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The effect of organic nitrogen and glucose on the production of recombinant human insulin-like growth factor in high cell density *Escherichia coli* fermentations

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SUMMARY

Two kinds of fed batch fermentation processes were compared at a 10-liter scale to examine their effect on recombinant human insulin-like growth factor (IGF-1) gene expression in *Escherichia coli*. The difference between the two processes was the feed medium composition and whether the process used a single or dual feed during the course of the fermentation. In the dual feed system, organic nitrogen was delivered at a higher rate (50 g/h) than in the single feed system (5 g/h). The dual feed process resulted in a significant increase in IGF-1 yield. 30 mg IGF-1/g dry cell weight was synthesized in the dual feed system compared to 3 mg IGF-1/g dry cell weight obtained in the single feed system. The presence of high levels of organic nitrogen during the induction period may enhance IGF-1 synthesis by protecting the IGF-1 from proteolytic degradation. The IGF-1 yield decreased to 17 mg/g dry cell weight when the glucose supply was decreased from 17 g/h to 8 g/h during the induction period; however, an increase in glucose supply from 17 g/h to 50 g/h during the induction period did not enhance the IGF-1 synthesis. Thus, the enhancement of IGF-1 gene expression in the dual feed process was mainly dependent on a high level of organic nitrogen and an appropriate level of glucose in the medium during the induction period.

INTRODUCTION

Human insulin-like growth factor (IGF-1) is a 70-amino-acid polypeptide hormone [7,13]. It is usually found in human serum and promotes mitogenic activity [15]. In vivo, by direct infusion, the polypeptide hormone stimulates hypophysectomized rat growth [14]. Because IGF-1 is believed to mediate many of the effects of growth hormone [3,17], it is also referred to as somatomedin C. Future studies on the clinical attributes of recombinant IGF-1 will depend in part on the development of high-yield fermentation techniques. The *Escherichia coli* strain used in this study carried a synthetically constructed IGF-1 gene. The codons used in the synthesis of the IGF-1 gene were those found most frequently in highly expressed *E. coli* proteins

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[6]. The only methionine in mature IGF-1 (at residue 59) was replaced with a threonine using an ACC codon to create a 70-amino-acid analog [11]. The gene was also fused to an eight-amino-acid leader peptide for better stability. After removal of the eight-amino-acid leader peptide by cyanogen bromide treatment, the resulting threonine IGF-1 analog was as active as native IGF-1 both in a radioimmunoassay and in a radioreceptor assay [11]. It was also mitogenic for Balb/C 3T3 cells [11].

The expression vector was a multicopy plasmid proprietary to Amgen and used for many of its polypeptide products [1]. In fermentation, however, the IGF-1 expression level was significantly lower than many other foreign eukaryotic genes expressed using the same vector system and a high density fermentation protocol. Other researchers have also found low yields of IGF-1 in *E. coli* using a similar thermoinduction system [2]. Low expression has been shown to result from proteolytic degradation of the product during thermoinduction [2]. Protease induction is now known as an unavoidable consequence of the heat shock response in wild-type *E. coli* strains [5]. Recent studies by Buell et al. [2] demonstrated that a protease-impaired mutant of *E. coli* (i.e., *lon, htp*R) produced a significantly higher level of IGF-1 compared to lon^+ and $htpR^+$ strains [2]. However, the use of protease-deficient strains has been a problem in industrial high cell density fermentation. In this study, we have found that manipulation of nutrient conditions during the fermentation can also be used to improve the yield of IGF-1, even in a wild type (lon^+, $htpR^+$) *E. coli* host.

MATERIALS AND METHODS

Organism and fermentation

The host strain was a wild-type *Escherichia coli* K-12. The gene for IGF-1 was synthesized and cloned onto a multicopy plasmid having runaway copy number control. The gene was placed under the transcriptional control of the lambda phage p_L promoter. The lambda repressor gene (cI_{857}) was cloned onto a second single copy plasmid. The repressor protein and plasmid are both sensitive to temperature at 42°C. Upon shifting the temperature to 42°C, three effects ensue: derepression of transcription, amplification of the plasmid bearing the recombinant IGF-1 gene, and cessation of replication of the plasmid encoding the lambda repressor.

The fermentations were carried out in a fedbatch mode in 16-l fermentors. The inoculum was prepared by aseptically transferring 200 μl of a frozen 17% glycerol stock culture into 500 ml of Luria broth media in a 2-l flask. The culture was grown to late exponential or stationary phase overnight at 30°C on a rotary shaker. The culture was then transferred into a fermentor containing 8 l of batch medium. The pH of the medium was controlled at 7.0 using concentrated ammonium hydroxide and phosphoric acid. Temperature was controlled at 30°C during the growth phase and shifted to 42°C for the production phase. The feed medium was sterilized separately and aseptically connected to the fermentor using silicon rubber tubing. The supply of feed medium was controlled by a peristaltic pump. Aeration was maintained at 10 lpm. The dissolved oxygen level was controlled at 50% of air saturation by an impeller speed controller. Dissolved oxygen was also controlled by the manual manipulation of the back pressure within the fermentor.

Analytical methods

Culture turbidity was measured at 600 nm using a Perkin Elmer Model 35 spectrophotometer after dilution in 0.15 M sodium chloride. Glucose concentration was measured using a Yellow Springs glucose analyzer. Acetate concentration was measured using a glass column packed with Poropac S in a Hewlett Packard S-790 gas chromatograph equipped with a flame ionization detector. The cell dry weight was determined by the method described previously [4]. The IGF-1 concentration was determined by quantitative SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. The IGF-1 band on the gel was scanned with an E-C Apparatus Densitometer and Shimadzu reporting integrator. The concentration of IGF-1 was obtained by comparison to a purified standard.

RESULTS AND DISCUSSION

Two fermentation processes for cell growth and product synthesis were compared in this study. The difference between the two methods was the feed medium composition and whether the process used single or dual feeding during the course of fermentation. In the single feed system, which is typically used at Amgen for recombinant protein production, the feed medium (Table 1) was added to the culture after the glucose level in the batch medium dropped to less than 1 g/l. This normally occurred at a cell density of 3 g cell dry weight/l and about 1 h prior to induction. The feed rate was increased based on cell density. The initial feed rate was 10 ml/h and was increased stepwise to 15 ml/h, 25 ml/h and 40 ml/h, as indicated in Fig. 1. At a cell density of approximately 6 g cell dry weight/l, the culture was induced by raising the temperature to 42°C. IGF-1 was synthesized to a final concentration of 3 mg/g of cell dry weight. As shown in Fig. 1, the cells were grown under glucose-limited conditions to a final density of 14 g of dry cell weight/l. Residual glucose and acetate were lower than 1 g/l during the course of induction (Fig. 1).

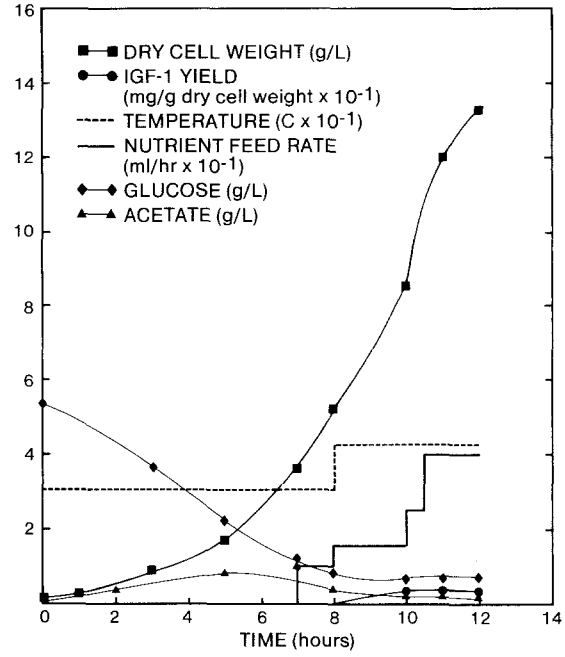

Fig. 1. Fermentation profile for single feed system. One kind of feed medium was used in this fed batch fermentation. The feed was initiated at a cell density of 3.5 g dry cell weight/l. Feed rate was changed according to the increasing cell density. The culture was thermoinduced at a cell density of 6 g dry cell weight per liter.

Table 1

The medium composition in single feed fed batch fermentation

Chemicals	Batch medium	Feed medium
Glucose	5 g/l	430 g/l
Bactotryptone	25 g/l	110 g/l
Yeast extract	5 g/l	–
K_2HPO_4	7 g/l	–
KH_2PO_4	8 g/l	–
$(NH_4)_2SO_4$	4 g/l	5 g/l
$MgSO_4 \cdot 7H_2O$	1 g/l	8 g/l
Trace metals solution[a]	2 ml/l	10 ml/l
Vitamin solution[b]	2 ml/l	10 ml/l

[a] Trace metals solution: $FeCl_3 \cdot 6H_2O$, 27 g/l; $ZnCl_2 \cdot 4H_2O$, 2 g/l; $CaCl_2 \cdot 6H_2O$, 2 g/l; $Na_2MoO_4 \cdot 2H_2O$, 2 g/l; $CuSO_4 \cdot 5H_2O$, 1.9 g/l; H_3BO_3, 0.5 g/l; concentrated HCl, 100 ml/l.

[b] Stock vitamin solution: riboflavin, 0.42 g/l; pantothenic acid, 5.4 g/l; niacin, 6 g/l; pyridoxine, 1.4 g/l; biotin, 0.06 g/l; folic acid, 0.04 g/l.

In the dual feed system, the first feed (Table 2) was used to grow the cells to the point of induction (6 g cell dry weight/l), using a feed rate similar to the single feed system. At induction, the first feed was replaced with the second feed (Table 2), and the feed rate was shifted to 150 ml/h as indicated in Fig. 2. This feed rate was maintained for the remainder of the fermentation. The second feed contained a rich concentration of soluble organic nitrogen and a low concentration of glucose (Table 2). The second feed was delivered at a 3.7-fold higher rate to allow the glucose supply to be the same as in the single feed system during the induction phase. The IGF-1 synthesis in the dual feed system reached a final level of 33 mg/g dry cell weight. Using dark phase microscopy, a small inclusion body was observed in each cell. That the inclusion body fraction contained product was supported by the fact that IGF-1 activity was recoverable in the particulate fraction of the cell lysate. Cell growth

Table 2

The medium composition in dual feed fed batch fermentation

Chemical	Batch medium	Feed medium 1	Feed medium 2
Glucose	5 g/l	430 g/l	110 g/l
Yeast extract	5 g/l	–	110 g/l
Bactotryptone	–	–	220 g/l
K_2HPO_4	7 g/l	–	–
KH_2PO_4	8 g/l	–	–
$(NH_4)_2SO_4$	4 g/l	110 g/l	–
$MgSO_4 \cdot 7H_2O$	1 g/l	8 g/l	–
Trace metal solution[a]	2 ml/l	10 ml/l	–
Vitamin solution[a]	2 ml/l	10 ml/l	

[a] Trace metal solution and vitamin stock solution are the same as in Table 1.

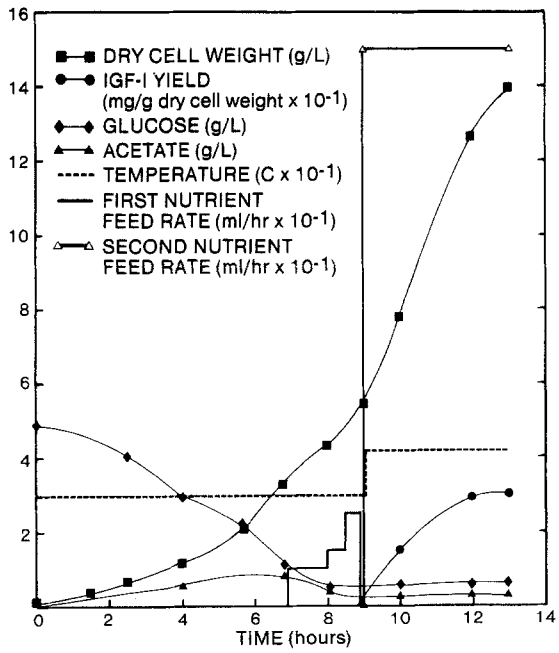

Fig. 2. Fermentation profile for dual feed fed batch fermentation. Two kinds of feed were used in the fed batch fermentation. The first feed (minimal medium) was fed at a cell density of 3 g dry cell weight per liter and increased according to the cell density. The culture was thermoinduced and shifted to second feed at a cell density of 6.5 g dry cell weight per liter. The second feed, which contained 110 g/l of glucose, 110 g/l of yeast extract and 220 g/l of bactotryptone, was fed at a single rate of 150 ml/h.

was very similar to that found in the single feed system with a rate of mass increase of 2.3 g dry cell weight/l/h. The final cell density reached 14 g dry cell weight/l. Little residual acetate or glucose were detected during the induction period (Fig. 2).

In other experiments, the effect of glucose concentration on IGF-1 synthesis was investigated using the dual feed approach described above. The glucose concentration in the second feed medium was adjusted to 55 g/l, 110 g/l and 330 g/l so that under a constant feed rate of 150 ml/h during induction, the level of glucose supplied to the fermentor was in effect 8 g/h, 17 g/h and 50 g/h (Table 3). The level of nitrogen supply was constant at 50 g/h. The results of this experiment are shown in Table 3 and Fig. 3. The results indicate that a glucose feeding rate higher than 17 g/h (the rate used in the experiments of Figs. 1 and 2) did not enhance the yield of IGF-1 (Table 3). The yield of IGF-1 was 30 mg/g of dry cell weight at a final cell density of 20 g dry cell weight/l (Table 3). At the highest glucose feed rate, the acetate level quickly increased to a final level at 9 g/l (Fig. 3; Table 3). The presence of this high a level of acetate did not appear to affect the IGF-1 synthesis. The high level of glucose feeding during the induction period resulted in an increased rate of cell mass accumulation (3.3 g dry cell weight/l/h). The growth finally leveled off due to the high concentration of acetate in the culture (Fig. 3). However, when the glucose feed rate was decreased to 8 g/h (Table 3) during the induction period, the yield of IGF-1 was found to be lower. The growth rate following induction was about 2.3 g dry cell weight/l/h. Approximately 18 mg IGF-1/g cell dry weight was synthesized with the lower glucose feed rate (Table 3). Small inclusion bodies were found in each cell. The low yield of IGF might reflect the negative effect of a lower energy supply which may induce proteolytic activity during the induction period.

The host *E. coli* cell is a wild type which contains the normal complement of heat shock genes [9]. The requirement of the 42°C induction temperature to express IGF-1 in our system induces the heat shock response [5,9]. At least one of the heat shock genes, the *lon* gene, is known to encode for a protease

Table 3

The effect of glucose and organic nitrogen feed rate during the induction period on the expression level of IGF-1

Fermentation	Glucose feed rate at induction (g/h)	Organic nitrogen feed rate at induction (g/h)	Yield of IGF-1 (mg/g dry cell wt.)	Inclusion bodies	Final cell concentration (g dry weight/l)	Final acetate level (g/l)
Single feed	17	5	3	no	13	< 1
Dual feed	8	50	18	yes	17	< 1
Dual feed	17	50	33	yes	14	< 1
Dual feed	50	50	30	yes	20	9
Dual feed	50	0[a]	0	no	15	3

[a] $(NH_4)_2SO_4$ as nitrogen source.

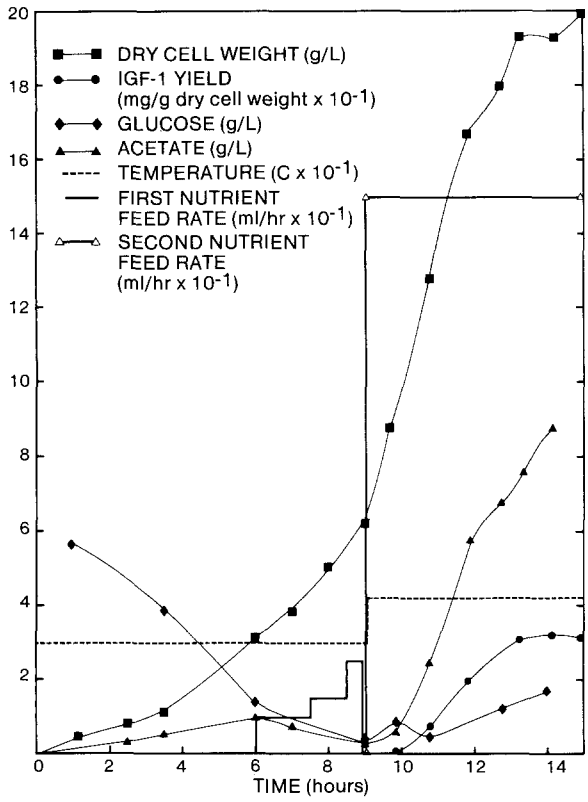

Fig. 3. Fermentation profile of a dual feed fed batch fermentation. Two kinds of feed were used in the fed batch fermentation. The first feed (minimal medium) was used to grow the cells to a density of 6 g dry cell weight per liter. The culture was then thermoinduced and shifted to a second feed medium. The second feed medium which contained 330 g/l of glucose, 110 g/l of yeast extract, and 220 g/l of bactotryptone was delivered at a single rate of 150 ml/h.

which can degrade foreign protein [5,12]. In addition, the presence of the *htp*R gene, that regulates the transcription of heat shock genes in the host cell [10,16], may also trigger the induction of heat shock genes which encode other proteases capable of degrading IGF-1. Buell et al. [2] reported in a similar expression system significant improvement in IGF-1 accumulation by using mutants of *lon*; *htp*R, and *lon, htp*R. The enhancement of IGF-1 synthesis was 2.6-times in a *lon* mutant, 2.3-times in an *htp*R mutant and 4.1-times in a *lon, htp*R mutant [2]. This clearly indicated that the IGF-1 accumulation is affected by the protease activities in the host cells.

In this study, manipulation of fermentation condition was found to affect IGF-1 accumulation. When one compares the single feed system with the dual feed system, the culture was supplied with a much higher concentration of organic nitrogen during the induction period. Thus, the significant increase of IGF-1 expression found with the dual feed system appears to be related to the high level of organic nitrogen present during the induction period. The overall rate of protein breakdown increases many-fold when the bacteria are deprived of a carbon or organic nitrogen source such as amino acids [8]. IGF-1, as a small foreign peptide, may be more susceptible than larger recombinant proteins to proteolytic degradation, particularly in a low organic nitrogen medium and in a medium

where glucose levels are limiting after induction. This hypothesis is supported by the fact that IGF-1 is not synthesized in inorganic nitrogen medium using ammonium sulfate as the source of nitrogen (Table 3). A larger peptide, such as IFN-αCon_1, was expressed at a high level in minimal media [1]. However, it is worthy of note that the expression of IFN-αCon_1 is increased 50–75% by the use of the dual feed method (data not shown). Therefore, it is also possible that the presence of a high concentration of amino acids during the induction period could repress protease activity and enhance expression of larger polypeptides.

Buell has also reported that a high rate of IGF-1 synthesis results in higher levels of IGF-1 accumulation [2]. Our results indicated that the enrichment of organic nitrogen in fermentation media can also result in a 10-fold improvement in IGF-1 accumulation in wild-type *E. coli* host cells. The organic nitrogen used in the dual feed method is in the form of yeast extract and bactotryptone which can directly provide amino acids as precursors and may enhance the rate of IGF-1 synthesis. The formation of inclusion bodies which results from the high expression of IGF-1 may also protect the product from proteolytic degradation in the wild-type host cells.

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