MAXIMIZING EXPRESSION OF GRANULOCYTE COLONY STIMULATING FACTOR IN *PICHIA PASTORIS* THROUGH HOST SELECTION, PROCESS OPTIMIZATION AND GENE DOSAGE STRATEGY

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by

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Submitted

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Dedicated to
My beloved Parents & Teachers

CERTIFICATE

This is to certify that the thesis entitled "**Maximizing expression of Granulocyte Colony Stimulating Factor in Pichia pastoris through host selection, process optimization and gene dosage strategy**" being submitted by **Mr. Ashwani Gautam** to the **Indian Institute of Technology Delhi**, for the award of the degree of '**Doctor of Philosophy**', is a record of the bonafide research work carried out by him, which has been prepared under my supervision and guidance in conformity with the rules and regulations of the 'Indian Institute of Technology, Delhi'. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

Dr. Saroj Mishra

Professor
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(**Ashwani Gautam**)

ABSTRACT

Filgrastim (non-glycosylated), PEG-Filgrastim (chemically modified Filgrastim) and Lenograstim (glycosylated) are the commercially available forms of G-CSF out of which the first two are the non-glycosylated forms available from *Escherichia coli*. The glycosylated form is more stable and has high specific activity compared to the non-glycosylated form. The objectives of the present study were to devise strategies, at genetic and bioprocess level, to enhance the yield of G-CSF in *Pichia pastoris* attempted to design discrete approach for effectively improving the G-CSF production in *P. pastoris*.

Out of three host strains, namely, SMD1168, GS115 and X33, maximum production of G-CSF was obtained in X33 *Mut*^{S} clones. For each vector-host combination, several clones were screened at shake flask level and the `best clone' (in terms of cell biomass and extracellular protein) selected. For X33 *Mut*^{S} transformants, maximum extracellular protein levels varied from 37.3 to 47.3 mgL^{-1}. The experimental data at small scale indicated that the production of G-CSF negatively affected the growth parameters of the host strains during the induction phase. The selected clones were also evaluated at bioreactor level for which a typical four phase fermentation was carried out comprising of batch and fed-batch glycerol, transition phase followed by the terminal methanol induction phase. A mixed feed of methanol and sorbitol was employed during the induction phase. The results indicated maximum G-CSF production of 250 mgL^{-1} with total extracellular protein level of ~480 mgL^{-1} in X33

host against *Mut*^{S} background. A comparative analysis of various strains in terms of protein and G-CSF yield, volumetric and specific productivity was also carried out. Among different parameters studied, specific growth rate (μ) showed a distinct effect on protein titer as the pre-set value of μ decreased from 0.05 h^{-1} to 0.01 h^{-1} with respect to final G-CSF titer which was 466 mgL^{-1} and specific productivity which was 0.0179 $mgg\text{-}dcw^{-1}h^{-1}$. Based on these data, two sequential phases with different values of μ were implemented during the induction phase resulting in ~ 19% increase in G-CSF titer. Effect of gene dosage was also investigated and the data indicated considerable increase in the value of G-CSF transcripts which lead to increase in G-CSF titer during early phase of induction but the system was limited on account of availability of chaperones and other folding pathway enzymes. Thus, a balance needs to be created between folding and transcription to achieve high yield in multi-copy expression cassettes.

सार

Filgrastim (गैर-ग्लाइकोसिलेटेड), पीईजी-फिल्मेंसिस्टिम (रासायनिक रूप से संशोधित Filgrastim) और लेनोगार्स्टिम (ग्लाइकोसिलेटेड) जी-सीएसएफ के व्यावसायिक रूप से उपलब्ध रूप हैं, जिनमें से पहले दो एस्चेरिशिया कोली से उपलब्ध गैर-ग्लाइकोसिलेटेड फॉर्म हैं। ग्लाइकोसिलेटेड फॉर्म अधिक स्थिर और गैर-ग्लाइकोसिलेटेड रूप की तुलना में उच्च विशिष्ट गतिविधि है। वर्तमान अध्ययन के उद्देश्यों में, पीचिया में जी-सीएसएफ की पैदावार बढ़ाने के लिए, जेनेटिक और बायोप्रोसैस स्तर पर रणनीतियों को तैयार करना, पी। पादरीस में जी-सीएसएफ उत्पादन को प्रभावी ढंग से सुधारने के लिए असतत दृष्टिकोण को डिजाइन करने के लिए पेस्टोरिसटैम्पट किया गया था।

एसएमडी 1168, जीएस 115 और एक्स33 में तीन मेजबान तनावों में से, जी-सीएसएफ का अधिकतम उत्पादन एक्स33 में मिला गया था। प्रत्येक वेक्टर-होस्ट संयोजन के लिए, शेक फ्लास्क स्तर पर कई क्लोन दिखाए गए थे और `सर्वश्रेष्ठ क्लोन '(सेल बायोमास और बाह्य कोशिका के संदर्भ में) चयनित X33 MutS ट्रांसफार्मेंट के लिए, अधिकतम बाह्य प्रोटीन स्तर 37.3 से 47.3 एमजीएल-1 से भिन्न होता है। छोटे पैमाने पर प्रयोगात्मक डेटा ने संकेत दिया है कि जी-सीएफएफनेग के उत्पादन में अवरोध चरण के दौरान मेजबान तनाव के विकास मापदंडों को प्रभावित किया गया था। चयनित क्लोनों का भी मूल्यांकन किया गया था बायोरिएक्टर स्तर पर जिसके लिए बैच और फेड बैच ग्लिसरॉल, ट्रांज़िशन चरण के बाद एक सामान्य चार चरण किण्वन किया गया था, टर्मिनल मेथनॉल इन्डेक्शन चरण के बाद। प्रेरण चरण के दौरान मेथनॉल और सोर्बिटोल की एक मिश्रित फ़ीड को नियोजित किया गया था। परिणामस्वरूप अधिकतम जी-सीएसएफ का उत्पादन 250 एमजीएल-1 के

साथ-साथ कुल सेक्यूलर प्रोटीन स्तर ~ 480 एमजीएल-1 एक्स X33 मेजबान MutSbackground के खिलाफ था। प्रोटीन और जी-सीएसएफ उपज, वॉल्यूमेट्रिक और विशिष्ट उत्पादकता के मामले में विभिन्न उपभेदों का तुलनात्मक विश्लेषण भी किया गया था। विभिन्न मापदंडों में अध्ययन किया गया, विशिष्ट वृद्धि दर (μ) ने प्रोटीन टिटर पर एक अलग प्रभाव दिखाया, जैसा कि μ के पूर्व-निर्धारित मूल्य में 0.05 एच -1 से 0.01 एच -1 1 से अंतिम जी-सीएसएफ टेटर के संबंध में घटकर 466 एमजीएल -1 और विशिष्ट उत्पादकता जो 0.0179 एमजीजी-डीसीडब्ल्यू -1 एच -1 था। इन आंकड़ों के आधार पर, एमडी के विभिन्न मूल्यों के साथ दो अनुक्रमिक चरणों में शामिल किया गया था, जिसके परिणामस्वरूप जी-सीएसएफ टिटर में ~ 1 9% की वृद्धि हुई। जीन खुराक के प्रभाव की भी जांच की गई और डेटा में जी-सीएसएफ टेप की वैल्यू में काफी वृद्धि का संकेत दिया गया, जिससे प्रारंभिक चरण में जी-सीएसएफ टिटर में वृद्धि हो सकती है, लेकिन सिस्टम सावधानी और अन्य तहमार्ग के मार्ग की उपलब्धता के कारण सीमित था एंजाइमों। इस प्रकार, बहु-प्रतिलिपि अभिव्यक्ति केट में उच्च उपज प्राप्त करने के लिए तह और प्रतिलेखन के बीच संतुलन बनाए जाने की आवश्यकता है।

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