OVERPRODUCTION OF FOREIGN GENE PRODUCT IN RECOMBINANT *ESCHERICHIA COLI* BY *IN SITU* ADSORPTION OF TRYPTOPHAN

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For two stage culture with control of tryptophan concentration, rapid *in situ* removal of tryptophan by adding adsorbent was investigated. Activated carbon, BAC, could adsorb a larger amount of tryptophan than other adsorbents. From test tube cultures, it was found that 20 g/l of BAC was necessary to remove large portions of tryptophan in a medium containing 20 mg/l of tryptophan and induce the β-galactosidase gene expression. Two stage culture was performed using a jar fermentor in which tryptophan concentration was kept at 20 mg/l in the first stage for cell growth and then almost totally removed by direct addition of 20 g/l of BAC to the medium. One hour after addition of BAC, an increase in enzyme activity was observed. Maximum specific enzyme activity reached a final value of 320 μkat/g-protein, which corresponded to a level 12 times that of the control culture.

Introduction

For overproduction of foreign gene products in recombinant *Escherichia coli*, various techniques have been developed such as high level expression of the foreign genes and high cell density culture of recombinant cells. In high level expression using a strong promoter, such as *trp*,[7] *lac*,[5] *tac*[2] or λ phage P_RP_L[11], a decrease in the growth rate and plasmid instability due to the stress of foreign gene expression need to be overcome. In high cell density cultures using fed-batch culture techniques, it is necessary to avoid inhibition of the cell growth owing to the accumulation of metabolic inhibitors. To solve these problems, two-stage fed-batch culture with strong inducible promoter has been proposed[6,8,9,13].

In the two-stage culture for overproduction of foreign gene products under control of *trp* promoter, the gene expression can be regulated by controlling tryptophan concentration in the medium: it is repressed by tryptophan and derepressed without tryptophan. The gene expression can also be stimulated by adding an inducer such as 3-β-indole acrylic acid (IAA)[14]. IAA was, however, not so effective in the medium containing high tryptophan concentration and it was not preferable because of its toxicity[9]. Therefore, it is more desirable to establish a technique involving rapid removal of tryptophan.

We have investigated a few methods to remove tryptophan, such as application of enzymatic degradation of tryptophan[9] and the development of a fermentation system equipped with a cross-flow filtration apparatus[6]. In enzymatic degradation, however, the removal was not so rapid. A few hours were required to decrease tryptophan concentration in the medium after feeding-off. For effective induction by this method, the measurements of carbon source and tryptophan concentration in the medium and their control are required, because expression of tryptophanase closely depends on the glucose and tryptophan concentrations in the first stage. Although cross-flow filtration was an effective method for tryptophan removal, this system was not so simple and a volume of fresh medium was needed for replenishment. Substituting for these methods, *in situ* removal of tryptophan is expected to be more suitable and practical.

In this paper, we will report a new removal method involving direct addition of activated carbon. By applying this method, quick and *in situ* removal of tryptophan was possible and a foreign gene product was overproduced in recombinant *E. coli*.

1. Experimental

1.1 Microorganism and cultivation

Two plasmids, pMCT98 and pRLK13, were used[6] which contain the β-galactosidase gene fused to *trp* promoter and the cloned *trp* repressor, respectively. *E. coli* C 600 (F^-, *thr-1*, *leuB6*, *thi-1*, *hsdS*, *lacY1*, *tonA21*, λ^-, *supE44*) harboring these two plasmids was used. FB-medium supplemented with 0.5 g/l of leucine, 3 g/l of

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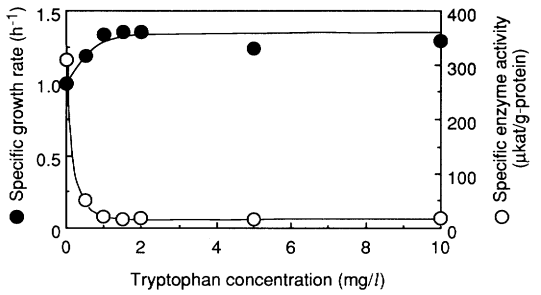

Fig. 1 Effect of tryptophan concentration on specific growth rate and specific enzyme activity

threonine, 5 mg/l of thiamine, 100 mg/l of ampicillin, 30 mg/l of chloramphenicol and 2.5 g/l of casamino acids was used and tryptophan was added to the medium for preculture and jar–fermentor culture. Glucose and the amino acids were supplemented intermittently as determined previously[12]. A seed culture (100 ml) cultivated for 6 hours in the same medium was transferred to a jar–fermentor (working volume: 1 L, type MB, Iwashiya Bio–Science Co.) and cultivated at 37 °C and pH 7.0. Agitation speed was varied between 400 and 600 rpm so that the dissolved oxygen concentration (DO) was controlled at 2 – 3 ppm. In the middle and the late stages of cultivation, pure oxygen gas was supplied to keep the DO level constant. Growth of organisms was monitored by measuring the optical density at 660 nm (OD_{660}). In the control culture to repress *trp* promoter activity, tryptophan concentration was maintained by feeding a tryptophan solution of 4 g/l, which was estimated by monitoring both the cell concentration and tryptophan concentration.

1.2 Adsorbent

Granular activated carbon, BAC (Bead Activated Carbon ϕ 0.56 – 0.71mm) was donated by Kureha Chemical Industry Co., Ltd. A synthetic resin, Amberlite XAD–2 and 4 (ϕ0.30 – 0.85mm), was obtained from Japan Organo Co., Ltd. Diaion HP–20 (ϕ 0.30 – 1.20mm) was obtained from Mitsubishi Kasei Corp. Zeolite A3 (ϕ 0.5 – 1.3 mm) was purchased from Wako Pure Chemicals. Adsorbents were immersed in methanol and then washed several times with sterile water before use.

1.3 Analyses

β–Galactosidase activity and tryptophan concentration were measured as described in a previous paper[6]. For β–galactosidase activity, *o*–nitrophenyl–β–D–galactopyranoside (ONPG) was used as a substrate according to the method described by Bittner and Vapnek[1]. One kat of enzyme activity corresponds to 1 mol of ONPG hydrolyzed per second at 37 °C.

Tryptophan concentration was determined colorimetrically by the reaction with *p*–dimethylamino benzaldehyde as described by Kupfer and Atkinson[10].

Protein concentration was determined by the dye–binding method as reported by Bradford[4].

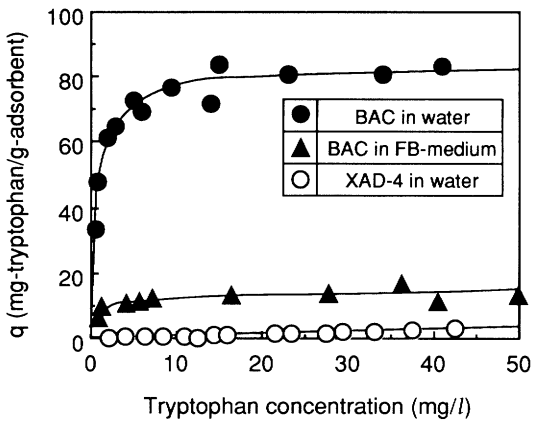

Fig. 2 Adsorption isotherm of tryptophan to adsorbent

2. Results and Discussion

2.1 Effect of tryptophan concentration

At first, we confirmed that the gene expression of β–galactosidase could be induced by decreasing tryptophan concentration. **Figure 1** shows the specific growth rate and specific enzyme activity under various initial tryptophan concentrations in the medium. Cultivation was done in an L–type test tube. In order to limit tryptophan consumption to as little as possible, the cells were cultivated for short periods. In the exponential growth phase (OD_{660} is less than 0.5) after only 1.5 to 2 hours cultivation, cells were harvested and were used for the measurement of specific enzyme activity.

As shown in Fig. 1, it was found that gene expression was induced at less than 0.5 mg/l of tryptophan. The specific growth rate, however, decreased at a low tryptophan concentration and reached a maximum value at above 1mg/l of tryptophan concentration. From these results, it is concluded that two stage culture is suitable for overproduction of foreign gene product and it is necessary to keep tryptophan concentration above 1mg/l in the first–stage for cell growth.

2.2 Comparison of adsorbent on adsorption isotherm

In order to carry out two stage culture by direct addition of adsorbent in a jar–fermentor, the following properties are required as criteria for the selection of adsorbent; no or little disruption by impeller agitation, autoclavable to prevent contamination, reusability, easy handling for separation and recovery after the culture and high adsorption capacity for tryptophan. From the criteria, we selected some adsorbents, such as BAC, XAD–2, XAD–4, Zeolite A3 and Diaion HP–20. Among them, BAC and XAD–4 was found to adsorb the tryptophan at relatively high levels from preliminary experiments.

Adsorption capacities of the two adsorbents were compared from the experiment on adsorption isotherms at 37 °C. Adsorption experiments were at first carried out in an aqueous solution containing tryptophan only. One hundred milligram of BAC was added per liter of solution for various concentration (less than 50 mg/l) of tryptophan. As shown in **Fig. 2**, adsorption isotherms of BAC and

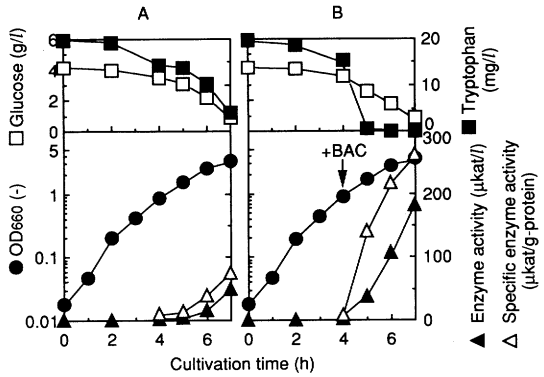

Fig. 3 Batch culture in test tube for inducible enzyme production

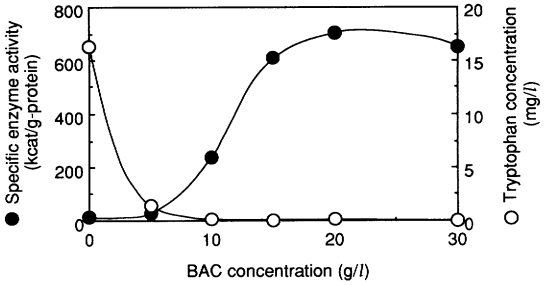

Fig. 4 Effect of BAC concentration on specific enzyme activity

XAD–4 were found to be expressed by Langmuir and Freundlich equation, respectively.

For BAC,

$$q = \frac{1.4 \times 82\, C}{1 + 1.4\, C}$$

For XAD–4,

$$q = 0.0642\, C$$

where q (mg/g) shows the adsorbed amount of tryptophan per g–adsorbent and C (mg/l) shows the equilibrium concentration of tryptophan in the solution. In the case of XAD–4, it is likely that the Freundlich equation as mentioned above appears in a part of the Langmuir relationship because the adsorption amount is relatively low.

It was also found that adsorption capacity of BAC was higher than that of XAD–4 by two to three orders of magnitude. Therefore, BAC was selected as a tryptophan adsorbent and used in the following experiments.

The adsorption isotherm of BAC in FB–medium was also examined. As shown in Fig. 2, the adsorption isotherm was expressed by the following equation.

$$q = \frac{1.7 \times 13\, C}{1 + 1.7\, C}$$

The adsorption capacity of tryptophan was found to decrease to about one sixth of that in the aqueous solution. This seems because other components can adsorb to BAC in the case of FB–medium.

2.3 Induction of gene expression by BAC addition in test tube culture

In order to assure the induction of gene expression by BAC addition, test tube cultures were done with and without the use of BAC.

Figure 3–A shows the time course of control culture without BAC. Initial tryptophan concentration was 20 mg/l. The enzyme activity increased gradually after about 5 hours. At this point, tryptophan concentration was 13 mg/l. This did not coincide with the results shown in Fig.1. As reported in our previous paper[6], such incomplete repression of *trp* promoter was observed at above OD_{570} = 10 in the case of fed–batch culture in which tryptophan was kept at 20 to 30 mg/l. This seems to be because the aporepressor concentration becomes relatively low in the late logarithmic phase and the repression of *trp* promoter becomes incomplete. From this result, we decided to keep tryptophan concentration at 20mg/l in a jar–fermentor culture in order to repress the gene expression completely.

Figure 3–B shows the cultivation results for inducible production. Five gram per liter of BAC was added after 4 hours. Tryptophan concentration decreased quickly and the gene expression was induced.

Figure 4 shows the effect of BAC concentration on inducible production of β–galactosidase when the FB–medium containing 20 mg/l of tryptophan was used. After 1 hour cultivation in a T–shape test tube containing 100 ml of the medium, various concentrations of BAC were added. Cells were harvested after 1.5 hour for assay of the enzyme activity. At this point, cells were in the exponential growth phase and OD_{660} was less than 0.5. It was found that final cell concentration after cultivation was almost the same. This means that the amount of BAC did not affect the cell growth rate in the region of BAC concentrations shown in Fig. 4.

High activity of β–galactosidase, 660 μkat/g–protein, was obtained above 20 g/l of BAC. This is about twice the level compared with that without tryptophan shown in Fig. 1. Although the reason is not yet clear, it may be that BAC adsorbed some components which inhibit gene expression.

At 5 g/l of BAC, tryptophan in the medium still remained at a level of about 2 mg/l. From the equilibrium results shown in Fig. 2, tryptophan concentration in the medium was evaluated to be 0.25 mg/l. This means that the adsorption equilibrium was not attained after 1.5 hour incubation or that some metabolic products lowered the adsorption capacity of BAC. This phenomenon is considered to occur in a jar–fermentor culture in which BAC is directly added. Therefore, a BAC concentration of 20 g/l was selected for the jar–fermentor culture from the results shown in Fig. 4, not Fig. 2.

2.4 Fed–Batch culture without BAC in jar–fermentor

In a jar–fermentor culture, tryptophan concentration should be maintained at 20 mg/l in the first stage for cell growth. Therefore, tryptophan uptake rate was studied in fed–batch culture in which glucose and tryptophan were independently fed to the fermentor. In both of two independent cultures, tryptophan concentration could not be

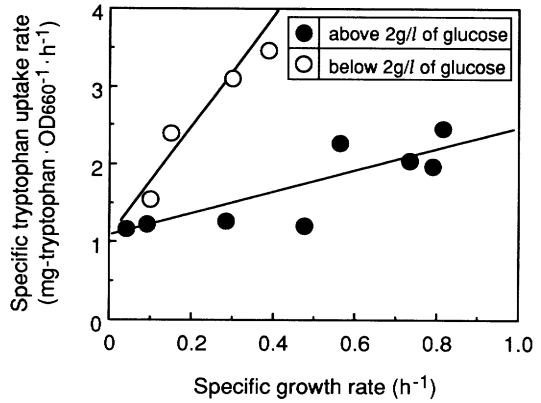

Fig. 5 Tryptophan uptake rate calculated from results of high tryptophan concentration. The data were calculated from the time-dependent results of two independent fed-batch culture

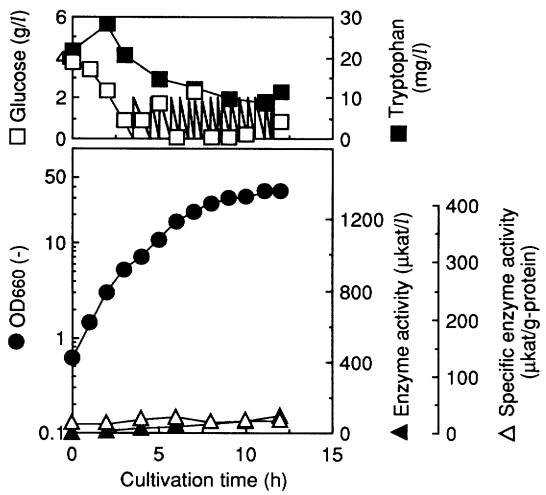

Fig. 6 Fed-batch culture at high tryptophan concentration

kept at a constant level in the late phase of cultivation. Based on the results of both cultures, tryptophan uptake rate was calculated from both tryptophan concentration in the medium and feeding rate of tryptophan solution.

Figure 5 shows the relationship between specific growth rate, μ, and specific tryptophan uptake rate. We found that two straight lines were clearly obtained depending on glucose concentration. Growth yield on tryptophan consumption changed from 0.7 in the case of high glucose concentration (higher than 2 g/l) to 0.16 OD_{660}/mg–trp in the case of low glucose concentration (less than 2 g/l). It seems that the tryptophan uptake rate can be regulated by catabolite repression. Tryptophanase from *E. coli*, which is one of the key enzymes of tryptophan assimilation, has been reported to be inducible and sensitive to catabolite repression[3]. In our previous paper[9], we also confirmed that tryptophanase production was induced by changing the feed of glucose to glycerol in fed–batch culture. Therefore, it is likely that the tryptophan uptake rate is enhanced under low glucose concentration. Based on these results, we decided

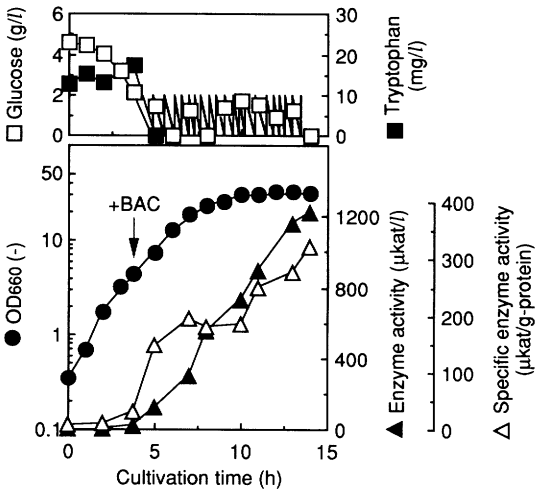

Fig. 7 Two-stage fed-batch culture using adsorbent BAC

to change the tryptophan feeding rate depending on glucose concentration.

Figure 6 shows the time course of the control culture, a fed–batch culture at high tryptophan concentration without addition of BAC. Tryptophan concentration in the medium was monitored intermittently and it was kept between 10mg/l and 30mg/l as shown in Fig. 6. β–Galactosidase was never induced and the maximum specific enzyme activity was 27 μkat/g–protein.

2.5 Inducible production with BAC in fed–batch culture

Figure 7 shows the time course of two–stage fed–batch culture using adsorbent BAC for inducible enzyme production. Twenty grams per liter of BAC was added to the jar–fermentor directly after about 4 hours. As shown in this figure, almost all of the tryptophan in the culture was adsorbed for at least 1 hour after BAC addition. The enzyme production was immediately induced and specific enzyme activity increased quickly to about 170 μkat/g–protein. After about 11 hours, it increased gradually. A similar trend has been observed in the case of medium change by cross–flow filtration. The reason is not yet clear but this may mean that the intracellular tryptophan level decreases gradually.

The maximum specific enzyme activity reached 320 μkat/g–protein. This value was about 12 times higher than that of the control fed–batch culture. It corresponded to one half of the result of Fig. 4. One possible reason may be that some inhibitors such as acetate accumulated in the medium because of the high cell concentration and then repressed the gene expression.

In this fermentor culture, the BAC concentration was determined based on the preliminary cultivation in a T–shape test tube containing 100 ml of the medium. Therefore, the induction level in the jar–fermentor culture may be improved by the optimization of BAC concentration in the fermentor culture.

In this paper, we have reported *in situ* inducible expression of recombinant gene by *trp* promoter. For this

purpose, tryptophan starvation by the feeding–off of tryptophan is one possible strategy. For this method, the measurement of tryptophan concentration in the medium and its control are required. However, the on–line monitoring of tryptophan concentration is actually difficult and enzymatic systems for sensing have not yet been developed. On the other hand, using the method by adsorbent addition as reported in this paper, it is possible to remove the tryptophan in the medium at any culture time, even if the accurate concentration can not be estimated. In addition, this method is suitable for two–stage fed–batch culture because BAC is autoclavable and easy to handle. The maximum specific enzyme activity obtained in this paper was comparable with that with cross–flow filtration[6]. The response time of the enzyme induction was also about the same. The change of growth rate after addition of BAC was not so serious while the rate decreased by 70% in the case of cross–flow filtration. These results show that this method is applicable for overproduction of foreign gene product because quick and *in situ* removal of tryptophan is possible.

Conclusion

For overproduction of cloned gene regulated by *trp* promoter, two stage culture by direct addition of adsorbent for tryptophan removal was investigated. The adsorbent, BAC (Bead Activated Carbon), could adsorb tryptophan. In test tube culture, it was found that the addition of 20 g/l of BAC was enough to remove 20 mg/l of tryptophan in the medium and induce the expression of cloned gene coding β–galactosidase as a model product.

Jar–fermentor culture with or without BAC were carried out. In the control culture, in which the tryptophan concentration was kept between 10 to 30 mg/l, the enzyme production was repressed perfectly. In two–stage culture by direct addition of BAC for *in situ* removal of tryptophan, inducible production was observed only 1 hour after BAC addition. Maximum specific enzyme activity of 320 μkat/g–protein was obtained after cultivation. This was 12 times higher than that of the control culture.

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