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Novel fed-batch strategy for the production of insulin-like growth factor 1 (IGF-1)

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The production of Long-R^3-IGF-1 (an IGF-1 fusion analog) by constant-rate, fed-batch fermentation of *Escherichia coli* yielded 2.6 g fusion protein/L, corresponding to an actual IGF-1 concentration of 2.2 g/L. A novel strategy employing three distinct feeding stages was developed which raised product concentration to 4.3 g/L (3.6 g/L of IGF-1) while minimising glucose and acetate accumulation. This improved productivity was not accompanied by an increase in inclusion body size.

Introduction

Fed-batch fermentation has been commonly used to achieve high-cell-density cultivation. There are several feeding strategies that can be applied to achieve different goals (Yamane and Shimizu, 1984; Lee, 1996). Exponential feeding has been successfully applied for the high-cell-density culture of several non-recombinant and recombinant *E. coli* strains (Strandberg and Enfors, 1991; Yee and Blanch, 1993; Helmulth *et al.,* 1994) and leads to less acetate formation.

An analog of insulin-like growth factor 1, termed Long-R^3-IGF-1 (Francis *et al.,* 1992), is produced as a solid inclusion body (IB) when expressed at high levels in recombinant *E. coli*. This protein is produced by GroPep Pty Ltd, Adelaide, and sold for use as a mammalian cell-culture supplement. Demand for culture media and consequently this protein is expected to increase as new biopharmaceutical products approach phase III clinical trials. Additionally, the fusion protein can be easily cleaved to give pure IGF-1, which has several clinical uses. There is consequently a need to improve the productivity of the current growth factor production process, preferably through process intensification. A key unit to be optimised for higher yield is the fermenter. The existing strategy uses a constant-rate fed-batch system and achieves approximately 20 g cell dry weight (CDW)/L. The resulting inclusion body is small, with a typical median diameter of 0.3 μm, making separation of the inclusion bodies from the cellular debris difficult following high-pressure homogenisation.

This paper details our initial attempts to increase the fermenter productivity and inclusion body size through improved control and feeding strategies. A series of fermentations was conducted where the feeding strategy was altered. A novel 3-stage feeding strategy was developed. Oxygen transfer was also enhanced through the use of oxygen-enriched air feed.

Materials and methods

Strains and Growth

E. coli strain JM101 [SupE thiD (lac$^-$ proAB) F′[traD36 proAB$^+$ lacI^q lacMZ M15]] containing the strictly-regulated plasmid p[Met^1]-pGH(1–11)-Val-Asn-[Arg^3]-IGF-1 (Francis *et al.,* 1992) was grown on a modified C1 media agar plate (containing 100 mg Amp/L) and incubated at 37°C for 24 h.

Shake Flask Culture

An inoculum for the fermenter was prepared in a shake flask containing 20 mL of sterile modified C1 media. This shake flask was inoculated with a single colony from the plate, then incubated at 37°C in a shaker at 200 rpm for 11 hours.

Fed-batch Fermentation

Fermentation was conducted in a 20-L fermenter with an initial volume of 12 L of modified C1 media (see below). A volume of inoculum was added to the fermenter and left overnight to give 2.4 g CDW/L at 9 am on the following day. The pH of the culture was maintained at 6.9 using 25% ammonia solution. Temperature was 37°C and dissolved O_2 concentration

(DOC) was greater than 60% saturation. Aeration was switched to oxygen-enriched air (mixture of 25% air and 75% oxygen) when the DOC dropped below 60%, at an approximate cell concentration of 3 g CDW/L. Nutrient feeding commenced when glucose exhaustion occurred, at an approximate cell concentration of 13.5 g CDW/L. After 4 h of feeding, the culture was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the fermentation was terminated 5 h later. Two different feeding strategies were employed; constant-rate feeding and a novel 3-stage feeding strategy. For constant feeding, nutrient feed was pumped at a rate of 9.7 mL/min or 11.4 mL/min. 3-stage feeding was PLC-controlled. The initial stage consisted of exponential feeding for 4 h to give a constant specific growth rate of $\mu = 0.25\ h^{-1}$ prior to induction. Immediately following induction, a linearly-decreasing feed rate was used for 0.5 h, followed by exponential feeding for 4.5 h at a specific growth rate of $\mu = 0.1\ h^{-1}$. OD_{600}, dry cell weight (DCW), glucose concentration, and acetate concentration were measured throughout the fermentation. Glucose concentration was measured using a YSI analyser (2700 SELECT, Yellow Springs Instruments, USA). Acetate concentration was determined using an enzyme assay kit obtained from Boehringer Mannheim (Catalog Number: 148261). Inclusion body size was measured using a Joyce Loebl Disc Centrifuge (Middelberg *et al.,* 1990). Recombinant protein concentration was determined by High Performance Liquid Chromatography (Falconer *et al.,* 1997).

Modified C1 media

D-Glucose.H_2O, 2.96 g/L (for shake flask) and 40.0 g/L (for fermenter); NH_4Cl, 2.58 g/L; KH_2PO_4, 2.54 g/L; Na_2HPO_4, 4.16 g/L; K_2SO_4, 1.94 g/L; $MgSO_4.7H_2O$, 0.67 g/L; $FeSO_4.7H_2O$, 20 mg/L; $MnSO_4.H_2O$, 5.0 mg/L; $ZnSO_4.7H_2O$, 8.6 mg/L; $CuSO_4.5H_2O$, 0.76 mg/L; trisodium citrate, 88 mg/L; thiamine, 48 mg/L.

Nutrient feed

D-Glucose.H_2O, 620 g/L; KH_2PO_4, 5.3 g/L; Na_2HPO_4, 79.0 g/L; K_2SO_4, 45.0 g/L; $MgSO_4.7H_2O$, 8.24 g/L. In yeast extract-supplemented nutrient feed, nutrient feed as above was supplemented with 65 g/L yeast extract.

Results and discussion

Fermentation profiles for the constant-rate feeding strategy are presented in Figure 1. Glucose accumulated to high concentrations (up to 40 g/L), and relatively low cell-densities were obtained. The highest OD_{600} previously achieved using this standard production method was approximately 80 (unpublished data). High acetate accumulation following induction is also typically observed (Figure 1). An inclusion body size of 0.32 μm and a fusion-protein concentration of 2.6 g/L, corresponding to an actual IGF-1 concentration of 2.2 g/L, were obtained for the fermentation reported in Figure 1a.

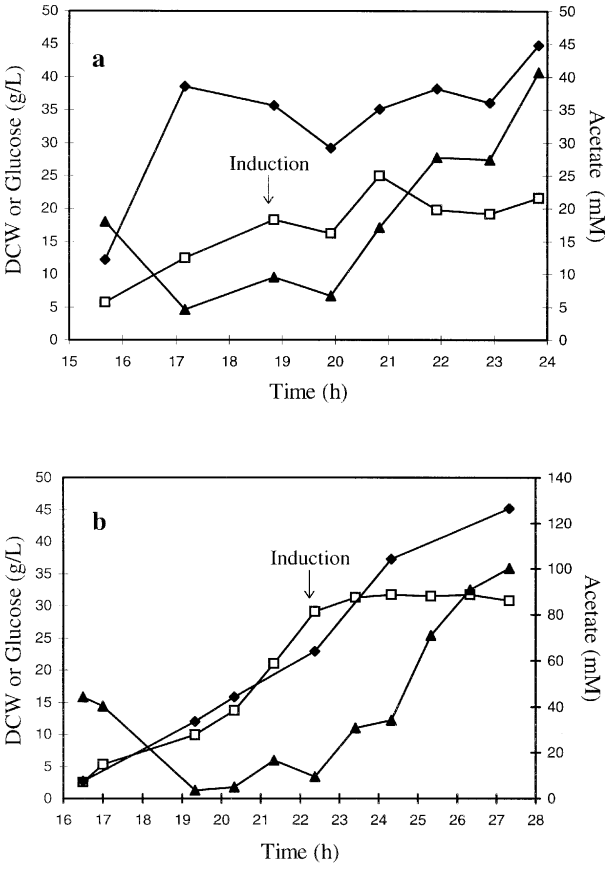

Figure 1 Fed-batch fermentation profiles using constant-rate feeding. □, dry cell weight (DCW); ◆, acetate concentration; ▲, glucose concentration. (**a**) feed rate = 9.7 mL/min; (**b**) feed rate = 11.4 mL/min.

The high acetate and glucose concentrations in Figure 1 are due to a rapid decrease in specific growth rate following induction (Lee and Ramirez, 1992). A feeding strategy employing three different stages was therefore designed to overcome this problem. In stage 1, a high specific growth rate ($0.25\ h^{-1}$) was used to generate a high cell density before induction, and hence a large cell population for the production of recombinant protein. In stage 2, a rapidly-decreasing feedrate that is designed to approximate the rapid drop in metabolic activity following IPTG induction was employed. Finally, the lower and variable metabolic activity following induction was approximated with a glucose

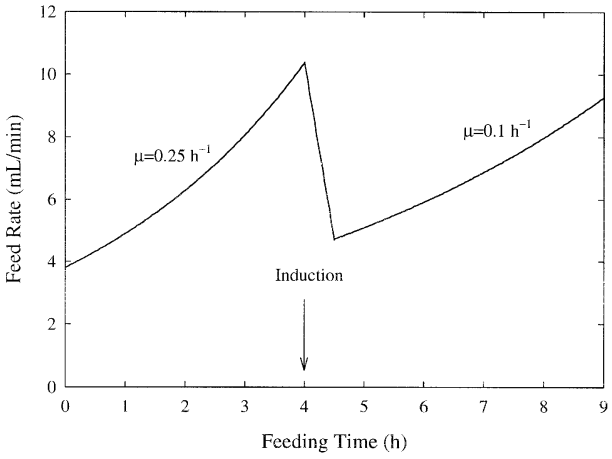

Figure 2 The novel 3-stage feeding strategy.

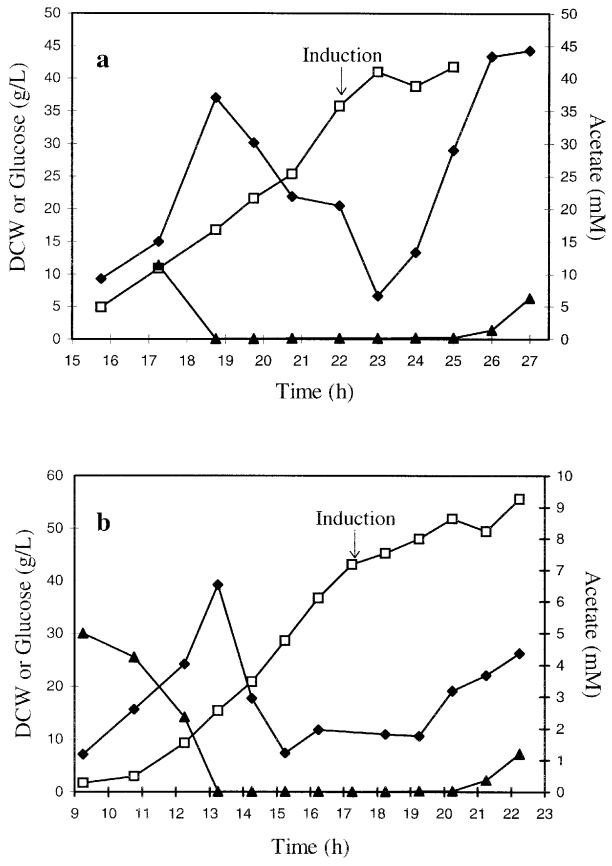

Figure 3 Fed-batch fermentation profiles using exponential feeding. □, dry cell weight (DCW); ◆, acetate concentration; ▲, glucose concentration. (**a**) without yeast-extract supplementation; (**b**) with yeast-extract supplementation.

feed rate designed to support a constant specific growth rate of 0.1 h^{-1}. The feed profile is summarised in Figure 2. Stages 2 and 3 are an approximation to the changes in growth rate observed due to induction shock, as described by the shock-recovery model of Lee and Ramirez (1992).

The fermentation profiles for 3-stage feeding are presented in Figure 3a. An OD_{600} of 150, corresponding to a dry cell weight of 42 g/L, was achieved. This was nearly double that obtained using constant-rate feeding. Glucose accumulated in fermentation broth was decreased to 6.2 g/L, approximately 15% of the glucose level using constant-rate feeding. This method therefore overcomes glucose accumulation problems. After induction, acetate gradually accumulated and reached a level of 44 mM at the end of fermentation. This acetate concentration is still excessive, but can be reduced by further optimisation of the feed profile. For example, the drop in metabolic activity post induction could be better approximated using three stages: a linearly decreasing stage; a stage with μ = 0.1 h^{-1}; and a stage with some constant μ < 0.1 h^{-1}.

A recombinant protein concentration of 4.3 g/L corresponding to an actual IGF-1 concentration of 3.6 g/L was achieved, with an inclusion body size of 0.33 μm. The recombinant protein concentration was much higher than that obtained using constant-rate feeding. There was, however, no improvement in inclusion body size. This suggests the increase in total product is solely due to the increased biomass concentration. There are several possible reasons why we did not see an increase in the specific ratio of product to biomass. Further optimisation of the feed profile to restrict acetate below the inhibitory concentration may be necessary. Optimisation of other key parameters (e.g., IPTG concentration) may also be necessary, although previous tests suggest relative insensitivity (data not shown). We have previously observed translational limitation for this strong promoter (Jorgensen *et al.,* 1997), so this is a likely limit to further productivity increases. To test this, a 3-stage fed-batch fermentation with yeast-extract-supplemented nutrient feed after induction was conducted.

The fermentation profiles for exponential feeding with yeast-extract-supplemented nutrient are presented in Figure 3b. An OD_{600} of 163, corresponding to a dry cell weight of 56 g/L, was achieved. This was higher than that obtained using exponential feeding without yeast extract supplementation. Glucose accumulation was reduced to a level of 7.3 g/L. After induction, acetate gradually accumulated and reached a level of

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4.4 mM at the end of fermentation. This is much lower than for the previous fermentations. A recombinant protein concentration of 3.7 g/L, corresponding to an IGF-1 concentration of 3.0 g/L, and an inclusion body size of 0.32 μm were achieved. Yeast extract supplementation did not improve inclusion body size. A lower recombinant protein level was also obtained. The presence of yeast extract may enhance the growth of cells and thus reduce the rate of protein expression, or may simply alter the amino acid balance. This will influence the metabolic flux through amino acid pathways and may reduce overall protein expression.

The novel 3-stage feeding procedure reported in this paper improved the productivity of IGF-1 fusion protein using recombinant *E coli* JM101. Glucose and, consequently, acetate accumulation were greatly decreased. Recombinant fusion protein concentration was increased from 2.6 g/L to 4.3 g/L, corresponding to an actual IGF-1 concentration of 3.5 g/L. However, inclusion body size was not altered by the novel feeding strategy nor by the supplementation of yeast extract. Further investigation of factors altering inclusion body size and protein expression level, and further optimisation of the feeding strategy, has the potential to further increase the already-high IGF-1 concentration achieved in this study.

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