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High-cell-density fermentation studies of a recombinant *E. coli* that expresses atrial natriuretic factor

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SUMMARY

Studies are presented on the fermentation of recombinant *Escherichia coli* that express rat atrial natriuretic factor (ANF) as a fusion protein. Our objective was to achieve high cell density while maintaining ANF expression at the same level as observed in shake flasks. Improved fermentation conditions included: maintaining glucose concentrations at 1 g/l, using an enriched medium, adding concentrates of medium throughout the fermentation, and blending oxygen for adequate aeration. Cell densities of 12 g/l (dry weight) were achieved, which represented a 10-fold increase over non-improved conditions, while maintaining ANF levels at 7 mg/g of dry cell mass. When galactose was used as an initial carbon source or as a feed supplement, there was a 2–3-fold increase in the expression of ANF from these high-cell-density fermentations. The recombinant ANF was biologically active.

INTRODUCTION

Recombinant DNA technology coupled with large-scale fermentation of genetically engineered organisms has provided the opportunity to derive large quantities of proteins that would be difficult or impossible to obtain otherwise. One such compound is atrial natriuretic factor (ANF). This polypeptide hormone is produced by heart atrial muscle and has diuretic and hypotensive action [4,5]. Through the application of genetic engineering, the gene for rat ANF has been cloned in *E. coli* [13,14,17,20].

Traditionally, batch fermentations of *E. coli* have yielded only 1–2 g/l (dry weight), due to limitations in nutrients and oxygen. By using a fed-batch fermentation process, one can achieve a significant increase in cell mass. However, there has been little published information on the application of fed-batch techniques to *E. coli* expressing foreign genes.

The objective of our studies was to express a recombinant-derived fusion protein containing atrial natriuretic factor (ANF) at commercially econom-

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ical levels, requiring a cell yield of approximately 8 g/l dry weight for a recombinant *E. coli*. To achieve this concentration of cells, we utilized a fed-batch approach. In addition, cell yields of a non-expressing host strain and a wild-type *E. coli* were compared to the organism expressing the ANF.

MATERIALS AND METHODS

Bacteria and plasmids

Wild-type *E. coli* strain W was obtained from the ATCC (Cat. No. 9637). Plasmid p180 is derived from pUC13 [18] and encodes the amino-terminal 88 amino acids of the *E. coli* CheY protein [10] fused to the rat ANF gene by a synthetic oligonucleotide linker. Transcription of the CheY-ANF-gene is under control of the *lac* promoter-operator region of pUC13.

Media

Luria agar plates were obtained from REMEL (Lenexa, KS). Modified "L" broth used for seed flask cultures contains (per liter): yeast extract (Difco) 5 g, Hy Soy® peptone (Sheffield) 10 g, and NaCl 10 g. The compositions of culture media are listed in Table 1. Medium formulations 1 and 2 are modifications of Luria [9] and Bauer [2], respectively. Formulation 3 is essentially a combination of formulations 1 and 2 and is based in part upon considerations of mass balance for a high-cell-density fermentation of *E. coli* [6]. UCON LB625 (Union Carbide) served as the antifoam agent.

Table 1

Formulations of media[a]

Component	Formulation 1 (modified Luria)	Formulation 2 (modified Bauer)	Formulation 3
Yeast extract	10.0 g	5.0 g	10.0 g
Hy Soy® peptone	20.0 g	–	25.0 g
K_2HPO_4	2.5 g	3.1 g	3.5 g
KH_2PO_4	–	3.1 g	3.5 g
$(NH_4)_2SO_4$	–	–	3.0 g
$MgSO_4 \cdot 7H_2O$	–	2.0 g	2.0 g
Glucose	2.5 g	5.0 g	30.0 g
NaCl	15.0 g	–	–
NH_4Cl	–	1.0 g	–
UCON LB625	2 ml	2 ml	2 ml
Trace elements solution [2]	3 ml	3 ml	3 ml

[a] Quantities per liter.

Mass culture

Seed inocula were prepared by streaking a frozen stock culture onto agar plates and incubating the plates at 35°C for 18 h. Wild-type and host strains were streaked onto Luria plates without ampicillin, while JM105(p180) was streaked onto ampicillin-containing Luria plates. As a seed flask, 1 l of modified "L" broth in a 2 l Erlenmeyer flask was inoculated with a loop of bacteria and incubated at 35°C, 300 rpm for 18 h. For inoculation of fermenters, 100 ml of broth from the seed flask was used. The 2 l fermenters (New Brunswick Scientific Co. Model C-32), having a working volume of 1.25 l, were operated at 35°C, 600 rpm, and 1 l/min air flow. Dissolved O_2 was maintained at 20–50% of saturation by blending pure O_2 into the air stream as needed. The pH was maintained at 7.0–7.5 during the fermentation through the controlled addition of 9 N H_3PO_4 and 14.8 M NH_4OH. Following the initial consumption of glucose down to 1 g/l, the glucose concentration was maintained between 0.2 and 1.2 g/l by adding a 25% (w/v) solution of glucose. In all fermentations of JM105(p180), expression of recombinant ANF was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1–10 mM and increasing the pH to 7.8–8.0. For the JM105 host strain and the wild-type culture, no IPTG was added. In fed-batch fermentations, media concentrates were added after growth had slowed. Total fermentation times ranged from 24 to 30 h.

Analytical

Cell growth was monitored by measuring absorbance at 660 nm (A_{660}). Glucose concentrations were measured with a Beckman Glucose Analyzer 2. Dry cell weight was determined by filtration of

culture fluids followed by drying of the cells for 5 min in a microwave oven. Cell weights reported in this paper are g/l dry weight.

The relative abundance of fusion protein in cell pellets was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [8] followed by staining with Coomassie Brilliant Blue. Cell pellets equivalent to 5 A_{660} units were resuspended in 200 μl to a final concentration of 0.125 M Tris (pH 6.8), 1% SDS, 10% glycerol, 0.004% bromophenol blue, 0.02 M DDT, and then boiled for 5 min. Purified ANF was quantitated through analysis by high performance liquid chromatography (HPLC) and tested for biological activity [16].

RESULTS

Batch fermentations of recombinant *E. coli* using modified "L" broth typically have produced cell yields of 1–2 g/l (Table 2). To increase the cell mass, a fed-batch process was developed using different culture media (Table 1). Use of the modified Bauer formulation supported a final yield of 9 g/l, while use of the modified Luria medium provided a yield of 8 g/l (Table 2). To achieve even greater densities, a third formulation was used (formulation 3), yielding a mass of 12 g/l. In order to further increase either cell mass or expression of the fusion protein, formulation 3 was supplemented with purines and pyrimidines [19]. A final concentration of 10 mg/l each of adenine, guanine, thymine, cytosine, and uracil was added at 9 h post-inoculation. When the growth rate had begun to decline at 10.5 h post-inoculation, an additional 40 mg/l concentration of each component was added. Neither an increased yield of cells nor an increased expression of ANF was achieved (data not shown).

Since the activity of the *lac* promoter is repressed by glucose, we examined the effect of galactose on the expression of ANF. In the first experiment (galactose fermentation No. 1), glucose was used as the

Table 2

Cell yields in different media using batch and fed-batch fermentations with JM105(p180)

Media formulation	Type of fermentation	Final optical density (A_{660})	Cell yield (g/l)
Modified "L" broth	batch	4.9	2
1 (modified Luria)	fed-batch	22.0	8
2 (modified Bauer)	fed-batch	20.0	9
3	fed-batch	22.0	12
3 (containing galactose)[a]	fed-batch	25.0	10
3 (containing galactose)[b]	fed-batch	25.0	9

[a] Galactose fermentation No. 1 (see Results).
[b] Galactose fermentation No. 2 (see Results).

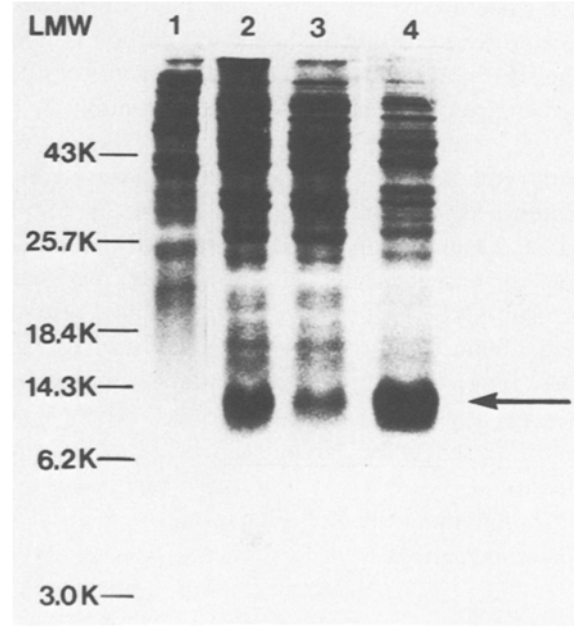

Fig. 1. Expression of recombinant-derived fusion protein during fed-batch fermentations of *E. coli*. Equally sized pellets of *E. coli* were prepared in SDS-PAGE sample buffer, electrophoresed in 12.5% polyacrylamide gels [8], and stained for protein with Coomassie Brilliant Blue. Proteins are from (1) untransformed JM105 in formulation 3, (2) JM105(p180) in formulation 1, (3) JM105(p180) grown in formulation 3, and (4) JM105(p180) in formulation 3 with 10 g/l galactose in place of glucose (galactose fermentation No. 2). The arrow indicates the fusion protein. Molecular weight standards, in daltons, are ovalbumin (43 K), α-chymotrypsinogen (25.7 K), β-lactoglobulin (18.4 K), lysozyme (14.3 K), bovine trypsin inhibitor (6.2 K), and α and β insulin chains (3 K).

initial carbon source and was added to maintain a concentration of 1–2.5 g/l. After 9 h, when the cells were still growing and glucose had been depleted, galactose was added to a concentration of 10 g/l; the fermentation then continued for an additional 18 h. In the other experiment (galactose fermentation No. 2), galactose was used as the initial carbon source and at the same concentration as glucose. During the first 9 h, galactose was added simultaneously whenever glucose was added to the first fermenter. At 9 h, 10 g/l galactose was added and the fermentation continued for an additional 18 h. In both experiments, the cell mass and expression of ANF were similar (Table 2 and Fig. 1). However, the expressed fusion protein was 2–3-times more abundant when cells were grown on either glucose plus galactose or galactose alone than when cells were grown using glucose as the sole carbon source (Fig. 1). Fig. 1 shows an SDS-PAGE profile of cell extracts from selected fed-batch fermentations.

We observed that pH was critical for ANF expression; if the pH dropped below 7.5 during induction, expression was not detectable by SDS-PAGE. Quantitation by HPLC resulted in a final yield for non-galactose fed fermentations of 7 mg purified ANF per g of dry cell mass. These results were obtained consistently (data not shown) for shake flask, batch, and fed-batch fermentations. In experimental studies, this purified ANF was demonstrated to be biologically active (data not shown).

To determine whether cell densities were a function of the particular host strain used, fed-batch fermentations were performed with the untransformed JM105 host strain and wild type *E. coli* using formulation 3 (Table 3). Both JM105(p180) and the untransformed JM105 produced identical cell yields of 12 g/l. When the wild-type culture was grown under the same conditions, the yield was 30 g/l, a 2–3-fold increase in mass above that of the cultures of JM105.

Table 3

Cell yields of three strains of *E. coli*

Strain	Final optical density (A_{660})	Cell yield[a] (g/l)
E. coli W	52	30
JM105	23	12
JM105(p180)	22	12

[a] Cells were grown in fed-batch fermentations utilizing formulation 3.

DISCUSSION

We have demonstrated that by utilizing an improved culture medium and periodically feeding nutrients, a cell yield of 12 g/l could be achieved by recombinant *E. coli* that express ANF. This is in contrast to the 1–2 g/l yield for a batch fermentation. By applying a fed-batch approach, nutrient limitation is overcome and a significant increase in cell mass can be obtained.

When fed-batch methods have been applied to non-recombinant *E. coli*, cell yields of 55–125 g/l have been reported [12,15]. Genetically engineered organisms have existed for a relatively short time and generally are used in proprietary applications. Therefore, little published information exists on the application of fed-batch techniques to *E. coli* expressing foreign genes. Using a gradient feeding technique, Allen and Luli [1] cultivated recombinant *E. coli* to a yield of 80 g/l. However, no mention is made of induction or expression of a foreign gene product. Fieschko et al. [7] have reported a cell yield of 18 g/l for an *E. coli* that expressed a human alpha interferon analogue. Mizutani et al. [11] achieved high-cell yields of 63 g/l with an *E. coli* strain that expressed a leucine gene; however, at this density the specific activity (S.A.) of the gene product was depressed severely due to high β-lactamase activity that degraded penicillin and thus removed selective pressure. To achieve a reasonably high cell yield of 32 g/l and improve S.A., they added additional leucine to the medium. Thus, by making various changes in culture medium, they demonstrated that good cell yields and gene expression could be obtained.

Similarly, when we experimented with the ad-

dition of galactose in place of glucose, a 2–3-fold increase in ANF expression occurred. The resultant cell yields were equivalent to those obtained when glucose was the carbon source. The increased ANF expression with galactose is probably due to the availability of a non-repressing carbon and energy source during induction.

To ensure that the presence of the recombinant plasmid was not compromising cell growth, the untransformed host strain was grown under the same fed-batch conditions as JM105(p180); both cultures grew similarly. However, when a wild-type *E. coli* was grown under the same conditions, the cell mass obtained was more than twice that of the recombinant strains, indicating that the limitation on increased mass was strain-dependent. Neither the presence of the plasmid nor the increased expression of fusion protein adversely affected cell growth in the presence of galactose. The effect of increased protein expression upon cell yield is unpredictable. However, for many applications, wild-type *E. coli* may be unsuitable in not permitting tightly regulated expression of plasmid-borne promoters. For applications in which regulated expression of a plasmid-borne *lac* promoter is desired, a strain such as JM105, which carries the $lacI^q$ mutation, is suitable [18].

Since one goal of this project was to achieve an economical process, one must examine the costs of fermentation and isolation. The cost for galactose is substantially higher (10×) than the cost of glucose. However, based upon other commercial processes, the increased cost of a medium component has a negligible effect on the total cost of the process. This is especially valid when the medium component can enhance gene expression of a high-value product such as ANF and still provide suitable cell yields.

During these studies, we demonstrated that cells grown either in batch or fed-batch processes using glucose as a carbon source had equivalent expression of ANF. By cultivating cells to high cell densities, protein expression was not affected. A higher specific activity was achieved when cells were grown on galactose instead of glucose. Cell densities were similar for both glucose- and galactose-grown cultures. These studies indicate that a high-cell-density culture of recombinant *E. coli* can be grown and ANF can be purified in yields which are commercially acceptable.

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