

**STUDIES ON INTERFERON- α 2b
PRODUCTION USING *PICHIA PASTORIS***

By

ANAND GHOSALKAR

Department of Biochemical Engineering and Biotechnology

*Submitted
in fulfillment of the requirement of the degree of*

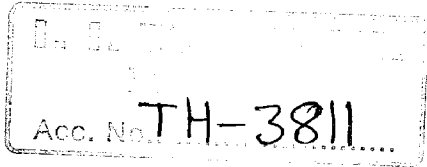
DOCTOR OF PHILOSOPHY

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

APRIL 2008



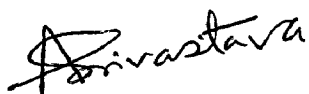
TH
577.245
G40-S

DEDICATION

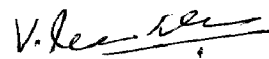
to my Uncle, late Shri. G. K. Pradhan

CERTIFICATE

This is to certify that the thesis entitled “Studies on interferon- α 2b production using *Pichia pastoris*” being submitted by Mr. Anand Ghosalkar 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 our supervision 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. Aradhana Srivastava



Dr. Vikram Sahai

ACKNOWLEDGEMENTS

From the very beginning of my Ph.D. project till its culmination to the present form, I have been extremely fortunate to receive the help, interest, and support of many people. Since I value these people highly, it is appropriately iconic to thank them at the very beginning of this thesis.

In particular, I wish to express my gratitude to my supervisor Dr. Aradhana Srivastava, for her intellectual input and continued encouragement during this work. Even though her teaching load is considerable and her research, and administrative responsibilities manifold, she has always given generously of her time and insights, and not a word of mine has appeared in papers or in this dissertation which she has not read and reflected and commented on. I feel privileged to have been her student, and sincerely thank her for her invaluable suggestions and respect the moral and intellectual honesty, with which she handles challenges and problems of any kind.

I gratefully thank Dr. Vikram Sahai for his often crucial hints and sharp linguistic judgment. His knowledge of bioprocess engineering as well as his practical advice concerning the fermentation processes was highly valuable. Despite of having several research projects and various responsibilities, he managed to guide me whenever I needed direction; he pushed me for publications and reviewed my manuscripts critically. I will forever remain grateful for his interest and encouragements expressed at various occasions.

I warmly thank Prof. V. S. Bisaria, Prof. Saroj Mishra and Dr. Nivedita Karmakar for accepting to be members of my student research committee and for their critical suggestions at various occasions. I thank all the members of departmental research committee and all faculty members for ensuring smooth conduct of research activities at the Department of Biochemical Engineering and Biotechnology, Indian Institute of

Technology Delhi (IIT Delhi). Many thank to IIT Delhi for providing me the funding, which not only allowed me to work uninterruptedly for five years but also made it possible for me to attend several national and international conferences.

I owe a special debt of gratitude to Dr. Millind Gore, Deputy Director, National Institute of Virology (NIV) Pune, who spontaneously agreed to provide the facilities for gene isolation and antiviral assay at NIV. I will forever remain indebted to all his staff and students who taught me the basics of animal cell culture and virus handling. I thank the editors and referees of various journals for their concrete suggestions which helped me improve my articles in aspects of presentation as well as content before publication.

Without people seeing to it that practical problems are solved, work would become difficult in any workplace. I specially thank our librarian Mrs. Neera Verma for providing various books and thesis for referencing without delays, all the administrative and technical staff of the department, especially Mr. V. K. Ghosh, Mr. Mukesh Anand, Mrs. Renu Sethi, Sharmaji, Ranaji, Khan Sahab, Yadavji, Bhagwansinghji, Dahiyaji and Hardeepji for making things work around the Labs. Some people who tend to go unacknowledged are the once who work in numerous ways to make our environment so much more comfortable and efficient. In this regard, I acknowledge the assistance provided by Kishanji, Santramji, Ram Gopalji, Sitaramji, Ratanji, Harishji, Sohrabji, Harilalji, Meharchandji, Shahdab and Vikas.

I thank all my colleagues at the department for their regular expressions of best wishes. I warmly thank Lalit, Rahul, Raju, Ashish, Asif, Rajib, Alok, Sarma, Vinod, Mohit, Richa, Roohi, Smita, Saurabh, Aditi and Vikas for their support and dear friendship. I will always be indebted to my seniors, Salony, Gunjan, Amalendu, Ashwini and Shilpi, for teaching me the basic ropes of experimental techniques.

I have been lucky in meeting a host of people more than willing to take an active interest in my work and who made my five years in the department a very memorable and pleasant experience. Many thanks to Parul, with whom I not only shared the lab, but also discussed my experiments and every scientific and non-scientific issue. It was because of her expertise in protein purification and active involvement in various fermentation experiments that I could do this work with greater efficiency. Her continued support in proofreading manuscripts and the thesis, and pushing me whenever I got stuck just proved what a difference a good friend can make. Special thanks to dear friends Anjali and Bhawna for exchanging various ideas and tricks on cloning experiments. Working with these people was truly a very special and enriching experience and I will always cherish the moments I spend with them, specially the full variety of home made food which otherwise is a very rare privilege for a guy living in a hostel. Amongst the people outside the department, I would like to thank dear friend Shijo for adding fun to our life. Your initiative in organizing trips to movies, golf course and amusement parks were especially effective in reenergizing us after long experiments.

No words can describe the level of gratitude I feel for my aunt Sarojani, my parents Seema and Ramesh, and my sisters Preeti, Suchita and Kavita for their unconditional support and encouragement through out my studies. While we all miss my uncle, Late Mr. Ganpati Pradhan for last four years, his love and affection for us and high values in life will continue to inspire me.

At last a million thanks to the grace of almighty God for his constant blessings.


Anand Ghosalkar

Abstract

The objective of the thesis was to study the production of human interferon- α 2b (IFN- α 2b) in *Pichia pastoris*. Gene coding for IFN- α 2b was cloned and expressed in *P. pastoris* under the control of alcohol oxidase promoter (*AOXI*) using three different secretion signals. Native secretion signal of IFN- α 2b, *Saccharomyces cerevisiae* MF- α factor prepro sequence and a mutated α prepro sequence without the Glu-Ala (EAEA) repeats were used separately for directing the secretion of IFN- α 2b into the culture medium of *P. pastoris*. The mutated α prepro sequence secreted a maximum of 200 mg/L IFN- α 2b into the culture medium, with the same amino acid sequence as that of the native IFN- α 2b secreted by human lymphocytes.

A chemically defined medium was optimized for maximum biomass production of recombinant *P. pastoris* in the fermentor cultures using glycerol as the sole carbon source. The response surface methodology was used for the optimization of nutritional requirements of salts, trace metals and vitamins for the growth of recombinant *P. pastoris*. Using the optimized medium biomass yield of 0.55 g dry cell weight/g of glycerol was achieved in a batch culture. In the chemically defined medium, recombinant IFN- α 2b was produced by fed-batch fermentation of recombinant *P. pastoris* in three phases; batch phase, transition phase (glycerol fed-batch phase) and methanol fed-batch phase. Exponential feeding of glycerol was used in the transition phase and a step increasing feeding of methanol was used during the methanol fed-batch phase. Maximum IFN- α 2b production of 363 mg/L was achieved by maintaining a specific growth rate of 0.08 h⁻¹ during the transition phase.

CONTENTS

Certificate	i
Acknowledgement	ii
Abstract	v
Contents	vi
List of figures	xi
List of tables	xii
List of abbreviations	xiii
1. Introduction and Objectives	1-6
2. Review of Literature	7-38
2.1 Interferons	8
2.1.1 Types of IFN	8
2.1.2 Biological properties of IFNs	9
2.2 IFN-α	11
2.2.1 IFN-α2b	13
2.2.2 Natural IFN-α production	14
2.2.3 Production of recombinant IFN-α	15
2.2.3.1 Prokaryotic expression systems	15
2.2.3.2 Eukaryotic expression systems	17
2.3 <i>Pichia pastoris</i>	18
2.3.1 Methanol metabolism	19
2.3.2 <i>AOX1</i> promoter	20
2.4 <i>Pichia pastoris</i> strains and vectors	21
2.4.1 Methanol utilization phenotype	22
2.4.2 Integration of expression vectors in <i>P. pastoris</i> genome	23
2.4.3 Multicopy integration of expression cassette	25
2.5 Post translational modification of secreted proteins	26
2.5.1 Secretion signal selection	26
2.5.2 O-Linked glycosylation	28

2.5.3	<i>N</i>-Linked glycosylation	29
2.6	Fermentation media and operational conditions	30
2.7	Optimization of medium components by statistical methods	33
2.7.1	Response surface methodology	34
2.8	High cell density fed-batch cultivation of <i>Pichia pastoris</i>	35
2.8.1	Glycerol batch phase	37
2.8.2	Transition phase	37
2.8.3	Methanol induction phase	38
3	Materials and Methods	39-59
3.1	Materials	40
3.1.1	Strains and plasmids	40
3.1.2	Cell line and virus strains	41
3.2	Methods	42
3.2.1	Gene isolation	42
3.2.1.1	Isolation of Mononuclear Leukocytes (MNL)	42
3.2.1.2	Induction of IFN-α in MNL	42
3.2.1.3	IFN production in MNL	43
3.2.1.4	Total RNA isolation	43
3.2.1.5	Reverse Transcription and polymerase chain reaction (RT-PCR)	43
3.2.2	Molecular biology techniques	44
3.2.2.1	Cloning of IFN-α2b gene with native secretion signal into pPIC9	44
3.2.2.2	Cloning of IFN-α2b gene with α prepro secretion signal into pPIC9	45
3.2.2.3	Cloning of IFN-α2b gene with mutated α prepro signal sequence into pPIC9	46
3.2.2.4	Transformation in <i>Pichia pastoris</i> GS115	47
3.2.3	Screening of His⁺ transformants for methanol utilization phenotype	47

3.2.3.1	PCR analysis of <i>Pichia pastoris</i> transformants	47
3.2.3.2	Expression of IFN-α2b in recombinant <i>Pichia pastoris</i> strain	48
3.2.4	Purification of recombinant human IFN-α2b	49
3.2.5	Optimization of synthetic medium for biomass production	49
3.2.5.1	Inoculum preparation and shake flask studies	50
3.2.5.2	Experimental design and statistical analysis	50
3.2.6	Batch cultivation of recombinant <i>Pichia pastoris</i> to study substrate inhibition	53
3.2.6.1	Inoculum development	53
3.2.6.2	Substrate inhibition studies	53
3.2.7	Production of IFN-α2b in <i>Pichia pastoris</i> by fed-batch fermentation	54
3.2.7.1	Fed-batch fermentation according to <i>Pichia</i> fermentation guidelines by Invitrogen	54
3.2.7.2	Fed-batch fermentation by exponential feeding of glycerol during the transition phase	55
3.2.8	Analytical techniques	57
3.2.8.1	Biomass estimation	57
3.2.8.2	Glycerol and metabolite estimation	57
3.2.8.3	Methanol estimation	58
3.2.8.4	Protein estimation	58
3.2.8.5	Estimation of IFN-α2b	58
3.2.8.6	Antiviral assay of IFN-α2b	58
3.2.8.7	Western blotting	59
4	Results and Discussion	60-95
4.1	Results	61
4.1.1	Gene isolation	61

4.1.1.1	Induction of IFN- α in MNL	61
4.1.1.2	RNA isolation and RT-PCR	62
4.1.2	Construction of expression plasmids	63
4.1.3	Transformation in <i>Pichia pastoris</i> and determination of methanol utilization phenotype	65
4.1.4	Expression of IFN- α 2b in <i>Pichia pastoris</i>	67
4.1.5	MALDI-TOF analysis and peptide mass fingerprinting	70
4.1.6	Biological activity of recombinant IFN- α 2b	73
4.1.7	Statistical analysis of the results obtained by experimental design	73
4.1.8	Optimization of the medium	76
4.1.9	Verification of the optimization	78
4.1.10	Substrate inhibition studies	78
4.1.11	Fed-batch fermentation according to <i>Pichia</i> fermentation guidelines by Invitrogen	79
4.1.11.1	Batch phase	79
4.1.11.2	Transition phase	80
4.1.11.3	Methanol fed-batch phase	80
4.1.12	Fed-batch fermentation by exponential feeding of glycerol	81
4.1.12.1	Batch phase	81
4.1.12.2	Transition phase	81
4.1.12.3	Methanol fed-batch phase	83
4.1.12.4	Production rates, specific production rates and specific consumption rates obtained in the fed-batch process	87
4.2	Discussion	89
5	Conclusions	96-99
	References	100-122
	Annexure	123-139
	Annexure I- Chemicals/consumables & equipments	124

Annexure II- <i>Pichia</i> media recipes	128
Annexure III-Protocols	130
Annexure IV-Vector maps and sequencing results	135
Biodata of author	140-142