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Construction of intein-mediated hGMCSF expression vector and its purification in *Pichia pastoris*

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Abstract

As a novel attempt for the intracellular recombinant protein over expression and easy purification from *Pichia pastoris*, the therapeutic cytokine human granulocyte macrophage colony stimulating factor (hGMCSF) gene was fused to an intein–chitin-binding domain (gene from pTYB11 vector) fusion tag by overlap extension PCR and inserted into pPICZB vector, allowing for the purification of a native recombinant protein without the need for enzymatic cleavage. The fusion protein under the AOX1 promoter was integrated into the *P. pastoris* genome (SMD 1168) and the recombinant *Pichia* clones were screened for multicopy integrants. Expression of hGMCSF was done using glycerol and methanol based synthetic medium by three stage cultivation in a bioreactor. Purification of the expressed hGMCSF fusion protein was done after cell disruption and binding of the solubilized fusion protein to chitin affinity column, followed by DTT induced on column cleavage of hGMCSF from the intein tag. In this study, final biomass of 89 g dry cell weight/l and purified hGMCSF of 120 mg/l having a specific activity of 0.657×10^7 IU/mg was obtained. This strategy has an edge over the other—His or—GST based fusion protein purification where non-specific protein binding, expensive enzymatic cleavage and further purification of the enzyme is required. It distinguishes itself from all other purification systems by its ability to purify, in a single chromatographic step.

Keywords: *Pichia pastoris*; Intein; rhGMCSF; Fusion tag purification; Recombinant protein

Human granulocyte–macrophage colony stimulating factor is an important therapeutic cytokine used in enhancing hematopoietic recovery after cancer chemotherapy and bone marrow transplantation [1]. Clinical indications for the use of this protein have expanded considerably and its therapeutic application in areas such as fungal infections, HIV infections, and anti-tumor therapy has been reviewed [2]. Due to its biological and pharmaceutical importance the production of recombinant hGMCSF[1] has been available from different recombinant expression systems [3]. In recombinant *Escherichia coli* the expression of hGMCSF has led to the formation of inclusion bodies, and the yield after refolding of the protein are low (20 mg/l) [4]. In the same host the periplasmic expression of hGMCSF was poor (4 mg/l) due to the toxicity of hGMCSF resulting in cell lysis upon induction [5]. Also, the hGMCSF expression was carried out in different hosts such as *Saccharomyces cerevisiae* [6], baculovirus [7], transgenic animals [8] and plant cell system [9] and the expression levels of hGMCSF were typically less than 5 mg/l. In *P. pastoris* the expression of hGMCSF have shown higher productivity in both inducible and constitutive expression [10,11]. However, new methods are under constant development for over expression and easy purification of recombinant hGMCSF.

The use of protein fusion and affinity technology has simplified the purification of recombinant proteins. However, a major problem encountered with fusion technology is an additional chromatography step for protease removal after separation of the affinity tag and protease. The use of intein as a fusion technology simplifies the protein purifica-

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[1] *Abbreviation used*: hGMCSF, human granulocyte macrophage colony stimulating factor.

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tion [12,13] wherein the DTT induces self-cleavage of intein and thereby separates the recombinant protein from the intein tag.

In the production of recombinant proteins, the use of methylotrophic yeast, *P. pastoris* as an expression system has become increasingly popular in recent times [14]. *P. pastoris* is easier to genetically manipulate and can be grown to very high cell densities using simple minimal media. Further, the availability of strong promoters to drive the expression of foreign genes of interest enables the production of large amounts of the pharmaceutically important proteins by fermentation [15].

In the current work, an attempt was made for higher expression of biologically active hGMCSF, using the methylotrophic yeast *P. pastoris*. As the extracellular expression of the recombinant hGMCSF leads to *Pichia*-specific glycosylations, [16] which may have an adverse pharmacological side effects, intracellular expression was tried as an alternative. Further, for the first time in *P. pastoris* the intein fusion tag has been tried for easier purification of intracellular rhGMCSF.

Materials and methods

Strains and plasmids

The *E. coli* Top 10F' strain was used as maintenance host. *P. pastoris* SMD 1168 (*his, mut*$^{+}$*, pep4*), used as the expression host, was purchased from Invitrogen. The media and procedures, used for *P. pastoris* growth and transformation, were described in *Pichia* protocols [17].

pTYB11 Vector was purchased from New England Bio Labs (Beverley, USA) and pPICZB vector from Invitrogen (San Diego, CA). The parental clone pVM1 (pRSETB: hGMCSF) was kindly provided by Dr. V. Murugan, Centre for Biotechnology, Anna University.

PCR amplification and fusion of hGMCSF and CBD-intein tag

The hGMCSF gene (408 bp) was amplified from the parental clone pVM1 (pRSETB:hGMCSF) by PCR with the following primers: GMCSF (intein) forward 5′-CAGG TTG TTGTACAGAACGCACCCGCCCGCTCGCC C-3′ and GMCSF reverse 5′-TGC TCTAGATCACTCC TGGACTGGCTCCCA-3′. The gene sequence coding for the chitin binding domain-intein tag was amplified using the plasmid pTYB11 and the primers: intein forward 5′-CCGGAATTCATGTGCTTTGCCAAGGGTAC-3′. intein (GMCSF) reverse 5′-GGGCGAGCGGGCGGG TGCGTTCTGTACAACAACCTG-3′. The hGMCSF forward primer and the CBD-intein reverse primer were designed to contain a short stretch of complementary sequences and so by using the overlap extension PCR of the two amplification products, the overlapping ends of the two fragments annealed and got extended. The fusion product had EcoRI site at the 5′ end and XbaI site at the 3′ end. The PCR products were analyzed and purified by Qiagen gel extraction kit (Germany).

Construction of recombinant shuttle vector

Recombinant DNA methods were performed essentially as described in Molecular cloning [18]. EcoRI, XbaI double digestion of the purified PCR product was carried out and this was inserted in the multiple cloning site of pPICZB resulting in pPICZB/hGMCSF plasmid. Transformation of this plasmid was carried out in *E. coli* TOP10F' and the transformants were screened on low salt LB agar plates containing 30 μg/ml Zeocin. Lysate PCR of the transformants was carried out to confirm the presence of the gene.

Transformation to P. pastoris SMD1168 (his4, pep4)

The recombinant plasmid pPICZB/hGMCSF was linearized using SacI and transformed to *P. pastoris* SMD 1168 by electroporation using an Eppendorf Electroporator 2510 at 2000 V with a 0.2 cm cuvette. After electroporation, 1 ml of ice-cold 1 M sorbitol was immediately added to the cuvette and incubated for 1 h at 30 °C. The cells were then plated on YPDS with increasing concentrations of zeocin (for screening multicopy integrants) and incubated at 30 °C for 2–3 days until the colonies appeared. The genomic DNA was isolated from the transformants and the integration of the gene was confirmed with insert specific primers.

Fermentation of recombinant P. pastoris

The fermentation culture was carried out in a 2 L bioreactor (Bioengineering KLF 2000) with 1.2 L basal salts medium: (composition per liter) 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4{\cdot}2H_2O$, 18.2 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, 40.0 g glycerol and 4 ml/l PTM1 solution: (composition per liter) 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CaSO_4{\cdot}2H_2O$, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin, 5 ml concentrated H_2SO_4. A 50 ml BMGY medium, grown for 36 h was used as inoculum and the initial batch cultivation was carried until the exhaustion of glycerol. A fed-batch phase using 50% glycerol at the feed rate 15 ml/h was then initiated and after 4 h, the feed was switched to 100% methanol (containing 1 ml/L PTM1 solution) at an initial feed rate 2 ml/h. This feed rate was then increased stepwise to maintain residual concentration of 0.5% methanol in the reactor. The pH during entire fermentation was adjusted and controlled at 5.0 with the addition of 28% ammonia solution and the temperature maintained at 28 °C. Samples were withdrawn at regular intervals and analyzed for the biomass, expression and activity.

Affinity purification

The fermentation broth was harvested by centrifugation and the cell pellet was disrupted by ultrasound after resus-