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Gene Expression using *nar* Promoter under Anaerobic Condition with Recombinant *E. coli*

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The *nar* promoter as an inducible promoter was characterized for the process development for the gene expression and the protein production under anaerobic condition. The LB medium was selected as a main culture medium showing the enzyme activity of 18,000 units/min/g cell in the flask cultivation. The optimum concentration of nitrate was 1%. Under anaerobic conditions, the gene expression was fully induced in the presence of nitrate.

Key words: *E. coli*, *nar* promoter, anaerobic condition, nitrate

INTRODUCTION

Biotechnology industry has been rapidly developed by the recombinant DNA technology. Many useful materials can be obtained through recombinant cells. The process development is very important to accomplish the mass production. Especially, *Escherichia coli* can grow in the very cheap medium and the growth time is very short, thus the biological process can be controlled easily [1, 2]. The many inducible promoters of plasmid used in *E. coli* have been developed such as *lac*, *trp*, *tac*, λP_L, *VHb*, *aniG*, and so on. *lac* and *tac* promoters are induced by IPTG. λP_L promoter is induced by the regulation of temperature [3]. *aniG* promoter is induced by the regulation of pH in the anaerobic condition [4]. *VHb*, *frd*, *nif*, *nar*, *OXYPRO* promoters are induced by the regulation of the dissolved oxygen [5-11]. Using the expensive inducer like IPTG decreases the economical efficiency and, in the case of induction by the regulation of temperature, the expressed protein can be denatured by heating. In the case of the *nar* promoter system under the anaerobic condition, the expensive inducer is not required anymore and the denaturation of product can be avoided. When oxygen, nitrate, and fumarate dissolve in the same medium, the nitrate is used as an electron acceptor by the redox potential if the oxygen is exhausted firstly [12-14]. The oxygen inhibits the synthesis of nitrate reductase at the transcriptional step [15]. The regulation of anaerobic respiratory gene by the *nar* operon system is described as follows (Figure 1) [16-19]. The NarX protein exsiting in the cell membrane activates the NarL protein by recoginizing the presence of nitrate and molybdate, and then the signal is transmitted to *nar* operon and the nitrate reductase is synthesized. The Fnr protein activated in the anaerobic condition induces the gene expression while it plays a role of a repressor in the aerobic condition. The nitrate can induce the *nar* promoter and inhibit the production of the fumarate reductase. The molybdate is necessary for the anaerobic gene expression and increasing the expression of nitrate reductase. But the necessary concentration of molybdate for the induction is very low [20]. When the gene of the target material is inserted to the *nar* operon system, the maximization of the gene expression can be accomplished under the anaerobic condition in the presence of nitrate and molybdate in medium. Production process using *nar* promoter in the recombinant cell system can be manipulated easily to induce the gene expression under the anaerobic condition without applying the expensive inducer or changing the temperature.

In this study, the characteristics of the *nar* operon system for the high productivity were investigated.

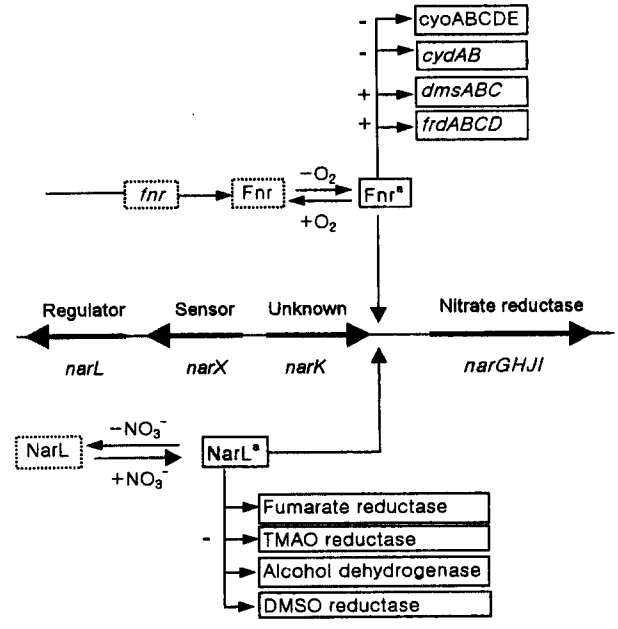

Fig. 1. Model for the regulation of anaerobic respiratory gene product by NarL and Fnr (Superscript "a" means the activated form. +: activation, −: repression).

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The optimum concentrations of nitrate and molybdate and the optimum induction conditions were studied to maximize the enzyme activity in the flask cultivation.

MATERIALS AND METHODS

Strain and Plasmid

The host strain used in this study was *Escherichia coli* strain MV1190 (△(*lac-pro*AB), *thi*, *sup*E, △(*srl-recA*)306::Tn10(*tet*^{r}) [*F':tra*D36, *proAB*, *lacI*^{q}Z△M15]). The plasmid pXR8971 containing *nar* promoter was drived from pSL800 and the 5'-terminal was connected with *lac*Z gene. The size was 7698 base pairs [18, 21]. Both strain and plasmid were kindly donated by Professor DeMoss at the University of Texas Medical School, Texas, U.S.A.

Media and Cultivation

The media used in this study were LB medium (10 g/L trypton, 5 g/L yeast extract, 10 g/L NaCl) and M9 minimal medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.493 g/L $MgSO_4$, 2 g/L glucose, 0.011 g/L $CaCl_2$). In order to obtain the color reaction, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 40 mg/L) dissolved in DMSO (dimethyl sulfoxide) was added to the agar plate. The flask culture was accomplished with 50 mL working volume in a 250 mL Erlenmeyer flask at 37°C and 250 rpm in a shaking incubator (Vision Scientific Co., VS-8480SR). And the inoculum size was 2% (V/V). The initial pH was adjusted to 7.0 with 1 N NaOH and 1 N HCl.

Assay

The optical density for the cell concentration was measured at 600 nm. Glucose kit (Sigma, 510-A) was used for the determination of glucose concentration. The assay of β-galactosidase specific activity was followed by Miller method [22].

RESULTS AND DISCUSSION

The determination of optimum medium was very important to obtain the high cell density and productivity. Nitrate (1%) and molybdate (0.1 M) were added at the start of the cultivation. When the cell was cultured in LB and M9 media, the cell concentration was similar to each other while the enzyme activity was obtained 18,000 units/min/g cell and 4,000 units/min/g cell, respectively (Figure 2). In the case of LB medium, the enzyme activity was continuously increased during 6 hours after the O_2 limit induction was applied. According to these results, the main culture medium was selected as LB medium. Figure 3 showed the comparison of activity when the O_2 limit induction was applied at the various growth phase in LB medium. When the gene expression was induced at the stationary growth phase (8 hours) by stopping shaking to make the anaerobic condition, the enzyme activity was slightly increased. However, the enzyme activity was already 5,000 units/min/g cell before the O_2 limit induction. The reason was the fact that the anaerobic condition was made in part because the surface aeration in the flask cultivation was not sufficient for the cell growth at the exponential growth phase. That was, the gene expression was partially induced, resulting in

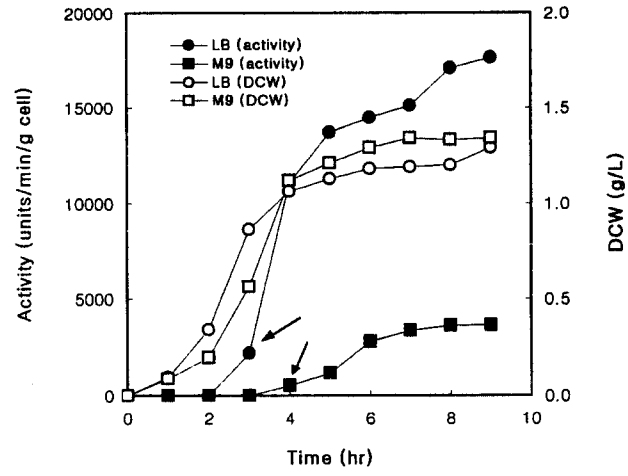

Fig. 2. Effects of various media on activity and dry cell weight (Nitrate and molybdate were added at the start of the cultivation. Arrows show O_2 limit induction).

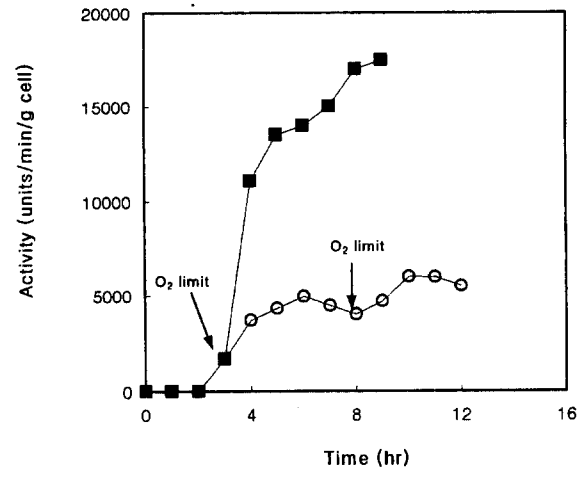

Fig. 3. Comparison of activity with different induction time in LB medium (Nitrate and molybdate were added at the start of the cultivation).

showing the considerable enzyme activity. When the gene expression was induced at the mid-exponential growth phase (3 hours) by making the anaerobic condition, the enzyme activity was rapidly increased upto 17,500 units/min/g cell showing the full induction. Thus, it was known that the dissolved oxygen was a very important factor in the induction of the gene expression utilizing the *nar* promoter.

Since the nitrate was used as a terminal electron acceptor under the anaerobic condition, the effect of nitrate on the gene expression was important. By the nitrate reductase, the nitrate was reduced into the nitrite which was very toxic to the cell growth. Thus, it was necessary of elucidating the optimum concentration of nitrate for the maximum gene expression. The effect of nitrate was studied by varying of the nitrate concentration from 0% to 2.0%. The cell concentration and the enzyme activity were continuously increased upto 23 hours (data not shown). Figure 4 showed the effect of the nitrate concentration on the enzyme activity and the cell concentration at 23 hours of the cultivation. The induced enzyme activity was increased with increasing the nitrate concentration, while the cell concentration was decreased. The maximum productivity was 31,000 units/L.min when the

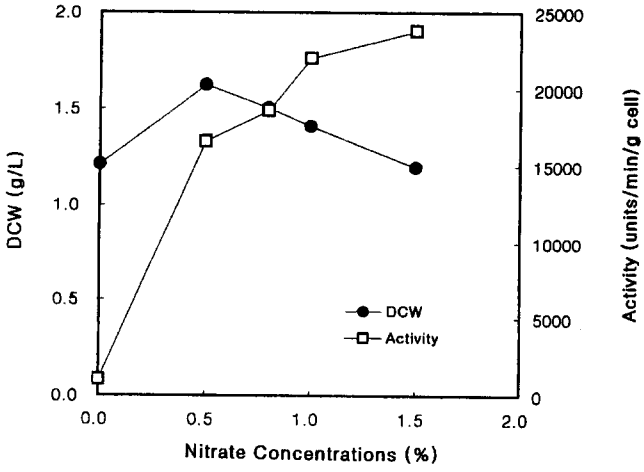

Fig. 4. Effect of nitrate concentration on dry cell weight and activity.

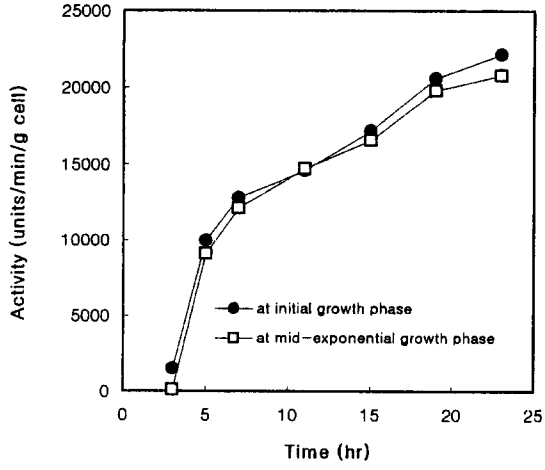

Fig. 5. Profiles of activity with different adding time of nitrate and molybdate. O_2 limit induction was applied at the mid-exponential growth phase (3 hours).

nitrate concentration was 1%. Thus, the optimum concentration of nitrate was 1%. The variation of the molybdate concentration did not significantly affect the gene exptression (data not shown). Thus, the molybdate concentration of 0.1 mM was used. When nitrate and molybdate were added at the start of cultivation in the anaerobic condition, the enzyme activity was not significantly different from that at the mid-exponential growth phase (3 hours) (Figure 5). When nitrate and molybdate were added at the start of cultivation, the enzyme activity of 22,000 units/min/g cell was obtained at 24 hours of cultivation.

In the *nar* operon system, many factors like nitrate, molybdate, and oxygen would affect the gene expression. Nitrate and molybdate were added at 3 hours when the O_2 limit induction was applied, if necessary. As shown in Figure 6, the enzyme activity was very low without the addition of nitrate whether or not the O_2 limit induction was applied (A&B, E&F). On the other hand, the enzyme activity was rapidly increased upto 9,000 units/min/g cell in the case of the addition of nitrate only or both nitrate and molybdate without the application of O_2 limit induction (C&D). However, behind 2 hours after induction, the enzyme activity remained at that level without increasing anymore. The reason was that the anaerobic condition was not completely established due to the surface aeration caused by shaking, even if the enzyme activity was fairly high through the anaerobic condition made in part by the oxygen depletion due to the cell growth at the mid-exponential growth phase. Thus, it was known that the nitrate was a very important factor in the gene expression.

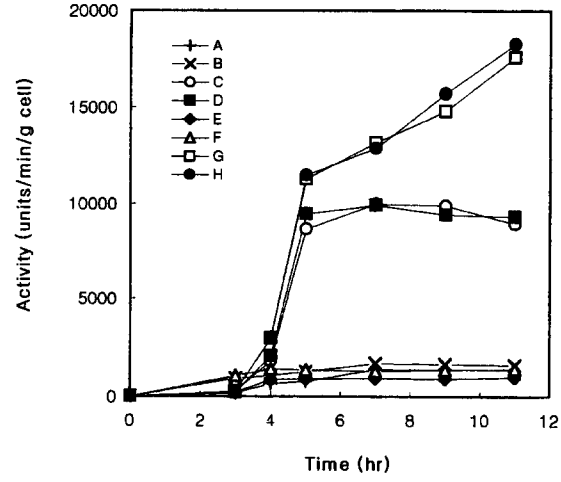

Fig. 6. Effects of nitrate and molybdate on activity under aerobic or anaerobic condition. Nitrate and molybdate were added at the mid-exponential phase when O_2 limit induction was applied (3 hours).
(A) without O_2 limit & without $NO_3^- + MoO_4^-$
(B) without O_2 limit & with MoO_4^-
(C) without O_2 limit & with NO_3^-
(D) without O_2 limit & with $NO_3^- + MoO_4^-$
(E) O_2 limit & without $NO_3^- + MoO_4^-$
(F) O_2 limit & with MoO_4^-
(G) O_2 limit & with NO_3^-
(H) O_2 limit & with $NO_3^- + MoO_4^-$

When the anaerobic condition was completely established in the presence of nitrate and molybdate (G&H), the enzyme activity was rapidly increased for 2 hours after the O_2 limit induction. And then, the enzyme activity was consistently increased upto 18,000 units/min/g cell. This meant that the nitrate would largely affect the gene expression under the anaerobic condition rather than under the aerobic condition. Thus, the addition of nitrate and molybdate might be the primary factor in the gene expression rather than the anaerobic condition caused by the O_2 limit. However, while the enzyme activity was not fully induced without the application of O_2 limit (C&D), it was rapidly increased under the anaerobic condition, resulting in showing the full induction (G&H). Therefore, the O_2 limit causing the anaerobic condition would be the primary factor in the gene expression induction rather than the addition of nitrate and molybdate.

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