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Fed-batch Cultivation of *Escherichia coli* YK537 (pAET-8) for Production of *phoA* Promoter-controlled Human Epidermal Growth Factor

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Abstract Secretion of the expressed heterologous proteins can reduce the stress to the host cells and is beneficial to their recovery and purification. In this study, fed-batch cultures of *Escherichia coli* YK537 (pAET-8) were conducted in a 5-L fermentor for the secretory production of human epidermal growth factor (hEGF) whose expression was under the control of alkaline phosphatase promoter. The effects of feeding of glucose and complex nitrogen sources on hEGF production were investigated. When the fed-batch culture was conducted in a chemically defined medium, the cell density was 9.68 g/L and the secreted hEGF was 44.7 mg/L in a period of 60 h. When a complex medium was used and glucose was added in pH-stat mode, the secreted hEGF was improved to 345 mg/L. When the culture was fed with glucose at a constant specific rate of 0.25 $gg^{-1}h^{-1}$, hEGF reached 514 mg/L. The effects of adding a solution containing yeast extract and tryptone were further studied. Different rate of the nitrogen source feeding resulted in different levels of phosphate and acetic acid formation, thus affected hEGF expression. At the optimal feeding rate, hEGF production achieved 686 mg/L.

Keywords: human epidermal growth factor, *phoA* promoter, fed-batch culture, *Escherichia coli*, phosphate, secretion

INTRODUCTION

Escherichia coli is the most frequently used bacterial host for the expression of foreign genes, and usually the expressed heterologous proteins are accumulated in the cytoplasm but not secreted into the medium [1]. Secretion of the expressed proteins not only reduces stress to the host cells, but also is beneficial to product isolation and purification. Several secretive expression systems of *E. coli* have been developed to produce heterogeneous proteins extracellularly. The *phoA* system is one of the most successful secretive systems, in which expression of the gene under the control of *phoA* promoter is induced by lowering the phosphate concentration in the culture. Therefore, it is an inexpensive system for induction [2-4].

Fed-batch culture is the most popular operation mode for the production of recombinant proteins. Various feeding strategies have been proposed to obtain a high cell density and high level of recombinant protein. Feeding strategies such as pH-stat [5], DO-stat [6], controlling glucose concentration [7], controlling specific growth rate [8,9], *etc.* have been adopted to control nutrient supply, cell growth, and by-product formation. In the cultivation for production of a foreign protein whose expression is controlled by the *phoA* promoter, cell growth is limited by phosphate during the expression phase. At the same time, supply of glucose should be strictly controlled to meet the requirement for gene expression and maintenance metabolism while formation of the harmful metabolite, acetate, should be avoided. However, detailed reports on fed-batch culture of recombinant *E. coli* to produce a protein controlled by the *phoA* promoter are scarce [10,11].

In this study, we used a recombinant strain of *E. coli* for secretive production of human epidermal growth factor (hEGF) whose expression was under the control of the *phoA* promoter. hEGF is a polypeptide hormone comprised of 53 amino acids, which is considered to be identical with human urogastrone [12]. It is a potent inhibitor of gastric acid secretion [13] and promotes epithelial cell proliferation both *in vitro* and *in vivo* [14]. The present investigation focused on feeding of glucose and complex nitrogen sources to enhance hEGF production.

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MATERIALS AND METHODS

Strain

E. coli YK537 (*supE44, hsdR, hsdM, recA1, phoA8, LeuB6, thi, lacY, rpsL20, galK2, ara-14, xyl-5, mtl-1*) [4] was used as the host organism. The gene coding for hEGF was chemically synthesized and was inserted into the vector pTZ-18R behind the alkaline phosphatase signal sequence which was under the control of the alkaline phosphatase promoter [15], and the dual terminators *rrnB* T1T2 were linked to the hEGF gene to obtain the plasmid pAET-8. Competent *E. coli* YK537 cells were transformed with pAET-8 according to standard protocols [16]. The recombinant strain was stocked in 25% glycerol at -20°C.

Media

The LB medium for inoculum culture contained (per liter): 10 g of tryptone (Oxoid, UK), 5 g of yeast extract (Oxoid, UK) and 10 g of NaCl. The defined medium contained (per liter): 0.176 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.011 g of $CaCl_2$, 0.2 mL of 1% (w/v) vitamin B_1, 10 g of glucose, 0.4 g of proline and 0.4 g of leucine. The production medium contained (per liter): 30 g of tryptone, 30 g of yeast extract, 2 g of NH_4Cl, 2 g of $(NH_4)_2SO_4$, 2 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl and 5.0 g of glucose. The pH for all the media was adjusted to 7 with 2 M NaOH prior to sterilization. The complex feed medium contained 240 g of tryptone and 120 g of yeast extract per liter.

Culture Conditions

The primary preculture was prepared by transfer of 1 mL of the stock culture to 30 mL of LB medium supplemented with 100 mg/L of ampicillin in a 250-mL flask, and was incubated at 37°C and 200 rpm for 10 h. The secondary preculture was prepared by transfer of 0.7 mL of the primary preculture to 70 mL LB medium in a 500-mL flask, and was cultivated under the same conditions for 9 h. The contents of two 500-mL flasks were combined to inoculate into 2.5 L of the production (defined or complex) medium in a 5-L fermentor.

The 5-L fermentor (RIBE-5, ECUST, China) was controlled by a personal computer with a software program (Tophawk Fermentation Control System, National Center for Biochemical Engineering Research, Shanghai, China). The fermentation conditions were: pH, 7 (with 2 M NaOH and 1 M H_2SO_4); temperature, 37°C; impeller speed, 450~1,100 rpm; aeration rate, 4 L/min; dissolved oxygen, above 20% of air saturation by manual adjustment of the agitation speed. All the fermentation data were collected in the computer.

Analytical Methods

Cell density was determined by measurement of the optical density of appropriately diluted culture sample at 660 nm (OD_{660}). Cell dry weight was estimated according to a linear relationship between the cell dry weight and OD_{660}. Acetic acid was analyzed by gas chromatography (GC112A, ShangFen, China) with a column packed with styrene-divinyl benzene (Chromosorb 101, Dikma, Lapoc, CA, USA), and detected with an FID detector [17]. Glucose concentration was measured with an enzymatic assay kit (Institute of Biological Products, Shanghai, China) containing glucose oxidase. Phosphate was analyzed by using a diagnostic kit (RS, Shanghai, China) based on the reaction between phosphate and ammonium molybdate to form phosphomolybdic acid, which in turn forms a blue substance with ammonium ferrous sulfate [18]. Proteins in the samples were precipitated by trichloroacetic acid prior to phosphate analysis to avoid interference. hEGF in the supernatant of the culture sample was measured with enzyme-linked immunosorbent assay (ELISA) with a diagnostic kit (DEG00, Quantikine, R&D, USA), and routinely measured by SDS-PAGE.

RESULTS AND DISCUSSION

Fed-batch Culture in the Defined Medium

The growth of *E. coli* YK537 needs vitamin B_1 and leucine according to its genotype, but we found that *E. coli* YK537 can only grow in a defined medium containing proline as well. In the chemically defined medium (Materials and Methods), *E. coli* YK537 (pAET-8) grew slowly with a maximum specific growth rate of 0.29 h^{-1}. At the end of the batch culture (20 h), the cell density only reached 1.69 g/L. A feed solution containing glucose (400 g/L), leucine (3 g/L), and proline (1.28 g/L) was fed to the fermentor as the initial glucose was depleted. At 60 h, the cell density achieved 9.68 g/L and the secreted hEGF was only 44.7 mg/L. Since *E. coli* YK537 (pAET-8) grew too slowly in the chemically defined medium, it seemed difficult to achieve a high cell density and high hEGF expression level within a relatively short period of time. Therefore, the complex production medium composed of tryptone and yeast extract was used to examine the effect on hEGF production.

Fed-batch Culture in the Complex Medium with pH-stat-based Glucose Feeding

In the culture process using a complex medium for the production of a *phoA* promoter-controlled gene product, supply of glucose is necessary not only to meet the energy requirement for maintenance metabolism and gene expression, but also for phosphate uptake [19], even though some energy could be provided through the catabolism of some nitrogen-containing organic compounds such as amino acids. Our preliminary experiments showed that after exhaustion of the initial phosphate in the complex production medium, the phosphate concentration gradually increased up to 3.2 mmol/L when glucose had been used up in a batch culture. This accumulation of phosphate

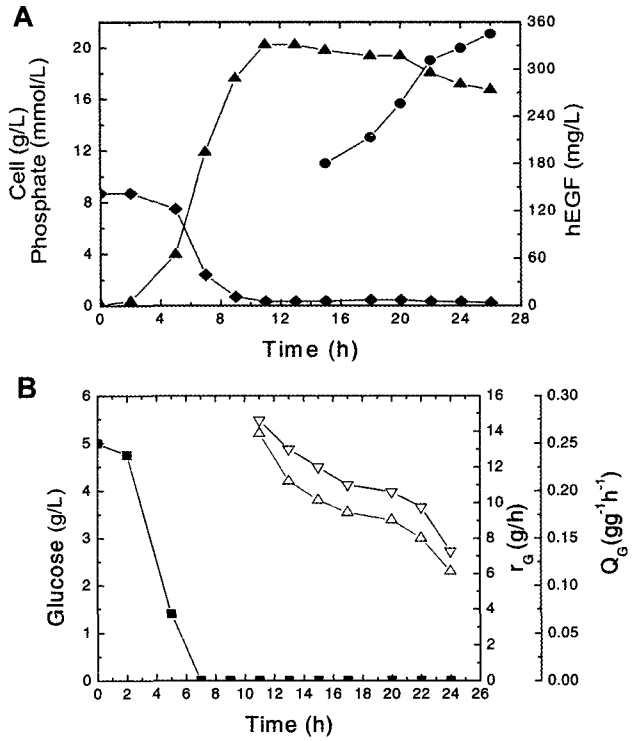

Fig. 1. Time courses of phosphate (◆), cell (▲) and hEGF (●) (A); glucose (■), specific consumption rate (Q_G, Δ) and feeding rate (r_G, ∇) of glucose (B) in fed-batch culture carried out in a 5-L fermentor using a complex medium. Glucose feeding was based on pH-stat mode.

was caused by the hydrolysis of phosphate esters present in yeast extract, and depressed expression of hEGF. Addition of glucose promoted uptake of the released phosphate, and resulted in a decrease in phosphate concentration (data not shown).

In the culture process, when glucose is depleted, the cells start to metabolize amino acids as energy sources to liberate ammonia. This causes a rise of pH, and can be served as a signal for the feeding of glucose. Therefore, the pH-stat feeding mode, a simple and reliable strategy, was adopted to provide glucose. A glucose solution (500 g/L) was supplied to the fermentor as an acid to maintain the culture pH of 7.0. The recombinant cells grew at a maximum specific growth rate of 0.66 h^{-1} and the cell density reached the maximum of 20.2 g/L at 11 h as phosphate in the culture was nearly exhausted (Fig. 1). Feeding of the glucose solution was initiated at 5.5 h as an abrupt increase in dissolved oxygen occurred, during which the residual glucose was maintained at an undetectable level and no accumulation of acetate was observed (not shown). The secreted hEGF attained 345 m/L at 26 h. The amount of cytoplasmic hEGF was 19.4 mg/L, which accounted for only 5% of total expressed hEGF. A similar quantity (17.8 mg/L) of hEGF was found in the periplasmic space. Therefore, most of the expressed hEGF was secreted into the medium.

In the pH-stat fed-batch culture, the phosphate concentration was effectively controlled at low levels (0.2~0.32 mmol/L), and the secreted hEGF was much higher than that obtained in the defined medium. However, with the gradual utilization of amino acids in the complex medium, the rate of ammonia release, the driving force to feed glucose, gradually reduced as well. This resulted in a decrease of glucose feeding rate and specific consumption rate (Fig. 1B). The initial feeding rate of glucose was 14.6 g/h at 11 h, which was reduced to 7.25 g/h at 24 h. This reduction in glucose feeding rate was probabley responsible for the decrease in the hEGF production rate, which declined from an average rate of 18.7 $mgL^{-1}h^{-1}$ between 15 and 22 h to 8.5 $mgL^{-1}h^{-1}$ after 22 h.

Fed-batch Culture with Constant Glucose Feeding

To improve glucose supply during the expression phase, feeding of glucose with a constant rate of 15 g/h, similar to the initial feeding rate in the pH-stat fed-batch culture, was executed after the initial glucose was depleted. The cell density reached the maximum of 20.9 g/L at 12 h, similar to that obtained in the pH-stat fed-batch culture. The total glucose addition was 706 g, 1.79-fold of that in the culture using the pH-stat mode. The secreted hEGF attained 465 mg/L at 26 h, 1.35-fold of that obtained in the culture using the pH-stat feeding mode. After exhaustion of phosphate, the cell density showed a slight decline due to dilution caused by feeding, while the total cell mass in the fermentor increased. The hydrolysis of some organic phosphorous compounds in the complex medium resulted in the release of phosphate and additional growth, and this in turn caused a reduction of the specific consumption rate of glucose when glucose was added at a constant flow rate.

Culture Fed with Glucose at a Constant Specific Rate

In the present fed-batch culture, the glucose feeding rate was calculated according to the total amount of cell mass in the fermentor and a constant specific glucose consumption rate of 0.25 $gg^{-1}h^{-1}$. This rate was similar to the initial specific supply rate in the pH-stat fed-batch culture. The result of the fed-batch culture with the constant specific glucose supply rate is shown in Fig. 2. Again, the cell density reached the maximum of 20.3 g/L at 11 h, while the level of secreted hEGF was 514 mg/L at 25 h, higher than those obtained in the previous experiments.

Cultures Fed with Glucose and Nitrogen Sources

As mentioned above, *E. coli* YK537 needs leucine and proline for growth. Although in the fed-batch cultures the growth of the *E. coli* YK537 (pAET-8) cells was limited by phosphate, the synthesis of proteins might be limited by both leucine and proline, or either of them, as well. In the fed-batch culture with a constant specific glucose supply rate, the decline of hEGF production rate (Fig. 2) might be caused by a shortage of those amino acids. Preliminary experiment showed that addition of proline and

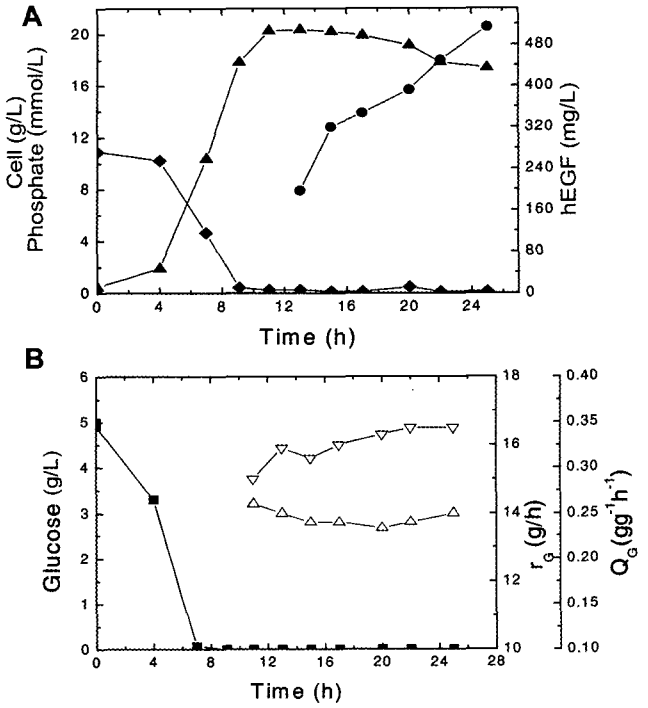

Fig. 2. Time courses of phosphate (◆), cell (▲) and hEGF (●) (A); glucose (■), specific consumption rate (Q_G, Δ) and feed rate (r_G, ∇) of glucose (B) in the fed-batch culture with a constant glucose supply rate.

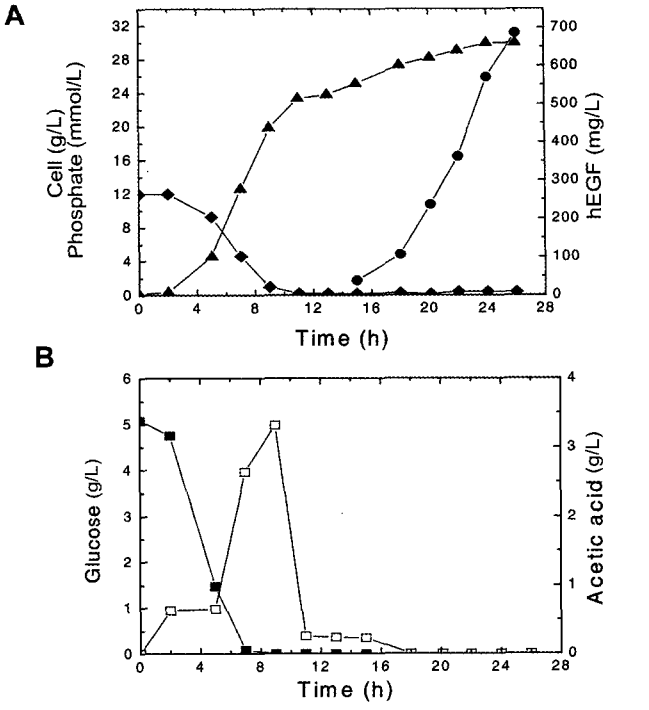

Fig. 3. Time courses of phosphate (◆), cell (▲) and hEGF (●) (A); glucose (■) and acetic acid (□) (B) in the culture fed with the complex nitrogen source solution at 24 mL/h (medium feeding rate). Glucose was added at a constant specific rate of 0.25 $gg^{-1}h^{-1}$. Addition of both feeds was started as the initial glucose was exhausted.

leucine improved hEGF production (data not shown). Therefore, a solution containing tryptone and yeast extract (see Materials and Methods) was continuously added to the fermentor to examine the effect on hEGF production. Glucose was continuously supplied at a constant specific rate of 0.25 $gg^{-1}h^{-1}$, while the nitrogen source solution was added at a constant rate. Three experiments were conducted, where the complex nitrogen source solution was added at a low (19 mL/h), medium (24 mL/h), or high (35 mL/h) rate, respectively. The results are shown in Table 1 and Fig. 3 (medium feeding rate).

Although the maximum cell density attained at the high feeding rate was respectively 1.41- and 1.66-fold of those attained at the medium and low feeding rates, the maximum production of secreted hEGF was only 32% and 36% of those obtained at the medium and low feeding rates, respectively. The average specific hEGF formation rate at the medium feeding rate was the highest; it was, 1.04- and 7.4-fold of those at the low and high feeding rates, respectively.

In the culture fed with the nitrogen sources at the low rate, 247 mL of nitrogen source solution were added to the fermentor, and the maximum cell density reached 25.5 g/L. After the depletion of the initial phosphate, the cells showed continual growth (with an average specific growth rate of 0.013 h^{-1}) caused by the phosphate supply from the added nitrogen sources. At the same time, the phosphate concentration maintained a low level around 0.2 mmol/L, similar to those in the previous cultures fed with glucose alone. Acetic acid reached 9.87 g/L at the end of the batch culture phase (8 h), but was then rapidly taken up as feeding of glucose started. The secreted hEGF reached 607 mg/L, obviously higher than that obtained in the fed-batch culture with a constant specific glucose supply rate.

In the culture fed with the nitrogen sources at the medium rate, 312 mL of the nitrogen source solution were added, and the cells reached a higher density (29.9 g/L) with an average specific growth rate of 0.034 h^{-1}. There was a little fluctuation in the phosphate concentration between 0.2 and 0.4 mmol/L in the later stage of the fed-batch culture. After the start of glucose feeding, acetate formed in the batch culture phase was rapidly consumed, then maintained at very low levels (Fig. 3B). The secreted hEGF reached 686 m/L at 26 h, higher than that in the culture fed with nitrogen sources at the low rate. The secreted hEGF in all fermentation experiments was routinely measured by SDS-PAGE, but resulted in a higher value. Therefore, data obtained by ELISA are reported in this work.

In the culture fed with the nitrogen sources at the high rate, 455 mL of the nitrogen source solution were added, and the cell density further increased to 42.2 g/L with an average specific growth rate of 0.050 h^{-1}. The phosphate concentration maintained around 0.5 mmol/L, higher

Table 1. Summary of the results of the cultures fed with the complex nitrogen source solution at different rates. Glucose was added at a constant specific supply rate of 0.25 $gg^{-1}h^{-1}$

Feeding rate of the nitrogen source solution (mL/h)	19 (low rate)	24 (medium rate)	35 (high rate)
Volume of the added nitrogen source solution (mL)	247	312	455
Added tryptone (g)	59.3	74.9	109.2
Added yeast extract (g)	29.6	37.4	54.6
Average specific growth rate in the expression phase (h^{-1})	0.013	0.034	0.050
Maximum cell density (g/L)	25.5	29.9	42.2
Maximum hEGF production (mg/L)	607	686	221
Average specific hEGF production rate ($mgg^{-1}h^{-1}$)	2.27	2.37	0.32

than those fed with the nitrogen source solution at the low and medium rates. No glucose was detected during the feeding phase. However, acetic acid was accumulated throughout the entire culture process. In the batch culture phase, the concentration of acetic acid achieved a peak of 8.66 g/L at 7 h, and then decreased to about 0.2 g/L after the start of glucose feeding. After 22 h acetate concentration increased again and reached 4.6 g/L at 28 h even though the specific glucose supply rate was not changed. The relatively high phosphate and acetate levels resulted in a low hEGF level of 221 mg/L.

Yeast extract and tryptone contained plenty of phosphate and phosphate esters, and the latter could be hydrolyzed to release phosphate by some extracellular enzymes produced by the *E. coli* cells. Assimilation of phosphate is energy dependent [19]. When the feeding rate of the nitrogen sources was high enough while the glucose feeding rate remained unchanged, limited glucose supply resulted in the limitation of phosphate uptake and a higher residual phosphate concentration in the culture. At the high feeding rate (35 mL/h), the phosphate concentration was higher than those as fed at a medium or low rate, thus expression of hEGF could not be fully derepressed. Moreover, under a condition of carbon source limitation, the carbon backbone of some amino acids can be degraded *via* β-oxidation to meet the energy requirement. In the culture fed with nitrogen sources at the high rate, the accumulation of acetate in the later period could be attributed to the catabolism of amino acids and unbalanced utilization of formed acetate. When the nitrogen sources were added at the medium rate (24 mL/h), no acetate was accumulated, thus the specific glucose supply rate of 0.25 $gg^{-1}h^{-1}$ seemed to adequately meet the energy requirement of the cells.

Feeding of tryptone and yeast extract supplied additional phosphate and caused further cell growth during the expression phase. A different feeding rate of nitrogen source resulted in a different specific growth rate. Many studies point out that specific growth rate greatly affects the expression of recombinant proteins [8,9]. In the expression phase of the fed-batch culture with the medium feeding rate of the nitrogen source solution, the average specific growth rate, 0.034 h^{-1}, was the most suitable for the production of hEGF (Table 1). The relationship between the specific hEGF productivity and specific growth rate in different periods of the fed-batch cultures was examined and the result is summarized in Fig. 4. It can be seen that the specific growth rate around 0.035 h^{-1} was suitable for the production of hEGF, at which a specific hEGF production rate of 2.7 $mgg^{-1}h^{-1}$ was obtained. A too low or too high growth rate resulted in lower specific production rate. A lower average specific hEGF production rate of 2.37 $mgg^{-1}h^{-1}$ was obtained in the culture fed with nitrogen sources at the medium rate, because the real specific growth rate could not be exactly maintained at the average value of 0.34 h^{-1} throughout the feeding process. It is noteworthy that the characteristic of *E. coli* YK537 (pAET-8) shown in Fig. 4 may be only valid when the cells are supplied with glucose at a specific rate of 0.25 $gg^{-1}L^{-1}$. If the glucose supply is changed, such a relationship may change accordingly.

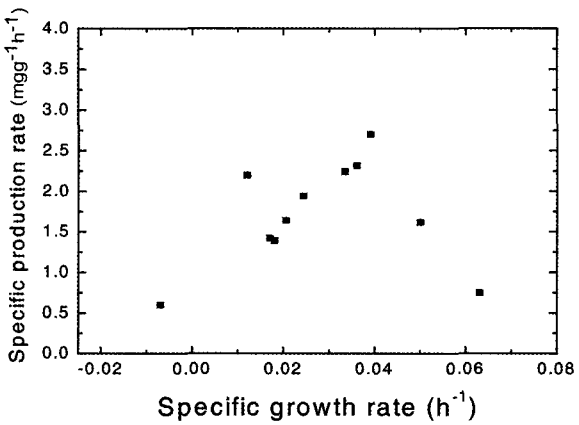

Fig. 4. Relationship between the specific hEGF productivity and specific growth rate.

CONCLUSION

In the fed-batch culture of *E. coli* YK537 (pAET-8) in the complex production medium, hEGF production was higher when glucose was supplied at a constant specific rate of 0.25 $gg^{-1}h^{-1}$ than that when added with a constant feeding rate or using a pH-stat feeding mode. Supply of

yeast extract and tryptone could further improve hEGF production. At the constant specific glucose supply rate of 0.25 $gg^{-1}h^{-1}$, the feeding rate of the nitrogen sources affected the residual phosphate concentration and acetate formation, thus exerted influence on hEGF production. At the optimal feeding rate of the nitrogen sources, a high hEGF production of 686 mg/L was realized.

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