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Enhancing toxic protein expression in *Escherichia coli* fed-batch culture using kinetic parameters: Human granulocyte-macrophage colony-stimulating factor as a model system

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The kinetics of recombinant human granulocyte-macrophage colony-stimulating factor (hGM-CSF) expression was studied under the strong T7 promoter in continuous culture of *Escherichia coli* using complex medium to design an optimum feeding strategy for high cell density cultivation. Continuous culture studies were done at different dilution rates and the growth and product formation profiles were monitored post-induction. Recombinant protein expression was in the form of inclusion bodies with a maximum specific product formation rate (q_p) of 63.5 mg g^{-1} DCW h^{-1} at a dilution rate (D) of 0.3 h^{-1}. The maximum volumetric product concentration achieved at this dilution rate was 474 mg l^{-1}, which translated a ~1.4 and ~1.75 folds increase than the values obtained at dilution rates of 0.2 h^{-1} and 0.4 h^{-1} respectively. The specific product yield ($Y_{P/x}$) peaked at 138 mg g^{-1} DCW, demonstrating a ~1.6 folds increase in the values obtained at other dilution rates. A drop in q_p was observed within 5–6 h of induction at all the dilution rates, possibly due to protein toxicity and metabolic stress associated with protein expression. The data from the continuous culture studies allowed us to design an optimal feeding strategy and induction time in fed-batch cultures which resulted in a maximum product concentration of 3.95 g l^{-1} with a specific hGM-CSF yield ($Y_{P/x}$) of 107 mg g^{-1} DCW.

[**Key words:** *Escherichia coli*; Continuous culture; Recombinant human granulocyte-macrophage colony-stimulating factor; Specific product formation rate; Fed-batch; High cell density]

Human granulocyte–macrophage colony-stimulating factor (hGM-CSF) is a hematopoietic growth factor that mediates differentiation and proliferation of granulocyte and macrophage colonies in the bone marrow (1). This lymphokine plays a vital role in various functions of the immune system, including responses to inflammation and infection, as well as in hematopoiesis (2). Therefore, a variety of potential clinical applications of this cytokine are as a vaccine adjuvant in immunotherapy of various malignancies or to reduce infection risk during bone marrow transplantation via acceleration of neutrophil formation (3). There are no reports of a systematic study of the kinetics of hGM-CSF expression and its large scale production in *Escherichia coli* at bioreactor level due to its extremely poor expression in various recombinant hosts ranging from bacterial, plants, insects to mammalian cells due to its toxicity toward host cells (4–7).

E. coli remain the workhorse for the production of recombinant proteins; however, the wide variability in proteins expression levels play a critical role in process scalability during high cell density cultivation (8). Fed-batch cultures have been used to achieve high cell densities to improve the volumetric productivity of various "difficult to express" proteins (9,10). In some studies, it has been reported that the induction of recombinant protein synthesis strongly inhibits the specific substrate and oxygen uptake rate of host cells (11). In recombinant *E. coli* cultures, a strong correlation between higher pre-induction specific growth rate (μ) and specific product formation rate (q_p) has been reported by many groups (12–14). However, in other reports, recombinant protein expression declined with increase in pre-induction μ (15,16), while others found no correlation between μ and q_p (17,18). Thus, the correlation between μ and q_p appears to be empirical and specific to each recombinant system depending on the nature of the protein, promoter and host–vector combinations (19). Moreover, heterologous protein expression triggers a stress response in the host cell and thus, it is very difficult to control post-induction specific growth rate which often becomes an intrinsic property of the host cell (20). It has also been shown that the biomass concentration at the time of induction can have considerable effects on the final product yields (21,22).

The availability of an exogenous supply of essential nutrients like amino acids is also a critical factor in recombinant protein expression since amino acid biosynthesis is often the rate limiting step in protein synthesis (23). Therefore, designing an appropriate fed-batch strategy with an optimized feed medium composition has an important role in determining final productivities (24). However, the nature of stress when recombinant protein

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expression is in the form of inclusion bodies (25,26) is quite different from that of expression in soluble form (27). Toxic proteins which interfere with the host cell machinery tend to reduce, or even in some cases stop the cell growth post-induction (28,29). However, in many cases, it is neither the inducer concentration nor the metabolic load but the amino acid composition that determines the final yields of recombinant protein (30).

We have previously reported an efficient expression system for high-level expression of recombinant hGM-CSF in shake-flasks culture (31). To overcome the toxic effects, we used a host–vector combination approach which produced hGM-CSF as inclusion bodies thereby blocking its interaction with the host cell machinery. In spite of significant improvement in the productivity, the toxic effects persist as evident from the sharp fall in growth rate post-induction. In present study therefore, we attempted to design bioprocess strategies which would take care of these toxic effects while scaling up expression to the bioreactor level. Measurement of post-induction kinetic parameters of growth and product formation in transient state continuous culture was done at different dilution rates ($D = 0.2$ h^{-1}, 0.3 h^{-1} and 0.4 h^{-1}) to design a robust feeding and induction strategy to achieve gram level hGM-CSF production in *E. coli* fed-batch cultures.

MATERIALS AND METHODS

Host strains and expression vector *E. coli* DH5α strain (Amersham Biosciences, USA) was used for plasmid propagation, while expression studies were conducted in *E. coli* BLR(DE3) cells (Novagen, USA). The expression plasmid pETA-GM having the hGM-CSF gene under the T7 promoter and asparaginase gene signal sequence at the N-terminus has been reported earlier where protein expression was obtained in the form of inclusion bodies (31). Moreover, in our previous report the purification and refolding steps were optimized which resulted to a refolding and purification yield of 45% and 95%, respectively (31).

Cultivation media and chemicals *E. coli* cells were grown routinely in LB medium containing 10 g l^{-1} bactotryptone, 5 g l^{-1} yeast extract and 5 g l^{-1} NaCl. The batch and feed media used during continuous culture studies (CSTR) was TB glucose medium which contained 24 g l^{-1} yeast extract, 12 g l^{-1} bactotryptone and 5 g l^{-1} glucose, pH 7.2 whereas 100 ml of separately autoclaved buffer (2.31 g l^{-1} KH_2PO_4, and 12.54 g l^{-1} K_2HPO_4) was added to make up the final volume to l L. For antibiotic selection, kanamycin was added at a concentration of 75 mg l^{-1}. All media constituents were obtained from Hi-Media, India, while kanamycin and IPTG were purchased from Sigma–Aldrich, USA.

Cell biomass and glucose measurement Cell density was measured by dry cell weight (g DCW l^{-1}) via drying the PBS washed cell pellet from a 1 ml culture to a constant weight at 80°C. The cell concentration was also monitored by OD_{600} values measured in triplicates and average values had been reported. The linearity of the relationship between OD_{600} and DCW was invariant during different cultivation strategies. The glucose concentration in the fermentation broth was measured using a glucose meter in mg dl^{-1} (Boehringer Mannheim, USA).

SDS-PAGE analysis For SDS-PAGE analysis, 50 μl of the induced culture was pelleted by centrifugation and re-suspended in 80 μl MilliQ water after which 20 μl of the standard 5× denaturing gel loading buffer [25 mM Tris–HCl (pH 6.8), 0.1 M 2-mercaptoethanol, 10% SDS and 50% glycerol] was added. Samples were heated at 100°C for 5 min and 10 μl of the prepared samples were loaded on the gel.

hGM-CSF quantification using ELISA The hGM-CSF ELISA Duo set (R&D system, USA) was used for the quantification of recombinant protein as per the manufacturer's instructions. The hGM-CSF concentration was quantified using a standard curve for hGM-CSF (R&D system) with appropriately diluted samples to measure readings in the linear range of ELISA. Samples were taken in triplicate and the standard experimental error was approximately ±5%. The detailed preparation of samples had been described earlier (32).

Bioreactor operation as CSTR CSTR studies were conducted in TB glucose medium in a 2 L bioreactor using 1 L working volume with pH and dissolved oxygen concentration (DO) control (in cascade with agitation rate, i.e., rpm and gas mixing). For primary inoculum development, the *E. coli* BLR(DE3) cells containing the pETA-GM plasmid were inoculated in 15 ml TB glucose medium containing 1.5× (75 mg l^{-1}) kanamycin and grown at 37°C with constant shaking at 250 rpm (rotation per minute) for 12 h. For secondary inoculation, 5 ml of this overnight grown culture was further inoculated in 100 ml TB glucose medium containing kanamycin and grown till a dry cell weight of 1.2 g l^{-1} ($OD_{600} \sim 3$) for seed culture to the bioreactor. This seed culture was used to inoculate a bioreactor containing 900 ml of TB glucose medium and 100 ml buffer with 1.5× of kanamycin. Cells were grown in batch mode till $OD_{600} \sim 10$ after which feeding was started at the pre-determined dilution rates (i.e., $D = 0.2$, 0.3 and 0.4 h^{-1}; $D = F/V$, where F is flow rate in ml h^{-1} and V is the working volume of the bioreactor). Simultaneously, the outflow pump was started and the system was allowed to reach pre-induction steady state. Approximately five reactor volumes were allowed to flow through to obtain a pre-induction steady state which could be checked by constant biomass concentration (OD_{600}), agitation rate in rpm, residual glucose concentration and the dissolved oxygen concentration (DO) values. The cultivation studies were conducted at pH 7.2 and 37°C with a constant air flow rate of 1 vvm (v v^{-1} min^{-1}) and the DO was controlled at 40% saturation. Throughout induction phase, a 0.5 mM IPTG concentration was maintained in the feed medium.

Bioreactor operation as fed-batch The batch and fed-batch cultivation studies were carried out in an Infors AG computer controlled 2 L bioreactor with 1 L working in starting. The working volume of bioreactor was 1 L. The batch medium contained TB glucose medium having 75 mg l^{-1} of kanamycin. The cultivation conditions for inoculum preparation were similar to CSTR. For fed-batch operation, the feeding rate (9% yeast extract, 9% tryptone, 13.5% glucose) was increased exponentially to maintain a specific growth rate of 0.3 h^{-1} by using a variable speed peristaltic pump. The feed was increased using an exponential feeding equation ($F = F_0\, e^{\mu t}$), where F_0 is the initial flow rate, F is the flow rate at any given time, μ is specific growth rate (i.e., 0.3 h^{-1}) and t is time in hours. As per the requirement, the air flow was supplemented with pure oxygen to maintain a constant dissolved oxygen concentration of 40% to avoid any oxygen limitation at high cell density. The substrate uptake rate fell continuously in the post-induction period and thus the post-induction feeding rate was also reduced gradually so as to control the residual glucose concentration around 100 mg dl^{-1} to avoid acetate accumulation in culture.

RESULTS

Production of hGM-CSF in batch culture In batch fermentation, cells were grown in 1 L TB glucose medium till a biomass concentration of 4 g l^{-1} ($OD_{600} = 10$) after which induction was

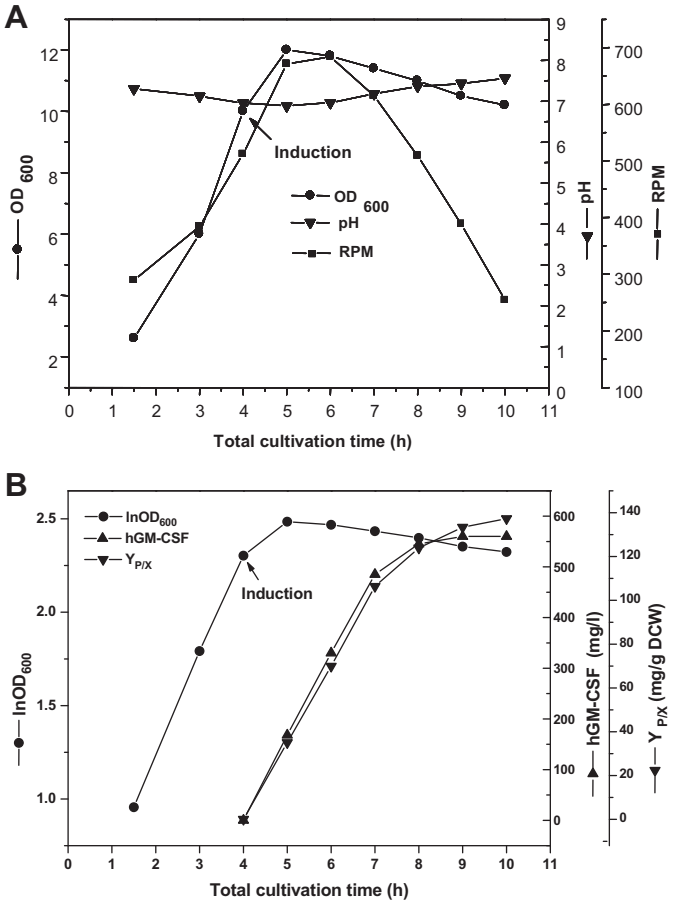

FIG. 1. (A) Time profile of growth (OD_{600}), pH and rpm in batch cultivation of *E. coli* BLR(DE3) cells having pETA-GM recombinant plasmid and (B) plot showing growth ($lnOD_{600}$), volumetric and specific product concentration of hGM-CSF in *E. coli* batch culture induced at OD_{600} of 10.