Pressurized Culture of Escherichia coli for a High Concentration

Toru Matsui, Haruto Yokota, Seigo Sat0, Sukekuni Mukataka and Joji Takahashi

Institute of Applied Biochemistry, University of Tsukuba, Tsukuba-shi, Ibaraki 305, Japan

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The cultural conditions for obtaining a high concentration of Escherichia coli K12 cells were investigated. A synthetic medium containing high concentrations of mineral salts was designed so as to support a high rate of cellular growth and the accumulation of a high cell concentration, without any additional feeding of inorganic nutrients, but with feeding of solid glucose.

The pasteurization of the above mentioned medium at 65°C was found to be advantageous for the cellular growth since usual sterilization at 121°C caused denaturation of the medium, which was inhibitory for cellular growth.

The accumulation of acetate, a well-known inhibitory product for the growth of E. coli, increased as the dissolved oxygen (DO) concentration decreased. When the DO concentration was maintained at the optimum value of 6.5 ppm throughout the cultivation by pressurizing the culture system with oxygen gas, 134 g (dry basis)/l of cells was accumulated in 12 hr.

The above culture conditions were applied to the cultivation of a recombinant E. coli strain transformed with pBR322trpAB, and 102 g/l of recombinant cells was obtained with a stably maintained recombinant plasmid in 9 hr under nonselective pressure.

As a response to the recent remarkable progress in gene engineering, various techniques have been developed for the production of useful proteins using recombinant Escherichia coli strains on an industrial scale. Among them, high cell concentration cultivation has been shown to be one of the most promising ways to increase the productivity.

For a high cell concentration culture of E. coli, it is necessary to supply sufficient amounts of nutrients. In addition, it is necessary to reduce the accumulation of acetate, a well-known metabolite inhibitory for cellular growth.1,2) Mori et al.3) reported the accumulation of 125 g (dry basis)/l of E. coli B cells in a fed-batch culture, to which several kinds of nutrients including glucose were intermittently supplemented. Landwall and Holme4) obtained 144 g/l of E. coli B/r cells in a dialysis culture, growth inhibitory metabolites such as acetate being removed from the culture broth. It is also desirable if a high concentration of cells can be obtained through the use of a cheap synthetic medium composed of inorganic nutrients and free from organic nutrients, except glucose.

In this study, therefore, a synthetic medium containing sufficient amounts of inorganic nutrients was developed, and the cultural conditions for reducing the accumulation of acetate were examined with E. coli K-12. The optimum cultural conditions determined were applied for E. coli pBR322trpAB cells to obtain a high cell concentration under nonselective pressure. The phenotypic stability of the recombinant plasmid and the expression of the gene were also examined.

Materials and Methods

Bacterial strain and plasmid. Escherichia coli K12 IFO 3301 was used as the wild type strain. This strain was transformed with plasmid pBR322trpAB by the method of Cohen et al.5) Plasmid pBR322trpAB was constructed by inserting the tryptophan synthase gene derived from E. coli K12 under the control of the tryptophan promoter into the EcoRI site of pBR322.
**Composition of culture media.** The composition of the synthetic medium designed is shown in Table 1, the amounts of nutrients being sufficient to support growth up to a high concentration. A trace element solution containing iron(II) sulfate heptahydrate, 10 g, zinc sulfate heptahydrate, 2.25 g, copper(II) sulfate pentahydrate, 1 g, manganese sulfate tetra- or pentahydrate, 0.5 g, calcium chloride dihydrate, 2 g, sodium borate decahydrate, 0.023 g, and ammonium molybdate, 0.01 g, in 1 l of distilled water was used for preparing the medium. The medium for seed cultures contained glucose, 5 g, diammonium hydrogenphosphate, 10 g, potassium sulfate, 2 g, magnesium sulfate heptahydrate, 0.2 g, sodium chloride, 0.3 g, and trace element solution, 400 μl, in 1 l of distilled water. The pH was adjusted to 7.2 with 6N NaOH and 5 mg of tetracycline was added to 1 l of the medium in the case of recombinant cells.

**Shaking culture.** For the preparation of seed cultures, one loopful of a one-day-old slant culture was inoculated into 50 ml of the medium in a 500 ml shake flask, followed by incubation at 37°C on a reciprocal shaker at 125 oscillations/min for 12 hr.

**Feeding of powdered glucose.** For glucose concentrations from 1 to 20 g/l during cultivation, the supplementation of powdered glucose was necessary. Since manual feeding of powdered glucose became impossible as the pressure increased, the glucose feeder shown in Fig. 1 was developed. When powdered glucose was simply fed under pressure from the glucose feeder attached to the upper flange, powdered glucose wetted by sparged broth adhered to the glucose feeding port and sometimes blocked it. Therefore, a method by which powdered glucose was first dissolved in a portion of the broth drawn from the fermentor and then the broth was re-fed into the fermentor, was developed, as follows. When valve (3) only was opened, and valves (2), (4) and (5) were closed, a portion of the broth was drawn into chamber (6) owing to the pressure imbalance between the culture system and the chamber (6), then valve (3) was closed and valve (5) was opened so that chamber (6) was restored to normal pressure. Next, a prescribed amount of powdered glucose was fed from reservoir (1) by opening valve (2) and dissolved in the broth in chamber (6). After chamber (6) had been pressurized by closing valves (2) and (5), and opening valve (4), the broth containing glucose

| Table 1. COMPOSITION OF THE CULTURE MEDIUM |
|------------------|--------|
| Glucose          | 20 g   |
| (NH₄)₂HPO₄      | 25 g   |
| K₂SO₄           | 5 g    |
| NaCl             | 0.75 g |
| MgSO₄·7H₂O       | 5 g    |
| FeSO₄·7H₂O       | 750 mg |
| ZnSO₄·7H₂O       | 170 mg |
| CuSO₄·5H₂O       | 75 mg  |
| MnSO₄·4·5H₂O     | 38 mg  |
| CaCl₂·2H₂O       | 150 mg |
| Na₂B₄O₇·10H₂O    | 17 mg  |
| (NH₄)₆M₂O₇·4H₂O  | 7.5 mg |
| Distilled water  | 1000 ml|
| pH               | 7.0    |

**Fig. 1. Apparatus for Feeding Glucose.**
1, glucose reservoir; 2 and 3, ball valves; 4, pressure valve; 5, exhaust valve; 6, chamber for sampled broth.

**Fig. 2. Schematic Diagram of the Pressurized Fermentor System.**
1, oxygen gas cylinder; 2, gas filter; 3, jar fermentor; 4, antifoam reservoir; 5, aqueous NH₄OH reservoir; 6, pH controller; 7, DO meter; 8, pH probe; 9, oxygen probe; 10, flow meter; 11, pressure regulator.
was returned to the fermentor by opening valve (3). By repeating these operations, powdered glucose could be conveniently fed, even under increased pressure.

Aerated stirred culture. Cultivations were carried out in a 2.61 laboratory fermentor (Fig. 2), equipped with an automatic pH controller and a dissolved oxygen (DO) meter, at an agitation speed of 1000 rpm with a supply of air and/or oxygen gas at a mass flow rate ranging from 1 to 2vvm. For inoculation, bacterial cells harvested from a shaking culture were added to the fermentor at the concentration of 3.5 g (dry basis)/l. During the cultivation, the pH was maintained at 7.0 by automatic titration with 15N NH₄OH and the temperature was maintained at 37°C. By using the system schematically shown in Fig. 2, DO could be controlled at any desired level by supplying air or oxygen-enriched air. When the cell mass had increased to such an extent that DO could no longer be maintained at a desired level, even by supplying pure oxygen gas, the culture system was pressurized to increase the driving force of oxygen transfer and DO could be maintained at the desired level to the end of cultivation.

Analytical methods. The cell concentration was determined from the optical density of the culture broth at 660 nm and also from the dried cell weight per unit volume. One optical density unit corresponded to 0.48 g/l of dried cells.

The glucose concentration was measured by a modified Somogyi method and by using a test paper impregnated with glucose oxidase (Glucose Pretest, Wako Pure Chemical Industries Co., Ltd.), with which residual glucose could be quickly (within 30 sec) determined during cultivation.

The acetate concentration was measured with a gas chromatograph equipped with a flame ionization detector and a 3 mm i.d. × 2 m length glass column packed with Thermon 3000 (Shimadzu Seisakusho Co., Ltd.), and expressed as grams of acetic acid per unit volume.

Plasmid stability was determined by counting ampicillin resistant cells and by measuring the specific activity of tryptophan synthase as a cloned gene product during the cultivation. Thus, 100 colonies on LB agar plates incubated overnight, after spreading appropriately diluted broth, were transferred with toothpicks to LB agar plates containing 50 µg/ml of ampicillin, and then the number of growing cells was counted. The activity of tryptophan synthase was assayed by the method of Smith and Yanofsky.

Results and Discussion

Examination of the sterilization conditions for the medium

Since the medium shown in Table I was designed to support the growth of 200 g/l of cells, it contains very high concentrations of mineral salts compared with synthetic media used in the past. In particular, the contents of trace metal elements in this medium correspond to 200~700 times those in the medium reported by Gray and Tatum. Also, the phosphate source, which is required at an extremely high concentration, is added in the form of the ammonium salt, because cellular growth is markedly inhibited in the presence of a high concentration of sodium or potassium phosphate.

When the cultivation was carried out in a medium sterilized under the usual conditions of 121°C for 10 min, the cellular growth was slow and it stopped at the cell concentration of 48 g/l at 8 hr, as shown in Fig. 3(a). Therefore, the medium components were divided into three, glucose, magnesium sulfate and other inorganic nutrients, and each solution was separately sterilized at 121°C for 10 min. After cooling the solutions, they were mixed and inoculated with the seed culture. The cellular growth obviously improved and the cellular concentration reached 91 g/l at 8 hr, as shown in Fig. 3(b).

In contrast, in the case of a medium sterilized at 65°C for 30 min, the cell concentra-

![Fig. 3. Effect of the Sterilization Conditions on Cellular Growth.](image-url)
tion reached 113 g/l at 8 hr, as shown in Fig. 3(c). Contamination was not observed. Consequently, it was decided to pasteurize the culture medium at 65°C for 30 min.

Effect of the glucose concentration on cellular growth

A high concentration of glucose is well known to inhibit cellular growth, and the fed-batch culture method, in which glucose is fed as it is consumed for cellular growth, has usually been employed to attain a high cell concentration.

In the case of growing the K12 strain in a glucose-fed-batch culture, as shown in Fig. 4, little difference was observed in the growth curves between case (a), in which the glucose concentration was maintained in the range of 1 to 10 g/l, and case (b), in which the glucose concentration was kept at 1 to 20 g/l. Cell concentrations of more than 100 g/l were obtained in 8 hr in both cases. In contrast, when the glucose concentration was kept at 20 to 50 g/l, as in case (c), cellular growth was obviously inhibited and the cell concentration reached after 8 hr was only 67 g/l.

The above results indicate that control of the glucose concentration between 1 and 20 g/l by feeding powdered glucose was preferable for obtaining a high cell concentration.

Effect of the DO concentration on cellular growth

To examine the effects of the DO concentration on cellular growth and the accumulation of acetate, cultivations were carried out at various DO concentrations, ranging from 1 to 32 ppm. In every run, the DO concentration was kept constant through the cultivation. The culture system was pressurized, if necessary, at the later stage of the cultivation to supply oxygen required by the high concentration of cells. Figure 5 shows the final cell concentrations attained and the concentrations of acetate accumulated with various DO concentrations. At DO concentrations higher than 10 ppm, cellular growth was increasingly suppressed as the DO concentration increased, so that the cell concentration attained was only 20 g/l at a DO of 32 ppm, and the accumulation of acetate was 2.5 g/l. On the other hand, at DO concentrations less than 6.5 ppm, the accumulation of acetate increased as the DO concentration decreased, and cellular growth was inhibited by the accumulated acetate. When the cultivation was carried out at a DO of 6.5 ppm, the acetate accumulation decreased and the cellular growth reached a
High Concentration Cultivation of \textit{E. coli}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Effects of the Dissolved Oxygen Concentration on Cellular Growth and Acetate Accumulation. O, dried cell weight; \(\triangle\), acetate concentration.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Typical Time Course of a High Concentration Culture of \textit{E. coli}. O, dried cell weight; \(\triangle\), acetate concentration.}
\end{figure}

maximum of 134 g/l in 12 hr. Thus, the optimum DO for a high concentration culture of this strain was found to be 6.5 ppm.

Figure 6 shows typical time courses of cellular growth and acetate accumulation under the optimum cultural conditions. The cultivation was started with an inoculum size of 3.5 g (dry basis)/l at a DO concentration of 6.5 ppm with a supply of air at normal pressure. Glucose was intermittently fed to maintain its concentration in the range of 1~20 g/l. From the middle of the cultivation, the DO concentration was maintained at 6.5 ppm by supplying oxygen gas-enriched air at normal pressure, and then the culture system was pressurized with oxygen gas to maintain the DO concentration at 6.5 ppm. At the end of the cultivation, the pressure of the culture system was around 1.4 atm.

\textbf{Effect of the mineral salt concentrations on cellular growth}

To reexamine the optimal contents of inorganic nutrients in the culture medium, cultivations were carried out with various concentrations of inorganic nutrients under the same conditions as in Fig. 6.

As is clear on comparing growth curves II and III with growth curve I in Fig. 7 (growth curve in Fig. 6), when the contents of mineral salts were decreased to 0.4 and 0.8 times those in Table I, the final cell concentrations were lower, though the growth rate at the initial stage was unchanged. On the other hand, when the contents of mineral salts were increased to 1.2 times, the growth rate decreased, as shown by growth curve IV, though the final cellular concentration was the same as in the case of growth curve I. When the contents of mineral salts were increased to 1.6 times, both the growth rate and the final cell concentration decreased, as shown by growth curve V.

The above results confirm that the culture medium shown in Table I is properly designed to support a high rate of cellular growth and the accumulation of a high concentration of...
Fig. 8. High Concentration Cultivation of Recombinant E. coli Cells on the Synthetic Medium.

○, dried cell weight; △, specific activity of TSase; ●, ampicillin resistant cells.

Application to recombinant cells

The above techniques were applied for the cultivation of recombinant cells, in which tryptophan synthase from E. coli K12 had been inserted by using plasmid vector pBR322.

The cultivation was carried out under the optimum conditions determined for the K12 strain and also under nonselective pressure to check the stability of the recombinant plasmid. As shown in Fig. 8, cellular growth of as high as 102 g/l was attained in 9 hr, though the growth rate slightly decreased, compared with that in the case of K12 cells shown in Fig. 6. A loss of ampicillin resistant cells was not observed during the cultivation. The specific activity of tryptophan synthase, as the cloned gene product, was rather increased at the later stage of the cultivation, reaching 160 U/mg-protein.

The above results show that the present culture method is applicable to the high concentration cultivation of various recombinant E. coli cells.

References