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[J. Ferment. Technol., Vol. 66, No. 2, 187–191. 1988]

Fed-Batch Cultures of Recombinant *Escherichia coli* with Inhibitory Substance Concentration Monitoring

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Fed-batch cultures of recombinant *Escherichia coli* HB101 were investigated to obtain high cell density and large amounts of β-galactosidase (β-gal). *E. coli* HB101 was transformed with a hybrid plasmid pTREZ1, which contained a β-gal gene controlled by the *trp* promoter. In fed-batch cultures of recombinant *E. coli*, when the cell concentration reached around 13 g/*l*, the cell growth stopped and large amounts of inhibitory substances have accumulated in the broth. These inhibitory substances were isolated and identified. Acetate produced by the cells was evidently the main inhibitor of cell growth and β-gal production. Since the cells proved to assimilate acetate, the feed rate was controlled with acetate concentration monitoring in the fed-batch culture. As a result, the acetate concentration was maintained at a low level and cells grew smoothly without acetate-induced inhibition. Cell concentration and β-gal quantity reached high values of 28 g/*l* and 64 U/ml, respectively.

Recombinant DNA technology allows large-scale production of valuable materials which otherwise might only be obtained in minute quantities from natural sources. And studies on highly-concentrated cell cultivation and high levels of expression for cloned genes are very important to obtain large amounts of valuable materials using host cells harboring hybrid plasmids.

Highly-concentrated cultivation of recombinant *Escherichia coli* by fed-batch cultures was investigated to achieve overproduction of heterologous proteins. Fed-batch cultures[1] and dialysis cultures[2] for *E. coli* B have already yielded high cell densities of 125 g/*l* and 144 g/*l*, respectively. In this study, *E. coli* HB101 harboring the hybrid plasmid pTREZ1, which contained a β-galactosidase (β-gal) gene controlled by the *trp* promoter, was used as a recombinant. Fed-batch cultures were used to obtain high cell density and large amounts of β-gal.

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This study deals with inhibitory substances produced by recombinant *E. coli* and fed-batch cultures with acetate concentration monitoring for overproduction of gene products.

Materials and Methods

Bacterial strains and plasmid DNA *E. coli* HB101 was used as a carrier for the recombinant plasmid pTREZ1.[3] pTREZ1 carries the coding sequence for a large part of β-gal, joined to the sequence for eight N-terminal amino acids of the *trp* E polypeptide.

Cultivation The *E. coli* strain harboring plasmid pTREZ1 was cultivated in M9 medium containing 2.5 g/*l* casamino acids, 5 g/*l* glucose, 1.5 g/*l* yeast extract, 0.1 g/*l* proline, 0.1 g/*l* thiamine HCl, 20 mg/*l* tryptophan, and 50 mg/*l* ampicillin. Cultures were placed in 0.5-*l* shake flasks closed with cotton plugs (working volume 0.05 *l*). They were shaken on a reciprocal shaker at 115 oscillations/min with a 7-cm stroke at 37°C. Fed-batch cultures were grown in a 5-*l* jar fermentor with a 2-*l* initial working volume, including a 200-ml overnight seed culture, and 0.2 ml of antifoam (1705-W, Lion Co. Ltd.). pH was

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maintained at 7, temperature at 37°C, gas flow rate at 2 *l*/min, and agitation speed at 400–1000 rpm. Aqueous ammonia (6 N) was used to adjust pH. The dissolved oxygen (DO) level was manually controlled at 1–3 ppm by changing the agitation speed. When the agitation speed reached the maximum of 1000 rpm, 50% oxygen gas was supplied to the fermentor instead of air. The feeding medium consisted of 200 g/*l* glucose, 100 g/*l* casamino acids, 60 g/*l* yeast extract, 0.4 g/*l* tryptophan, and 0.1 g/*l* ampicillin, at pH 7.0.

Analysis β-Gal activity, glucose concentration, and cell concentration were assayed as described previously.[3] Acetate concentration was measured with an isotachophoretic analyzer (Model IP-2A, Shimadzu, Ltd.) and a gas chromatograph (Model 663, Hitachi, Ltd.). In isotachophoresis, the leading electrolyte was 10 mM HCl·β-alanine at a pH of 3.1. The terminating electrolyte was 10 mM *n*-caproic acid. The electric current was 100 μA at 20°C. The gas chromatograph was used with a 2-m glass column packed with PEG 6000 10% Flusin P 30/60 (Gasukuro Kogyo, Inc.). Temperatures at the injection port, the column, and the FID detector were 150°C, 130°C, and 140°C respectively. The pH of culture supernatant was adjusted to below 3 with 6 N HCl before injection.

Results and Discussion

Inhibition of cell growth Highly-concentrated cell cultivation of *E. coli* harboring plasmid pTREZ1 by fed-batch culture was investigated. The DO concentration was used as a control indicator for medium feed, since the DO concentration increased when glucose in the broth was consumed.

The results of cultivating the recombinant *E. coli* in the 5-*l* jar fermentor are shown in Fig. 1. When the cell concentration reached around 13 g/*l*, cell growth stopped despite medium feed. The cell concentration was gradually decreased by dilution with the broth by medium feed. Furthermore, β-gal production could not be induced, though an inducer, 3-β-indolylacrylic acid (IA) was added together with casamino acids after 32 h of cultivation. Mori and colleagues[1] have reported a high cell concentration of 125 g/*l* in a fed-batch culture of *E. coli* B. However, since Sinclair and Stokes[4] and Landwall and Holme[2] have described the accumulation of inhibitory substances in *E. coli* B cultivation, these results seem to indicate that inhibitory substances accumulating in the broth stopped cell growth.

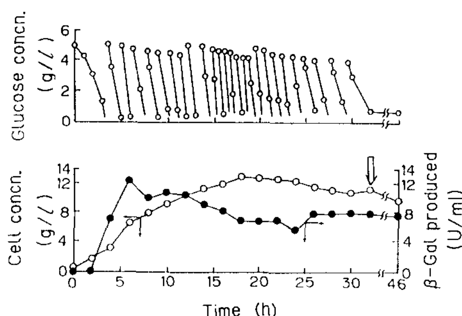

Fig. 1. Highly-concentrated cell cultivation of recombinant *E. coli* by fed-batch culture.
Initial tryptophan concentration was 10 mg/*l*. Arrow indicates the addition of 15 mg/*l* 3-β-indolylacrylic acid (IA) and 2.5 g/*l* casamino acids.

The inhibition of cell growth by the supernatant was investigated. Feeding medium and fresh cells were added to a shake flask with the supernatant after 12 h of cultivation, when cell growth still continued. They were also added to a shake flask with the supernatant after 19 h of cultivation, when cell growth had stopped. The cells did not grow at all in the flask. Figure 2

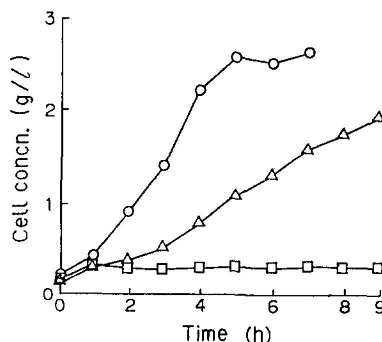

Fig. 2. Inhibition of cell growth by supernatant.
One and a quarter ml of feeding medium and 5 ml of fresh cells were added to shake flasks containing 43.75 ml of the supernatant. A control flask contained M9 salt medium instead of the supernatant. ○, control; △, supernatant at 12 h of cultivation; □, supernatant at 19 h of cultivation.

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shows these results. For the supernatant at 12 h of cultivation, the specific growth rate of cells was about half that measured for a control without adding supernatant. For comparison, the frozen and stored cells were inoculated into fresh medium to examine growth. The specific growth rates of the cells at 12 h of cultivation, the cells at 19 h of cultivation, and fresh cells were 0.36, 0.32, and 0.45 1/h, respectively. The growth of the cells was not lowered as much. Furthermore, adding IA induced β-gal production. These results show that inhibitory substances accumulated in the broth as cultivation proceeded.

Isolation and identification of inhibitory substances The supernatant at 19 h of cultivation, when cell growth had stopped, was used to isolate and identify inhibitory substances in the broth. Liquid fractions were obtained by fractionating the supernatant by molecular weight. The fraction of molecular weight of 1000 or less inhibited cell growth. Next, anionic substances obtained from the fraction using ion-exchange regins inhibited cell growth. This indicates that substances inhibiting cell growth are low-molecular anionic substances such as organic acids, which *E. coli* cells are known to produce.[5]

The solution containing low-molecular anionic substances (solution L) was analyzed for organic acids with an isotachophoretic analyzer. The isotachopherograms of the solution L and of authentic organic acids are shown in Fig. 3. It was found that component A had accumulated in the solution L by comparison with the isotachopherogram of the feeding medium components (data not shown). The isotachopherogram of the authentic organic acids indicates that component A is acetic acid. The isotachopherogram of the solution L to which authentic acetic acid was added supported this indication (data not shown). Acetic acid concentration in the supernatant at 19 h of cultivation was measured at a high value of 0.55 M. Gaschromatograph analysis of the supernatant in the broth further confirmed acetic acid accumulation.

Various amounts of ammonium acetate were added to a medium to investigate the influence of acetate on cell growth and β-gal production. Results are shown in Fig. 4. Both the specific growth rate and β-gal production decreased when the acetate concentration was 0.17 M and more. It can be concluded that acetate accumulated in the broth was the primary inhibitor of cell growth and β-gal production.

Acetate production by recombinant *E. coli* is shown in Fig. 5. In the fed-batch culture with DO concentration monitoring, cell growth stopped at 14 h of cultivation, when acetate concentration reached 0.23 M. The cell concentration was 11.6 g/*l*. The growth yield was 0.26 g-cells produced/g-glucose

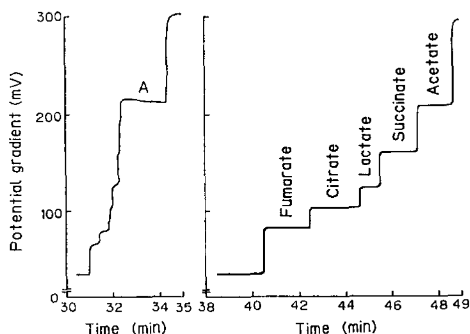

Fig. 3. Isotachopherograms of low-molecular anionic solution and authentic organic acids.
The low-molecular anionic solution was diluted ten times with distilled water. A mixture of 0.02 M authentic organic acids solution was used.

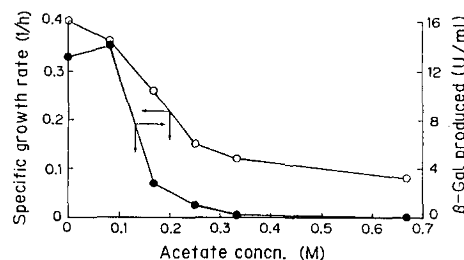

Fig. 4. Inhibition of cell growth and β-gal production by acetate.
Fifteen mg/*l* IA and 2.5 g/*l* casamino acids were added after 15 h of cultivation. β-Gal production was assayed after 24 h of cultivation.

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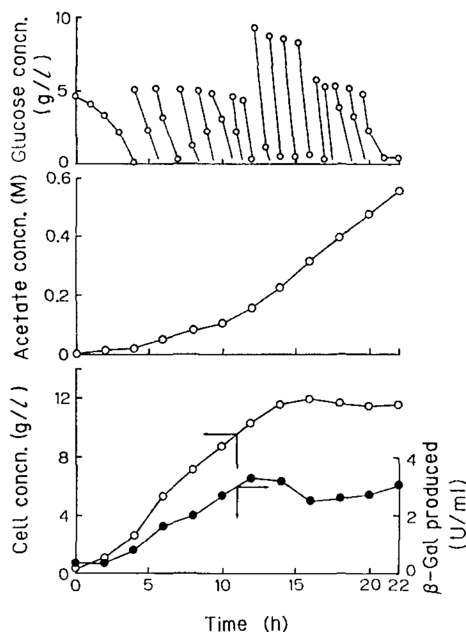

Fig. 5. Acetate production by recombinant *E. coli*.

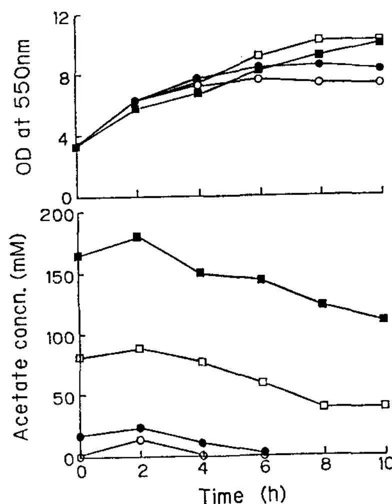

Fig. 6. Acetate assimilation by cells.
Ammonium acetate concentration: ○, no addition (control); ●, 17 mM; □, 83 mM; ■, 167 mM.

consumed. Acetate production causes this very low growth yield. The cells continued to produce acetate after 14 h of cultivation. Smirnova[6] has reported that cell growth stopped at an average acetate concentration of 0.15 M in fed-batch cultures of *E. coli* K12. Furthermore, Yano and colleagues[7] have described that the cell growth of *E. coli* B was completely inhibited at 0.18 M of acetate. The variant inhibition of cell growth by acetate concentration seems to be due to differences in *E. coli* strains.

These results show that acetate produced by *E. coli* was the main inhibitory substance.

Recombinant *E. coli* cultivation with acetate concentration monitoring Recombinant *E. coli* produced acetate in the broth. This acetate inhibited cell growth. Therefore, acetate production by cells must be prevented to attain highly-concentrated cell cultivation. Since a high glucose concentration in the broth was considered to accelerate acetate production by expression of the Crabtree effect,[8] the feeding medium was fed continuously to the fermentor to maintain glucose concentration in the broth under 1 g/*l*. However, cells produced a great deal of acetate in a manner similar to that indicated by Fig. 5.

The assimilation of acetate by cells was investigated to decrease acetate concentration in the broth, as shown in Fig. 6. Ammonium acetate was added to M9 medium in amounts of 17, 83, and 167 mM. Glucose concentration in the M9 medium was 1 g/*l*. Acetate concentration in the broth increased for glucose catabolism at the beginning of cultivation. After 2 h of cultivation, acetate concentration gradually decreased in all cases and cell concentration increased. This shows that cells assimilated acetate and converted it into cell mass.

In fed-batch culture, the feed rate was controlled and acetate concentration was monitored. Medium feed was stopped or the feed rate was decreased when acetate accumulated in the broth. This allows cells to assimilate acetate. Medium feed was resumed or the feed rate was increased when the acetate concentration in the broth decreased. The results of fed-batch culture with acetate concentration monitoring are shown in Fig. 7. Acetate concentration in the broth was predetermined under 33 mM

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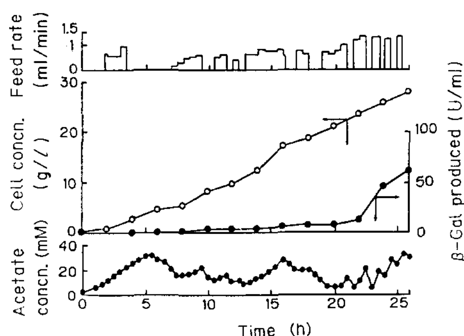

Fig. 7. Fed-batch culture with acetate concentration monitoring.

and measured with the gas chromatograph every 30 min. Medium feed was stopped when acetate accumulated in the broth. As a result, the acetate concentration level was kept low and cells grew smoothly without acetate-induced inhibition. At 26 h of cultivation, the cell concentration reached a high value of 28.1 g/*l*. Growth yield reached a high value of 0.53 g/g. Since the feeding medium used contained 0.4 g/*l* tryptophan, β-gal production was repressed. However, β-gal was produced by derepression of the *trp* promoter at the end of cultivation, since tryptophan in the broth was consumed as nutrient upon stoppage of the medium feed. These results indicate that high cell density could be obtained by using feed rate control with acetate concentration monitoring.

β-Gal was produced using fed-batch culture with acetate concentration monitoring. β-Gal production was induced by exchanging a feeding medium containing tryptophan for one containing no tryptophan, rather than by adding IA and casamino acids during cultivation. Iijima and colleagues[9] have reported that tryptophan in the broth was removed by the cross-flow filtration and β-gal gene was expressed. In this work, tryptophan was consumed by cells. β-Gal production results of feeding-medium exchange are shown in Fig. 8. β-Gal production started as soon as the feeding medium was exchanged at 12 h of cultivation. At the end of cultivation, 64 U/ml of β-gal were

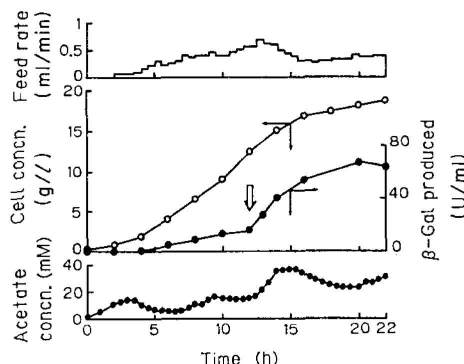

Fig. 8. β-Gal production by feeding-medium exchange.
Arrow indicates when a feeding medium containing tryptophan was exchanged for one containing no tryptophan.

produced, and the cell concentration was 18.8 g/*l*. The glucose concentration in the broth ranged from 0.09 to 0.98 g/*l*. The growth yield was 0.49 g/g. β-Gal per cell mass was 3.4 U/mg. This is a very large amount of β-gal compared with that previously reported.[8]

These results show that fed-batch culture with acetate concentration monitoring yielded high cell density and high β-gal production.

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(Received September 28, 1987)

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