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RESEARCH NOTES

Production of Human Epidermal Growth Factor in Fed-batch Culture of Acetate-tolerant *Escherichia coli**

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Abstract An acetate-tolerant mutant of *Escherichia coli* DH5α, DA19, was used for secretory production of human epidermal growth factor (hEGF) whose expression was under the control of *phoA* promoter. The recombinant cells were cultured in a chemically defined medium, and glucose was added at different specific provision rates during the growth and expression phases. It was found that pH had a significant effect on the extracellular hEGF production. The extracellular hEGF concentration was 75.5mg·L^{-1}, 5.2-fold of the level reached at pH 7.0, even though more acetate was produced. Nitrogen source was limited in the later growth phase. Supplementation of ammonium promoted the consumption of phosphate and reduced the time to exhaust phosphate, but the extracellular hEGF production was similar to that without supplementation of ammonium.

Keywords human epidermal growth factor, acetate-tolerance, *Escherichia coli*, pH

1 INTRODUCTION

Escherichia coli is a valuable organism for the high level production of recombinant protein in high cell density culture. The expressed heterologous proteins are usually accumulated inside the cells, which can be easily degraded by cellular protease[1]. Moreover, foreign proteins can form inactive inclusion bodies [2] caused by misfolding, and the refolding is quite inefficient and expensive. Secretion of the heterologous proteins reduces not only the toxic effects on the host cells, but also the difficulties and cost in recovery and purification.

Another disadvantage of *E. coli* is accumulation of acetate during cultivation, which inhibits cell growth and the foreign gene expression [3,4], and it is more deleterious to the recombinant cells[5]. Many strategies have been adopted to reduce the formation of acetate by process control[3,6] and metabolic engineering[7—9]. An acetate-tolerant mutant of *E. coli* DH5α, DA19, was isolated by Zhu *et al.*[10], which forms less acetate on glucose than DH5α and grows better in the presence of acetate. This feature is especially useful in large-scale fermentors, where uniform distribution of the added concentrated glucose feed is more difficult than in small ones.

Human epidermal growth factor (hEGF) is a peptide composed of 53 amino acids. It can promote proliferation of epidermal cells and inhibit the secretion of gastric acid, and has wide cosmetic and medical applications. We have studied hEGF production in *E. coli* YK537 using complex media, and the secreted hEGF concentration reached 686mg·L^{-1}[11,12]. A preliminary study has been carried out with DH5α and DA19 in shake flask cultures, which indicates that DA19 produces 7.54mg·L^{-1} of secreted hEGF in a complex medium, compared with 3.98mg·L^{-1} produced by DH5α[13]. The presence of large amount of proteins in complex medium increases the difficulty in downstream separation and purification of target protein, but study on foreign protein production under control of the *phoA* promoter in defined media is rare. In this study, hEGF was produced by the acetate-tolerant strain DA19 in a chemically defined culture medium. It was found that pH had a significant effect on *phoA* promoter-controlled gene expression, and hEGF production was improved remarkably at an alkaline pH even though the acetate level was elevated at the same time.

2 MATERIALS AND METHODS

2.1 Strain

The host strain used in this study was *E. coli* DA19, which was isolated in continuous culture of *E. coli* DH5α with acetate as the selective pressure [10]. The plasmid pAET8 carried the gene coding for ampicillin resistance and that encoding hEGF which was behind the *phoA* (alkaline phosphatase) signal sequence under the control of *phoA* promoter [14]. DA19 was transformed with pAET8, and the transformant was selected and stocked in mass concentration of 40% glycerol at −20℃.

2.2 Media

The Luria-Burtani (LB) medium contained (per liter): tryptone (Oxoid, UK), 10g; yeast extract (Oxoid), 5g; and NaCl, 10g. The M9 medium[15] for seed culture contained (per liter): glucose, 4g; $Na_2HPO_4·12H_2O$, 15.12g; KH_2PO_4, 3g; NaCl, 0.5g; NH_4Cl, 1g; $MgSO_4·7H_2O$, 0.5g; $CaCl_2$, 0.011g; and mass concentration of 1% vitamin B_1, 0.2ml. The main culture medium was a modified M9 medium and

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was consisted of (per liter): glucose, 10g; $Na_2HPO_4 \cdot 12H_2O$, 2.27g; KH_2PO_4, 0.45g; NaCl, 0.5g; NH_4Cl, 1g; $MgSO_4 \cdot 7H_2O$, 0.5g; $CaCl_2$, 0.011g; mass concentration of 1% vitamin B_1, 0.2ml; and trace elements solution, 0.4ml. The trace elements solution contained (per liter): $FeSO_4 \cdot 7H_2O$, 40g; $MnSO_4 \cdot nH_2O$, 10g; $AlCl_3 \cdot 6H_2O$, 10g; $CoCl_2$, 4g; $ZnSO_4 \cdot 7H_2O$, 2g; $Na_2MoO_4 \cdot 2H_2O$, 2g; $CuCl_2 \cdot 2H_2O$, 1g; and H_3BO_4, 0.5g. The vitamin and trace elements solutions were filter-sterilized (0.22μm) before use. The feed solution was mass concentration of 25% glucose supplemented with 0.6g·L^{-1} of $MgSO_4 \cdot 7H_2O$.

2.3 Cultivation

The stock culture (300μl) was inoculated into 30ml LB medium in a 250-ml flask, and was incubated overnight at 37℃ with shaking at 250r·min^{-1}. Then, 1-ml aliquots of this primary culture were transferred into three 500-ml flasks each containing 70ml of M9 medium and shaken at 250r·min^{-1} and 37 ℃ for 10h. The secondary inoculum cultures were combined and transferred to 2.36L of the main culture medium in a 5-L fermentor (RIBE-5, ECUST, China) controlled by a personal computer with a software program, Tophawk (the National Center for Biochemical Engineering, Shanghai, China). The temperature was controlled at 37℃ and the pH was controlled by addition of mass concentration of 14% ammonia solution. The initial aeration and agitation rates were 3L·min^{-1} and 600r·min^{-1}, respectively, which were manually adjusted to maintain the dissolved oxygen (DO) level above 20% of air-saturation.

2.4 Assay

The cell density was estimated by measuring the optical density of appropriately diluted culture sample at 600nm (OD_{600}), and the dried cell mass (DCM) was calculated according to a linear relationship between OD_{600} and DCM. The concentration of glucose was determined by using an enzymatic assay kit (KeXin, Shanghai, China). Ammonium was determined by using the Berthelot method. Phosphate was determined based on the blue color formed by its reaction with molybdenum salt [16]. Acetic acid was measured by using gas chromatography with a column filled with Chromosorb 101 (Dikma, Lampoc, CA, USA).

hEGF was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Schagger[17]. The gel system contained a separating gel, a spacer gel, and a stacking gel with polyacrylamide concentrations of 15%, 10%, and 5%, respectively. The gel was stained with Coomassie brilliant blue R250, and quantified using an image analysis system (FuRi, Shanghai, China). Extracellular hEGF concentration was directly measured with the culture supernatant. The cell pellets were dissolved in the solubilization buffer[17] and heated in a water bath at 100℃ for 20min, and then was applied for intracellular and periplasmic hEGF analysis.

3 RESULTS AND DISCUSSION

3.1 Fed-batch cultures controlled at pH 7.0

Initially, the cultivation was carried out in a batch mode. When glucose initially added in the medium was depleted, feeding of glucose was started to allow the total cell mass in the reactor to increase exponentially. To maintain a constant specific growth rate of 0.2h^{-1}, the feeding rate of limiting substrate, glucose, is given by

$$F = \frac{X_0 V_0 \exp(\mu t)}{Y_{X/S} S_F},$$

where F is the volumetric feeding rate (L·h^{-1}), S_F is the glucose concentration in the feed solution (g·L^{-1}), μ is the specific growth rate (h^{-1}) to be controlled, $Y_{X/S}$ is the cell yield coefficient based on consumed glucose at the μ to be controlled, X_0 and V_0 are the biomass concentration (g·L^{-1}) and culture volume (L) at the start of feeding. When the specific growth rate was 0.2h^{-1}, a specific glucose supply rate of 0.19g·g^{-1}·h^{-1} was obtained according to the above equation. The biomass concentration in the culture was measured every hour, and the quantity of glucose to be added until next sampling was calculated according to the above formula but was added at a constant volumetric rate to deliver the same amount of glucose.

The preliminary fed-batch culture of DA19 (pAET8) was carried out in the defined main culture medium with pH controlled at 7.0 during the whole fermentation process. The initial glucose of 10g·L^{-1} was exhausted at 8.25h indicated by a sudden increase of DO, and glucose was added with an average specific supply rate of 0.19g·g^{-1}·h^{-1}. At 14.85h, the phosphate concentration decreased to 1.6mmol·L^{-1}, at which point the specific growth rate of 0.2h^{-1} could not be maintained any more despite continued addition of the glucose feed. Meanwhile, acetate started to accumulate, which indicated that nutrients other than glucose were limiting. In the expression phase, glucose was added at the same specific supply rate, and the residual glucose was gradually increased up to 6.1g·L^{-1}, while the NH_4^+ concentration was very low. The hEGF production level was 16.1mg·L^{-1} (data not shown).

This preliminary experiment indicated that in the expression phase the glucose feeding rate was too high. Fig.1 shows the typical trends of biomass (DCM), glucose, phosphate, ammonium, and extracellular hEGF concentrations in fed-batch culture with reduced glucose feeding rate in the expression phase. The process could be divided into four phases. Phase I is the batch culture period that lasted until exhaustion of the initial glucose; Phase II is an exponential growth period, during which glucose was added according to the above-mentioned method until the phosphate concentration decreased to about 1.6mmol·L^{-1}; in Phase III glucose was added at a constant rate of 6.6g·h^{-1} until phosphate in the medium was nearly consumed; and Phase IV was the expression period in which glucose was added at a constant specific rate of 0.16g·g^{-1}·h^{-1}. Because of the reduced feeding rate of glucose, no residual glucose was detected during the