen evolution rate of the recombinant *E. coli* HK-8(pCBH4) was 0.13 μ*M*/h·mg-cell, and that was about 1.6-fold higher than those of *E. coli* C600 or *C. freundii* TIT0101. During 20 h of operation, hydrogen evolved by the recombinant *E. coli* was 1.4 μ*M*/mg-cell, and that was about 1.4-fold higher than those of C600 or TIT0101.

Then, we attempted to use these immobilized cells to the repeated batch hydrogen evolution from glucose (Fig. 6). The maximum hydrogen evolution rate observed was in the first batch of the recombinant *E. coli*,

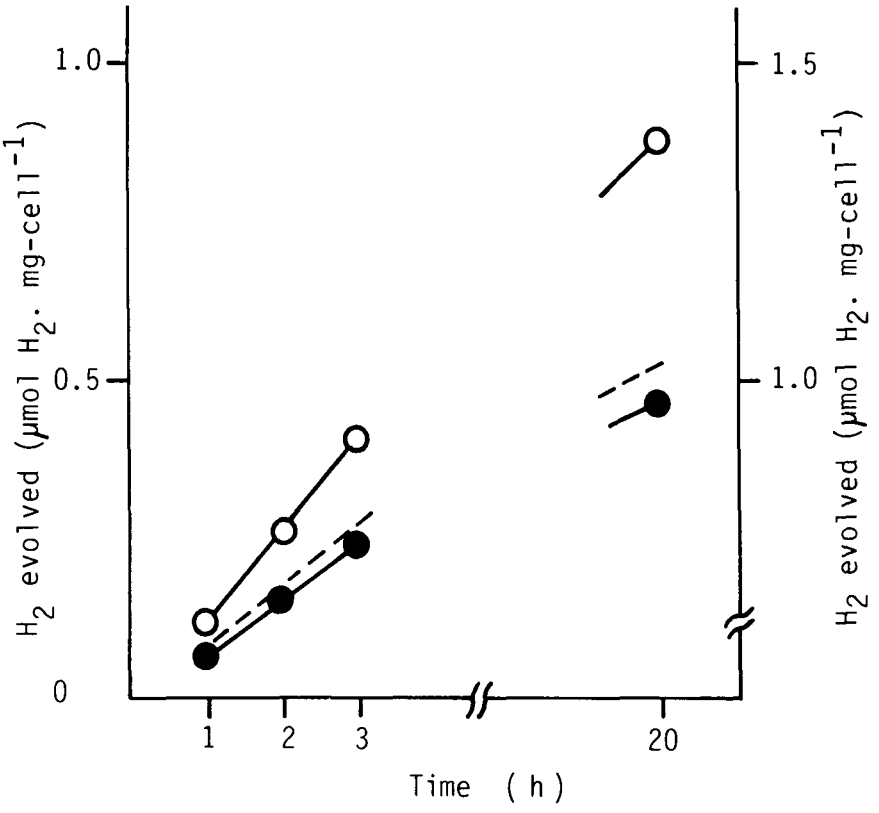

Fig. 5. Hydrogen evolution from glucose by immobilized cells. Experimental procedure was the same as that described in Fig. 3, but immobilized cells were used instead of washed whole cells. Hydrogen evolved was expressed in μ*M* hydrogen·mg-cells^{-1}. O—O, *E. coli* HK-8(pCBH4); ●—●, *E. coli* C600; ----, *C. freundii* TIT0101.

and that was 0.12 μ*M*/h·mg-cell. The hydrogen evolution rate was decreasing gradually, however, during more than 1 mo of operation, the hydrogen evolution rate of the recombinant *E. coli* was always twofold higher than that of C600 or TIT0101. The half-life time of hydrogen evolving activity was about 5 d. This might be because of the inactivation of the whole glycolitic pathway, including hydrogenase. Because in this experiment the medium used contained no N-source, the cells were not able to grow. Therefore, during these experiments, the enzyme system was gradually denaturated.

Hydrogen Evolution by Immobilized Growing Cells

The hydrogen evolution by immobilized cells was attempted using culture medium, L-broth. The results are shown in Fig. 7. The maximum hydrogen evolution rate was also observed in the first batch of the recombinant *E. coli*, and that was 0.18 μ*M*/h·mg-cell. Compared with hydrogen evolution from glucose, it was about 1.5-fold higher. The half-life time was about 20 d. We assumed that by using L-broth instead of glu-

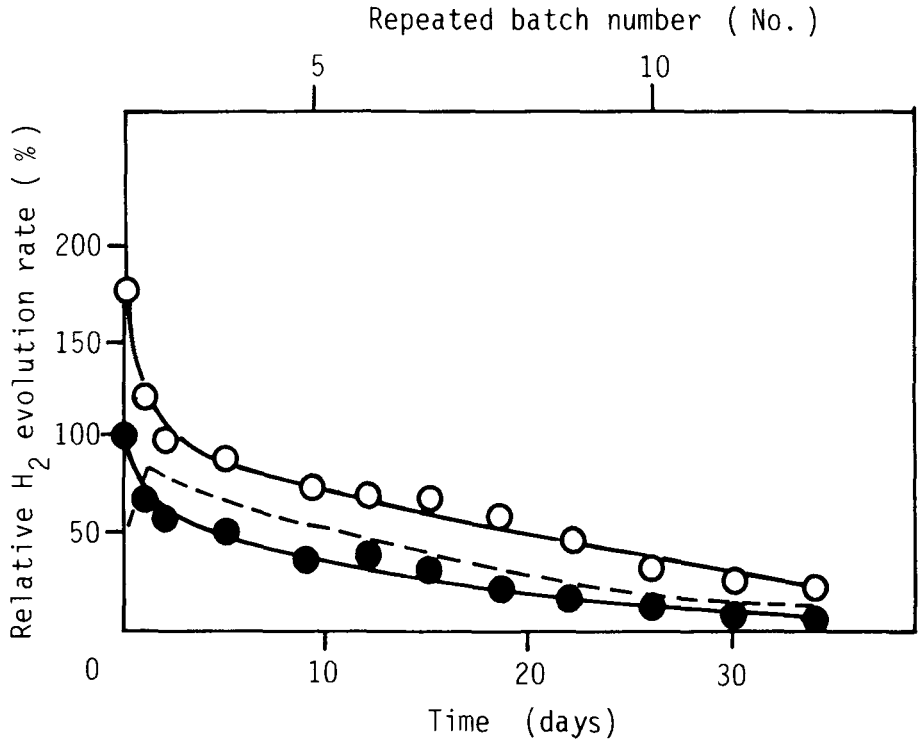

Fig. 6. Repeated batch hydrogen evolution from glucose by immobilized cells. Experimental procedure was the same as that described in Fig. 5. Immobilized cells were washed with saline and reused in fresh medium. hydrogen-evolution rates were expressed as a percentage using that of *E. coli* C600 to express 100%. ○—○, *E. coli* HK-8(pCBH4); ●—●, *E. coli* C600; ----, C. *freundii* TIT0101.

cose for hydrogen evolution, the cells were kept in a highly viable state and, consequently, a higher hydrogen evolution rate and longer half-life time were achieved. It was also obvious that the hydrogen evolution rate of the recombinant *E. coli* from L-broth was about twofold higher than that of C600. From these results, this recombinant *E. coli* was considered to be applied for a continuous hydrogen evolution process, both for its high hydrogen evolution rate and stability. In addition, feeding fresh culture medium, L-broth, to a continuous hydrogen evolution process might improve the half-life time of hydrogen evolving activity.

CONCLUSIONS

Basic studies of hydrogen evolution by the recombinant *E. coli* was investigated. The optimum culture temperature of the recombinant *E. coli* cells for hydrogen evolution from glucose was in the neighborhood of 18°C. The plasmid retention stability of the recombinant *E. coli* was considerably high, especially plasmid pCBH4, which was almost completely retained without any restriction. The hydrogen-evolving activity of the

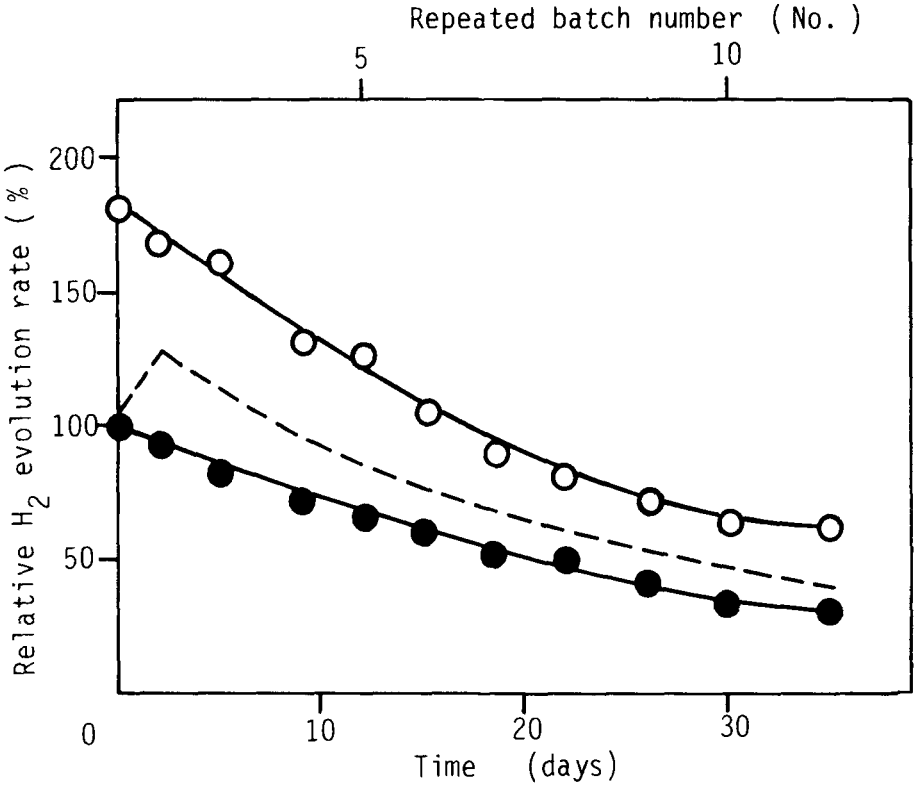

Fig. 7. Repeated batch hydrogen evolution from L-broth immobilized cells. Experimental procedure was the same as that described in Fig. 6, but L-broth was used instead of glucose solution. hydrogen-evolution rates were expressed as a percentage using that of *E. coli* C600 to express 100%. ○—○, *E. coli* HK-8(pCBH4); ●—●, *E. coli* C600; ----, *C. freundii* TIT0101.

immobilized recombinant *E. coli* was maintained for a long time. Therefore, this recombinant *E. coli* was considered to be applied for a continuous hydrogen evolution process.

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