

Short Communication

Optimization of secretory expression of recombinant hGM-CSF in high cell density cultivation of recombinant *Escherichia coli* using Taguchi statistical method

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Abstract

Human granulocyte macrophage colony stimulating factor (hGM-CSF) has many therapeutic applications. In this study, in order to verify the purification process, the effect of carbon source, IPTG concentration and post-induction time on the secretion of recombinant hGM-CSF into the culture medium by recombinant *Escherichia coli* during high cell density cultivation were evaluated by using the Taguchi statistical method. The results indicated that glucose, 1mM IPTG and a time of 6 h post-induction, represented optimum conditions. The secreted hGM-CSF, overall volumetric productivity and purified hGM-CSF were 373 mg/l, 18 mg/l/h and 63 mg/l, respectively.

Keywords: *Escherichia coli*; Fed-batch; High cell density cultivation; Human granulocyte macrophage colony stimulating factor; Optimization; Purification

Human granulocyte macrophage colony stimulating factor (hGM-CSF) is a glycoprotein that stimulates the proliferation and differentiation of granulocyte and

macrophage progenitor cells (Metcalf, 1985). The hGM-CSF has considerable therapeutic applications such as treatment of aplastic anemia, myelodysplastic syndrom and AIDS. It is also used after cancer chemotherapy and bone marrow transplantation and inhibits the progress of breast cancer (Klingemann 1989; Eubank *et al.*, 2009).

High cell density cultivation (HCDC) is a powerful technique to maximize volumetric productivity during recombinant protein production. The combination of HCDC with recombinant DNA technology has made it possible to produce recombinant proteins, such as GM-CSF, in amounts suitable for different purposes (Shojaosadati *et al.*, 2008). Fed-batch cultivation is the most commonly used technique to obtain high cell densities within short cultivation times (Chuanliu *et al.*, 2000; Shojaosadati *et al.*, 2008).

Escherichia coli is one of the most studied and well characterized microorganism in terms of its molecular genetics, physiology and expression systems, and it is also a commonly used host for laboratory and industrial recombinant protein production (Choi and Lee 2004 a, b; Matsui *et al.*, 2008). Among various statistical experimental designs, the Taguchi method offers distinct advantages by which several factors can be examined simultaneously and much quantitative information can be obtained with a few experimental trials

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(Yari *et al.*, 2009). In this study, in order to verify the purification of the recombinant hGM-CSF secreted into the culture medium, some variables were optimized during HCDC using the Taguchi statistical method.

The *Escherichia coli* BL21 (DE3) strain and chemically inducible expression vector pET (+26) containing the *gm-csf* gene and also the *pelB* signal sequence, were used for secretory expression of the hGM-CSF (Zomorodipour *et al.*, 2003). Inoculum preparation was carried out using sub-cultivation of recombinant *E. coli* on LB-agar. A single colony from the resulting LB culture was transferred to a 1 liter shake flask containing 200 ml of defined F medium (Table 1), incubated for 12 h at 30°C with shaking at 180 rpm and was used for the subsequent inoculation of the bioreactor. Solid and liquid media were supplemented with Kanamycin (30 µg/ml) as a selective marker.

Fed batch culture was carried out in a stirred tank Bioflo 3000 bioreactor (New Brunswick Scientific, USA) containing 2 liter of F medium (Table 1) at 30°C. The bioreactor was capable to control the pH, temperature, agitation, dissolved oxygen and level. The pH of the fermentation medium was adjusted to 6.9 by automatic addition of aqueous ammonia (25% w/w) and HCl (20% v/v). Dissolved oxygen (DO) was maintained at 40% saturation by blending of air and oxygen and agitation speed range from 500 to 900 rpm. Foaming was prevented by adding sterilized silicon oil as antifoam. At the end of the batch process, as indicated by the increase in DO and pH, feeding (Table 1) was initiated with an exponential strategy and using a

fixed volume operation (Fatemi *et al.*, 2004). In order to induce recombinant hGM-CSF expression, different concentrations of IPTG were used.

At the end of the fermentation process, culture medium was separated from the biomass by centrifugation at 4000 rpm for 10 min. The supernatant and biomass contained soluble recombinant hGM-CSF secreted into the medium and the periplasmic space, respectively.

Cell density was determined by measurement of optical density at 600 nm (one unit of OD is equal to 0.5 g cell dry weight/l) (Fatemi *et al.*, 2004). Glucose concentration was analyzed using an enzymatic kit (Chem Enzyme, Iran). Expression analysis was performed by SDS-PAGE using the Laemelli method (Rastegar-jazii *et al.*, 2007) and gels were visualized by staining with Coomassie Brilliant Blue R-250. Recombinant hGM-CSF concentration was determined using high performance liquid chromatography (HPLC) by applying a C4 reverse-phase column. The column was eluted with 0.1% trifluoroacetic acid (TFA) in water (solvent A), and 0.1% TFA in acetonitrile (solvent B), at a flow rate of 1 ml/min. A gradient controller was used to provide linear gradients of 0-100% solvent B (Shu and Yang, 1996). Separation of recombinant hGM-CSF from other proteins was carried out after 37 min which was detected at 280 nm by using a UV spectrophotometer.

In order to optimize expression of the recombinant hGM-CSF secreted into the culture medium, the effects of three variables including carbon source, inducer concentration and post-induction time were

Table 1. Composition of the defined F medium (developed at the NIGEB) for seed, batch and feeding solutions.

Components	Seed medium (g/l)	Batch medium (g/l)	Feeding solution (g/l)
Glucose	10.0	25.0	500.0
Glycerol	30.0	30.0	500.0
KH ₂ PO ₄	19.9	19.9	-
(NH ₄) ₂ HPO ₄	6.0	6.0	-
MgSO ₄ .7H ₂ O	1.2	1.2	20.0
EDTA	14.1×10 ⁻³	14.1×10 ⁻³	13.0×10 ⁻³
CoCl ₂ .6H ₂ O	2.5×10 ⁻³	2.5×10 ⁻³	4.0×10 ⁻³
MnCl ₂ .4H ₂ O	15.0×10 ⁻³	15.0×10 ⁻³	23.5×10 ⁻³
CuCl ₂ .2H ₂ O	1.5×10 ⁻³	1.5×10 ⁻³	2.3×10 ⁻³
H ₃ BO ₃	3.0×10 ⁻³	3.0×10 ⁻³	4.7×10 ⁻³
Na ₂ MoO ₄ .2H ₂ O	2.1×10 ⁻³	2.1×10 ⁻³	4.0×10 ⁻³
Zn(CH ₃ COO) ₂ .H ₂ O	33.8×10 ⁻³	33.8×10 ⁻³	16.0×10 ⁻³
Fe(III) citrate	100.8×10 ⁻³	100.8×10 ⁻³	40.0×10 ⁻³

Table 2. L4 orthogonal array in the Taguchi experimental design for optimization of human granulocyte macrophage colony stimulating factor expression and secretion into the culture medium.

Trial number	IPTG (mM)	Carbon source	Post-induction time (h)
1	1	Glucose	5
2	1	Glycerol	6
3	3	Glucose	6
4	3	Glycerol	5

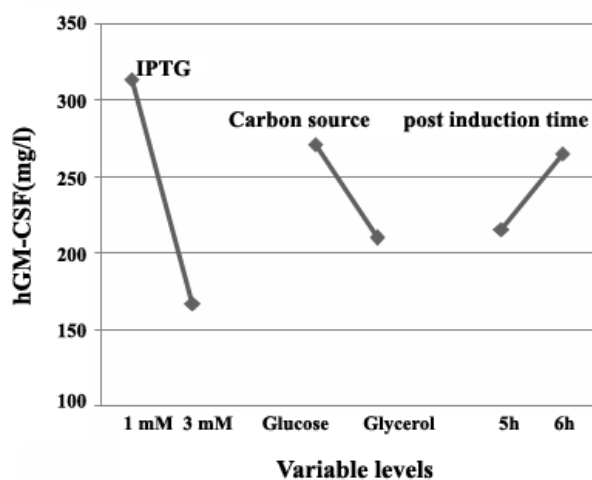


Figure 1. Main effects of IPTG concentrations, carbon sources and post-induction times on the secretory expression of the recombinant human granulocyte macrophage colony stimulating factor during high cell density cultivation (HCDC).

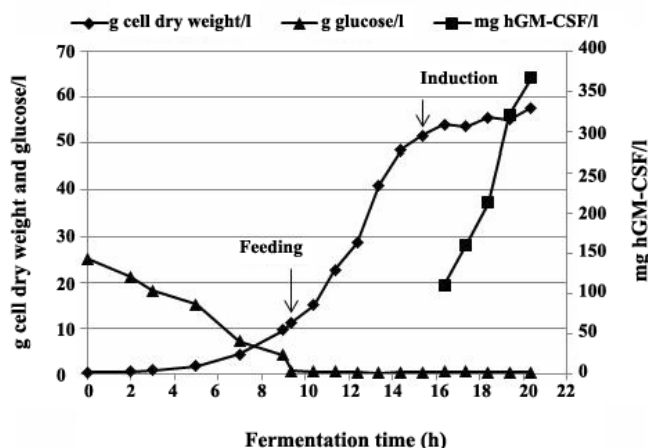


Figure 2. Glucose (▲), Cell dry weight (◆) and human granulocyte macrophage colony stimulating factor (■) concentration profiles during HCDC under optimized conditions (1 mM IPTG and 6 h post-induction time).

evaluated using the Taguchi statistical method during high cell density cultivation. An L4 orthogonal array was selected for 3 factors at 2 levels by the Taguchi experimental design (Table 2). Analysis of variance (ANOVA) revealed and identified the effects of each factor, optimum conditions and estimated the performance of the optimum condition. In order to investigate the effects of variables on the secretion of the expressed recombinant hGM-CSF into the culture medium by HCDC, the Taguchi statistical design was applied. The standard L4 orthogonal array was used for optimization of 3 variables including carbon source, inducer concentration and post-induction time at two levels (Table 2). All cultures were induced at cell densities higher than 50 g/l ($OD_{600} \sim 100$).

The main effects of variables showed that the IPTG at a concentration of 1 mM, glucose as carbon source and 6 h post-induction time, are the optimum conditions for maximum secretion of the recombinant hGM-CSF into the culture medium during HCDC (Fig. 1).

Analysis of variance (ANOVA) of the results showed that IPTG (77.5%) is more effective variable than the carbon source (13.5%) and post-induction time (9.0%). The expected value under optimum conditions for the recombinant hGM-CSF secreted into the culture medium, as suggested by Taguchi methodology was 369 mg/l.

In order to evaluate the expected expression and secretion of recombinant hGM-CSF into the culture medium, an HCDC experiment was performed using the optimized conditions (Fig. 2). Under these conditions, 373 mg/l of recombinant hGM-CSF was obtained, which was in good agreement with the expected Taguchi value.

Purification of recombinant hGM-CSF secreted into the culture medium was carried out with two-steps column chromatography; ion exchange and gel filtration. The culture medium from the fermentation process was subjected to centrifugation at 2680 g for 10 min at 4°C. The supernatant was concentrated using ammonium sulfate precipitation and dialyzed against dialysis buffer (50 mM Tris-HCl, 20 mM NaCl, pH 6.5). The dialyzed solution was passed through a DE-52 column (Whatman, USA) equilibrated with dialysis buffer. Subsequently, the column was eluted by elution buffer (50 mM Tris-HCl, pH 6.5, 120 mM NaCl), and the resulting fraction was concentrated, dialyzed (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) and passed through a sephacryl S-100 column (2.6 cm × 100 cm Pharmacia, Sweden) equilibrated with dialysis buffer. The column was then washed and eluted (50 mM Tris-

HCl, 150 mM NaCl, pH 8) at flow rate of 12 ml/h and 2.5 ml fractions were collected. The presence of protein in the fractions was confirmed by SDS-PAGE according to the method of Laemmli (Fig. 3 b). As a result of this procedure, 63 mg of pure recombinant hGM-CSF per one liter of culture medium was obtained. Periplasmic recombinant hGM-CSF was obtained by a modified cold osmotic shock process (Rastgar Jazii *et al.*, 2007).

Production and purification of the recombinant hGM-CSF using an easier and optimized process is an important step in biopharmaceutical industry. Purification of the periplasmic recombinant hGM-CSF expressed during HCDC using a defined medium has been previously performed using a simplified chromatography procedure (Rastegar-jazii *et al.*, 2007). However, some recombinant hGM-CSF was found in the culture medium (data not shown). Therefore, in order to facilitate the purification process, the effects of IPTG concentration, carbon source and post induction time on recombinant hGM-CSF expression and secretion into the culture medium were evaluated by HCDC using the Taguchi statistical design.

Following induction, along with recombinant hGM-CSF expression, growth rates were decreased, eventually approaching constant levels. Also, substrate concentration decreased during the batch fermentation stage and reached zero at the end of the process and

maintained during the exponential feeding stage.

As shown in Figure 1, IPTG at 1 mM was more effective than the 3 mM concentration with regard to expression and secretion of the recombinant hGM-CSF into the culture medium. IPTG is toxic to cells and at higher concentrations may lead to the expression of lower levels of recombinant hGM-CSF. Nevertheless, IPTG concentration may have to be optimized for every protein under the conditions used (Turner *et al.*, 2004). Post-induction time is an important variable for expression of recombinant proteins (Lee, 1997); our results indicated that at 6 h after induction, there is a greater probability for the expression of recombinant hGM-CSF and its translocation to the culture medium. This study also showed that glucose was a better carbon source than glycerol for secretion of the hGM-CSF into the culture medium. Analysis of variance for optimization of effective variables showed that IPTG concentration plays the most important role in secretory expression of the recombinant hGM-CSF.

In this study, 373 mg/l and 241 mg/l of recombinant hGM-CSF were secreted into the culture medium and the periplasmic space under optimized conditions, respectively (614 mg total recombinant hGM-CSF/l). In fact the total amount of recombinant hGM-CSF is much higher than the periplasmic production of soluble recombinant hGM-CSF (390 mg/l) in HCDC

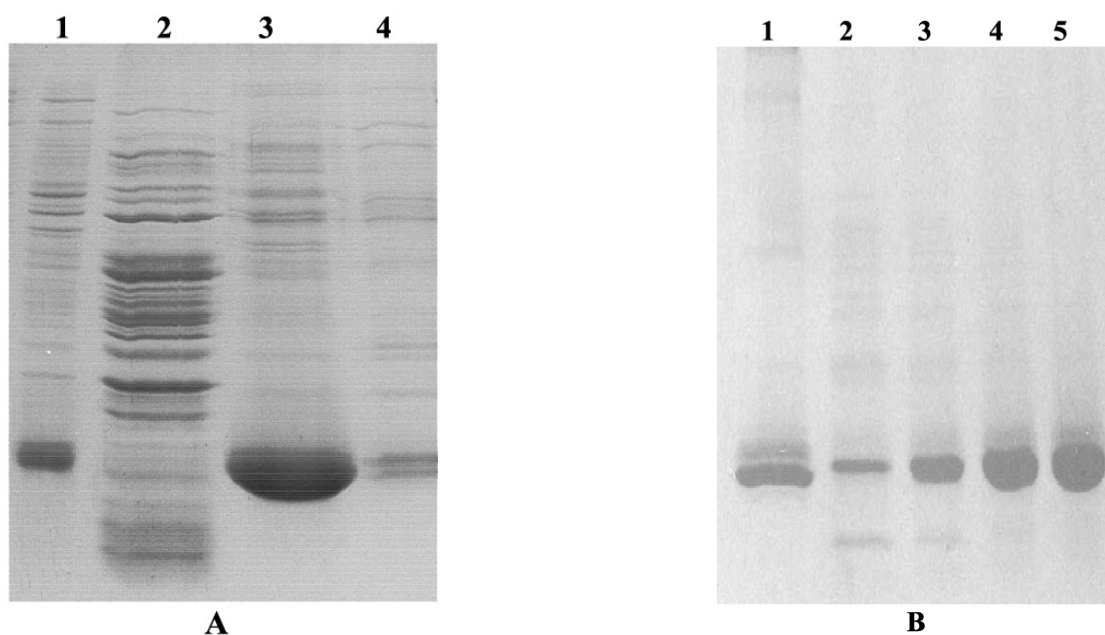


Figure 3. SDS-PAGE analysis of recombinant hGM-CSF purification stages. A: Fractions obtained by ion exchange chromatography and B: gel filtration from high cell density cultivation (HCDC) at the optimum condition. A: Lane 1; total recombinant proteins before injection to the column, lane 2; washed fraction with 20 mM NaCl, lane 3; eluted fraction with 120 mM NaCl, lane 4; eluted fraction with 700 mM NaCl, B: lanes 1; standard hGM-CSF, lane 2-5; different fractions after gel filtration.

($OD_{600}=190$), previously observed in a similar genetic structure using a semi-complex medium (Sletta *et al.*, 2007). The quantity of recombinant hGM-CSF obtained in this research is also higher than the periplasmic expression of recombinant hGM-CSF by *E. coli* under continuous fermentation using a semi-complex medium (89 mg/l) (Azimi, 2007) and fed-batch fermentation of *E. coli*, also using the semi-complex medium (311 mg/l) (Fatemi *et al.*, 2004). Also, this quantity is higher than the secretory expression of hGM-CSF into culture medium by plant suspension culture using defined medium (0.783 mg/l) (Lee *et al.*, 2002) and fed-batch fermentation of *Pichia pastoris* using defined medium (131 mg/l) (Bhattacharya *et al.*, 2007).

Complex and semi-complex culture media, containing an animal carbon source (tryptone), yeast extract and undefined components, are not reliable for the purpose of biopharmaceutical production. Extraction and purification of the recombinant hGM-CSF secreted into culture medium are also easier and preferable than its periplasmic situation because of shorter down-stream processing stages and a better folding environment without intracellular proteolysis, (Choi and Lee, 2004). Although *E. coli* is considered as weak in secretion of proteins into the culture medium, but small periplasmic proteins are frequently released into culture medium. However, in most cases, outer membrane disruption is necessary for release of the proteins into culture medium. Various strategies have been applied to promote secretory production of recombinant proteins in *E. coli*. Periplasmic leakage resulting from cell lysis or osmotic pressure difference between the periplasm and culture medium could cause the release of periplasmic recombinant proteins into the culture medium. Co-expression of the colicin E1 lysis protein (Kil) or gene coding for the third topological domain of the transmembrane protein TolA, could also release proteins into the culture medium. Using certain chemical strategies, such as adding magnesium, calcium, EDTA, glycine and Triton X-100; and mechanical methods, including ultrasound and enzymatic procedures such as, lysozyme, increase the permeability of the outer membrane, which can enhance the secretion of recombinant proteins into the culture medium. Secretion could also be increased by verifying physical and chemical parameters such as temperature, culture medium composition, pH etc. (Mergulhao *et al.*, 2005; Choi and Lee 2004).

Expression and secretion of several recombinant proteins into the culture medium using *E. coli* with

mentioned methods have been studied (Choi and Lee 2004). To our knowledge, this is the first report that uses the Taguchi statistical design for optimizing the secretory expression of the hGM-CSF by recombinant *E. coli* during HCDC to facilitate the extraction and purification processes.

In conclusion, release of the recombinant hGM-CSF from the periplasm into the culture medium could be due to the enhancement of the permeability of the outer membrane as a result of the magnesium and EDTA presence in the culture medium. In addition, osmotic pressure difference between periplasm (with accumulated recombinant hGM-CSF) and culture medium can be a driving force for transportation of the proteins across the outer membrane. The results of this research could lead to attractive prospects for the pharmaceutical industry.

Acknowledgments

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