TO ENHANCE THE SECRETORY EFFICIENCY OF GRANULOCYTE COLONY STIMULATING FACTOR IN *PICHIA PASTORIS VIA* MANIPULATION OF SECRETORY SIGNAL SEQUENCES

SAKSHI AGGARWAL

DEPARTMENT OF BIOCHEMICAL ENGINEERING & BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

SEPTEMBER 2020

TO ENHANCE THE SECRETORY EFFICIENCY OF GRANULOCYTE COLONY STIMULATING FACTOR IN *PICHIA PASTORIS VIA* MANIPULATION OF SECRETORY SIGNAL SEQUENCES

by

SAKSHI AGGARWAL

DEPARTMENT OF BIOCHEMICAL ENGINEERING & BIOTECHNOLOGY

Submitted

In the fulfilment of the requirements for the degree of doctor of philosophy

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI

SEPTEMBER 2020

DEDICATION

This research is dedicated to my beloved parents and my supervisor…

CERTIFICATE

This is to certify that the thesis entitled **" To enhance the secretory efficiency of granulocyte colony stimulating factor in *Pichia pastoris via* manipulation of secretory signal sequences"** being submitted by **Ms. Sakshi Aggarwal** 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 her, which has been prepared under our 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.

Professor Saroj Mishra
Professor
Department of Biochemical Engineering & Biotechnology
Indian Institute of Technology Delhi

Date:
Place:

ACKOWLEDGMENTS

With ending this long journey of PhD, I would like to express my sincere gratitude and respect to my supervisor **Prof. Saroj Mishra** for her support, guidance, motivation and immense knowledge which gave me power and spirit to excel in this field. She guided me so nicely through the entire project with her wide knowledge and innovative ideas. She has always been more enthusiastic about the experimental results, her strong dedication towards research and regular lab visits fuelled me to excel in this area of research. Whenever an experiment fails, she reacts so positive and always have a way out. I am highly grateful to her for my never-ending fount of moral support. She has endlessly corrected my thesis with her discerning vision for which I am really thankful. Apart from this, she has been a counsellor with whom I could discuss all my anguishes and take advice. She is an epitome of wisdom, knowledge and perfection, an ideal person I have ever met in my career. She is very responsible and caring mentor, I feel myself lucky under her guidance.

I would like to acknowledge my SRC members Prof. Prashant Mishra, Prof. S.K. Khare and Dr. Ritu Kulshreshta for their invaluable comments and advice. I sincerely thank Dr. M.K Aggarwal and Dr. A.K Sau for always being there and helping me with all the important decisions of my life. I am very thankful to DST for providing me fellowship during PhD. upto 5-years.

I would give my special thanks to Mr. Sumeet Kapoor, for always helping me with access to instruments in the laboratory. Thanks to Rehmanji, Harilalji and Yogesh for providing me with all the lab consumables and clean glasswares, whenever necessary.

I am indebted to my seniors Sumbul Afreen, Swati Ohja, Sujata, Tushar, Tenzin kemzon and Anees K for their kind help and guidance. My sincere thanks to Anshul Sharma, who really introduced me to *P. pastoris* cultivation and process optimization experiments very nicely. A

very special thanks and love to my juniors Avijeet Jaiswal and Manju Khicher for their invaluable advice in addressing a research problem. I am indebted to Dr. Ayan Dutta, Scientist (Qiagen) for guiding me in my cloning experiments. He is such a humble person who has really helped me in troubleshooting PCR failures. I would always remember my friends Sumbul Afreen, Manju khicher and Aakanksha Ahlawat for the fun time and numerous tea break discussions on research topics to future plans. I would like to give special thanks to Neetu Singh and Kritika Goyal (sister cum best friend), for her unconditional love and support during my ups and down. Special thanks to my lab mates Rohit, Ashima, Radhika, Aishvi, Asmita for maintaining healthy environment in lab. I would give my special thanks to my friend Ruchika Bhatt for helping me in computational work. I am thankful to my batchmates Richa Sharma, Sonam Takkar, Gautam Anand for their kind help and support.

The unconditional love and support of my family members is really acknowledgeable. My heartfelt thanks to my family members: Mom, Dad, Lakshay, Akshay and Shriesty for their unconditional love and affection. My Dad, an ideal person, always motivated me to fulfil my dreams. His hard work and dedication is always an inspiration for me. My Mom (superhero) my best friend has a major role during my PhD, without her I would not be able to do it. Thanks for remembering me in your prayers for the ultimate success. In every step, she has supported and encouraged me throughout my life. Along with me, she had also spent sleepless nights during my PhD. She used to call me mid-nights when I had work in lab for the whole night. My heartiest thanks to my brother Lakshay for his numerous late-night visits to IITD, to take me home. It was all family love that refilled fresh energy after a failed experiment.

I would like to thank the Almighty for His blessings throughout my journey.

…Sakshi Aggarwal

ABSTRACT

The methylotrophic yeast *Pichia pastoris* has been widely used for the production of human therapeutics, but production of granulocyte colony-stimulating factor (G-CSF) in this yeast is low. In this study, improved extracellular production of G-CSF was carried out by introducing mutations in the α-mating type (MAT) secretory signal using a native cDNA (*WT-GCSF*) and a codon optimized *GCSF* gene (*CO-GCSF*). Mutations in the pro-region of the α-MAT (deletion (Δ) of the amino acids 57-70), resulted in an increase in extracellular production of G-CSF in both the cases with higher production from the *CO-GCSF*. Response to Δ30-43 and Δ47-49 deletions was different from the *CO-GCSF* and the *WT-GCSF* genes indicating higher rates of synthesis in the former to overcome the regulatory control exercised by these segments. The loss of secretion occurring due to Δ30-43 in the *WT-GCSF* was partially restored (by 60%) when the Δ57-70 was added. An important role of the 47-49 amino acids was also demonstrated. The role of the P1' position of the kex2 cleavage site in the α-MAT was demonstrated and specific substitutions by smaller amino acids lead to increased production of G-CSF. Secondary and tertiary structure prediction using I-TASSER indicated an important role of the 3rd alpha-helix in the pre-pro peptide. Presence of a minimum loop length and secondary structure on the pro-peptide region allowed enhanced extracellular production of G-CSF. Also, the role of several nutritional factors in controlling the morphology of recombinant *P. pastoris* was shown for the first time and the morphological switch was mediated through quorum sensing molecules.

सार

मिथाइलोट्रॉफिक खमीर, पिक्रिया पास्टोरिस का व्यापक रूप से मानव चिकित्सा विज्ञान के उत्पादन के लिए उपयोग किया गया है, लेकिन इस खमीर में ग्रैनुलोसाइट कॉलोनी-उत्तेजक कारक (जी-सीएसएफ) का उत्पादन कम है। इस अध्ययन में, α-संभोग (α-मेट) प्रकार स्रावी संकेत को एक देशी (डब्ल्यू टी-जीसीएसएफ) और एक कोडन अनुकूलित जीन (को-जीसीएसएफ) का उपयोग करके जीसीएसएफ के बेहतर बाह्य उत्पादन को अंजाम दिया गया। α-संभोग कारक के एमिनो एसिड (Δ५७-७०) के विलोपन से जीसीएसएफ के समर्थक क्षेत्र में को-जीसीएसएफ से परिणामस्वरूप उच्च उत्पादन के साथ दोनों मामलों में जीसीएसएफ के बाह्य उत्पादन में वृद्धि हुई है। Δ३०-४३ और Δ४७-४९ विलोपन का जवाब डब्ल्यूटी-जीसीएसएफ और को-जीसीएसएफ जीन से अलग था, जो इन खंडों द्वारा नियंत्रित नियामक नियंत्रण को दूर करने के लिए पूर्व में संश्लेषण की उच्च दरों का संकेत देता है। डब्ल्यूटी-जीसीएसएफ में Δ३०-४३ के कारण होने वाले स्राव की हानि आंशिक रूप से (६०%) द्वारा बहाल की गई थी जब Δ५७-७० जोड़ा गया था। '४७-४९' एमिनो एसिड की महत्वपूर्ण भूमिका का भी प्रदर्शन किया गया था। α-संभोग (α-मेट) में केएक्स२पी१ साइट की भूमिका का प्रदर्शन किया गया और संकेत दिया कि छोटे अमीनो एसिड जीसीएसएफ प्रोटीन उत्पादन को बढ़ावा देते है।आई-टेसर का उपयोग करके माध्यमिक और तृतीयक संरचना की भविष्यवाणी ने पूर्व-प्रोपेप्टाइड में तीसरी अल्फा-हेलिक्स की एक महत्वपूर्ण भूमिका का संकेत दिया। प्रो-पेप्टाइड क्षेत्र पर एक न्यूनतम लूप लंबाई और माध्यमिक संरचना की उपस्थिति ने जी-सीएसएफ के अतिरिक्त बाह्य उत्पादन की अनुमति दी। इसके अलावा, पुनः संयोजक पी. पस्टोरिस की आकृति विज्ञान को नियंत्रित करने में कई पोषण संबंधी कारकों की भूमिका पहली बार दिखाई गई और रूपात्मक संवेदी अणुओं के माध्यम से रूपात्मक स्विच की मध्यस्थता की गई।

CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

Abbreviations	Full forms
APS	Ammonium per sulphate
BME	β-Mercaptoethanol
BSA	Bovine serum albumin
BMGY	Buffered complex glycerol medium
BMMY	Buffered complex methanol medium
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
MALDI	Matrix assisted laser desorption ionization
PCR	Polymerase chain reaction
PDB	Protein data base
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
UV	Ultra violet

List of symbols

Symbols	Meanings
~	Approximately
bp	base pair
°C	Degree Celsius
µM	Micromolar
µg	Microgram
µl	Microliter
g	Gram
h	hours
Kb	Kilobase pair
kDa	Kilodalton
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
ng	Nanogram
Nm	Nano meter