Development of an expression system for heterologous protein expression in fungus

Thesis submitted under the Faculty of Science of the
Cochin University of Science and Technology
For the award of degree of

Doctor of Philosophy
in
Biotechnology

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January 2016

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08 January 2016

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Development of an expression system for heterologous protein expression in fungus**" is a *bona fide* record of the research carried out by **Mr. Aravind Madhavan** (Reg No. 4150) under my guidance and supervision at the Centre for Biofuels, Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India. I declare that all suggestions made by the audience during pre-synopsis seminar and recommended by the Doctoral committee have been incorporated in the thesis. I also declare that this work or no part of it has been submitted elsewhere for the award of any Degree, Diploma, Associateship or any other title or recognition

Rajeev Kumar Sukumaran

Thiruvananthapuram
08 January 2016

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Development of an expression system for heterologous protein expression in fungus"** is based on the original work done by me under the guidance of Dr Rajeev Kumar Sukumaran, Senior Scientist, Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India and the thesis or no part of it has been submitted elsewhere, for the award of any Degree, Diploma, Associateship or any other Title or Recognition.

Aravind Madhavan

Dedicated To My Teachers

Acknowledgements

I hereby place on record my deep sense of gratitude and respect to all those who made this work possible, directly and indirectly, and supported me in all aspects, for the successful completion of this endeavour. I thank God Almighty whose blessings and grace which showered upon me throughout my life, and which enabled me to be where I am today.

Foremost, I am grateful to Dr Rajeev K Sukumaran, my guide and mentor through this journey for his constant support, encouragement and supervision. He had granted me enough freedom to explore on my own and at the same time, the guidance to pull through when my steps faltered. I owe him lot of gratitude for having shown me the path of research. I believe it will be a driving force to accept the challenges cheerfully in all my future endeavours. Without his continuous and warm encouragement, insightful decisions, critical comments, and above all his inspiration and patient guidance, throughout my research period, it would have been difficult to complete my thesis.

My sincere thanks go to Prof Ashok Pandey, Head, Biotechnology Division, CSIR-NIIST. I will remember with gratitude, for his sincere advice, academic inspiration and affectionate encouragement he showed in the fruitful completion of this thesis.

I have immense thanks to Dr. Ajaya Ghosh, Director, and Dr Suresh Das, former Director, National Institute for Interdisciplinary Science and Technology for providing me necessary facilities in the Institute for the research work.

I would like to thank INSPIRE programme division, DST, Government of India for the financial support in the form of Junior and senior research fellowship. I also acknowledge the help provided by Dr Chamma Awasthi and Mr Nagesh Kumar, INSPIRE programme division, DST, New Delhi for resolving the problems associated with my fellowship release and disbursal.

I wish to place on record my heartfelt gratitude to Dr Saita G Bhat, Department of Biotechnology, CUSAT and expert member of my doctoral committee for her valuable suggestions and constant support which has helped me immensely during my research work.

I would also like to acknowledge with much appreciation Dr. Bernhard Seiboth, Head, Molecular Biotechnology, Institute of Chemical Engineering,Vienna, Austria for providing me with several protocols regarding fungal gene manipulation and transformation when I was really down with negative results.

I take this opportunity to thank Dr K Madhavan Nampoothiri, Dr Binod P, Dr Arumugham, Er M Kiran Kumar, Dr Leena D, and Dr Anil K Mathew for their valuable suggestions and timely support during the study. I express the most sincere gratitude to Dr Ramesh Kumar N for sequencing the amplicons and constructs. I am thankful to Dr Sindhu R for her help, encouragement and moral support. I wish to convey my thanks to all technical and non-technical staff, Mr Prakash KM, Radha Chechi, Rajalekshni Chechi, Sashikala Chechi for helping me in many different ways to carry out the research work.

I also want to acknowledge with thanks, the staff of purchase section especially Mr. Anil, Mrs Aswathy, Mrs Sandya, Thilaka Chechi, and Mr Ajayan for their administrative help and the authorities of the finance section especially Mrs Viji, and Mrs Remani for their immense help and support in various ways.

I thankfully remember all my teachers right from my KG to PG for it is with their effort I have come this far. I would like to specially thank Dr. Jayachandran K, Dr Keerthi TR and Dr Linu Mathew of School of Biosciences, MG University, Kottayam for introducing me to the wonderful world of science and research. My skills as a research scholar have been accomplished only with the efficient training by Dr Thakur Das Saha, Genome Analysis Laboratory, Rubber Research Institute of India, Kottayam and

Dr Toms C Joseph, Microbiology, Fermentation and Biotechnology Division, Central Institute of Fisheries Technology, Cochin during my M Sc dissertation and post M Sc engagement as research fellow.

The best part about belonging to the biotechnology division of CSIR-NIIST is the interactions with students doing research in diverse field of biotechnology, and I am proud to belong to the "Team Biotech". Many thanks go in particular to Leya Thomas, Sabeela Beevi, Meena S and Arjun for their benevolent friendship, love and unlimited support granted both in good times and bad. My seniors who helped me in setting a solid foundation in the initial years, Dr Rajasree KP, Dr Deepthy Alex, Dr Vidya and Dr Ushasree deserve special mention. I wish to thank all other seniors Dr. Arya Nandan, Dr Bindu Mol Ismail, Dr Nisha, and the unconditional cooperation of my other friends in biotech division, Rahul, Christina, Anusree, Varsha, Ramya Krishnan, Vani, Mandavi, Balendu, Parvathy, Dhanuja, Nisha, Sajitha, Koyna, Dileep, Ayman, Nishant, Karthik, Vivek, Raja, Kiran, Aswathi, Sujitha, Reshma, Resna, Aswathy, Prajeesh, Vaishaki, Emrin, Meera, Dilna, Vini, Anju, Dhanya, Vaishaki and Salman.

I am short of words in expressing my acknowledgement and gratitude to Arun K B for his endless patience, care and encouragement throughout this entire journey. He is gem of a person and a true friend. Without him, I would have struggled to find the inspiration and motivation needed to complete this work. "You are my best friend I have ever met"!

Special thanks to my M Sc classmates, Dr Farha AK, Dr Antu K Antony, Mrs Divya JB for their help and moral support.

I would like to thank Dr. P. Nisha, Agroprocessing and Natural Products Division, CSIR-NIIST for the technical support given for cytotoxicity study.

Words cannot express my deep sense of gratitude to all my family members for their unconditional love and support. Above all, I am forever indebted to the-two most important persons in my life, my parents. Their love and support in all that I have done until now are the keys of all my achievements.

Aravind Madhavan

List of Publications

Published in SCI Journals

1. **Madhavan A**, Sukumaran RK, 2014, Promoter and signal sequence from filamentous fungus can drive recombinant protein production in the yeast *Kluyveromyces lactis*, Bioresour Technol. 165:302-308.

2. **Madhavan A**, Sukumaran RK, 2015, Signal peptides from filamentous fungi efficiently mediate the secretion of recombinant proteins in *Kluyveromyces lactis*. Biochemical Eng. J. 102: 31-37.

3. **Madhavan A,** Sukumaran RK, 2015, Secreted expression of an active human interferon- beta (HuIFNβ) in *Kluyveromyces lactis* (Engineering in Life Sciences- Accepted)

Book Chapters

1. Rajeev K Sukumaran, Vani Sankar, **Aravind Madhavan**, Meena Sankar, Vaishaki Satheesh, Ayman Salih Omar Idris, Sabeel Beevi Ummalyama, 2015, Enzyme Technologies: Current and Emerging Technologies for Development of Novel Enzyme Catalysts, In: M Chandrasekaran (ed) Enzymes in Food and Bevarage Processing (ISBN: 13: 978-1-4822-2128-2), CRC Press, Ohio, USA. pp. 39-66.

Conference Papers/Presentations/Posters

1. **Aravind Madhavan** and Rajeev K Sukumaran, Promoter for heterologous gene expression in Aspergillus sp. Poster presented in International Conference on New Horizons in Biotechnology 2015 held at National Institute for Interdisciplinary Science and Technology, Trivandrum, India from 22-25th November 2015 (Best Poster Award)

2. **Aravind Madhavan** and Rajeev K Sukumaran, Signal peptides from filamentous fungi efficiently mediate the secretion of recombinant proteins in *Kluyveromyces lactis*. Poster presented in International Conference on Emerging Trends in Biotechnology 2014 held at Jawaharlal Nehru University, New Delhi, India from 6-9th November 2014.

3. Sukumaran RK, **Madhavan A**, Rajasree KP, Mathew A, Enzymes for Biomass Deconstruction. National Symposium on Bioenergy for Sutainable Development – The potential role of sugar crops, held at Coimbatore, 23-25 June, 2014.

4. **Aravind Madhavan** and Rajeev K Sukumaran, Promoter and signal sequence from filamentous fungus can drive recombinant protein production in the yeast *Kluyveromyces lactis*. Poster presented in International Conference on Advances in Biotechnology and Bioinformatics 2013 held at D.Y Patil Vidyapeeth, Pune, India from 25-27th November 2013.

5. Sukumaran RK, Mathew A, Rajasree KP, **Madhavan A.** Enzymes for deconstructing biomass: The centre for biofuels initiatives. In: The 5th India Korea Joint Workshop on Bioenergy, at CSIR-NIIST, Thiruvananthapuram, India. Sept 9-10, 2013

6. **Aravind Madhavan**, Ashok Pandey and Rajeev K Sukumaran, Functional validation of fungal promoter in the yeast- *Kluyveromyces lactis*. International Conference on & IX convention of the Biotech Research Society of India 2012 held at Punjabi University, Patiala, India from 21-23rd November 2012.

7. Sukumaran RK, Mathew A, Rajasree KP, **Madhavan A**, Gottumukkala LG. Studies for biomass hydrolysis: The centre for biofuels initiatives. International conference on Industrial Biotechnology and 1X convention of the Biotech Research Society, India, November 21-23, Patiala India. (2012)

8. Rajeev K Sukumaran, Abraham Mathew, Rajasree KP, **Aravind Madhavan**, Enzymes for Biomass Deconstruction: From moldy bran to tuning gene expression - The Centre for Biofuels Initiatives. National Symposium on Emerging Trends in Biotechnology, organized by the Cochin University of Science and Technology, December 12-14 (2012) Kochi, India.

CONTENTS

1. Introduction and Review of literature

1.1.Introduction

The genetic and metabolic versatility of filamentous fungi makes them outstanding microbial cell factories in industrial biotechnology. Their unexplored genetic diversity poses interesting prospects of exploiting them as a source of new genes and as expression hosts. The main advantages of using filamentous fungi as expression hosts include their natural abilities to secrete a variety of proteins in large quantities and their intensive use in bioprocessing. They can grow on relatively inexpensive substrates, and are able to produce and secrete tremendous amounts of recombinant proteins. Unlike the traditional *E.coli* or Yeast (*Saccharomyces cerevisiae , Pichia pastoris*) based expression systems, filamentous fungi as expression hosts offer the advantages of inexpensive cultivation, easy induction, efficient secretion, and a protein synthetic machinery closer in functioning to the higher order animals which has advantages in production of several proteins of interest in pharma and diagnostic industries. Probably the greatest advantage is in the production of low value high volume proteins which are uneconomical to produce using a conventional expression system. While there are expression systems based on filamentous fungi reported previously, there are none available commercially or in the public domain.

There are about 250 named species in the genus *Aspergillus* (Geiser *et al.,* 2007), and these include industrially important species like *Aspergillus niger, Aspergillus oryzae, Aspergillus awamori, Aspergillus sojae, Aspergillus terreus* etc and pathogenic species like *Aspergillus fumigatus, Aspergillus parasiticus, Aspergillus flavus* etc. The extraordinary secretion capacity makes *Aspergilli* suitable candidates for the production of a variety of industrially important products such as organic acids, pharmaceuticals, proteins and enzymes (Meyer 2008; Lubertozzi and Keasling 2009). *Aspergillus* occupies a unique position among other microbial production hosts like bacterial or yeast origin, due to its capability to withstand extreme cultivation conditions (Raper and Fennel 1965; Kis-Papo *et al.,* 2003; Machida and Gomi 2010). For example, *Aspergillus* can be cultivated over a wide range of temperatures (10–50 °C), pH (2–11), salinity (0–34%), water activity (0.6–1.0) and under oligotrophic or nutrient rich conditions. Therefore *Aspergillus* can be used for solid-state or submerged fermentations because of the availability of well established fermentation protocols of *Aspergillus* sp. for large scale production.

Genetic manipulation of different fungal host strains has also allowed the production of industrially relevant amounts of several recombinant proteins such as human lactoferrin, calf chymosin, or plant derived sweeteners-thaumatin and neoculin (Nevalainen and Peterson 2014). *Aspergilli* are classified as "generally regarded as safe" (GRAS). Existence of industrial facilities, availability of easy biomass separation procedures, and detailed knowledge about high yield cultivations represent further advantages for their use as cell factories in biotechnology.

The review focus on the genetic engineering tools for filamentous fungi, different types of expression vectors that have been employed for heterologous protein production in the recent years and possible genetic manipulation approaches to develop new and improved expression systems. Despite the importance of *Aspergilli* and other filamentous fungi as microbial cell factories in the food and bio-pharmaceutical industry, only a limited number of genetic tools are currently available for efficient overproduction and subsequent secretion of heterologous proteins. Thus an in-depth understanding of the physiology and metabolism of these organisms is required for development of novel genetic engineering tools for improved large-scale production strategies.

1.2. Filamentous fungi as expression hosts

Filamentous fungi are widely used host organism for the production of homologous and heterologous proteins. Secretion of the recombinant proteins has several advantages over intracellular localization. Extracellular expression stays induced for longer compared to the intracellular expression and is also advantageous for the expression of toxic proteins. Another advantage is the easy downstream processing, since the cell harvesting and disruption steps may be avoided and the protein product is relatively pure. The development of highly advanced fermentation techniques also allows the cultivation of fungi in large quantities in inexpensive media. The secreted levels of native proteins are high in optimal production conditions, for example, *T. reesei* can produce hydrolases at 40 g/l quantity (Zou *et al.,* 2012) and *A. niger* has been shown to secrete 25 g of glucoamylase per litre (Ward *et al.,* 2006). Productivity and secretion potential of filamentous fungi, which is found to be in the range of 30–40 g/l for homologous enzymes like cellulases and amylases, is considered to be superior to any other system (Demain and Vaishnav 2009). *A. nidulans* is the model organism among the fungi for studies on cell biology and gene regulation. *A. niger*, *A. oryzae* and *T.reesei* are the best natural filamentous fungal protein production hosts. Although

filamentous fungi share the status with bacteria and yeasts as microbial cell factories, the knowledge on genetic manipulation of fungi may not be considered as equivalent to other systems. Nevertheless, the past decade of research on filamentous fungi have led to significant advancements in the area of fungal molecular biology. Also, with lot of sequenced fungal genomes now available, genetic manipulations in fungi have become easier. Some patented systems under development feature *Aspergillus japonicas, Aspergillus sojae* and *A. oryzae* (Berka *et al.,* 1995; Heerikhuisen *et al.,* 2001; Hata *et al*., 2003). Several secondary metabolites were also successfully produced in *A. nidulans* and *A. oryzae* (Anyaogu and Mortensen 2015). Some disadvantages of filamentous fungi as heterologous protein hosts are their relatively low frequencies of transformation, morphological defects, and extracellular protease production (Kinghorn and Unkles1994; Radzio and Kueck 1997).

1.3. Transformation

Efficient genetic transformation techniques are a pre-requisite for the genetic manipulation of high yield production strains. The development of transformation techniques for *Aspergillus* and other filamentous fungi is hampered by the presence of complicated multicellular morphology, complex chitinous cell walls, and lack of extracellular plasmids compared to those for *E. coli* and *S. cerevisiae*. The first successful reports of gene cloning and transformation of *Aspergillus* were in early 80s (Kinghorn and Hawkins 1982; Ballance *et al.,* 1983; Tilburn *et al.,* 1983). Several methods for *Aspergillus* transformation are currently in use (Table 1.1). Best results have been obtained by transforming protoplasts, prepared from fungal mycelia or germinating conidia through treatment with cell wall lyzing enzymes such as Lysing Enzyme® from *Trichoderma harzianum*, chitinase from *Streptomyces griseus* and β-glucuronidase from *Helix pomatia* (de Bekker*et al.,* 2009). However, several transformation techniques have been developed that refined the transformation efficiency to 100 transformants per microgram DNA (Dawe *et al.,* 2000). Other methods include electroporation (Ward *et al.,* 1989; Sanchez and Aguirre, 1996) and biolistic techniques (Herzog *et al.,* 1996; Barcellos *et al.,* 1998). The plant transformation workhorse *Agrobacterium tumefaciens* has been used to transform *Aspergillus* and other fungi and has been successful in generating high numbers of transformants (Gouka *et al.,* 1997; de Groot *et al.,* 1998; Michielse *et al.,* 2008)

Table 1.1. Various methods used for transformation of *Aspergilli*

Methods	Organism	Advantage	Disadvantage	Reference
Electroporation	*A. niger* *A. awamori* *A. nidulans* *A. fumigates*	Different cell types used; simple and cheap method	Required specialized instrument	Ozeki *et al.,* 1994 Weidner *et al.,* 1998
Protoplast Mediated Transformation	*A. nidulans* *A. niger* *A. oryzae*	Different cell types used	Low and variable regeneration frequency of protoplast; laborious procedure	Tilburnl *et al.,* 1983
Agrobacterium Mediated Transformation	*A. awamori*	Transformation frequency high	Various parameters during co-cultivation affects transformation rate; Time consuming	Gouka *et al.,* 1999
Shockwave Mediated Transformation	*A. niger*	Efficient, easy	Physical method requiring Instrument	Loske *et al.,* 2014
Biolistic Transformation	*A. nidulans*	Recipient cells retain cell wall	Expensive; Requires specialised equipment	Fungaro *et al*., 1995

1.3.1. Selectable Markers for Fungal Transformation

Auxotrophy is commonly used as selection marker in filamentous fungal transformations. This requires a selectable marker gene that restores prototrophy. However, the disadvantage of using auxotrophy as a selectable marker is requirement of mutant strain. Nutritional markers are available for well studied model organisms like *Neurospora crassa*, *A. nidulans* and *T. reesei* which include pyrG (Gruber *et al.,* 1990) and argB (Penttila *et al.,* 1987). Dominant selectable markers that impart antibiotic resistance are commonly used for fungal transformation (Table 1.2). The hygromycin B resistance gene hph (Cullen *et al.,* 1987b) and the phleomycin resistance gene ble are highly efficient antibiotic resistance markers, which can be used in filamentous fungi (Austin *et al.,* 1990). More recently, strategies such as

transient expression of Cre recombinase were used to excise the marker gene from the transformants to develop recombinant strains free of antibiotic resistance genes (Florea *et al.,* 2009).

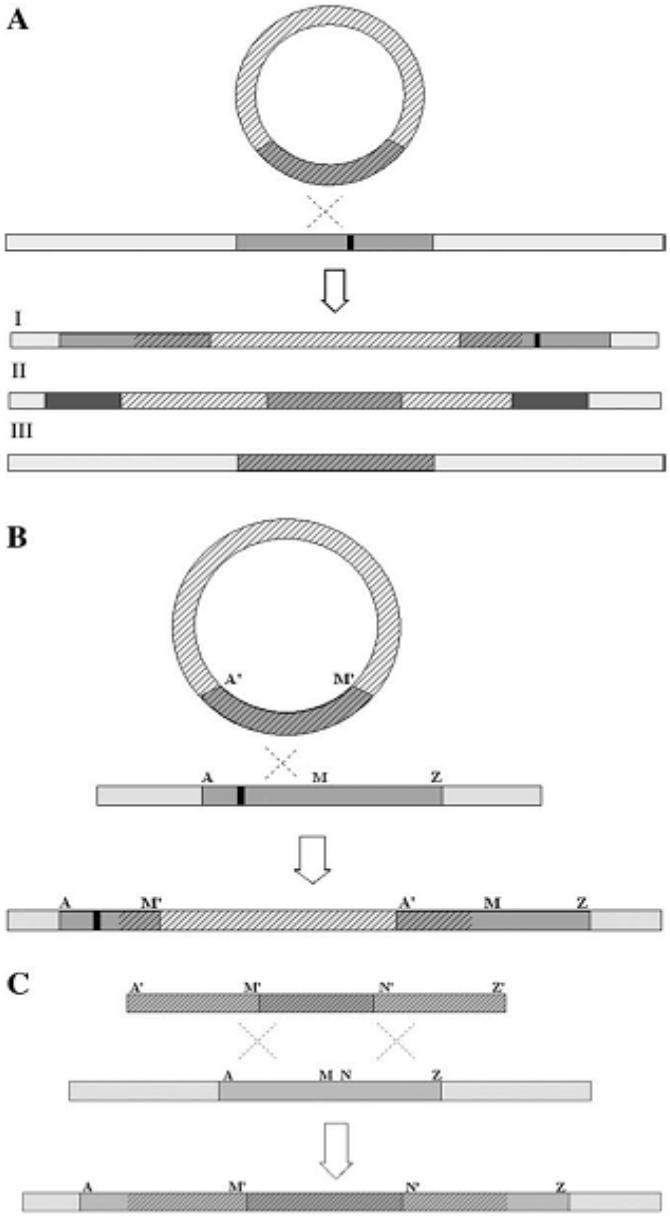
Table 1.2. Dominant selection markers in *Aspergilli* (Adapted from Dave *et al.,* 2015)

Selection marker	Marker gene function	Metabolic target	Species Transformed
Bar	Phosphinothricin acetyl transferase	Glutamine synthetase	*A. niger* *A. fumigates* *A. nidulans*
benA	Benomyl resistant β tubulin	Mitosis	*A. falavus* *A. parasitcus*
Ble	Phleomycin binding protein	DNA scission	*A. nidulans* *A. oryzae*
blmB	Bleomycin N acetyltransferase	DNA scission	*A. oryzae*
Cbx	Carboxin resistant succinic dehydrogenase mutant	TCA cycle	*A. oryzae* *A. parasiticus*
gliT	Gliotoxin sulfhydryl oxidase	Not Known	*A. fumigates*
Hph	Hygromycin B phosphotransferase	Translation	*A. niger* *A. nidulans* *A. syndowii* *A gignateus* *A. terreus* *A. ficcum*
Hsv-l tK	Thymidine kinase	Nucleotide metabolism	*A. fumigates*
oliC	Oligomycin resistant mitochondrial ATP subunit	ATP synthase	*A. nidulan* *A. niger*
ptrA	Thiamine biosynthetic enzyme	Thiamine antagonist	*A. oryzae* *A. nidulans* *A. terreus* *A. fumigatus*

1.3.2. Copy Number and Site of Integration

In filamentous fungi, successful transformation often results from the integration of transforming DNA at ectopic (non homologous sites) as well as at the homologous region (Hynes, 1996) (Fig 1.1). During fungal transformation, single or multiple copies of the targeted gene may be integrated into the chromosomal DNA (Jeenes *et al.,* 1991; Hynes 1996) and the target gene copy number and the sites of integration may vary in these transformants (Penttila *et al.,* 1987). Higher copy-number transformants does not always produce high amount of heterologous proteins (Jeenes *et al.,* 1991). In addition, certain chromosomal locations in filamentous fungi may be more favourable for heterologous expression than others, due to the efficient interaction of the local regulatory elements like transcription factors (Davis and Hynes, 1991). Homologous recombination events can be increased by overexpressing the genes involved like UvsC (Natsume *et al.,* 2004). Non-homologous integration occurs through a DNA repair system catalyzing the non-homologous end-joining of double-stranded DNA breaks. A major breakthrough in strain improvement was achieved on deletion of the ku70 and or ku80 genes of the heterodimeric Ku70-80 protein (which promotes non homologous recombination) (DeFazio *et al.,* 2002). Analysis of mutants of *A. niger*, *A. sojae* and *A. oryzae* deficient in the ku70/80 genes, revealed that the rate of homologous integrations was greatly increased from 1−10% in the wild type to about 60−80% in the ku70/80-disrupted strains (Takahashi *et al.,* 2006; Meyer *et al.,* 2007).

Fig.1.1. Chromosomal integration of vector DNA in *Aspergillus*.



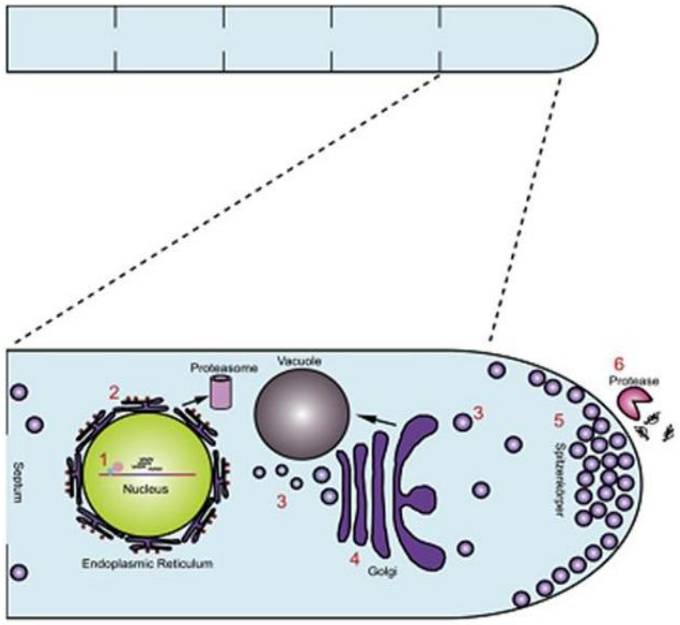
Adapted from Lubertozzi et al., 2009

(A) Integration of circular plasmid by single crossover. Plasmid (shaded) bearing wild type marker (gray) recombines with auxotrophic mutant host chromosome (gray with black bar indicating mutation). (I) Type I or homologous integration. (II) Type II or ectopic integration; the dark gray bars represent an interrupted gene sequence. (III) Type III gene replacement; here the chromosomal marker is replaced by the plasmid copy, restoring it to wild-type. (B) Integration of a plasmid bearing a truncated wild-type marker; only recombination at the homologous locus reconstitutes the functional marker gene. Sequence A′to M′on the plasmid is homologous to A to M in the target; the black bar indicates location of a mutation in auxotrophic host. (C) Insertion of linear DNA; here A′ to M′ and N′ to Z′ flanking the marker (darker gray) are homologous sequences corresponding to those at A to M and N to Z, respectively, in the chromosomal target (lighter gray).

1.4. Expression System

Genetic engineering of an organism requires various tools like the transformation technique, promoters (essential for making of primary transcript), gene(s) of interest, markers, reporter gene and signal peptide. Each component can be engineered to further refine the heterologous protein production (Fig. 1.2). The control of protein production in filamentous fungi mainly occurs at transcriptional level. Achieving high expression of heterologous genes requires a strong promoter to drive efficient transcription. In both the model filamentous fungi and industrially important fungi, a variety of either constitutive or inducible promoters are involved in diverse physiological processes in the host cell. Several reporter genes, including the green fluorescence protein GFP, laccase, β-galactosidase LacZ, and the glucuronidase GUS of *E. coli*, were used to monitor the activity of potential candidate promoters. Some of these reporters may also serve as visual selection markers as they allow transformants to be visually distinguished.

Fig.1.2. Pathways in filamentous fungi that can be engineered for enhanced heterologous gene expression.



Adapted from Su et al., 2012.

The numbers indicate the pathways that can be engineered. 1. Transcription/host defence/copy numbers/genome integration locus/intron structure; 2. Signal peptide/codon usage/mRNA stability/protein quality control; 3. Vesicle sorting; 4. glycosylation; 5. hyphal growth/membrane fusion; and 6. Extracellular proteolysis.

1.4.1. Promoters for Heterologous Gene Expression in Filamentous Fungi

1.4.1.1. Inducible Promoters

Inducible expression systems allow tight control of gene expression. Furthermore, high biomass formation without growth reduction can be obtained, in case those heterologous proteins have detrimental effects on the fungus. The promoter of the *A. niger* glucoamylase gene glaA and the *A. nidulans* alcohol dehydrogenase gene alcA and cbh1 in *T. reesei* are the most frequently used inducible promoters in *Aspergillus* expression systems. The gene GlaA is an amyloglucosidase that performs the successive hydrolysis starch molecule into glucose. The induction of glaA promoter is by starch, maltose, and low concentrations of glucose but repressed by xylose (Fowler *et al.,* 1990). Basal transcription of glaA requires only 214bp from the start codon while highly efficient expression needs upstream region including 562 and 318 bp respectively in the promoter region. The promoter region of glaA in *A. oryzae* and *A. niger* shared two regions in the promoter region (I and II) which is required for the high level expression when maltose as the inducer (Hata *et al.,* 1992). Three consensus CCAAT sites were identified within the region between−600 and−300 bp upstream from the ATG region of glaA promoter. These regions are the binding sites of AngCP protein of *A. niger* which is a member of CCAAT binding protein family and found to be involved in the enhancement of gene expression in fungi (Qiu *et al.,* 2002). Introduction of a series of CCAAT repeats into the most upstream located CCAAT box gradually increased transcriptional activity of PglaA (Liu *et al.,* 2003). AmyR protein a newly identified protein was recently found to be involved in the regulation of starch/maltose-induced expression of several amylolytic genes of *Aspergillus* (Vongsangnak *et al.,* 2009).

AlcA promoter is one of the strongest and commonly used promoters in the model fungus *A. nidulans* which have been used for high-level expression of homologous as well as heterologous genes (Nikolaev *et al.,* 2002). The *A. nidulans* alcA promoter is induced by ethanol and repressed by glucose. This promoters has been successfully employed in the expression of heterologous proteins including a plant adapted green fluorescent protein (GFP) (Fernandez-Abalos *et al.,* 1998), a monomeric red fluorescent protein (Toews *et al.,* 2004), human lactoferrin (Ward *et al.,* 1992b), and an *A. fumigates* dimodular non-ribosomal peptide synthetase in *A. nidulans* (Maiya *et al.,* 2006). AlcR is the regulator protein for alcA promoter and is strictly dependent on the presence of a co-inducer. The co-inducer is acetaldehyde, an intermediate produced from ethanol or related carbon catabolite compounds

by the alcohol dehydrogenase of the ethanol utilization pathway (Nikolaev *et al.,* 2002). AlcR is a Zn–DNA-binding protein and interacts as a monomer with inverted or direct 5′-CCGCA-3′ repeats. This binding of alcR results in the activation of alcA transcription. The number and the position of the AlcR-binding sites in the promoter region and the concentration of alcR proteins plays key roles in the strength of alcA promoter (Panozzo *et al.,* 1997). The high concentration of alcR proteins required for high strength of alcA promoter limits the multicopy integration. This effect could be partially overcome by increasing the copy number of the activator protein in the production strains (Gwynne *et al.,* 1989). CreA mediates the repression of the alcA promoter by competing directly with binding of the alcR activator to its binding sites within the alcA promoter. This has led to the development of improved strains with alcR gene expressed from a constitutive promoter (Mathieu and Felenbok1994).

Cbh1promoter of *T. reesei* is one of the strongest inducible promoters of fungal world and hence used for the construction of expression vectors (Nyyssonen and Keranen, 1995; Zou *et al.,* 2012). The promoter is induced by lactose and is under the tight regulation by several transcription factors (Kubicek *et al.,* 2009). Several reports have demonstrated use of other inducible promoters for high-level expression of heterologous proteins like the xylose-inducible *A. awamori* endoxylanase (exlA) promoter (Gouka *et al.,* 1997), and the thiamine-dependent *A. oryzae* thiA promoter. Meyer *et al.,* (2011) have described a very tight, tuneable Tet-on (induced by tetracycline) expression system for *A. niger*, that is dependent on the doxycycline concentration. The phosphate-inducible aphA promoter of the *A. nidulans* acid phosphatase was used to express human interferon-α-2 (MacRae *et al.,* 1993), and the *A. oryzae* sodM promoter of the superoxide dismutase gene was applied for heterologous expression by addition of small amounts of H_2O_2 (Ishida *et al.,* 2004). Another recent report mentions the use of sucA of *A. niger* (Roth and Dersch 2010). Recently, the *Aspergillus terreus* terrein gene cluster which encodes for specific transcription factor TerR that is indispensable for terrein cluster induction was identified and selected for development of a new heterologous expression system (Gressler *et al.,* 2015).

1.4.1.2. Constitutive promoters

Constitutive promoters were also exploited for the construction of highly efficient expression vectors for filamentous fungi. Constitutive promoters do not require expensive inducers and are not under the control of tight transcriptional regulation. The constitutive gpdA promoter of the glyceraldehyde-3-phosphate dehydrogenase of *A. nidulans* is the most commonly used

promoters among the filamentous fungi. Glyceraldehyde-3-phosphate dehydrogenase (GAPD) is an enzyme that catalyzes the sixth step of glycolysis. The gene gapd is constitutively expressed in *A. nidulans* (Punt *et al.,* 1990). The *A. nidulans* gapd promoter has been used successfully for expression of human interleukin-6 in *A. niger* (Broekhuijsen *et al.,* 1993), and the sweet protein thaumatin II in *A. awamori* (Moralejo *et al.,* 1999) and the *Phanerochaete chrysosporium* manganese peroxidases in *A. niger* (Punt *et al.,* 2002).

Among the constitutive promoters, which have been described for filamentous fungi are the alcohol dehydrogenase promoter PadhA of *A. niger*, the pyruvate kinase promoter Ppki of *Trichoderma reesei* (Limón *et al.,* 1999) the gdhA promoter of *A. awamori* glutamate dehydrogenase (Moralejo *et al.,* 1999), the pkiA promoter of the *A. niger* protein kinase A (Storms *et al.,* 2005; Rothand Dersch 2010) the tpiA promoter of the *A. nidulans* triosephosphate isomerise (Upshall *et al.,* 1987), mdhA promoter of *A. niger* malate dehydrogenase (Blumhoff *et al.,* 2013), gpdA promoter of *A. terreus* glyceraldehyde-3-phosphate dehydrogenase (Huang *et al.,* 2014). Different fungal recombinant proteins produced from constitutive/inducible proteins is summarised in Table 1.3.

Table 1.3. Recombinant proteins produced in Filamentous fungi

Organism	Promoter	Heterologous gene	Reference
*A.nidulan*s	glaA	Bovine chymosine	(Cullen *et al.,* 1987a)
A.nidulans	*alcA*	Human lactoferrin	(Ward *et al.,* 1992b)
A.nidulans	sodM	glucoamylase	(Ishida *et al.,* 2004)
A.nidulans	Tefl	polygalacturonase (pgaA, pgaB)	(Kitamoto *et al.,* 1998)
A.nidulans	adhA	human: tissue plasminogen activator (tPA)	(Upshall *et al.,* 1987)
*A.nidulan*s	glaA	Cytotoxin restrictocin	(Brandhorst *et al.,* 1994)
*A.nidulan*s	glaA	Human interleukin-6	(Carrez *et al.,* 1990)
*A.nidulan*s	amyB (*A. oryzae*)	*A. oryzae*: carboxypeptidases OcpA, OcpB, CpI	(Morita *et al.,* 2009)
A.awamori	amyA/amyB	bovine: prochymosin	(Korman *et al.,* 1990)

A.awamori	exlA	human: interleukin-6 *Cyamopsis tetragonoloba*: α-galactosidase *Thermomyces lanuginose*: lipase *Escherichia coli*: β-glucuronidase	(Gouka *et al.,* 1996, 1997)
A.awamori	gam/glaA	human: lactoferrin bovine: prochymosin B *Thaumatococcus daniellii*: Thaumatin	(Dunn-Coleman *et al.,* 1991) (Gouka *et al.,* 1997) (Faus *et al.,* 1998)
A.awamori	glaA	Human lactoferrin	(Ward *et al.,* 1995)
A.awamori	glaA	Bovine chymosine	(Ward *et al.,* 1990)
A.oryzae	glaA	Bovine chymosine	(Tsuchiya, *et al.,* 1994)
A. oryzae.	a-amylase	Human lactoferrin	(Ward *et al.,* (1992a)
A.oryzae	thiA	Bovine chymosine	(Yoon et al 2013)
A.niger	glaA	*Phanerochaete chrysosporium* manganese peroxidase	(Punt *et al.,* 2002)
A.niger	*alcA/aldA*	*Pleurotus eryngii*: peroxidase	(Eibes *et al.,* 2009)
A.niger	*adhA*	human: granulocyte macrophage colony stimulating factor GM-CSF	(Davies 1994)
A.niger	*amy (taka)*	*Thermomyces lanuginosus*:lipase	(Prathumpai *et al.,* 2004b)
A.niger	*aphA*	human: interferon-α-2	(MacRae *et al.,* 1993)
A.niger	*gpdA*	human:α1-proteinase inhibitor (antitrypsin), interleukin-6, hen: egg-white lysozyme HEWL	(Archer *et al.,* 1990) (Bohlin *et al.,* 2006)

A.niger	*gpdA*	*Trametes versicolor*: laccase	(Record *et al.,* 2002)
A. oryzae.	a-amylase	Human lactoferrin	(Ward *et al.,* 1992a)
A. terreus	Tet^R	Polyketide synthase	(Gressler *et al.,* 2015)
T. reesei	Cbh1	Bovine chymosin	(Harkki *et al.,* 1989)
T. reesei	Cbh1	*Phlebia radiate* laccase	(Saloheimo and Niku-Paavola 1991)
T. reesei	Cbh1	*Hormoconis resinae* glucoamylase	(Joutsjoki *et al.,* 1993)
T. reesei	Cbh1	Fab antibody fragment	(Keranen and Penttila 1995)
T. reesei	Cbh1	Barley endopeptidase B	(Saarelainen *et al.,* 1997)
T. reesei	Cbh1	*A. niger* acid phosphatase	(Miettinen-Oinonen *et al.,* 1997
T. reesei	Cbh1	*A. niger* lipase	(Qin *et al.,* 2012)
T. reesei	Pdc, eno	Homologous Xylanase11	(Li *et al.,* 2012)

1.4.2. Promoter Engineering to Enhance Heterologous Gene Expression in Filamentous Fungi

Promoters can be engineered for higher yields of heterologous proteins in filamentous fungi. Initially, there were trials that directly engineered tandem copies of the promoter fused upstream of a gene of interest. The introduction of multiple copies of inducible promoters though seemed to be an efficient strategy, was found to be a failure. The multiple copies of the promoter titrate out the available transcription activators in the cell. Introduction of an increasing number of PglaA copies, however, decreased the expression of a heterologous uidA gene in *A. niger* (Verdoes *et al.,* 1994). However, introducing multiple regions containing consensus binding sites for transcriptional activators or eliminating repressor binding sites can increase the level of transcription of the heterologous genes. Several studies reported the possibility of engineering the promoter for increased expression of heterologous genes. For example region III copy numbers in the gapd promoter in *A. oryzae* (Minetoki 1998), increasing the copy number of the CCAAT motif in the glaA promoter in *A. niger* (Liu *et al.,* 2003), and replacing the Cre1 binding sites with those of ACEII and Hap2 in the cbh1

promoter of *T. reesei* (Zou *et al.,* 2012). The introduction of heterologous transcriptional activator elements from one promoter to other promoters can lead to increased expression of heterologous genes. Fusion of the gapd box of the gapd promoter into the upstream region of the highly regulated *A. nidulans* amdS gene resulted in a 30-fold increase in expression of the lacZ reporter gene (Punt *et al.,* 1992). Promoter engineering by introduction of novel binding sites for useful transcription activators to enhance recruitment of RNA Polymerase II, could be used to improve heterologous gene expression in filamentous fungi.

1.4.3. Signal Peptides

Secretion signal peptides are the crucial elements for controlling heterologous protein production in fungus. Extensive research has been conducted to improve the secretary protein yield in yeast expression systems and still the problem is largely unresolved with highly unpredictable yield of secreted proteins. Signal peptide dependent secreted proteins contain a signal peptide (SP) at the N-terminus that directs the ribosomes to the rough endoplasmic reticulum (ER) for the completion of protein synthesis (Blobel *et al.,* 1975; Von Hejine *et al.,* 1990). The signal peptide, typically 15–50 amino acids in length and consisting of 15–20 hydrophobic amino acid residues, have three regions: n, h, and c and is cleaved off during translocation across the membrane. The positively charged n-region is at the N-terminus, varying widely in length. The h-region has a hydrophobic core, which has a block of 7–16 hydrophobic amino acids. The c-region is located at the C-terminus, with 4–6 relatively polar residues (Nothwehr and Gordon, 1990). Approximately 90% of the *A. niger* extracellular proteins identified by mass spectrometry contain classical N-terminal signal peptides (Tsang *et al.,* 2009). Although the amino acid sequences of the signal peptides are diverse, the functions of the signal peptides from different lineages of life are conserved. The native signal peptide of human interleukin-6 (hIL-6) directs secretion of hIL-6 into the extracellular fluid with low efficiency (0.4×10^3 U/ml) while the hiL-6 with the *A. niger* glaA signal peptide the yield is 11.8×10^3U/ml (Carrez *et al.,* 1990). The signal peptides from glucoamylase and chymosin showed comaparable efficiency in directing the chymosin to extracellular fluid in *A. nidulans* (Cullen *et al.,* 1987a). Use of leader peptide of an endogenous *A. awamori* endoxylanase in cutinase from *F. solani* during expression in *A. awamori* increased the production of the cutinase by two fold (van Gemeren *et al.,* 1996). For the expression of recombinant protein in filamentous fungi, a signal peptide from a highly

secreted protein such as *A. niger* glucoamylase and *T. reesei* CBHI, were frequently chosen to direct secretion of heterologous proteins (Ward, 2012).

The use of fungal secretion signal in driving recombinant protein production in yeasts has attained much attention recently. Most of the yeast expression systems contain the *S.cerevisiae* α factor pre pro signal as the secretion signal. The cellobiohydrolase I (CBH1) secretion signal from *T. reesei* is relatively small compared to other secretion signals, making this a suitable candidate for driving heterologous protein production in yeast. Very few secretion signals of fungal origin are used in yeast expression systems, including the signal peptide of *Rhizopus oryzae* amylase (Li and Wang, 2011) and *A. awamori* glucoamylase (Fierobe *et al.,* 1997). But all these signal peptides are typically used for expressing their own native proteins and not for other (heterologous) proteins. Signal peptides differ in their capacity to secrete proteins and the selected secretion signal should be compatible to a variety of proteins. Even though different signal peptides are capable of directing the translocation of heterologous proteins, they do not necessarily have the same efficiency. Analysis of the efficiency of different signal peptides for directing recombinant protein into extracellular fluid is essential for the development of higher production strains. There should be an optimized compatibility between signal peptide and the mature protein for high secretion of the protein of interest (Nothwehr and Gordon, 1990).

1.4. 4. Glycosylation: Expression and export and protein secretion

Protein glycosylation is one of the most important post-translational modifications and most of the all known proteins in eukaryotes are glycosylated (Apweiler *et al.,* 1999). Post-translational modifications of secreted recombinant proteins have been shown to influence the stability of protein, yield, and biological function. The therapeutic protein production by filamentous fungi for use in mammals has been restricted by the inherent difference in protein *N*-glycosylation and by the failure of the fungal cell to modify proteins with mammalian glycosylation patterns. However, both fungi and mammals attach a specific oligosaccharide to asparagine residues in the sequence Asn-X-Ser/Thr/Cys (where X represents any amino acid except proline), though the further processing of the attached glycan differs significantly between fungus and mammalian cells (Wildt *et al.,* 2005). Fungal oligo mannose type glycosylation makes the fungi unsuitable for pharmaceutical protein production. Compared to yeast which has the tendency to hyperglycosylate proteins, filamentous fungi are far more conservative with high mannose N linked glycosylation

(Deshpande *et al.,* 2008). However, fungi still lack the terminal sialic acid residues-the characteristic pattern of human glycosylation important for defining the function of the glycosylated protein. These problems have been addressed in several studies on the glycosylation pathway of filamentous fungi like *A. nidulans*, *A. niger*, *A. oryzae* (Kasajima *et al.,* 2006; Kainz *et al.,* 2008) and *T. reesei* (Maras *et al.,*1999; Zhong *et al.,* 2011). Glycoengineering of fungal production hosts is lagging behind the yeast even though the N-glycan structures of several secreted glycoproteins of *T. reesei* and *Aspergillus* sps have been elucidated (Moreman *et al.,* 1994; Maras *et al.,* 1997; Harison *et al.,* 1998). Attempts were made to modify fungal glycosylation structures by the insertion of glycan structure modifying enzymes (Nevalainen *et al.,* 2005). Considering the expression of recombinant proteins in filamentous fungi, glycosylation plays a key role in various aspects of the protein secretion pathway including folding, translocation, and sorting (Eriksen *et al.,* 1998).

1.4.5. Protein Secretion

Proteins entering the endoplasmic reticulum (ER) undergo a series of post-translational modifications including folding and maturation**.** In of the ER lumen, several folding assistants are present, like folding enzymes and molecular chaperones eg, BiP (Pedrazzini and Vitale, 1996; Gething, 1999). After proper modification and processing, the nascent polypeptide leave the ER by means of vesicle budding and is transported to the golgi apparatus for further modifications and then secreted through vesicular fusion with plasma membrane. During heterologous protein production, overexpression of heterologous gene leads to the heavy load of protein in ER and leads to the accumulation of unfolded or misfolded proteins (due to inefficient folding capacity of the cell or stress-causing agents). An increased load of proteins destined for secretion, altered metabolic conditions and depletion of Ca^{2+} can activate an intracellular signalling pathway referred to as the Unfolded Protein Response (UPR) (Kaufman, 1999; Rutkowski and Kaufman, 2004). Overexpression of genes encoding chaperones and foldases were performed to increase the folding capacity (Chapman *et al.,* 1998; Welihinda *et al.,* 1999; Conesa *et al.,* 2001) and thereby decreasing the accumulation of unfolded or misfolded proteins in the ER. Several genes for several ER chaperones and foldases have been isolated from fungi, such as bipA (Van Gemeren *et al.,* 1997), pdiA (Jeenes *et al.,* 1997; Ngiam *et al.,* 2000); and a family of calnexins (Conesa *et al.,* 2001) characterised and overexpressed to improve the heterologous protein production.

Filamentous fungi overproducing specific homologous or heterologous proteins had increased levels of bipA transcription, whereas bipA overexpressed strains had no significant effect on protein secretion yields (Punt *et al.,* 1998). BiP overexpression did not result in the increase of secreted levels of hIL-6 in *Aspergillus* (Gouka *et al.,* 1997) and pdiA overexpression did not increase secreted yields of HEWL in *A. niger* (Nigam *et al.,* 2000). Valkonen *et al.,*(2003) reported that the constitutive induction of the UPR pathway by overexpressing the transcription factor hacA in *A. niger var. awamori* leads to the enhanced production of *Trametes versicolor* laccase by up to 7-fold and of bovine pre pro chymosin by up to 2.8-fold in this biotechnologically important fungus. Disruption of a vacuolar protein sorting receptor gene in *A. oryzae* which is responsible for the aberrant vacuolar degradation enhanced production and secretion of the bovine chymosin and human lysozyme heterologous proteins (Yoon *et al.,* 2010).

1.4.6. Codon Optimization

When heterologous proteins are expressed in filamentous fungi, secretion yields are low compared to those of homologous proteins or proteins from closely related fungal species and generally do not exceed tens of milligrams per litre (Gouka *et al.,* 1997). Codon optimization is one of the most common approaches for improving the production level of heterologous proteins in host organisms (Gustafsson *et al.,* 2004). Codon preferences for effective transcription may vary from organism to organism. Heterologous gene expression through codon optimization resulted in improved heterologous protein production in filamentous fungal species (Table 1.4). Several studies of filamentous fungi have reported that codon optimization increased mRNA levels and corresponding increase in protein production (Scholtmeijer *et al.,* 2001; Koda *et al.,* 2005; Tanaka *et al.,* 2008). The effect of codon optimization was first studied by Gouka *et al.,* (1996) and expressed codon optimized synthetic plant α- glucosidase gene where 53 % of the codons were optimized for yeast codon bias, expressed in *A. awamori* (Koda *et al.,* 2005). Synthetic potato α-glucan phosphorylase gene was constructed by optimizing 39 % of the codons for codon usage in *A. niger* and was expressed in *A. niger*. The increase in mRNA after codon optimization was also reported in other filamentous fungal species such as *T. reesei* and *Schizophyllum commune* (Li *et al.,* 2007; Scholtmeijer *et al.,* 2001; Te'o *et al.,* 2000). A synthetic gene of the house dust mite allergen (Der f 7) from *Dermatophagoides farina*, had been optimized for *A. oryzae* codon usage and secreted protein comparison with native protein showed increase of production

level from three to five folds (Tanaka *et al.,* 2008). Most of the low-frequency codons of filamentous fungi contain A or U in their third positions. So codon optimization is an effective method for preventing premature polyadenylation during heterologous gene expression in filamentous fungi. Furthermore, the translation efficiency may be affected by the presence of rare codons in heterologous genes, although little information is available on this issue (Kinnaird *et al.,* 1991; Tuller *et al.,* 2010). Recently Straat *et al.,* (2014) overexpressed a codon-optimized gene for *cis*-aconitate decarboxylase in an oxaloacetate hydrolase and glucose oxidase deficient *A. niger* strain which led to highly increased yields and itaconic acid production titers.

Table 1.4. Codon optimization of heterologous genes in filamentous fungi

Gene	Source	Expression Host	Reference
Xylanase (xynB)	*Dictyoglomus thermophilum*	*Trichoderma reesei*	Te'o *et al.,* 2000
Hygromycin B resistance gene (hph)	*Escherichia coli*	*Schizophyllum commune*	Scholtmeijer *et al.,* 2001
Aequorin	*Aequorea aequorea*	*Neurospora crassa, Aspergillus niger, Aspergillus awamori*	Nelson *et al.,* 2004
α-Glucan phosphorylase	*Solanum tuberosum*	*Aspergillus niger*	Koda *et al*., 2005
Xylanase	*Orpinomyces* PC-2	*Hypocrea jecorina*	Li *et al.,* 2007
Luciferase	*Photinus pyralis*	*Neurospora crassa*	Gooch *et al.,* 2008
House dust mite allergen (Der f 7)	*Dermatophagoides farina*	*Aspergillus oryzae*	Tokuoka *et al.,* 2008
Endoglucanase	*Reticulitermes speratus*	*Aspergillus oryzae*	Sasaguri *et al.,* 2008
Der f 7	*Dermatophagoides farina*	*Aspergillus oryzae*	Tanaka *et al.,* 2012

1.4.7. Protease deficient strains

The main problem associated with extracellular protein production in filamentous fungi is the secretion of proteases by the host. Once the secreted protein arrives in extracellular fluid, they are immediately at the risk of being degraded. Filamentous fungi secrete large amounts of proteases, which degrade heterologous proteins (van den Hombergh *et al.,* 1997). The UV mutagenesis of the *A. niger* strain AB1.13 leads to the development of protease deficient strain. Recently, the affected gene was identified as a fungal specific Zn2Cys6 binuclear cluster transcription factor, which controls the activity of several protease-encoding genes (Punt *et al.,* 2008). Targeted inactivation of known extracellular proteolytic enzyme coding genes has been proven successful for the development of protease deficient strains. Human lysozyme production has been increased by the disruption of two proteinase genes (tppA and pepE) in *A. oryzae* (Jin *et al.,* 2007). Antisense RNA technology has been used to reduce the amount of serine-type carboxypeptidases to increase the production of human lysozyme in *A oryzae* (Zheng *et al.,* 1998). *A. niger* strain disrupted for three protease genes were evaluated for recombinants production (Van den Hombergh *et al.,* 1997). Specific mutants of *A. nidulans*, deficient in the aspartic protease gene, exhibited the ability to produce chymosin (Berka *et al.,* 2003). Yoon *et al.,* (2011) demonstrated how successive disruption of ten protease genes in *A. oryzae* was effective in enhancing heterologous production of human lysozyme and bovine chymosin production. Recent proteomic studies revealed that culture starvation, ie, depletion of carbon sources led to the secretion of higher amount of proteases (Adav *et al.,* 2010). Overexpression of *Aspergillus tubingensis* faeA in protease-deficient *A. niger* enables ferulic acid production from plant material (Zwane *et al*., 2014).

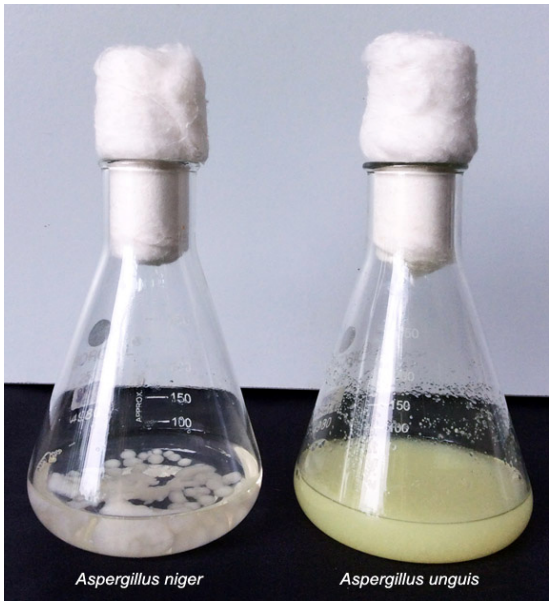
1.5. Objectives of the present Study

The attraction of filamentous fungi as production hosts is based on their natural ability to secrete large amounts of proteins (hydrolytic enzymes) into the extracellular fluid. Eukaryotic post-translational machinery is an added advantage for proteins requiring extensive post translational modifications like glycosylation, addition of multiple disulfide bonds etc.

One of the major problems associated with fungal cultivation for protein production is the peculiarity of its growth characteristics. Most of the fungi under submerged conditions grow as cotton like mycelial clumps which can assume the shape of large balls and can stick on to fermenter surfaces, hampering mass and oxygen transfer. Pelleted growth exhibited by

some fungi on the other hand can be highly advantageous since there is a uniform cell density which allows higher biomass and product formation (Liu *et al*., 2013). We had previously isolated a novel filamentous fungus belonging to the taxonomic group *Aspergillus unguis* which showed excellent growth characteristics under submerged fermentation make it attractive host for large-scale production of proteins. Under our culture conditions, the fungus grew as pellets in an even suspension, and did not form any large aggregates allowing better circulation of air and mixing which makes it as an attractive host for heterologous protein production (Fig 1.3). The fungus showed excellent growth characteristics in lignocellulosic biomass also.

Fig. 1.3. Pelleted growth of *Aspergillus unguis* suitable for large scale cultivation



Aspergillus unguis shows a pelleted growth with small sized mycelial pellets which allow it to attain high cell densities. In comparison, *A. niger* grows as large mycelial clumps and attains only a lower biomass density

The use of fungal signal peptides for driving recombinant protein secretion has currently attracted significant attention. Filamentous fungi are rich source of signal peptides which can be used as a carrier protein for directing secretion of heterologous proteins. Extensive research has been conducted to improve the secretary protein yield in yeast expression systems and still the problem is largely unresolved with highly unpredictable yield of secreted proteins.

The current work was therefore proposed to develop a filamentous fungus based expression system for expression of heterologous proteins in *Aspergillus unguis* NII 08123 available in NIST culture collection as host.

The specific objectives were

1. Construction of an integrative expression vector for *Aspergillus unguis* transformation and gene expression
2. Genetic transformation and analysis of the specific gene expression in *Aspergillus unguis* for establishing it as a production host
3. Profiling of *Aspergillus unguis* secretome for the prospecting of novel native promoters for efficient gene expression
4. Application of fungal signal peptides for developing highly efficient yeast expression systems

2. Materials and Methods

2.1. Microorganisms and culture conditions

The microorganism used for the current study was a filamentous fungus *Aspergillus unguis* NII 08123, previously isolated from soil sample, at the Biotechnology division of CSIR – National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram, India. Other fungi used in the study were *Trichoderma reesei* RUT C30, *Aspergillus nidulans* FGSC A4 and *Aspergillus niger NII 8121*. *T. reesei* RUT C30 was a kind gift from Prof. George Szakach, Technical University of Budapest, Hungary; *Aspergillus nidulans* FGSC A4 was supplied by Fungal Genetic Stock Centre, UK and *Aspergillus niger NII 08121* was from the CSIR-NIIST culture collection. Various fungal stock cultures were maintained as slants on Potato Dextrose Agar (PDA) or PDA + Hygromycin (50µg/ml). *Kluyveromyces lactis* was sourced from (Life Technologies, USA) and was maintained in YPD medium (Himedia, India). Culture was periodically subcultured.

For generating seed cultures, spores from stock cultures were inoculated on PDA slants. They were grown at 30 °C till the spore matured (4-5 days). Spores from seed slants were used for experiments either fresh or they were stored (for not more than a week) at 4 °C until used. Spore suspensions were prepared in sterile saline containing 0.05 % Tween 80. Sterile saline (3-5ml) was added to fully grown slants, and spores were dislodged into it by gentle pipetting using a 1.0 ml micropipette under aseptic conditions. The suspension was recovered by aspiration and transferred to a sterile 15 ml glass vial. Spores were counted under a phase contrast microscope using a hemocytometer to obtain the spore count. The suspension was appropriately diluted with sterile saline to obtain the required spore count (10^7 spores/ml unless otherwise specified) and was used as inoculum.

For genomic DNA preparation, mycelia were grown on potato dextrose broth in shake flasks at 30 °C for 48 h. Mycelia were harvested and washed with sterile distilled water, dried and stored at -20 °C until further use.

To test the mitotic stability of the transformants, they were subcultured for five consecutive generations on selection plates and for the sixth generation on non selection medium.

Escherichia coli strains DH5α, BL21 (DE3) and JM109 used for transformation studies were grown on Luria Bertani Agar at 37 °C and were stored at 4 °C for short term

preservation. The cultures were preserved as 50% glycerol stocks at -80 °C for long term storage. The bacterial strains and vectors used in the present study are listed in Table 2.1

Table 2.1. List of Bacterial strains and vectors used in the present study

Bacterial strains (*E.coli)*	Characteristics	Source
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	(Fermentas) Thermo Fisher, USA
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(lac-proAB)	(Fermentas) Thermo Fisher, USA
BL21(DE3)	*E. coli B dcm ompT hsdS* ($r_B^-m_B^-$) *gal*	New England Biolabs (NEB), USA
Vectors		
pTZ57R/T	Blue-White Screening, M13/pUCprimer sites, T7 promoter	(Fermentas) Thermo Fisher, USA
pUC19	2.686 kb vector, high copy number, Ampicillin resistance marker, lacZ selection and ColE1 compatibility group origin of replication.	(Fermentas) Thermo Fisher, USA

2.2. Protein Assay

Protein assay was performed according to the method of Bradford (1976) and the protein concentration was expressed as mg/ml.

2.3. Chromosomal DNA isolation from the fungus

Fungal spores (10^7 spores) were inoculated in 100 ml of potato dextrose broth (Himedia, India) medium with 1% glucose as carbon source in 250 ml Erlenmeyer flasks, and were incubated at room temperature (28 ± 2 °C) for 48 h. One gram wet-weight of the mycelium was frozen in liquid nitrogen and was ground to a fine powder. It was suspended in 10 ml of lysis buffer (250 mM NaCl, 25 mM EDTA, 0.5 % w/v CTAB and 200 mM Tris–HCl, pH 8.5). The suspension was incubated at 60 °C for 30 min with occasional gentle mixing. After centrifugation at 18,000g for 15min (4 °C), the supernatant was transferred to a new tube and

polysaccharides and proteins were precipitated by adding equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Clear aqueous fraction was transferred with wide bore pipette into a clean nuclease free centrifuge tube. DNA was precipitated by adding 2-3 volumes of chilled iso-propanol to aqueous fraction. The solution was gently mixed by inversion, placed at -20 °C for 20 min and DNA was recovered by centrifugation at 18000g for 15 min (4 °C). The DNA precipitate was washed twice with 70 % ethanol and was allowed to air dry after which it was resuspended in 10 mM Tris-EDTA buffer (pH 8.0). RNAase was added to a final concentration of 10 µg/ml at 37 °C (Doyle and Doyle, 1987). The DNA was purified again using phenol:chloroform: isoamyl alcohol precipitation method. After a final wash with ethanol, the DNA was allowed to air dry and was resuspended in 10mM Tris-EDTA buffer (pH 8.0). For long term storage, DNA was kept at -20 °C.

2.4. Plasmid DNA isolation

Plasmid DNA was isolated based on the alkaline lysis method (Bimboim and Doly, 1979). For DNA sequencing the plasmid DNA was isolated using Qiagen® "Mini Prep" Plasmid isolation kit following the manufacturer's instructions.

2.5. Nucleic acid quantitation

The concentration of purified DNA was determined by measuring absorbance of appropriately diluted DNA at 260 nm. An absorbance unit of 1.0 at 260 nm was taken to be equivalent to a concentration of 50 µg/ml of double stranded DNA (Sambrook *et al.*, 1989; Sambrook *et al.*, 2001). The purity of DNA was confirmed by measuring the 260/280 ratio and the acceptable limit was set to ~ 1.8.

2.6. Electrophoresis and Western Blot analysis

2.6.1. Poly Acrylamide Gel Electrophoresis (PAGE)

The molecular weight as well as the homogeneity of the protein was determined by Sodium Dedecyl PAGE (SDS PAGE) as well as Native PAGE using the method of Laemlli (1970). Gels with 12% strength of acrylamide were used for the electrophoresis. The total protein concentration in the culture fluid was determined by Bradford's method (1976). The protein

in the supernatant was precipitated using 10 volumes of 12% Trichloroacetic acid (TCA) in acetone, and the precipitate was recovered by centrifugation at 13400g for 20 min at 4 °C. The pellets were washed thrice with acetone; air dried and was resuspended in distilled water. Samples normalized for their protein content were loaded on either 12% Native or SDS poly acrylamide gels and were separated electrophoretically (Laemmli, 1970). Band intensities were measured using ImageJ software (Schneider *et al*., 2012).

2.6.2 Western Blot analysis

Western blotting was done with 20-25µg of total protein in a semi dry electro-blotting system (GE Healthcare, USA). The proteins were resolved by 12% SDS gel electrophoresis and transferred to a PVDF transfer membrane (Immobilon P™, Millipore®, USA). Pre-stained protein molecular weight markers were used to check the efficiency of transfer. After every step, the blots were washed in wash buffer. The blocking of blots was done in a blocking solution, (3% BSA in PBS) either overnight or for 3h at 37 °C. The blots were then washed and incubated with and appropriately diluted primary antibody solution and incubated for 4h on a rotary shaker at room temperature. To remove the unbound primary antibody, blots were washed using the wash buffer (composition). For detection of the blots, the secondary antibody solution was added and incubated for 2h at room temperature. The blots were later washed with wash buffer and were incubated with the chromogeneic substrate for developing the blot. Developed blots were washed and were documented using a Gel Documentation system (Chemidoc, Biorad, USA). Image analyses were performed using ImageJ software (Schneider *et al*. 2012).

2.6.3. Agarose Gel Electrophoresis

Agarose Gel electrophoresis of DNA was performed in a Biorad Horizontal Gel apparatus. 1% Agarose gel was used for separation of PCR amplicons and 0.8 % was used for Genomic DNA. Ethidium bromide was included in the gel for fluorescent visualization of DNA fragments under UV light. On every gel, 0.5µg of 1kb DNA ladder (Fermentas®, USA) was run as a molecular weight marker for determination of the approximate size of DNA fragments

2.7 Transformation of *E coli* cells

Transformation steps were performed according to standard procedures (Sambrook *et al.*, 2001)

2.8. Transformation of *K. lactis* cells

The *K. lactis* cells were first revived from glycerol stocks on YPD plates and allowed to grow for 2 days at 30 °C. The day before transformation the culture was prepared in 10 ml YPD media and grown overnight. The culture was then used for inoculating 50 ml YPD medium in 100 ml Erlenmeyer flask at initial OD_{600} of 0.05 and incubated at 30 °C at 200 rpm until OD_{600} reached 0.7 to 0.9. The cells were harvested at room temperature by centrifugation at 3000g, washed once with sterile water and resuspended in 10 ml YPD broth containing 25mM dithiothreitol (DTT) and 20 mM HEPES (pH 8.0) and incubated at 30°C for 15 min. They were centrifuged and washed twice with 50 ml sterile distilled water. The pellet was again washed with 25 ml distilled water and resuspended in 4.0 ml 1M Sorbitol, centrifuged at 4000g and finally resuspended in 1ml of 1M Sorbitol. The competent cells were stored at -80 °C until use.

Competent cells (100 µl) were mixed with 5 µg of linearized plasmid and placed on the ice for 15min. After linearization with SacII, the plasmids were transformed into *K.lactis* cells by electroporation using an electroporator (Genepulser Xcell, Biorad, USA) with a 2.0 mm electroporation cuvette. The conditions used were: a sample volume of 100 µl, charging voltage of 1.8 kV, and capacitance of 25 µF. Integration of the expression cassette into the genome occurred by homologous recombination into the lac4 locus. Transformed colonies were selected on YPD acetamide plates and were incubated at 30 °C for 3–4 days.

2.9. DNA manipulation

All DNA manipulations involving cloning and restriction digestions were performed following established procedures (Sambrook *et al.*, 2001). Restriction enzymes used for digestion of DNA fragments during construction of various expression vectors were from New England Biolabs (NEB) or MBI Fermentas. DNA fragments were extracted from

agarose gel pieces using GeneJET gel extraction kit (MBI Fermentas) according to procedure recommended by supplier.

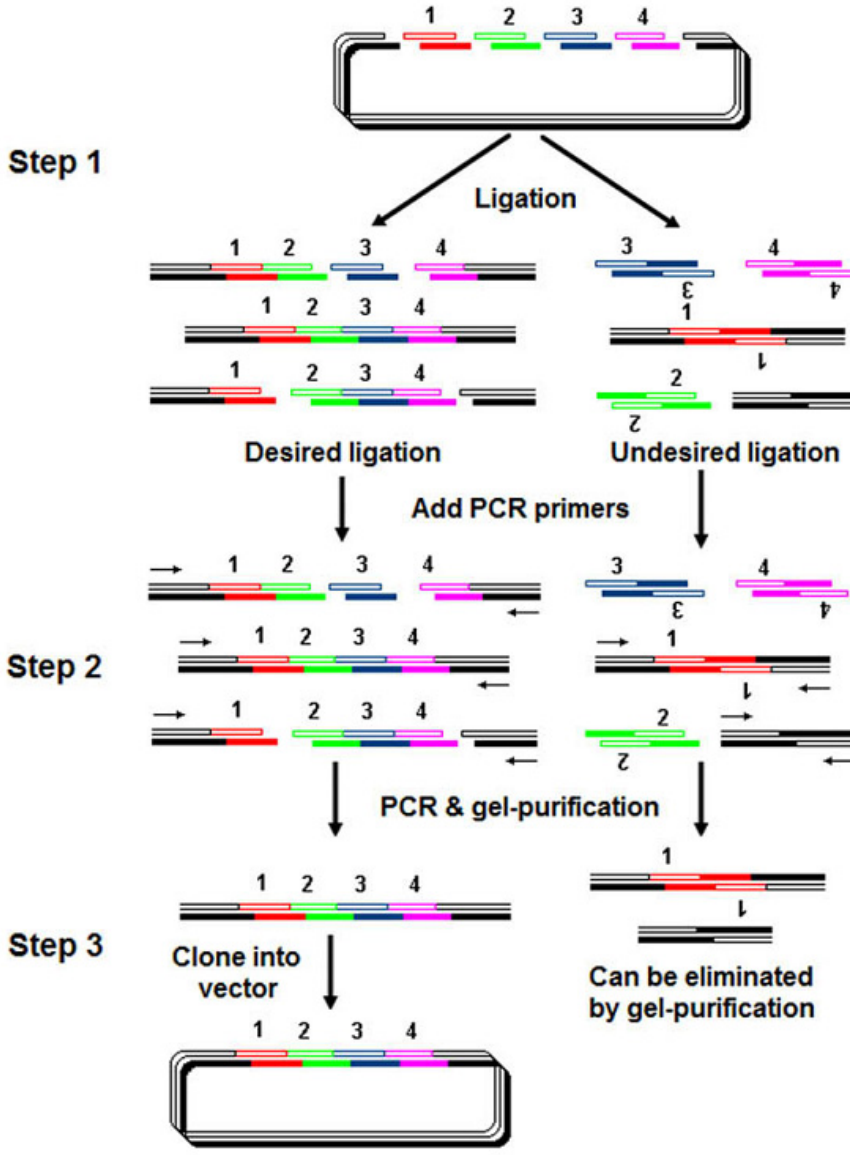
2.10. Restriction endonuclease digestion

DNA samples in appropriate reaction buffers were digested with restriction endonucleases (New England Biolabs, USA) at desired temperatures according to supplier's instructions. For analytical purpose, the reactions were set up in a volume of 20 µl. For preparative scale digestion, reactions were carried out in a volume of 60- 100 µl. Reactions were stopped by adding 6× gel loading buffer (final concentration of 1×), and mixture was subsequently heated at 65 °C for 5 min to inactivate the enzyme. The samples were subjected to agarose slab gel electrophoresis.

2.11. PCR-ligation method for cloning of multiple DNA inserts

For the assembly of multiple inserts for the construction of different vectors PCR-after-ligation method was used (An *et al*., 2010). After initial ligation of multiple inserts and vector, the ligation mixture is used as template for a PCR using a pair of primers flanking the cloning sites on the vector. The fragment with correct size is gel purified and inserted into the vector by conventional ligation and transformed in to *E. coli* DH5α cells (Fig.1.1)

Fig. 2.1. Outline of the PCR Ligation Method (An *et al*., 2010)



3. Construction of fungal expression vector and cloning

3.1. Introduction

Advances in genomics and proteomics have brought filamentous fungi in spotlight as microbial cell factory (Meyer, 2008). Endowed with high secretion capacity, good yields and favourable media requirements, *Aspergilli* are much sought after organisms for production of industrially important compounds as well as for use as hosts for expression of heterologous protein production (Lubertozzi and Keasling, 2009). For example, *Aspergillus niger* is an industrially important citric acid producer (Papagianni, 2007). Well established fermentation processes and 'generally regarded as safe' status make most of the *Aspergillus* sp attractive heterologous protein production hosts. For heterologous protein production, an expression system comprises of various components which plays crucial role in each step of recombinant protein production. Developing various components like promoters, markers, reporters, signal peptides and most importantly suitable host background therefore assumes importance.

Previous studies conducted at the Centre for Biofuels, CSIR-NIIST on prospecting of fungal strains producing cellulases had resulted in the isolation of a filamentous fungal isolate *Aspergillus unguis*. The growth characteristics of *A. unguis* under submerged fermentation make it attractive host for large-scale production of heterologous proteins. Under submerged culture conditions, the fungus grew as pellets in an even suspension and did not form large clumps/aggregates, allowing better aeration and mixing suitable for large scale production. Other *Aspergilli* like *A. niger* grew as mat or ball-like structures in shake flasks and larger non-uniform aggregates/cotton like mass in fermenter. Based on the above growth characteristics, *A. unguis* was chosen as a host strain for recombinant protein production.

To enable stable integration of the heterologous gene of interest into the chromosome of the host fungus, an expression vector is needed. For the construction of expression vector, glyceraldehyde 3 phosphate dehydrogenase (GAPD) promoter from *A. nidulans* and hygromycin (hph) as selectable marker for the transformants was used. Hygromycin B is a widely used selection marker in the transformation of fungi (Nakazawa *et al*., 2009; Kano *et al*., 2010).

Electroporation of germinated conidial cells was used for easy transformation of the filamentous fungus. Electroporation has been used to transfer DNA into many organisms. Usually, electroporation procedures for fungi require protoplasts or germinating conidia (Robinson and Sharon, 1999; Dobrowolska and Staczek, 2009). In this study, we describe the

construction of an expression vector developed for *A. unguis* and its genetic transformation and selection.

3.2. Materials and Methods

3.2.1 Strain, Plasmids and Media

Aspergillus unguis and *Aspergillus nidulans* were used for isolation of genomic DNA. *Escherichia coli* DH5α and JM109 (Fermentas, USA) was used as host for DNA manipulations. Vector pTZ57R/T (Fermentas, USA) was used for the routine cloning experiments and maintenance of target genes and regions. Potato dextrose agar and broth (Himedia, India) were used for the cultivation of *Aspergillus unguis* NII 08123 as described under section 2.4.1. *E.coli* was grown in Luria Bertani (LB) broth and transformants were grown in the same medium supplemented with 100µg/ml Ampicillin. For biomass estimation, the strain was grown on potato dextrose agar (PDA) medium for conidia production, and then conidia were harvested by washing the plate with 5 ml sterile saline, the conidia were resuspended and adjusted the concentration to 1×10^7 conidia per ml. Conidia (10^7 spores/ml) were inoculated in 100 ml of Mandel and Weber medium (Mandel and Weber, 1969) supplemented with 1% glucose. The fungal biomass was collected by filtration and then washed by distilled water for three times. The dry weights of different samples were obtained by determination the constant weight at 100°C. Protein content was quantified in the supernatants using Bradford's assay (1976).

3.2.2. General DNA Manipulation Techniques

All the DNA manipulation techniques were carried out using the standard procedures as described by Sambrook *et al.*, (1989). Chromosomal DNA isolation was performed as described in section 2.3 and this DNA was used as the template for PCR and for further studies.

3.2.3. Molecular cloning

PCR fragments obtained after conventional PCR were gel purified using QIAquick® Gel Extraction Kit (Qiagen, Germany) and the eluted products were cloned in to pTZ57R/T

vector followed by transformation into competent *E.coli* DH5α (Fermentas, USA). Transformation was performed using Transform Aid™ Bacterial Transformation Kit (Fermentas, USA). LB plates containing Ampicillin (100 µg/ml) were plated with the transformed *E. coli* and were incubated for 12-16 h at 37 °C. Ampicillin resistant colonies were selected as successfully transformed cells. One of the transformant colonies of *E. coli* was inoculated into LB Broth containing Ampicillin and was cultivated for 12-16 h at 37 °C with 200 rpm agitation in an incubator shaker. Plasmid DNA was isolated using a plasmid isolation kit (Miniprep, Qiagen, Germany). Presence and size of the inserts were determined by restriction digestion of the purified plasmids according to the enzyme manufacturer`s protocol and also by PCR amplification of the desired fragment from the plasmid using the same set of primers used for generating the amplicons. The generated amplicons were sequenced to confirm the presence of insert. Sequence reads were analyzed by NCBI-BLAST. Newly constructed gene cassettes were sequenced at each step of construction, cloned and propagated in *E coli* DH5α strain for long term storage.

3.2.4. Transformation of *Aspergillus unguis*

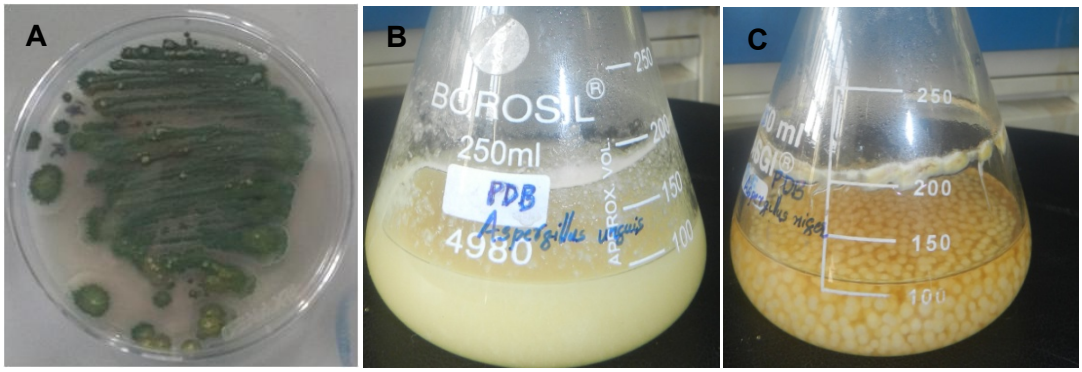
Conidia from strain *A.unguis* washed 5 times with 10 ml of distilled water were used to inoculate 100 ml of potato dextrose broth at a density of 1×10^7 conidia/ml and incubated at 30 °C in a rotary shaker (250 rpm) for 7 h. Conidia recovered by centrifugation (3000g for 5min at 4 °C) were resuspended in 50 ml of ice-cold sterile water, centrifuged again and was resuspended in 20 ml of osmotic medium (1.2 M $MgSO_4$, 10 mM Sodium phosphate buffer) supplemented with 70mg of lyzing enzyme from *Trichoderma harzinarum*. The cell wall weakening reaction was allowed to proceed for 90 min at 30 °C with gentle agitation (100 rpm). Treated conidia recovered by centrifugation (3000g for 5 min at 4 °C) were resuspended in ice-cold sterile water, washed twice and resuspended in 1 ml of ice-cold electroporation buffer (10 mM Tris-HCl (pH 7.5), 270 mM sucrose, 1 mM lithium acetate) and was kept on ice. For electroporation, 20µg of purified DNA was added to 100 µl of the ice cold conidial suspension. The mixture was incubated on ice for 30 min and then transferred to a 0.2 cm cuvette (Biorad, USA). Electroporation was performed using an electroporator (Gene Pulser™, Biorad, USA). Voltage was adjusted to 1500 V, capacitance to 25 µF and resistance was 400 Ω (pulse length varied between 5.1 and 5.8 ms). Following electroporation, 1 ml of ice-cold YPD with Sorbitol was added to the cuvette and the cell

suspension was transferred to a sterile 10 ml tube, kept on ice for 15 min and incubated at 30°C for 2h in a rotary shaker at 100rpm. Transformed colonies were selected on hygromycin plates and were incubated at 30 °C for 5-7 days. The transformants were analyzed by PCR amplification with the specific primers (Modified protocol of Sanchez *et al*., 1996).

3.3. Results and Discussion

Growth characteristics of this fungus make better strain for heterologous protein production (Fig.3.1). *A. unguis* was found to grow very well in lignocellulosic biomass and produce biomass hydrolysing enzymes especially glucose tolerant β glucosidases (Rajasree *et al*., 2013). The cell biomass increased with time and achieved maximum on day 4 in Glucose (7.5 g/l dry cell weight). The maximum protein content (1.8 mg/ml) also was obtained at the fourth day (Fig.3.2). Protease activity of the strain was measured and compared with the other well known heterologous expression hosts. The production was less during the initial 24 hours in *A. unguis* and later the production was comparable to *A. niger* and *T. reesei* (Fig.3.3). The pH profiling during the growth of the fungus revealed that the culture did not acidify the medium like other *Aspergilli*.

Fig. 3.1. Growth morphology of *Aspergillus unguis* NII 08123



(A) *A. unguis* in culture plate (B) *A. unguis* in submerged culture showing uniform pelleted growth (C) *A. niger* in submerged culture showing growth as large mycelial balls

Fig.3.2. Biomass and protein content analysis of *A. unguis*

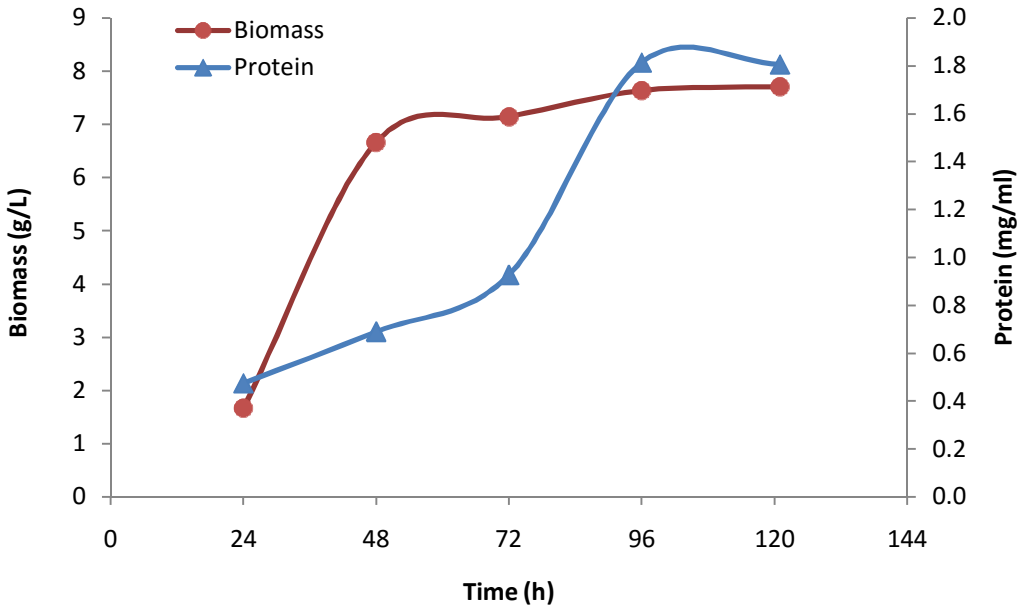
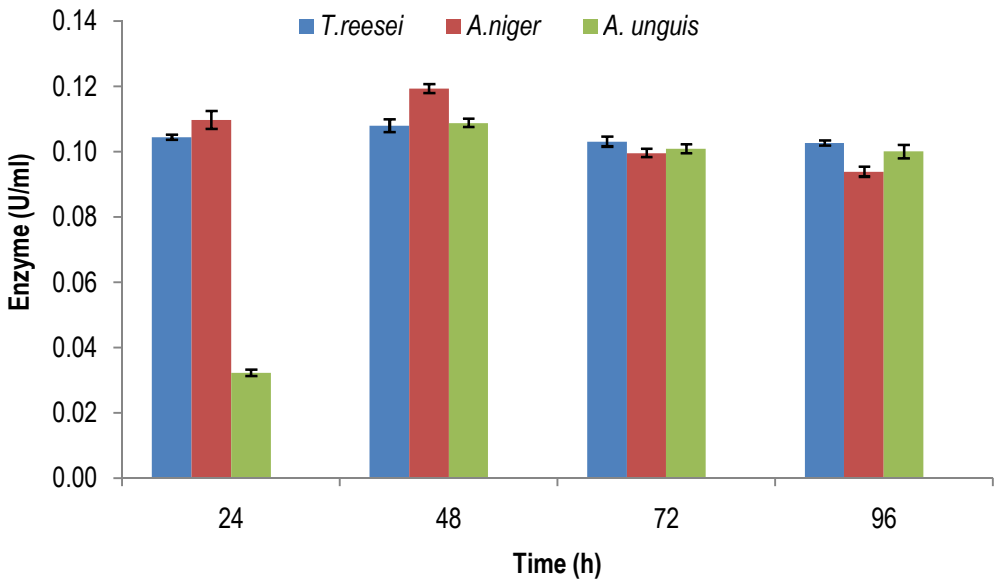


Fig.3.3. Protease profile of *A. unguis. T. reesei* and *A. niger*



3.3.1. *Chromosomal DNA isolation*

Fungal genomic DNA was isolated using CTAB method as per the protocol described in section 2.3 and was visualized in an agarose gel. The size of the genomic DNA was >10kb.

3.3.2 Construction of hygromycin expression cassette (Pgapd-hph-TtrpC)

The hygromycin gene (1020bp) was PCR amplified from the vector pSHKLx1 (mycobacterial shuttle vector) using the primer pairs HPHF and HPHR (Table 3.1, Fig. 3.4). The amplified gene was sequenced to confirm the identity. Antibiotic-resistance markers allow straight forward selection of strains with a high copy number of the integrated plasmid. The gapd promoter (2300bp) including regulatory elements for driving hygromycin expression was amplified from *Aspergillus nidulans* using GAPDHF and GAPDHR (Table 3.1, Fig. 3.5). The efficient transcription terminator trpC (770bp) was amplified from *A.nidulans* with the primer sets TRPCHF and TRPCHR (Table 3.1, Fig. 3.6). The Hph-TrpC construct was made by fusing hygromycin gene to the trpC terminator after digestion with restriction enzyme BamH1 using DNA ligase and was amplified from the ligation mixture by ligation PCR method using HPHF and TRPCHR (Table 3.1, Fig.3.7) and further linked to the gapd promoter by blunt end ligation. The whole cassette was PCR amplified by ligation PCR method using GPDHF and TRPCHR (Table 3.1, Fig.3.8). The constructed hygromycin expression cassette was sequenced to confirm whether the gene has been incorporated '*in-frame'* in the cassette (Fig.3.9).

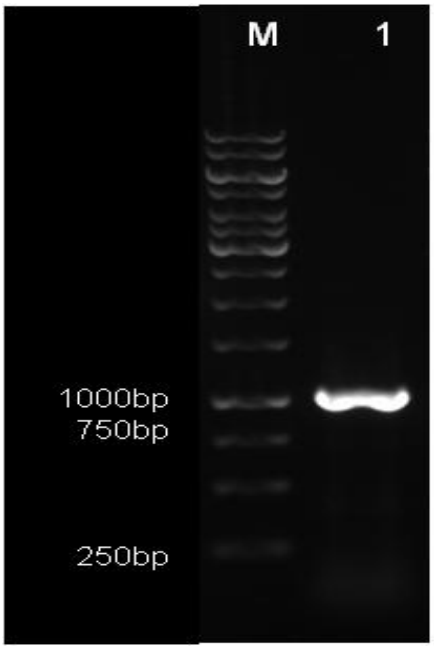
3.3.3. Construction of EGFP expression cassettes (Pgapd-EGFP-TtrpC)

Green Fluorescent Protein (GFP) over-expression vector was prepared by cloning an improved version of GFP gene-EGFP under the control of *A. nidulans* gapd promoter. The gene (720bp) was amplified using the primer set GFPF and GFPR (Table 3.1 and Fig. 3.10). EGFP carries a Ser 65 to Thr point mutation in the chromophore domain which results in an enhanced fluorescence with a single excitation and emission peak. gapd promoter and trpC terminator was amplified by the designed oligonucleotide primer pairs GAPDGF, GAPDGR and TRPCGF and TRPCGR (Table 3.1). For the vector construction, GFP was first ligated to trpC terminator after digestion with Not1. The GFP-TrpC cassette was amplified by ligation PCR with primer pairs GFPF and TRPCGR (Table 3.1, Fig. 3.11). The PCR product was then purified and further ligated to gapd promoter by blunt end ligation. The whole construct was PCR (ligation PCR) amplified with GAPDGF and TRPCR (Fig. 3.12) and sequenced to confirm the identity (Fig.3.13).

Table 3.1 Oligonucleotide primers used in the study

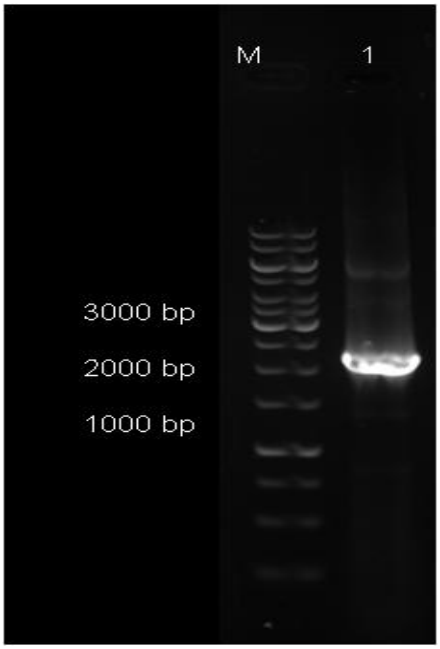
Primer	Sequence
GAPDHF	AATAGATCTGAATTCCCTTGTATCTCTACACACAG
GAPDHR	GGTGATGTCTGCTCAAGCGGGGTAGCTG
HPHF	ATGCCTGAACTCACCGCGACGTCTGTCGA
HPHR	TCGTCCGAGGGCAAAGGAATAGAGTAGATGCCGA
TRPCHF	AATGGTACCCCGCGGGATCCACTTAACGTTACTGAAA
TRPCHR	AATAAGCTTTCGAGTGGAGATGTGGAGTGGGCGC
GPDGF	AATAAGCTTGAATTCCCTTGTATCTCTACACACAGGC
GPDGR	AGCTACCCCGCTTGAGCAGACATCACC
GFPF	ATGGTGAGCAAGGGCGAGGAGCTG
GFPR	GCGGCCGCTCGGCATCTACTTTACTTGTACAGCTCGTCCAT
TRPCGF	AATCCGCGGGATCCACTTAACGTTACTGAAA
TRPCGR	AATTCGAGTGGAGATGTGGAGTGGGCGC
PUCF	AATAGATCTTCGACCTGCAGGCATGCAAGCTTGG
PUCR	AATATCGATGTAATCATGGTCATAGCTGTTTCCTGTG

Fig 3.4: PCR amplification of Hygromycin gene



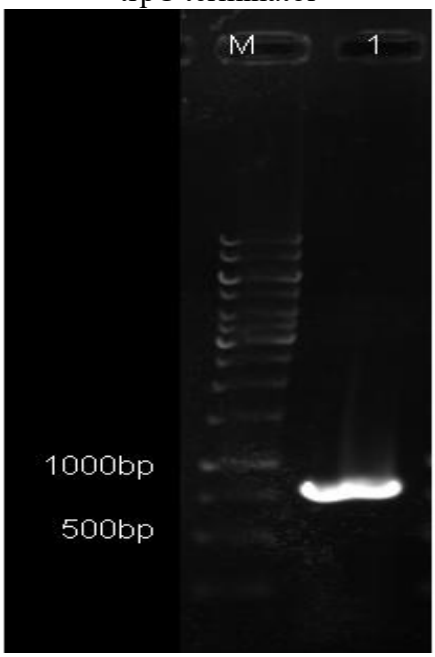
M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - PCR amplification of hygromycin gene (1020bp)

Fig 3.5: PCR amplification of gapd promoter



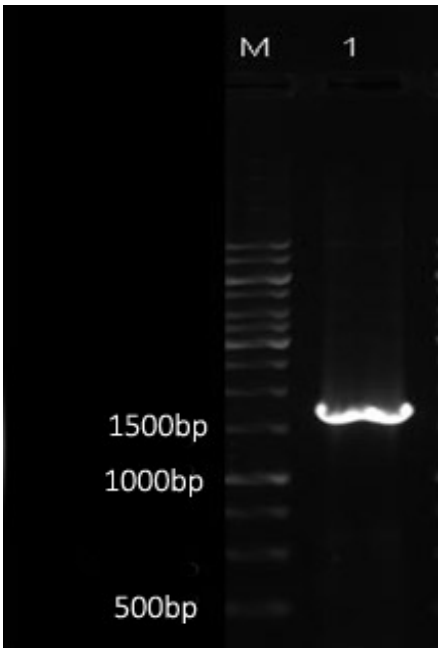
M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - PCR amplification of gapd promoter (2300bp)

Fig 3.6: PCR amplification of trpC terminator



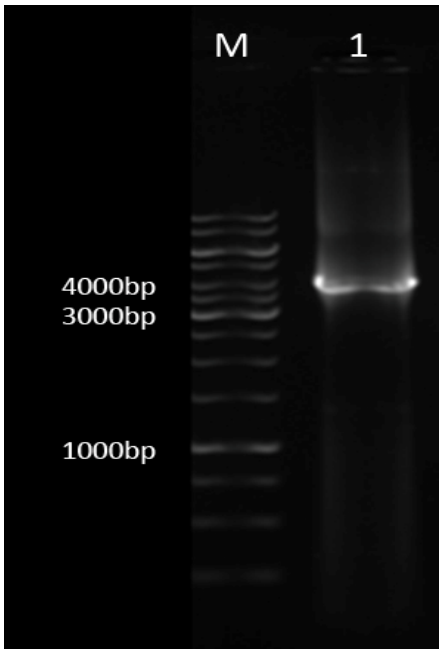
M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - PCR amplification of trpC gene (770bp)

Fig 3.7: Ligation PCR of hph-TtrpC construct



M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 -Ligation PCR product (hph-TtrpC) (1790bp)

Fig 3.8: Ligation PCR of full length hygromycin expression cassette



M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - Ligation PCR product of full length hph cassette (Pgapd-hph-TtrpC) (4090 bp)

Fig. 3.9. Sequencing of hygromycin expression cassette (Pgapd-hph-TtrpC)

GAATTCCCTTGTATCTCTACACACAGGCTCAAATCAATAAGAAGAACGGTTCGTCTTTTTCGTTTATATCTTGCA
TCGTCCCAAAGCTATTGGCGGGATATTCTGTTTGCAGTTGGCTGACTTGAAGTAATCTCTGCAGATCTTTCGACA
CTGAAATACGTCGAGCCTGCTCCGCTTGGAAGCGGCGAGGAGCCTCGTCCTGTCACAACTACCAACATGGAGTAC
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TTCAAGTACACCAACGCTCTTCTGATCCAGTCGATCATCCGCTGAAGGCGCTTTCGAATCTGGTTAAGATCCACG
TCTTCGGGAAGCCAGCGACTGGTGACCTCCAGCGTCCCTTTAAGGCTGCCAACAGCTTTCTCAGCCAGGGCCAGC
CCAAGACCGACAAGGCCTCCCTCCAGAACGCCGAGAAGAACTGGAGGGGTGGTGTCAAGGAGGAGTAAGCTCCTT
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TTGGTTGAATTTAGAACGTGGCACTATTGATCATCCGATAGCTCTGCAAAGGGCGTTGCACAATGCAAGTCAAAC
GTTGCTAGCAGTTCCAGGTGGAATGTTATGATGAGCATTGTATTAAATCAGGAGATATAGCATGATCTCTAGTTA
GCTCACCACAAAAGTCAGACGGCGTAACCAAAAGTCACACAACACAAGCTGTAAGGATTTCGGCACGGCTACGGA
AGACGGAGAAGCCACCTTCAGTGGACTCGAGTACCATTTAATTCTATTTGTGTTTGATCGAGACCTAATACAGCC
CCTACAACGACCATCAAAGTCGTATAGCTACCAGTGAGGAAGTGGACTCAAATCGACTTCAGCAACATCTCCTGG
ATAAACTTTAAGCCTAAACTATACAGAATAAGATAGGTGGAGAGCTTATACCGAGCTCCCAAATCTGTCCAGATC
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GCCAGACAGCTCTGGCGGCTCTGAGGTGCAGTGGATGATTATTAATCCGGGACCGGCCGCCCCTCCGCCCCGAAG
TGGAAAGGCTGGTGTGCCCCTCGTTGACCAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCATCGAATCAC
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CAGAAGTCCAATTGCTTCCGATCTGGTAAAAGATTCACGAGATAGTACCTTCTCCGAAGTAGGTAGAGCGAGTAC
CCGGCGCGTAAGCTCCCTAATTGGCCCATCCGGCATCTGTAGGGCGTCCAAATATCGTGCCTCTCCTGCTTTGCC
CGGTGTATGAAACCGGAAAGGCCGCTCAGGAGCTGGCCAGCGGCGCAGACCGGGAACACAAGCTGGCAGTCGACC
CATCCGGTGCTCTGCACTCGACCTGCTGAGGTCCCTCAGTCCCTGGTAGGCAGCTTTGCCCCGTCTGTCCGCCCG
GTGTGTCGGCGGGGTTGACAAGGTCGTTGCGTCAGTCCAACATTTGTTGCCATATTTTCCTGCTCTCCCCACCAG
CTGCTCTTTTCTTTTCTCTTTCTTTTCCCATCTTCAGTATATTCATCTTCCCATCCAAGAACCTTTATTTCCCCT
AAGTAAGTACTTTGCTACATCCATACTCCATCCTTCCCATCCCTTATTCCTTTGAACCTTTCAGTTCGAGCTTTC
CCACTTCATCGCAGCTTGACTAACAGCTACCCCGCTTGAGCAGACATCACCATGCCTGAACTCACCGCGACGTCT
GTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGT
GCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGAT
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AGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCT
GTTCTGCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCA
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CACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCC
GAGGACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGC
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CGCGTTTTCGGGTTTACCTCTTCCAGATACAGCTCATCTGCAATGCATTAATGCATTGACTGCAACCTAGTAACG
CCTTNCAGGCTCCGGCGAAGAGAAGAATAGCTTAGCAGAGCTATTTTCATTTTCGGGAGACGAGATCAAGCAGAT
CAACGGTCGTCAAGAGACCTACGAGACTGAGGAATCCGCTCTTGGCTCCACGCGACTATATATTTGTCTCTAATT
GTACTTTGACATGCTCCTCTTCTTTACTCTGATAGCTTGACTATGAAAATTCCGTCACCAGCNCCTGGGTTCGCA
AAGATAATTGCATGTTTCTTCCTTGAACTCTCAAGCCTACAGGACACACATTCATCGTAGGTATAAACCTCGAAA
TCANTTCCTACTAAGATGGTATACAATAGTAACCATGCATGGTTGCCTAGTGAATGCTCCGTAACACCCAATACG
CCGGCCGAAACTTTTTTACAACTCTCCTATGAGTCGTTTACCCAGAATGCACAGGTACACTTGTTTAGAGGTAAT
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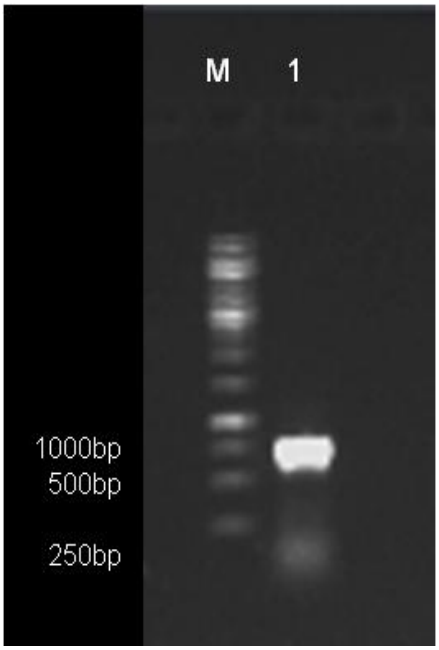
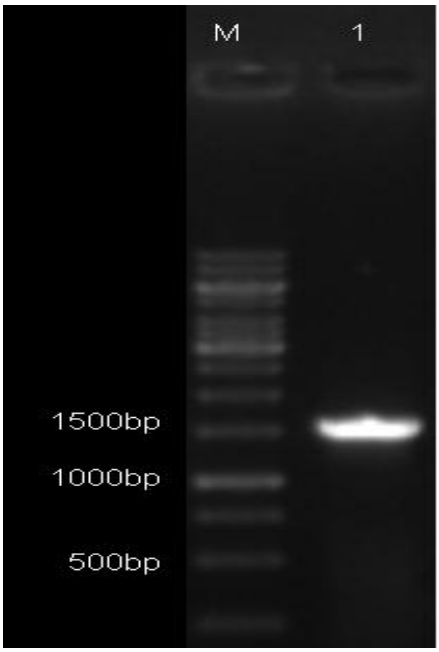
Fig 3.10: PCR amplification of EGFP gene



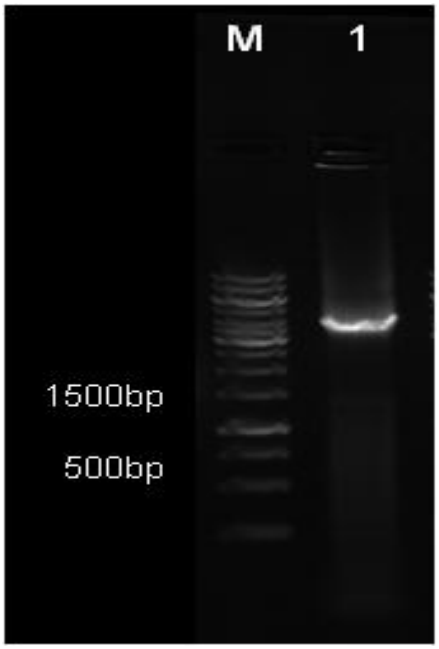
M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - PCR amplification of EGFP gene (720bp)

Fig 3.11: Ligation PCR of EGFP-TtrpC cassette



M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - PCR amplification of EGFP-TtrpC cassette (1490 bp)

Fig 3.12. Ligation PCR of Pgapd-EGFP-TtrpC expression cassette



M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - PCR amplification of Pgapd-EGFP-TtrpC cassette (3790 bp)

Fig.3.13. Sequencing of EGFP expression cassette (Pgapd-EGFP-TtrpC)

GAATTCCCTTGTATCTCTACACACAGGCTCAAATCAATAAGAAGAACGGTTCGTCTTTTTCGTTTATATCTTGCA
TCGTCCCAAAGCTATTGGCGGGATATTCTGTTTGCAGTTGGCTGACTTGAAGTAATCTCTGCAGATCTTTCGACA
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TTCAAGTACACCAACGCTCTTCTGATCCAGTCGATCATCCGCTGAAGGCGCTTTCGAATCTGGTTAAGATCCACG
TCTTCGGGAAGCCAGCGACTGGTGACCTCCAGCGTCCCTTTAAGGCTGCCAACAGCTTTCTCAGCCAGGGCCAGC
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CCTACAACGACCATCAAAGTCGTATAGCTACCAGTGAGGAAGTGGACTCAAATCGACTTCAGCAACATCTCCTGG
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ATGGTTGACCGGTGCCTGGATCTTCCTATAGAATCATCCTTATTCGTTGACCTAGCTGATTCTGGAGTGACCCAG
AGGGTCATGACTTGAGCCTAAAATCCGCCGCCTCCACCATTTGTAGAAAAATGTGACGAACTCGTGAGCTCTGTA
CAGTGACCGGTGACTCTTTCTGGCATGCGGAGAGACGGACGGACGCAGAGAGAAGGGCTGAGTAATAAGCCACTG
GCCAGACAGCTCTGGCGGCTCTGAGGTGCAGTGGATGATTATTAATCCGGGACCGGCCGCCCCTCCGCCCCGAAG
TGGAAAGGCTGGTGTGCCCCTCGTTGACCAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCATCGAATCAC
CGGCAGTAAGCGAAGGAGAATGTGAAGCCAGGGGTGTATAGCCGTCGGCGAAATAGCATGCCATTAACCTAGGTA
CAGAAGTCCAATTGCTTCCGATCTGGTAAAAGATTCACGAGATAGTACCTTCTCCGAAGTAGGTAGAGCGAGTAC
CCGGCGCGTAAGCTCCCTAATTGGCCCATCCGGCATCTGTAGGGCGTCCAAATATCGTGCCTCTCCTGCTTTGCC
CGGTGTATGAAACCGGAAAGGCCGCTCAGGAGCTGGCCAGCGGCGCAGACCGGGAACACAAGCTGGCAGTCGACC
CATCCGGTGCTCTGCACTCGACCTGCTGAGGTCCCTCAGTCCCTGGTAGGCAGCTTTGCCCCGTCTGTCCGCCCG
GTGTGTCGGCGGGGTTGACAAGGTCGTTGCGTCAGTCCAACATTTGTTGCCATATTTTCCTGCTCTCCCCACCAG
CTGCTCTTTTCTTTTCTCTTTCTTTTCCCATCTTCAGTATATTCATCTTCCCATCCAAGAACCTTTATTTCCCCT
AAGTAAGTACTTTGCTACATCCATACTCCATCCTTCCCATCCCTTATTCCTTTGAACCTTTCAGTTCGAGCTTTC
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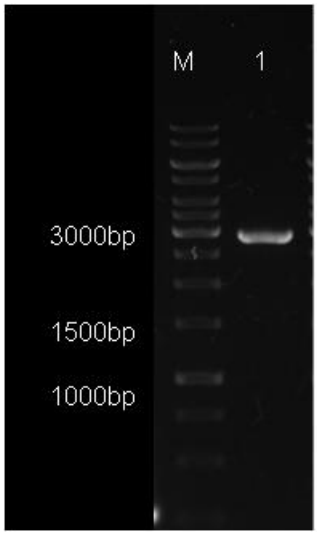
CCTTCTTTCTAGAAGTCCTCGTGTACTGTGTAAGCGCCCACTCCACATCTCCACTCGA

3.3.4. Construction of pUC19 backbone

The essential regions of the pUC19 vector which is required for the replication and selection in bacteria including replication origin and β-lactamase gene was PCR amplified from the pUC19 vector with primer pairs PUCF and PUCR, purified and sequenced (Fig.3.14 and 3.15).

Fig 3.14: PCR Amplification of pUC19 backbone



M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - pUC19 backbone PCR amplicon (2652 bp)

Fig.3.15. Sequence of pUC19 backbone

TCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC
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TCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCA
ACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGG
AGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTT
TTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACC
CCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAA
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CTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT
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GGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGG
TTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGC
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TCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAG
TCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGC
AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCAT
TAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCAC
ACAGGAAACAGCTATGACCATGATTAC

3.3.5. Construction of the full length expression cassette

For full length functional expression cassette construction, EGFP expression cassette (HindIII and ClaI digest), hygromycin expression cassette (BglII and HindIII digest) and pUC19 backbone (BglII and ClaI digest) was ligated together and transformed into *E.coli* DH5α cells. The cells with appropriate plasmids were isolated. The whole expression vector was sequenced to confirm the identity of the cassette. The overall method adopted for vector construction is outlined in the Figure 3.16. The vector constructed using the pUC19 backbone may be considered as an *E.coli- Aspergillus* shuttle vector which can replicate and maintain in *E.coli* and bear the fungal expression cassette. The vector named as pAsp isolated from transformed *E.coli* host and its linearized form (10532bp) are shown in Fig. 3.17A & B. The vector is pUC19 based with β-lactamase gene for positive selection in *E. coli* using Ampicillin. In addition, plasmids contain hygromycin resistance gene (hph) under the control of the *A. nidulans* gapd promoter for positive selection in *A. unguis* transformants. The *A. nidulans* trpC terminator (trpC) is also present to ensure proper mRNA termination. Despite the importance of *Aspergillus* as a cell factory for production of recombinant enzymes, the development of over-expression/producing systems are still in demand. The current study presents a novel host system which is compatible for heterologous expression and secretion in *Aspergillus*.

Fig.3.16. Schematic diagram showing the construction of *A. unguis* expression vector

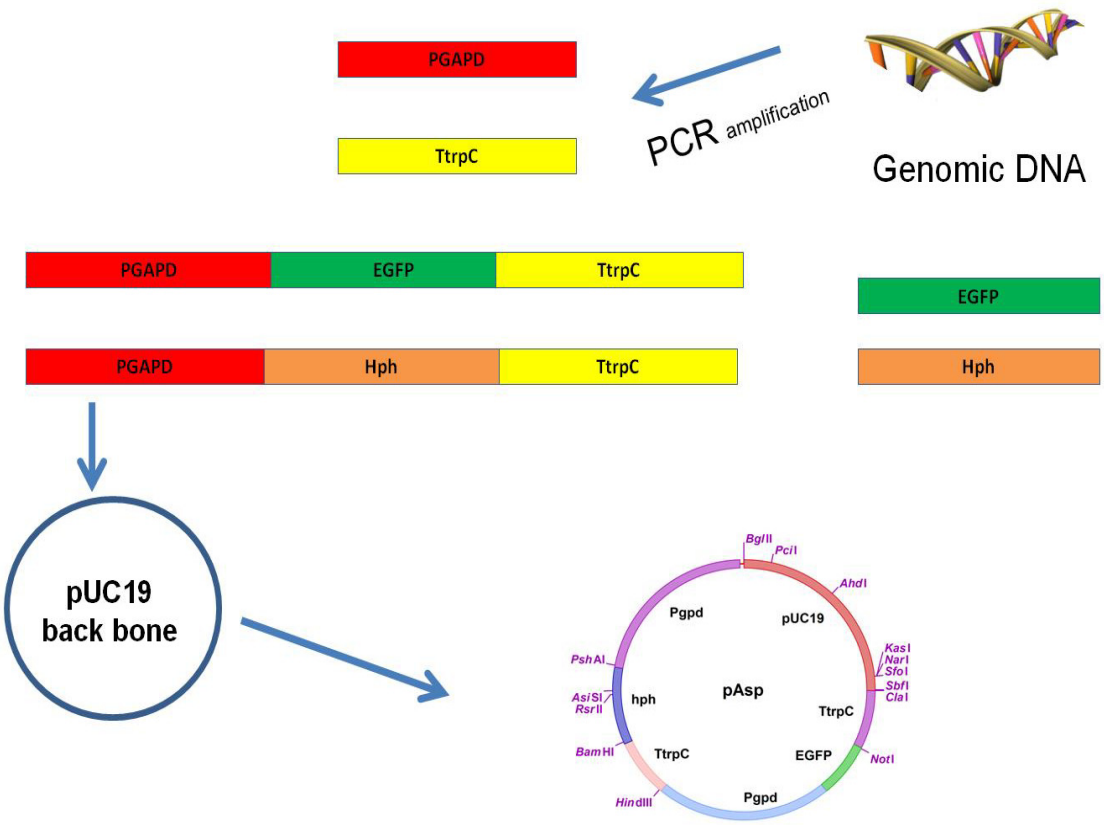
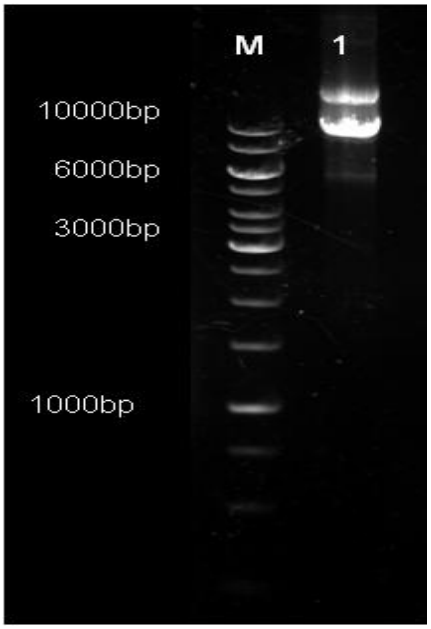
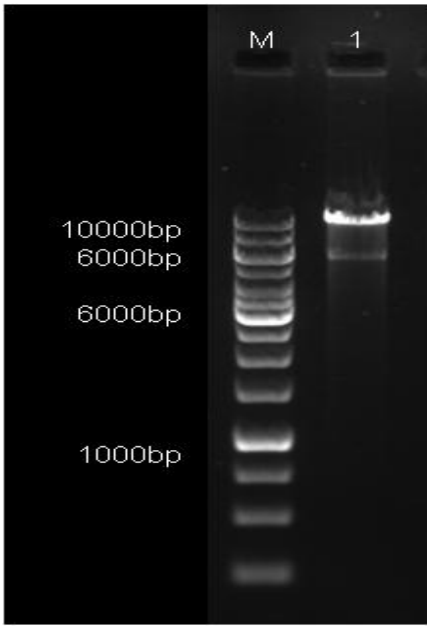


Fig 3.17: pAsp vector



M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - pAsp isolated from *E coli* host



M - GeneRuler™ 1kb DNA ladder (Fermentas)
1 - linearized pAsp vector (10532bp)

3.3.6. Transformation optimisation of the *Aspergillus unguis*

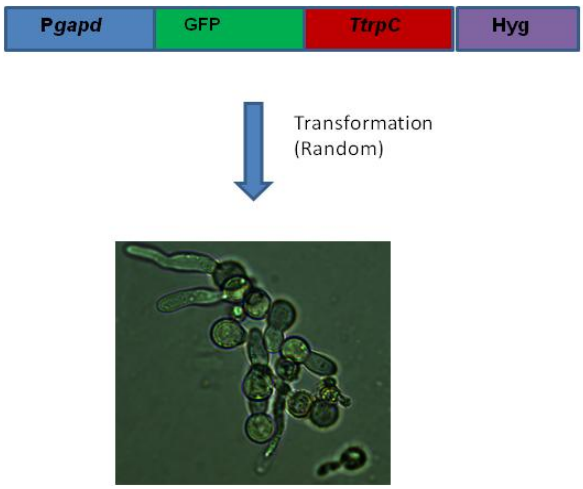
The minimal inhibitory concentration of hygromycin B for *A. unguis* was determined by inoculating the conidia in potato dextrose agar medium supplemented with 0, 25, 50, or 100 µg/ml hygromycin B. Growth was totally inhibited at 25 µg/ml concentration. On the basis of these tests, 50 µg/ml was chosen for the selection of resistant colonies. Transformation protocol for *A. unguis* was modified from the protocol described for *A. nidulans*. Lyzing enzyme from *Trichoderma harzinarum* has been used for the weakening of the germinated conidial cell wall. The modified protocol was found to be effective for the transformation of *A. unguis*.

3.3.7. Selection of transformants

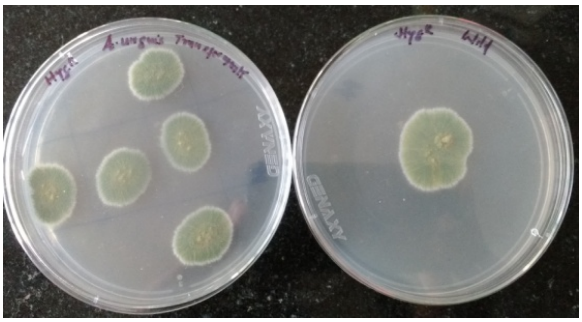
Immediately following electroporation, 1.0 ml of YPD medium supplemented with sorbitol was added to the cuvette, and the cells were resuspended rapidly, transferred to an appropriate tube, and incubated at 30 °C for an additional 2-3 h to improve the recovery of transformants. Transformants were selected using hygromycin B (100 µg/ml) on potato dextrose agar (PDA). Each positive transformant was used to create mono conidial cultures for genetic stability. All *A. unguis* strains were spread on PDA plates and were grown at 30°C for about 7 days and then stored at 4 °C after conidia formed. The conidia of the fungal transformants were collected from PDA plates and inoculated into 250 ml flasks containing 100 ml potato dextrose broth (PDB) and cultured for 2 days at 28 °C and 200 rpm on a rotary shaker for genomic DNA isolation. Using the protocol described above, hygromycin resistant colonies of the strain were clearly visible after five days incubation at 30 °C. All the ectopic integrants retained original phenotype after chromosomal integration indicated the successful transformation strategy in *A. unguis* (Fig.3.18). PCR products of the expected size (1000bp) for the hph gene were obtained from the transformants but not from the wild-type DNA. Subsequent sequencing analysis of the PCR products confirmed the presence of the chromosomally integrated hph gene in the *A. unguis* transformants (Fig. 3.19). These presumptive transformants remained resistant even after growth on non-selective agar media up to 15 generations. This indicated the mitotic stability of the transformants. Consistent with the observations of other workers on fungal transformation, two types of colonies, representing stable transformants and "abortive" derivatives, were observed (Lubertozi and Keasling, 2009). The former were characterized by stably integrated selectable genes whereas

in the latter category, the introduced genes were expressed transiently, giving rise to colonies incapable of sustained growth under selective conditions. Hygromycin is an aminoglycosidic antibiotic that inhibits protein synthesis in prokaryotes and eukaryotes by interfering with translocation and causing misreading (Dave *et al.*, 2015). We found that 50 µg/ml hygromycin B is the optimal concentration for selection of fungal transformants.

Fig.3.18. Transformation of *A. unguis* with expression cassette

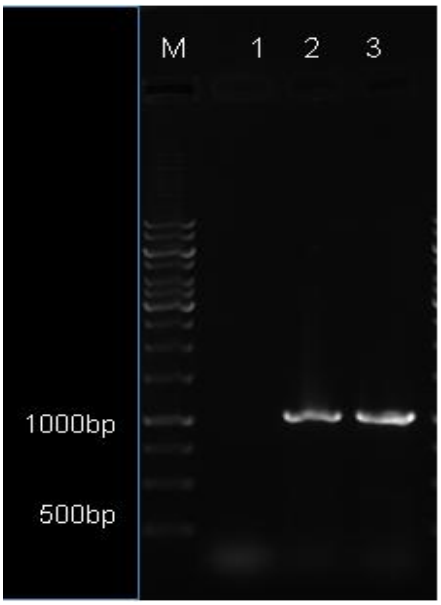


A: Strategy for transformation of *A.unguis* by electroporation of germinated conidia



B: Transformant (left) and wild type (right) colonies of *A.unguis*

Fig 3.19. PCR amplification of hygromycin gene from two independent hyg transformants



Lane Information

M - GeneRuler® 1kb ladder (Fermentas)
1 - Control (Wild type colony of *A. unguis*)
2-3 - PCR amplification of hygromycin gene from transformants

3.3.8. Optimization of Transformation

The effect of germination time on transformation frequency was evaluated. Data presented in Fig.3.20 showed that the number of transformants per microgram of DNA was higher in 7h of germination compared to those obtained after 4, 5, 6, 8 or 9h germination times, respectively. Therefore 7h germinated conidia were used in all further experiments. Effect of the duration of treatment with lyzing enzyme was studied and 90 min was found to the best (Fig. 3.21). Topology of the plasmids was also tested for increasing the transformation efficiency. Compared to the circular forms, linear forms of plasmids were found to be effective with increased transformation frequency perhaps reflecting an increased frequency of plasmid integration to the genomic DNA of *A. unguis* (Fig. 3.22).

Fig.3.20. Effect of conidial germination time on transformation efficiency

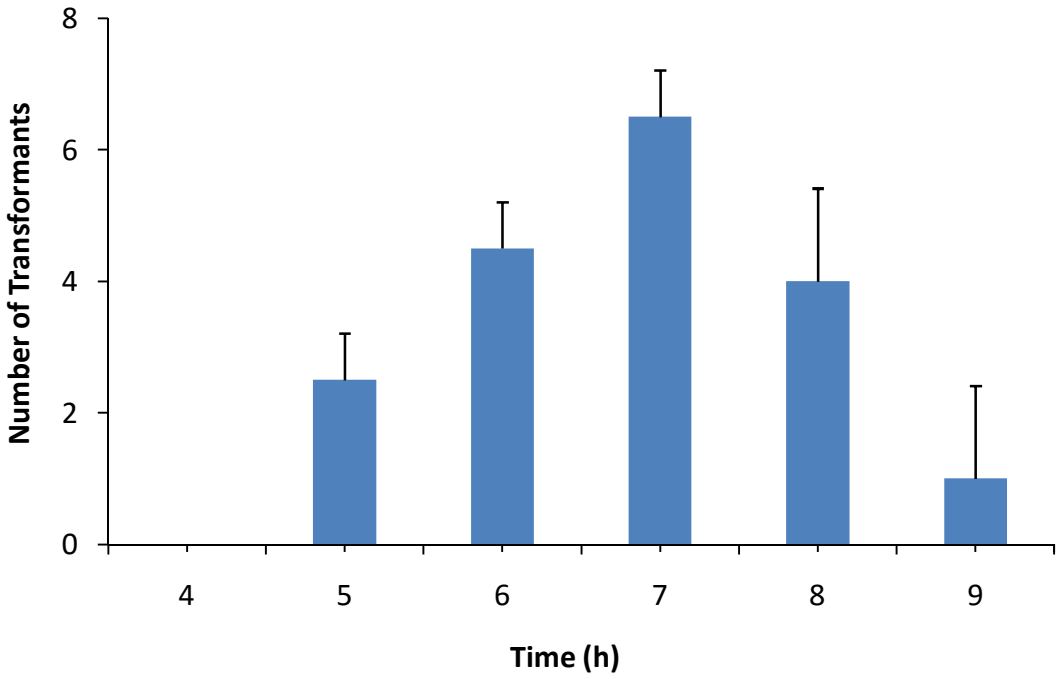


Fig.3.21. Effect of the duration of treatment with lyzing enzyme on transformation efficiency

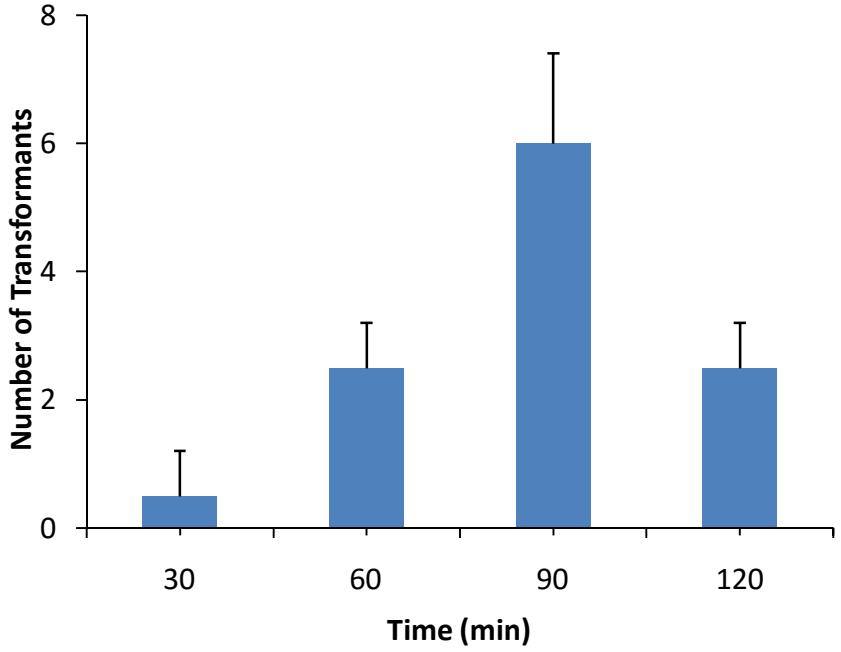
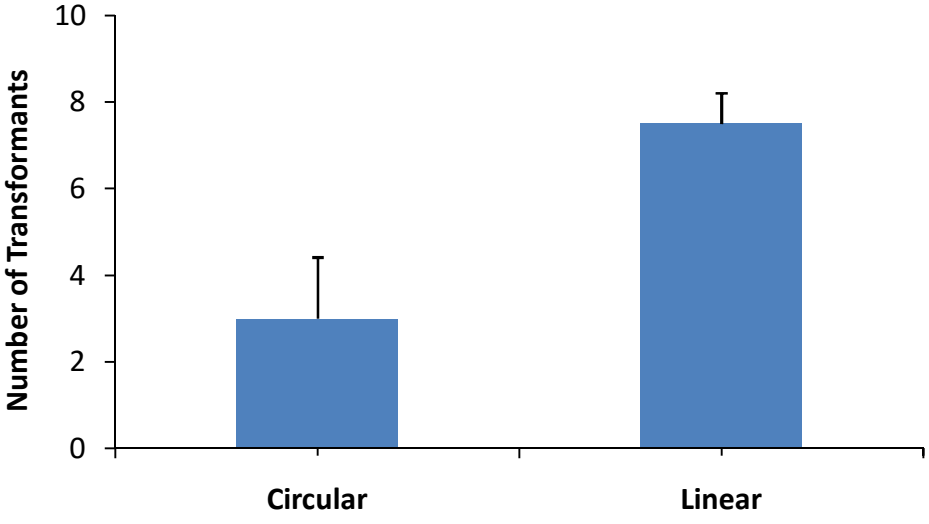


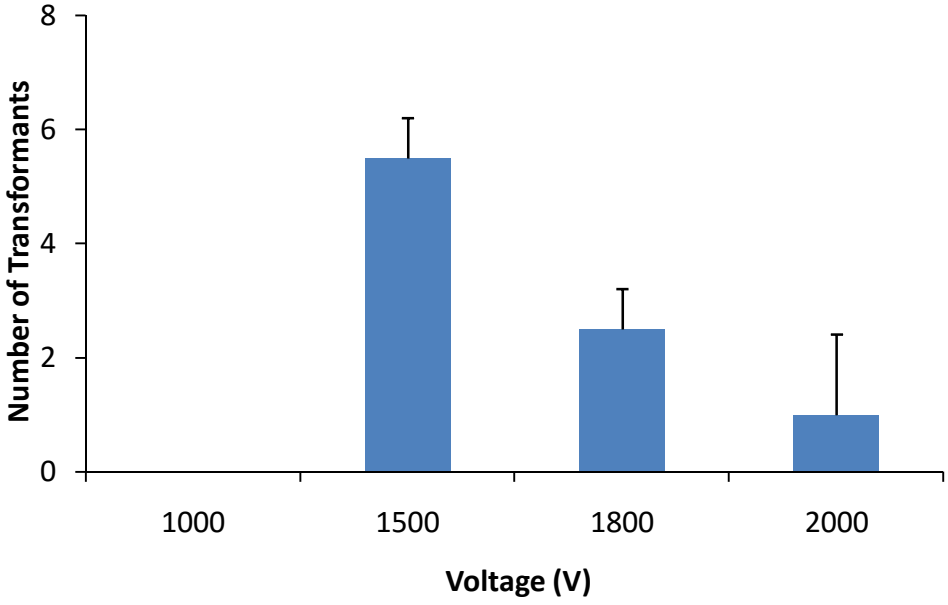
Fig.3.22. Effect of plasmid topology on transformation efficiency



The effect of varying field strength on cell viability and the recovery of transformants were also evaluated. No transformants were detected at 1000V per 0.2cm and the efficiency of transformation improved on increasing the field strength to 1500v in conjunction with 25 µF capacitance (Fig.3.23). Time constants under these conditions ranged from 5.1 to 5.8ms.

Optimal conditions comprised 1500V/0.2cm field strength, 25 µF capacitance, and 400-ohm resistance. The use of optimized protocol resulted in 10-11 transformants per 20µg of DNA.

Fig.3.23. Effect of voltage on transformation efficiency



3.4. Conclusion

Considering the differences between *A. unguis* and other *Aspergilli*, this strain is a very promising candidate as a host for protein production. The expression vector for *A. unguis* was constructed, sequenced and tested in *A. unguis*. The strain has a very high transformation frequency, which is convenient for the high-throughput screening of transformants and further genetic manipulation for strain improvement. The strain is highly susceptible to the antibiotic hygromycin and selection based on resistance to this antibiotic was highly effective. The culture does not acidify the growth medium and produces very low levels of extracellular protease activity, both of which contribute to an increased stability of the protein of choice in the extracellular fluid of this fungal species

4. Gene expression studies in *Aspergillus unguis*

4.1. Introduction

The availability of genome sequences of several industrially and medically important *Aspergilli* was a major breakthrough in filamentous fungal research. This resource has led to the growing interest in investigating the beneficial properties of industrially important fungi. A new lead towards the development of novel engineering tools for strain improvement and a deeper understanding of the physiology and metabolism of these organisms is possible now. *Aspergilli* have many applications in various industrial biotechnological processes like production of organic acids (e.g., citric acid and gluconic acid). Although *Aspergilli* have been exploited extensively for recombinant protein production, high-level synthesis of most heterologous proteins was a challenging task. However, in recent years, protease-deficient strains, transformation strategies, and crucial genetic tools have been developed to enhance recombinant expression of specific proteins by filamentous fungi.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used as a reporter protein for monitoring gene expression and protein localization studies in many organisms from prokaryotes to eukaryotes (Chalfie, 1995). A synthetic version of one of these variants sGFP (S65T) was developed for use in plants (Chiu *et al*., 1996; Haas *et al*., 1996) and has been expressed successfully in a number of fungi, including, *Candida albicans*, *Aspergillus nidulans*, *Aureobasidium pullulans* and *Ustilago maydis*, (Spellig *et al*., 1996; Cormack *et al*., 1997; Siedenberg *et al*., 1999). Furthermore, *gfp* gene expression is easily quantified in whole cultures via fluorimetry or in individual cells or sub-cellular compartments with confocal microscopy.

This chapter describes the potential of the *Aspergillus unguis* strain NII 08123 for heterologous protein production and demonstrates that fungal strains with better growth characteristics with respect to protein production already exist and could be a better starting point for strain improvement strategies than the strains that are currently used. We report the expression of EGFP from glyceraldehydes 3 phosphate dehydrogenase (GAPD) promoter. The results in this study demonstrate the potential of *A. unguis* as host for recombinant protein production. An attempt was made to produce the pharmaceutically important protein human Interferon β (HuIFNβ) in *A. unguis*.

4.2. Materials and Methods

4.2.1. Media strains and growth conditions

Maintenance of plasmids was routinely performed in *Escherichia coli* strains DH5α and JM109. *E. coli* strains were cultivated in Luria Bertani medium (Himedia, India). Mycelia were routinely grown from conidia in potato dextrose agar (Himedia, India). Transformants of *A. unguis* were selected on potato dextrose agar plates supplemented with 50 µg/ml hygromycin.

4.2.2. Construction of vectors for intracellular and extracellular expression in *A. unguis*

The signal peptide sequence of glucoamylase (glaA) and cellobiohydrolase 1 (cbh1*)* were examined by the SignalP 3.0HMM software to predict the potential signal peptides. To test the secretion efficiency of the host strain, glucoamylase and cbh1 signal peptides with Kex2 sites were fused *in-frame* to the 5' end of the EGFP coding sequence and PCR amplified (Table 4.1). The construct was introduced into *A. unguis* pAsp vector downstream of the gapd promoter.

4.2.3. Construction of synthetic gene for human interferon

Individual codons were analyzed using the codon usage database (http://www.kazusa.or.jp/codon/). Codons with a low percentage usage were replaced by codons that occur at higher frequency. The glucoamylase signal peptide with Kex2 cleavage site was fused to the 5' end of the codon optimized human interferon-β coding sequence by PCR using the designed oligonucleotide primers (Table 4.1). The construct was then introduced in to pAsp expression vector.

4.2.4. Transformation of *A. unguis*

Conidia from *A. unguis* NII 08123, washed 5 times with 10 ml of distilled water were used to inoculate 100 ml of potato dextrose broth at a density of 1×10^7 conidia/ml and incubated at 30 °C in a rotary shaker (250 rpm) for 7h. Conidia recovered by centrifugation (3000g for 5 min at 4 °C) were resuspended in 400 ml of ice-cold sterile water, centrifuged again,

resuspended in 20 ml of osmotic medium (1.2M $MgSO_4$, 10mM Sodium phosphate buffer) supplemented with 70 mg of lyzing enzyme from *Trichoderma harzinarum*. The mixture was incubated for 90 min at 30 °C and 100 rpm in a shaker incubator. Treated conidia were recovered by centrifugation (3000g for 5 min at 4 °C), resuspended in ice-cold sterile water, washed twice and were finally resuspended in 1 ml of ice-cold electroporation buffer (10 mM Tris-HCl (pH 7.5), 270 mM Sucrose, 1 mM Lithium acetate) and kept on ice. For electroporation, 20µg of purified DNA was added to 100µl of the ice cold conidial suspension. The mixture was incubated on ice for 30min and then transferred to a 0.2 cm cuvette. Electroporation was performed using an electroporation apparatus (GenePulser-Xcell™, Biorad, USA). Voltage was adjusted to 1500 V, capacitance to 25 µF and resistance was 400 Ω (pulse length varied between 5.1 and 5.8 ms). Following electroporation, 1 ml of ice-cold YPD with Sorbitol was added to the cuvette and the cell suspension was transferred to a sterile 10 ml tube, kept on ice for 15 min and incubated at 30 °C for 2h in a rotary shaker at 100 rpm. Transformed colonies were selected on hygromycin plates and were incubated at 30 °C for 5-7 days. The transformants were analyzed by PCR amplification with the specific primers.

4.2.5 Mitotic stability of the transformants

To test the mitotic stability, putative transformants were passaged for five consecutive generations on selection plate, and then grown for 6 generations on non-selective medium. The transformants were then plated on hygromycin (50 µg/ml) containing medium

4.2.6. Confocal microscopy

Intracellular expression of EGFP from the gapd promoter was monitored as the fluorescence emission due to GFP accumulation in the cells. Cells grown for 48h in medium were harvested by centrifugation (5000g for 15 min at 20 °C), washed twice with distilled water and were resuspended in distilled water followed by imaging of the intra cellular fluorescence using a BD path-way 855 Confocal microscope (BD Biosciences, USA).

4.2.7. DNA isolation and PCR

Several independent putative transformant fungal colonies obtained after the transformation was maintained on PDA agar plates with appropriate hygromycin B concentration. For genomic DNA isolation, untransformed wild-type strain or fungal transformants were inoculated in potato dextrose broth and grown at 30 °C for 48h in a rotary shaker. Genomic DNA was isolated from untransformed control and putative fungal transformants using fungal DNA isolation protocol (Doyle and Doyle, 1987). The putative fungal transformants were screened for the presence of transgenes using GFP specific primers (Table 1.1).

4.2.8. Protein extraction from *Aspergillus unguis*

Biomass (~500 mg wet weight) was harvested by filtration and was washed with phosphate buffered saline (PBS) to remove extracellular proteins and other contaminants. Cell disruption was accomplished by grinding the wet biomass in liquid nitrogen using a mortar and pestle. After cell disruption, the biomass was resuspended in 1 ml extraction buffer (10mM NaCl, 0.5 mM deoxycholate, 20mM Tris-HCl, pH 7.6). Cell debris was removed by centrifugation (22,000 × g, at 4 °C for 30 min) and the supernatant was treated for 15 min on ice with 7µL nuclease mix (0.5 mg/ml DNAase, 0.25 mg/ml RNAase, and 50 mM $MgCl_2$). Then, 10 µl/ml sodium deoxycholate (20 mg/ml) was added and the mixture was kept for at least 30 min on ice, followed by addition of 4 volumes of ice cold 10% Trichloro acetic acid (TCA) in acetone. This mixture was incubated overnight at -20 °C and the precipitated proteins were collected by centrifugation (22,000 g, at 4°C 20 min), washed three times with ice cold acetone and was dissolved in distilled water.

4.2.9. Preparation of the extracellular protein fraction

For preparation of the extracellular protein fraction, biomass was removed by centrifugation at 6000 g for 15 min at 4°C from the culture broth, and the supernatant was filtered through a 0.2 µm filter. Protein precipitation and further processing were carried out as described above (4.2.8)

4.2.10. SDS PAGE and Western blot

The total protein concentration in the culture fluid was determined by Bradford's (1976) method. Samples normalized for their protein content were loaded on either 12% SDS poly acrylamide gels and were separated electrophoretically (Laemmli, 1970). The proteins were transferred onto a PVDF membrane by semi-dry blotting (Novablot™ , GE, USA) and was detected using anti GFP antibody (Life Technologies, USA) or anti human Interferon β antibody (Life technologies, USA), with the chromogenic substrate 3,3'5, 5' Tetramethyl Benzidine (TMB).

4.2.11. Fluorescent Intensity analysis

Supernatant and cells were subjected to fluorescent intensity analyses using a multimode plate reader (Tecan Infinite Pro 200, Switzerland) with excitation and emission wavelengths of 488 and 520 nm respectively. Cell-free supernatant was mixed with refolding buffer (0.05M NaH_2PO_4, 0.1 M NaCl, and 0.5 M Imidazole) and the fluorescence intensity was measured.

Table 4.1. Oligonucleotide primers used for the study

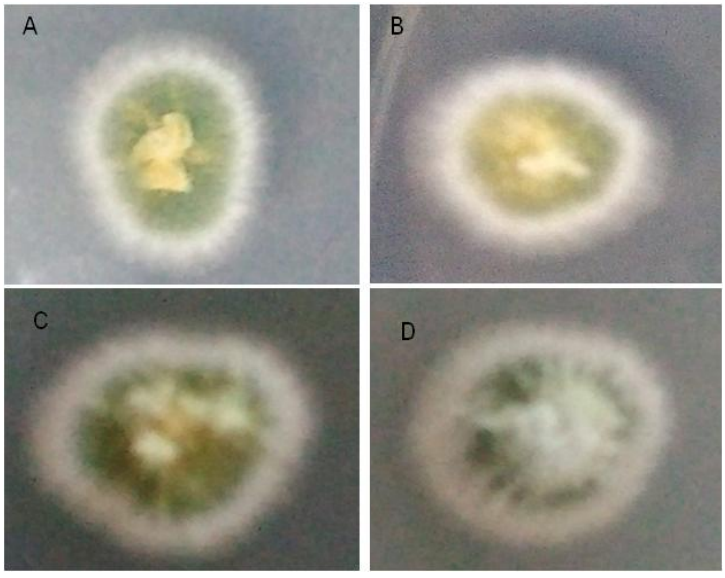
Primer	Sequence
GlUGFPR	ATACCATGGATGTCGTTCCGATCTCTTCTCGCCCTGAGCGGCCTTGTCTGCTCTGGGT TGGCAAGTGTGATTTCCAAGCGCATGGTGAGCAAGGGCG
CBGFPR	ATACCATGTATCGGAAGTTGGCCGTCATCTCGGCCTTCTTGGCCACAGCTCGTGCTCA GTCGGCCAAAAGAATGGTGAGCAAGGGCG
IFβF	CTTGCGGCCGCTCAGTTGCGGAGGTAACCGGTGAG
IFβR	ATACCATGGATGTCGTTCCGATCTCTTCTCGCCCTGAGCGGCCTTGTCTGCTCTGGGT TGGCAAGTGTGATTTCCAAGCGCATGTCCTACAACTTG
FGFPF	CTTGCGGCCGCTTACTTGTACAGCTCGTCCATGCC
EGFPF	AAGCTCGAGATGGTGAGCAAGGGCGAGGAGC
EGFPR	AAGGGTACCTTACTTGTACAGCTCGTCCATGC
IFNF	ATGTCCTACAACTTGCTC
IFNR	TCAGTTGCGGAGGTAACC

4.3. Results and Discussion

4.3.1. Molecular Analysis of EGFP Transformants and Intracellular expression

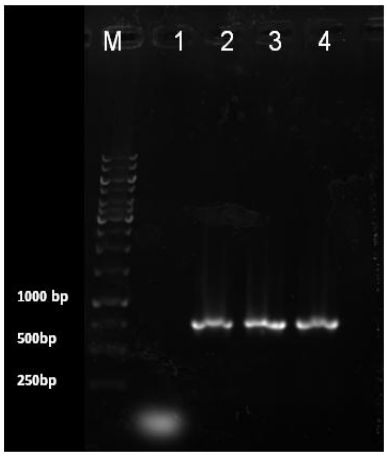
The morphology and sporulation pattern of all the transformants were found to be same as that of wild type *A. unguis* and no phenotypic change was observed (Fig. 4.1). The ability of *A.nidulans* gapd promoter sequence (Pgapd) to function in *A. unguis* was evaluated through expression of EGFP in the fungus. The newly constructed expression vector pAsp was introduced into the germinated conidia of the *A. unguis* through electroporation. The linearized gene expression construct upon random chromosomal integration gave rise to hygromycin resistant transformants. To evaluate the presence of integrated transgenes in putative fungal transformants, three randomly selected hygromycin B resistant fungal colonies were subjected to PCR evaluation of inserted gene. PCR amplification of GFP using specific primers was carried out on the total genomic DNA template extracted from putative fungal transformants, which yielded the amplicons of expected size. PCR products of expected size of 720bp for the EGFP gene were obtained from the transformants, but not from the wild-type genomic DNA (Fig.4.2).

Fig.4.1. *A.unguis* colonies transformed with EGFP expression cassette



A: Wild type colony; B, C, D: Transformant colonies

Fig.4.2. PCR amplification of the EGFP gene from the genomic DNA of transformed colonies of *A.unguis*



Lane Information

M	GeneRuler® 1kb ladder (Fermentas)
1	Control (Wild type colony of *A. unguis*)
2-4	PCR amplicons (720bp) from different transformant colonies using EGFP specific primers

Subsequent sequencing analysis of the PCR products confirmed the presence of EGFP gene in the *A. unguis* transformants. Few transformants were found to be mitotically stable without lose of selection marker, and these stable transformants were selected for further study. Confocal imaging was carried out in order to detect green fluorescence in fungal transformants and one of the strong GFP over-expressing fungal transformant was chosen for further experiments. Confocal imaging showed that the cells are indeed synthesizing GFP and accumulating at least a part of it inside the cells, since the fungal filaments showed fluorescence characteristic of GFP (Fig. 4.3). Intracellular protein content was analyzed by SDS PAGE analysis and time course study was done to observe the protein expression pattern as the time increases. Time course analysis of the production revealed that the production peaked at about 48-60h of growth and stabilized after that. Western blot analysis of the extracted protein confirmed the result (Fig.4.4).

Fig.4.3. Confocal image showing expression of GFP by the recombinant *A.unguis*

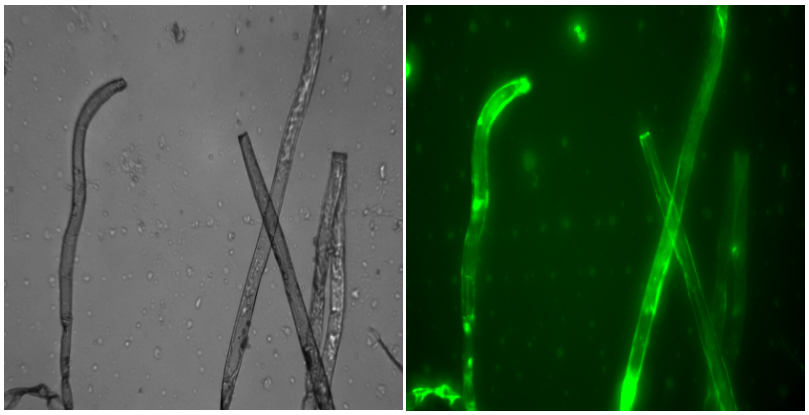
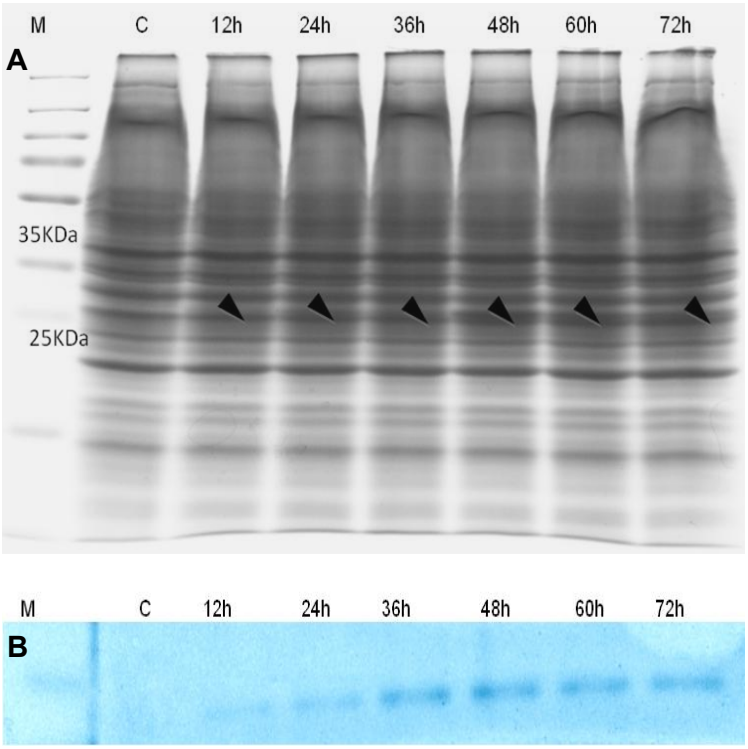


Fig.4.4. SDS PAGE and Western Blot showing intracellular EGFP expression at various time intervals



A –SDS PAGE of intracellular proteins from transformed *A. unguis* at different time intervals

B – Western Blot of the intracellularly expressed EGFP protein from transformed *A. unguis* at different time intervals

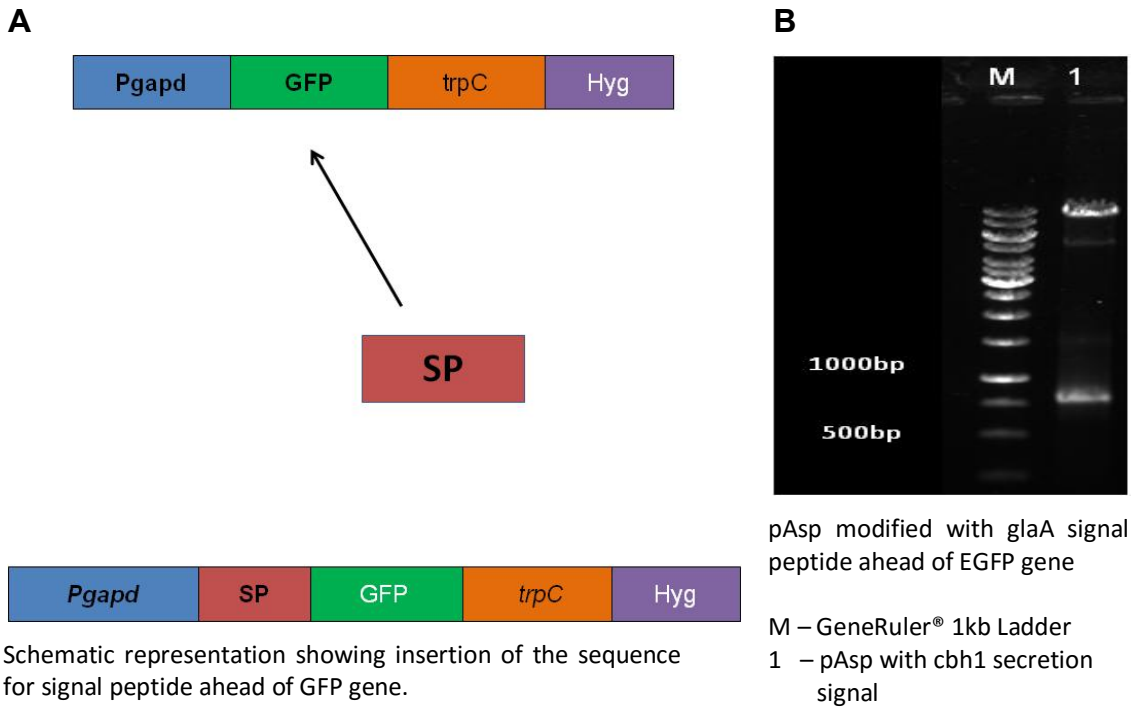
Lane Information

M	SDS PAGE Marker (Fermentas)
C	Intracellular proteins from wild *A. unguis*
12h-72h	Time course analysis of extracellular EGFP production

4.3.2. Secreted expression of EGFP in *A. unguis*

The glucoamylase (MSFRSLLALSGLVCSGLASVISKR) and cbh1 signal (MYRKLAVISAFLATARAQSAKR) peptides were further analyzed for putative secretion signal peptides and signal peptidase cleavage sites using the SignalP 3.0HMM software. Sequences for the signal peptides with Kex2 sites were fused to the N-terminus of green fluorescent protein (GFP) gene and was PCR amplified using oligonucleotide primers (Table 4.1). The resulting constructs were cloned into the pAsp expression vector (Fig.4.5). Modified plasmids were sequenced to confirm the identity. Linearized vector was electroporated into *A. unguis* cells and successful chromosomal integration was confirmed by PCR amplification of a 792bp fragment using specific primers. Sequencing results confirmed the identity of the gene present in the genome of the *A. unguis*.

Fig.4.5. Schematic representation of the construction of expression cassette for secreted expression of heterologous proteins



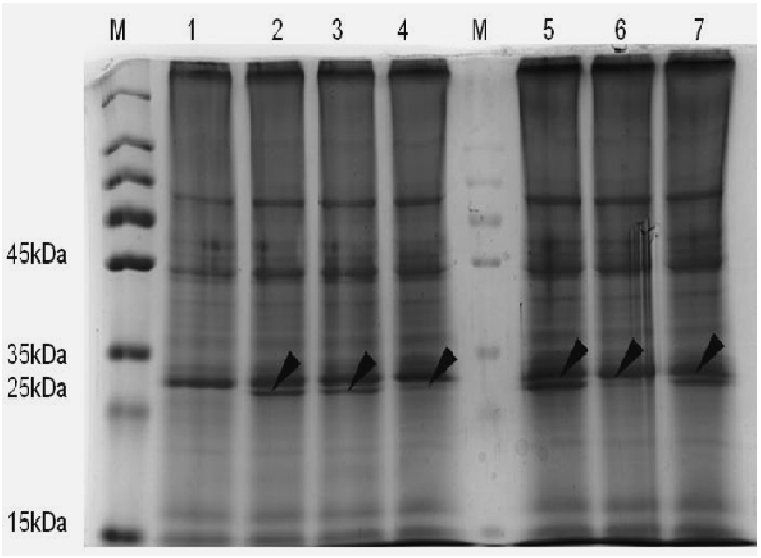
Schematic representation showing insertion of the sequence for signal peptide ahead of GFP gene.

pAsp modified with glaA signal peptide ahead of EGFP gene

M – GeneRuler® 1kb Ladder
1 – pAsp with cbh1 secretion signal

The recombinant *A. unguis* cells harboring the pAsp expression vector with EGFP gene and secretion signal sequences of either glaA or cbh1; secreted green fluorescent protein into the culture medium as evidenced by the fluorescence of the culture supernatants. Confirmation of the EGFP secretion into medium was done by detection of the protein in SDS PAGE and Western blots; and also through fluorescence intensity analyses. A protein of 25kDa, which is approximately equal to the EGFP's expected molecular weight was detected in the culture supernatant (Fig. 4.6). Fluorescent intensity analyses also confirmed the results (Fig. 4.7A &B). The glaA and cbh1 signal peptides supported comparable EGFP secretion. These are considered the strongest signal peptides among the filamentous fungi and by far, glaA is the most common secretion carrier used in filamentous fungi (Fleibner *et al*., 2010). Other promising signal peptides that have been successfully employed in other *Aspergillus* species are α-amylase (Nakajima *et al*., 2006) and endogenous signal peptides of secreted laccases or lipases (Tamalampudi *et al*., 2007; Yano *et al*., 2009). Usage of signal proteins demands subsequent removal of the carrier to regain full activity of the mature target protein. Introduction of KEX2 protease cleavage site allows the cleavage of signal peptide from the mature protein (Broekhuijsen *et al*., 1993). Thus glaA and cbh1 can be used as an efficient secretion signals in *A. unguis* for heterologous protein production.

Fig.4.6. Secreted Expression of EGFP in *A. unguis*: Clone to clone variation

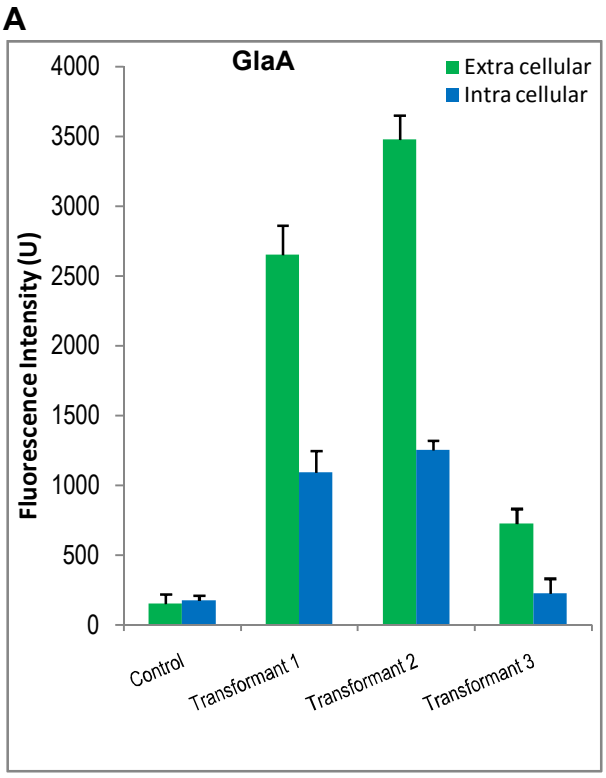


Lane Information

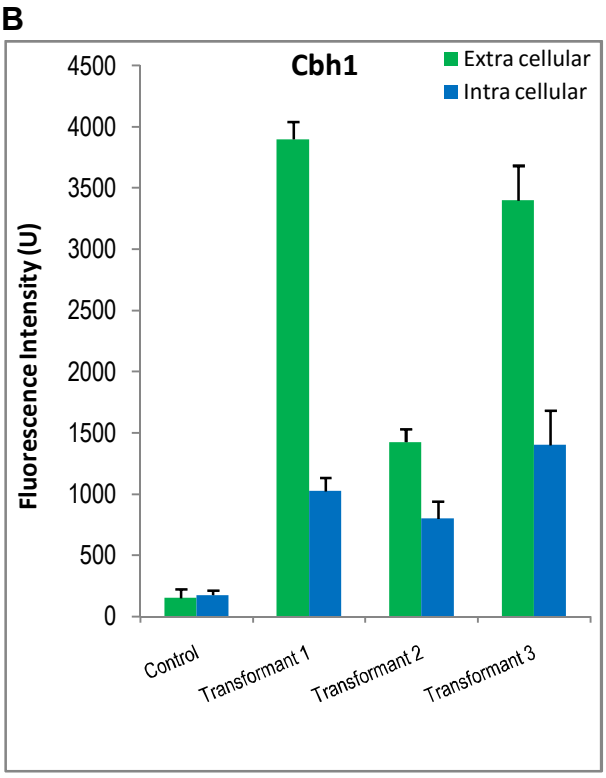
M	SDS PAGE marker (Fermentas)
1	Secreted proteins from wild *A.unguis*
2-4	Proteins secreted by different transformants (glaA signal peptide)
5-6	Proteins secreted by different transformants (cbh1 signal peptide)

Arrow heads indicate the EGFP band

Fig 4.7: EGFP secretion by *A. unguis* transformants using glaA and cbh1 signal peptides measured as fluorescence intensity of culture fluid



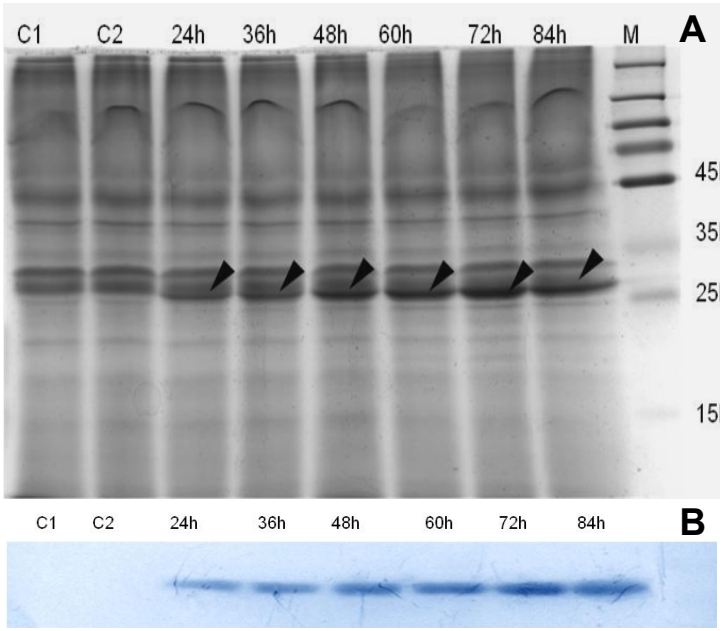
Intracellular and secreted EGFP expression by *A.unguis* from glaA secretion signal



Intracellular and secreted EGFP expression by *A.unguis* from cbh1 secretion signal

One important observation was the differences in expression pattern between the different transformants. Transformants carrying expression cassettes with the same promoter, exhibited different translation/transcription levels (eg. transformants 1, 2 and 3 for Pgapd using glaA and cbh1 signal peptide had differences in expression levels). Similar observations have been made with other filamentous fungal expression systems and the difference have been attributed to different sites of integration and different copy numbers of the integrated plasmids (Storms *et al*., 2005; Verdoes *et al*., 1994, Huang *et al*., 2014). Similarly a time course analysis of EGFP production revealed that the EGFP production commenced from 24 h onwards and peaked at about 60h of growth, beyond which it stabilized (Fig. 4.8A). During the time course study, no degradation of the protein was observed. Western blot analysis with Rabbit αGFP IgG-HRP conjugate confirmed that the band detected was indeed GFP (Fig. 4.8B).

Fig.4.8. SDS PAGE and Western Blot showing secreted EGFP expression at various time intervals



A –SDS PAGE of the proteins secreted by transformed *A. unguis* at different time intervals

B – Western Blot of the secreted EGFP protein from transformed *A. unguis* at different time intervals

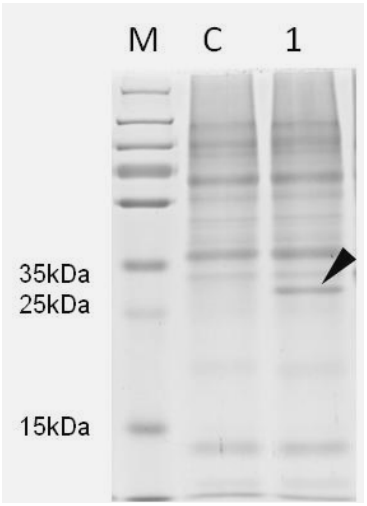
Lane Information

M	SDS PAGE Marker (Fermentas)
C	Extracellular proteins from wild *A. unguis*
24h-72h	Time course analysis of extracellular EGFP production

Aspergillus niger was transformed using the same expression cassette for comparison of the levels of extracellular protein production. Band intensity of EGFP secreted by *A.niger* was similar to that obtained from transformed *A. unguis* (Fig.4.9). It may therefore be concluded that the efficiency of transformed A. *unguis* in producing heterologous proteins is comparable to other *Aspergilli.* Fluorescent intensity analysis also confirmed the results (Fig. 4.10). The

above results confirmed the potential of *A. unguis* as a filamentous fungal host for heterologous protein production.

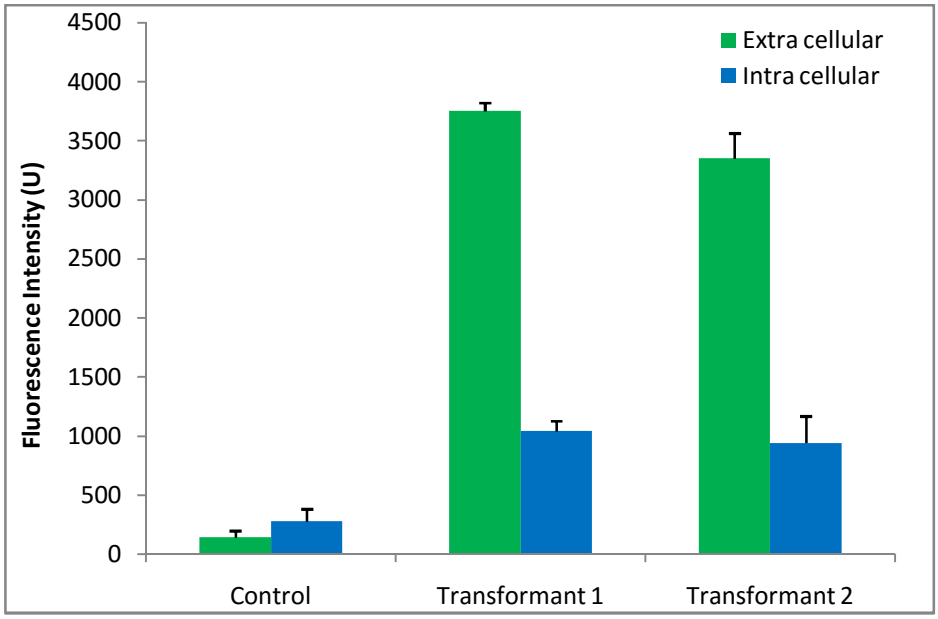
Fig.4.9. Secreted expression of EGFP in *A. niger* transformed with pAsp bearing gapd Promoter



Lane Information

M	SDS PAGE Marker (Fermentas)
C	Extracellular proteins from wild *A. niger*
24h-72h	Extracellular proteins from transformed *A. niger* showing EGFP production (arrowhead)

Fig.4.10. EGFP secretion by transformed *A.niger* measured as fluorescent intensity



4.3.3. Expression of human Interferon in *A. unguis*

Interferons are proteins of the cytokine family with antiviral activities, immune-modulation, and regulation of cell proliferation in higher vertebrates. Recombinant forms of interferon beta (IFNβ) are widely used as first-line treatment in relapsing forms of multiple sclerosis (MS).

4.3.3.1. Design and construction of codon-optimized human Interferon β.

Comparison of the codon usage of Human Interferon β (HuIFNβ) with that of *A. oryzae*- one of the closest relatives of *A. unguis;* using the codon usage database (http://www.kazusa.or.jp /codon/) indicated that the frequencies of individual codons in HuIFNβ are different from those commonly employed by *A. oryzae* for its proteins. The GC content of the coding region of native human interferon is 44%, while that of *A. oryzae* genes is 55% (http://www.kazusa.or.jp/codon/). Based on the codon usage preference of *A. oryzae*, a codon optimized HuIFNβ gene was designed. Codons infrequently used in *A. oryzae* were replaced by more frequently used codons, and portions of other codons were altered to reflect the usage of individual codons (Table 4.2). Consequently, 54% of the codons in native HuIFNβ were altered. Reconstitution of the gene by codon optimization resulted in a GC content of 57%. (Fig.4.11).

Table 4.2. Comparison of codon usage in *A. oryzae* genes with that of the native and codon-optimized HuIFNβ

Amino acid	Codon	Frequency of Native codon (No. in the gene)	Optimized
Ala	GCT	22.6 (2)	25.1 (GCC)
	GCC	25.1 (3)	25.1 (GCC)
	GCA	18.6 (1)	25.1 (GCC)
	GCG	0	0
Arg	CGT	0	0
	CGC	0	0
	CGA	10.1(1)	13.5 (CGC)
	CGG	0	0
	AGA	7.7 (5)	13.5 (CGC)
	AGG	6.9 (5)	13.5 (CGC)
Asn	AAT	16.6 (4)	20.6 (AAC)
	AAC	20.6 (8)	20.6 (AAC)
Asp	GAT	29.6 (2)	29.6 (GAT)
	GAC	26.2 (3)	29.6 (GAC)

Cys	TGT	6.0 (2)	7.1 (TGC)
	TGC	7.1 (2)	7.1 (TGC)
Gln	CAA	17.0 (3)	22.9 (CGC)
	CAG	22.9 (8)	22.9 (CGC)
Glu	GAA	26.8 (5)	33.3 (GAG)
	GAG	33.3 (8)	33.3 (GAG)
Gly	GGT	18.9 (1)	21.6 (GGC)
	GGC	21.6 (1)	21.6 (GGC)
	GGA	16.5 (2)	21.6 (GGC)
	GGG	11.8 (2)	21.6 (GGC)
His	CAT	12.8 (3)	12.8 (CAT)
	CAC	11.4 (2)	12.8 (CAT)
Ile	ATT	18.5 (5)	25.6 (ATC)
	ATC	25.6 (4)	25.6 (ATC)
	ATA	7.5 (4)	25.6 (ATC)
Leu	TTA	0	0
	TTG	16.8 (3)	20.9 (CTC)
	CTT	17.6 (3)	20.9 (CTC)
	CTC	20.9 (6)	20.9 (CTC)
	CTA	9.9 (2)	20.9 (CTC)
	CTG	19.9 (10)	20.9 (CTC)
Lys	AAA	16.8 (4)	29.4 (AAA)
	AAG	29.4 (7)	29.4 (AAA)
Met	ATG	-	-
Phe	TTT	14.5 (4)	23.9 (TTC)
	TTC	23.9 (5)	23.9 (TTC)
Pro	CCT	15.8 (1)	15.8 (1)
	CCC	0	0
	CCA	0	0
	CCG	0	0
Ser	AGT	10.8 (2)	16.7 (TCC)
	AGC	14.4 (5)	16.7 (TCC)
	TCT	15.0 (1)	16.7 (TCC)
	TCC	0	0
	TCA	11.6 (1)	16.7 (TCC)
	TCG	0	0
Thr	ACT	14.3 (2)	19.9(ACC)
	ACC	19.9 (3)	19.9 (ACC)
	ACA	14.0 (2)	19.9 (ACC)
Trp	TGG	15.0 (3)	15.3 (TGG)
Tyr	TAT	13.8 (4)	TAC (15.8)
	TAC	15.8 (6)	TAC (15.8)
Val	GTT	16.9 (1)	GTC (21.9)
	GTC	21.0 (3)	GTC (21.9)
	GTA	7.9 (0)	GTC (21.9)
	GTG	17.9 (1)	GTC (21.9)

Fig. 4.11. Codon optimized HuIFNβ gene

```
ATG TCC TAC AAC TTG CTC GGC TTC CTC CAG CGC TCC TCC AAC TTC CAG TGC
CAG AAG CTC CTG TGG CAG TTG AAC GGC CGC CTC GAG TAC TGC CTC AAG GAT
CGC ATG AAC TTC GAT ATC CCT GAG GAG ATC AAG CAG CTC CAG CAG TTC CAG
AAG GAG GAT GCC GCC TTG ACC ATC TAC GAG ATG CTC CAG AAC ATC TTC GCC
ATC TTC CGC CAG GAT TCC TCT TCC ACC GGC TGG AAC GAG ACC ATC GTC GAG
AAC CTC CTG GCC AAC GTC TAC CAT CAG ATC AAC CAT CTG AAG ACC GTC CTG
GAG GAG AAG CTG GAG AAG GAG GAT TTC ACC CGC GGC AAG CTC ATG TCC TCC
CTG CAT CTG AAG CGC TAC TAC GGC CGC ATC CTG CAT TAC CTG AAG GCC AAG
GAG TAC TCC CAT TGC GCC TGG ACC ATC GTC CGC GTC GAG ATC CTC CGC AAC
TTC TAC TTC ATC AAC CGC CTC ACC GGT TAC CTC CGC AAC TGA
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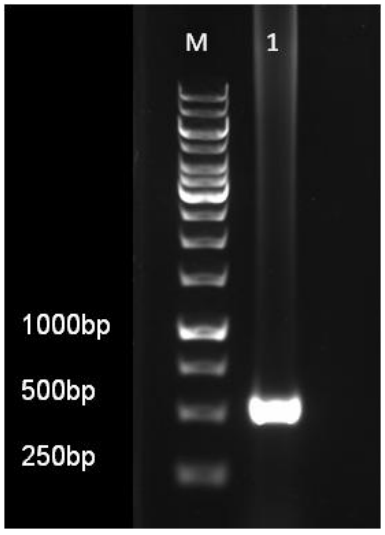
4.3.3.2. Secreted expression of HuIFNβ in *A.unguis*

In the present study, glucoamylase (glaA) signal peptide was cloned for secretion of HuIFNβ. The sequence for the signal peptide was fused to those corresponding to the N-terminus of the gene for synthetic HuIFNβ and was PCR amplified along with a nucleotide sequence encoding the KEX2 cleavage site (Fig. 4.12), and the resulting constructs were cloned into the *A. unguis* pAsp expression vector (Fig.4.13A &B). Linearized construct was electroporated into *A. unguis* cells and the successful transformant was confirmed by PCR amplification of a 500bp HuIFNβ fragment using specific primers (Fig.4.14). Twenty clones from 14 independent transformations were selected for screening. All the transformants were screened for human interferon β production by western blotting after SDS PAGE of the culture supernatants.

Fig. 4.12. PCR amplification of HuIFNβ gene

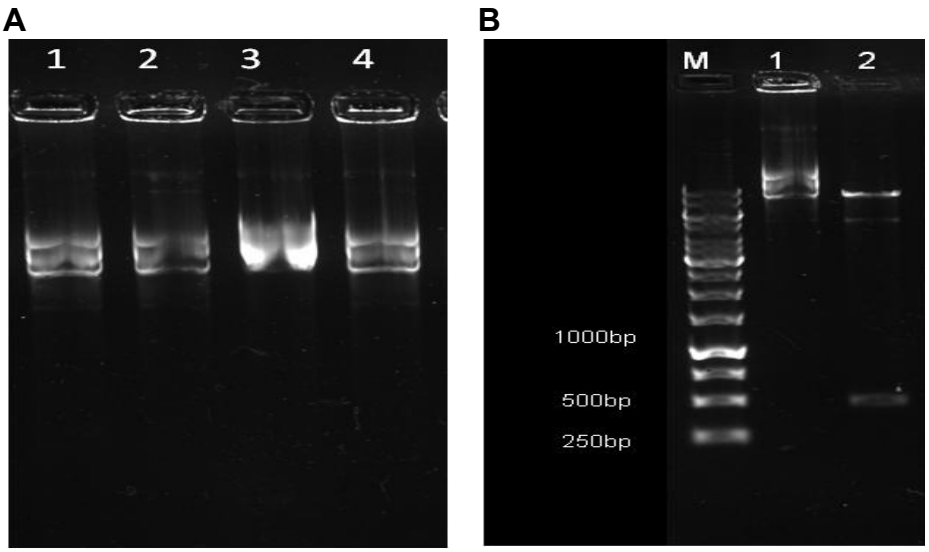


Lane Information

M GeneRuler® 1kb ladder (Fermentas)

1 Codon optimized HuIFNβ gene with secretion signal and KEX2 cleavage site sequences (573bp)

Fig.4.13. Confirmation of the successful construction HuIFNβ expression cassette



Lane Information

1,2,4 Native plasmid

3 Recombinant plasmid bearding the HuIFNβ expression construct

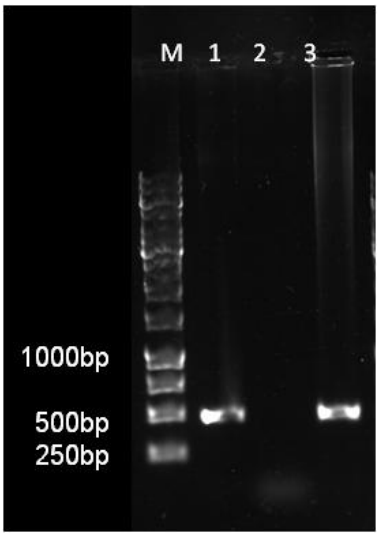
Lane Information

M GeneRuler® 1kb ladder (Fermentas)

1 Recombinant plasmid bearding the HuIFNβ expression construct

2 Double digestion showing the released HuIFNβ

Fig.4.14. Confirmation of the successful transformation of *A. unguis* with HuIFNβ construct



Lane Information

M GeneRuler® 1kb ladder (Fermentas)

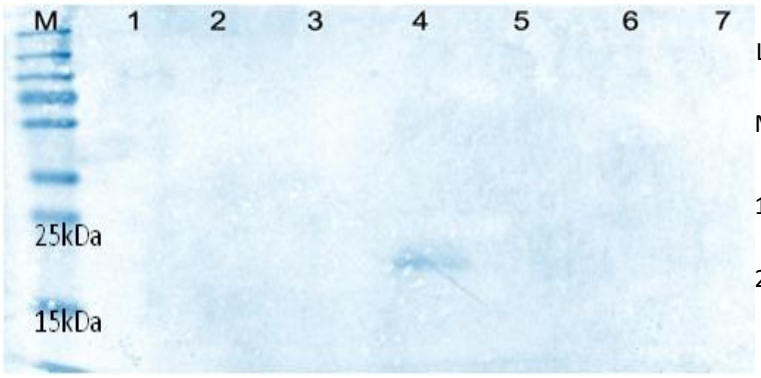
1,3 PCR amplicon of HuIFNβ gene using genomic DNA of transformant *A. unguis* as template

2 Negative Control- PCR amplification of HuIFNβ gene using genomic DNA of wild *A unguis*

Western blot analysis of the culture supernatants showed that one of the twenty transformants obtained was positive for HuIFNβ secretion with weak signals of desired protein. A 19 kDa protein which was the expected size for HuIFNβ was detected in the culture supernatants and

the identity of the protein was confirmed through western blot analysis using Rabbit α HuIFNβ-IgG-HRP conjugate (Fig.4.15).

Fig.4.15. Secreted expression of HuIFNβ from *A. unguis* confirmed through western blot analysis

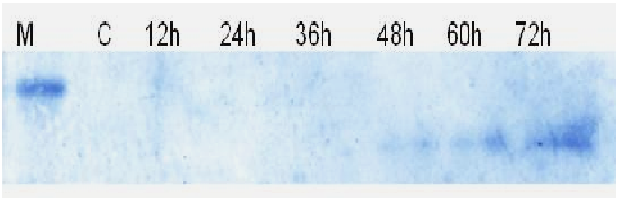


Lane Information

M	SDS PAGE marker (Fermentas)
1	Culture fluid of wild *A unguis*
2-7	Culture fluid of different *A unguis* transformants

Time course analysis of HuIFNβ production by the recombinant *A unguis* strain indicated that the protein was produced starting from 48h onwards and it continued till up to 72h till it was monitored (Fig.4.16).

Fig.4.16. Time course analysis of secreted HuIFNβ expression in *A. unguis* through western blot analysis



Lane Information

M	SDS PAGE marker (Fermentas)
C	Culture fluid of wild *A unguis*
12h-72h	Culture fluid of *A unguis* transformant at various time intervals

The concentration of in terms of band intensities on the western blot appeared to be less, indicating a lower expression or secretion of the protein. The mitotic stability of the transformants also appeared to be very less. Hygromycin resistance was lost from the transformants after 4-5 generations. The possible reason for lower production and mitotic instability might be the random ectopic integration of the expression cassette. Another reason could be the ineffectiveness of codon optimization, since it was based on codon usage

preferences for *A.oryzae* due to lack of genomic information of *A. unguis*. Effective codon optimization is possible only when the genomic information is available.

Codon optimization has been considered an effective strategy for improving the expression levels of heterologous genes that contain codons which are rarely used in the host organism (Gustaffson *et al*., 2004). It has been suggested that codon optimization does not affect transcript levels, but can alleviate the translation inefficiency often caused by ribosomal pauses at rare codons interrupting translation elongation (Sinclair and Choy, 2002; Hu *et al*., 2006). Codon optimization is generally believed to improve the translation efficiency of heterologous genes without affecting their mRNA levels. However, recently several studies have indicated that codon optimization causes an increase in the steady-state mRNA levels of heterologous genes in filamentous fungi (Tanaka *et al*., 2014). In the present study, it was demonstrated that the HuIFNβ altered to fit *Aspergillus* codon usage could produce the protein into the extracellular fluid albeit at lower concentrations.

4.4. Conclusion

A new filamentous fungus based expression system was developed using *A. unguis* as host and the expression of a reporter protein (EGFP) was demonstrated both as intracellular and in secreted form. Practical applicability of the host-expression system was demonstrated through expression of the human therapeutic protein- HuIFNβ (First ever report of hIFNβ expression in *Aspergillus*). Heterologous promoter Pgapd from *Aspergillus nidulans* and glucoamylase (glaA) signal peptide from *Aspergillus awamori* proved to be functional in *A.unguis*. This indicated the relative easiness in genetic manipulation of the system. The isolation of a native and efficient promoter from *A. unguis* may further improve the levels and rate of production of heterologous proteins compared to the heterologous promoter from another species. The system can be refined further through identification of highly active and safe integration sites for the expression cassette which may lead to maximal expression of the target proteins without affecting the normal phenotype and genotype of the organism. These chromosomal loci can be used for the development of specific integrative expression constructs to ensure high level expression of target proteins. In conclusion, the new expression systems presented in this study offer the features: (1) efficient transformation (2) secretion of protein (3) Growth characteristics of host offering specific advantages over existing filamentous fungal systems.

5. Profiling of *Aspergillus unguis* secretome for the prospecting of novel native promoters for efficient gene expression

5.1. Introduction

It is important to highly efficient promoters for effective genetic engineering of filamentous fungi. Use of efficient and active native promoters is desirable since they can be expected to function most effectively in a host and in several cases the regulation mechanisms can be made use of for controlling expression levels. For fine-tuning and regulated control of the gene expression, some inducible promoters have been reported, and are suitable for low or high expression from the same expression construct. These promoters respond to defined conditions for regulated high or low level expression of the gene of interest. Most of the promoters are dependent on the carbon or nitrogen sources in the culture medium and the examples include glucoamylase promoter *PglaA* of *A. niger* (Ganzlin and Rinas, 2008), the alcohol dehydrogenase promoter *PalcA* of *Aspergillus nidulans* (Waring *et al*., 1989), or the Taka-amylase A promoter PamyB of *Aspergillus oryzae* (Kanemori *et al*., 1999), acetamidase promoter *PamdS* from *Aspergillus nidulans* (Turnbull *et al*., 1990). Other promoters like thiamine promoter *PthiA* of *A. oryzae* (Shoji *et al*., 2005) or the promoter of human estrogen receptor *PhERα* (Pachlinger *et al*., 2005) are induced only in the presence of certain inducer compounds. More recently, Meyer *et al*., (2011) had described a tightly regulated, tuneable Tet-on (induced by tetracycline) expression system for *A. niger* that was dependent on the doxycycline concentration. The advantage of such systems is the possible fine regulation of gene expression, depending on the level of the inducer.

Constitutive promoters on the other hand, do not require expensive inducers and are not under the control of tight transcriptional regulation. The constitutive gapd promoter of the glyceraldehyde-3-phosphate dehydrogenase of *A. nidulans* is one of the most commonly used promoters among filamentous fungi (Hunter *et al*., 1992). Other constitutive promoters, which have been described for filamentous fungi include the alcohol dehydrogenase promoter *PadhA* of *A. niger* (Davies, 1994), the pyruvate kinase promoter *Ppki* of *Trichoderma reesei* (Limón *et al*., 1999) or the glyceraldehyde-3-phosphate dehydrogenase promoter *PgpdA* of *A. nidulans*.

In filamentous fungi, most studies have been focused on promoters of known genes with highly efficient transcription levels, such as the constitutive *gapd* promoter coding for glyceraldehyde 3 phosphate dehydrogenase and inducible glucoamylase promoter of

Aspergillus sp. which limited the screening of other highly efficient promoters. The advent of more comprehensive techniques, such as proteomic and transcriptomic analyses would make it easier to achieve this goal. The translated protein from a particular gene is highly correlated to the transcriptional efficiency of that gene. Thus the expressed protein complement of a gene is the efficient way to study the functional property of that gene. Therefore, it should be possible to find a new strong promoter of a highly expressed gene through proteomics analysis.

The proteome compositions of *Aspergilli* are still understudied. However, complete genome sequences of most of the *Aspergilli* are now publicly available, fostering gene manipulation and strain improvement. Protein identification based on peptide-mass fingerprinting is becoming a routine for *Aspergillus* sps and for other filamentous fungi. A comparative study on the secretome of *T. reesei* and *A. niger* during growth on sugar cane biomass has been carried out recently (Borin *et al*., 2015). A comparative study on the extracellular proteome of *A. niger* growing on different substrates has been carried out using LC/MS (Tsang *et al*., 2009). Recently Ogunmolu *et al*., (2015) reported the secretome analysis of *Penicillium funiculosum* by quantitative and label free quantitative mass spectrometry.

In proteomics one of the most important and widely used protein identification technique combines separation of the protein using gel electrophoresis and Mass Spectrometry (MS). The two ionization techniques mainly used in protein characterization using Mass Spectrometry are the Electron Spray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI). The ESI or MALDI is connected to Time Of Flight (TOF) analyzers. The protein of interest is subjected to proteolysis with specific protease(s) and the resultant unique peptides are then subjected to Mass spectrometry and their mass/charge ratios are compared with the theoretical masses derived from a sequence database. This data comparison and protein identification based on the masses is usually done with the help of software (eg. MASCOT). Main advantage of this technique compared to Edman degradation (Edman *et al*., 1950) is that only a small amount of protein is required for analysis, and the identification is very fast if the sequence database contains peptides with similar masses.

Proteomic study of the *A. unguis* proteins secreted in response to different carbon sources was performed using 2D gel electrophoresis (2DE) coupled with MALDI TOF MS/MS mass spectrometry. The identified proteins were shortlisted for further analysis for prospective promoter identification and functional analysis.

5.2. Materials and Methods

5.2.1. Strain and growth conditions

Aspergillus unguis was grown on Potato Dextrose Agar (Himedia) plates at 30 °C until sporulated. The spores were resuspended in sterile saline to prepare the inoculum as outlined in section 2.1. Submerged cultivation for proteomic study was carried out in 500ml Erlenmeyer flasks having 100 ml of Mandels and Weber, (1969) medium containing desired concentration of the carbon sources - glucose, lactose or maltose at 1% level. The contents of the flask were mixed thoroughly after addition of the basal medium and substrate, and the flasks were sterilized by autoclaving for 15 min at 121 °C and 15 lbs pressure. After cooling, the flasks were inoculated with 1 ml of a spore inoculum containing 1×10^7spores/ml. The flasks were incubated at 30 ± 2 °C and 200 rpm agitation for the specified time interval. The culture broth after 72 h of incubation was centrifuged at 8000 rpm for 20 min at 4 °C.

5.2.2. Preparation of Extracellular protein

For preparation of the extracellular protein fraction, biomass in culture broth was removed by centrifugation at 6000 × g for 15min at 4 °C and the supernatant was filtered through a 0.2µm filter. Four volumes of ice cold 10% TCA in acetone was added in to the supernatant and this mixture was incubated overnight at -20 °C and the precipitated proteins were collected by centrifugation at 22,000 × g , washed three times with ice cold acetone, air dried and was solubilised in the solubilization buffer containing 7M urea, 2M thiourea, 4% (w/v) CHAPS, 50mM DTT, 0.2% 100× biolyte 3/10 ampholyte, and 0.002% (w/v) bromophenol blue. The total soluble protein concentration was determined according to Bradford's (1976) method. The solubilized proteins were stored at -20°C until further analysis by two-dimensional gel electrophoresis.

5.2.3. Two dimensional gel electrophoresis

The first dimension based on the isoelectric point of proteins was run using a strip based Isoelectric Focusing System (Protean IEF®, Biorad, USA) at 20 °C with a current of 50µA per strip. 130µg of each protein sample was loaded onto IPG DryStrip™ gels of pH 3-10 (Biorad, USA) by in-gel rehydration. IEF was performed with the following conditions: 250V

× 20 min, 8000 V × 2.3h, and 8000V for 30000V-h. Prior to the second dimension (SDS-PAGE), the IPG strips were equilibrated for 15 min in 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), 0.375M Tris-HCl (pH 8.8), containing 2% (w/v) DTT, and then for 15 min in the same buffer additionally containing 2.5% (w/v) iodoacetamide. Equilibrated strips were transferred for the second dimension onto SDS-polyacrylamide gels. The proteins were separated on 12% gel. Subsequently, gels were stained using Coomassie Blue R-250.

5.2.4. MALDI TOF MS/MS

Gel pieces of the target protein were cut out and incubated in a buffer containing 200mM ammonium hydrogen carbonate and 40% (v/v) acetonitrile (ACN) at 37 °C for 10min. This was repeated until the gel was completely de-stained. Gel pieces were then vacuum dried and treated with 20µg/ml Trypsin, 40mM ammonium hydrogen carbonate, 9% (v/v) ACN, and 1 mM HCl. After incubation at 37 °C, gel fragments were removed and the supernatant was spotted on the plate for MALDI-TOF MS at a 1:1 ratio with 10–20 mg/ml DAHC. The analysis was performed using an MALDI-TOF/TOF (ultrafleXtreme ™, Bruker Daltronik) The obtained peptide mass results were then searched against the NCBI database using the MASCOT peptide mass fingerprinting search program (Matrix Science, Boston, MA).

5.3. Results and Discussion

Total extracellular proteins from the culture supernatant were separated by 2DE. The 2D profile with IPG strips of pH ranges 3-10 clearly indicated that most of the proteins had PIs <7 which suggested that the secretome of *A. unguis* consisted mainly of acidic proteins. *In-silico* digestion (theoretical digestion) of the known proteins deposited in the protein database (Swiss Prot) was performed with the help of MASCOT (Matrix Science, Boston, USA) and the peak values of theoretically calculated, experimentally observed and expected values were compared.

As promoter strength is often highly correlated to gene expression level, the 2-DE technique was firstly used to obtain the protein with the highest expression in the whole proteome. The protein was extracted from the wild strain cells which were grown in three different carbon sources- glucose, lactose and maltose for a comprehensive understanding of

the protein expression levels in response to different carbon sources. Differences in the secretome of *A. unguis* grown in glucose, maltose and lactose gauged by 2DE is shown in Fig.5.1, 5.2 & 5.3. Few highly expressed proteins were identified from the 2DE profile (Table 5.1). The positions of these protein spots on the gels are shown in Fig 5.1, 5.2 and 5.3. The spots were selected for protein identification based on their expression pattern, band intensity and differential response to the carbon source.

Fig.5.1. Representative 2DE map of the secretome of *A. unguis* grown on glucose

1.Uncharacterised Protein, 2.Putative Synaptic vesicle Transporter,
3.Putative uncharacterised protein, 4.Acetolactate synthase, 5.Spermidine synthase

Fig.5.2. Representative 2DE map of the secretome of *A. unguis* grown on lactose

1.Uncharacterised protein, 2.Probable glucan 1,3-beta glucosidase A, 3.Probable glucan 1,3-beta glucosidase A,4.Putative synaptic vesicle transporter, 5.Uncharacterised protein, 6.Spermidine synthase

Fig.5.3. Representative 2DE map of the secretome of *A. unguis* grown on maltose

1.Uncharacterised protein, 2.Probable glucan 1,3-beta glucosidase A, 3.Putative synaptic vesicle transporter, 4.Spermidine synthase

Table 5.1. Identification of highly expressed proteins of *A. unguis* secreted under growth utilizing different carbon sources

No.	Predicted protein	Score	Matching Peptide	Organism
1	Uncharacterised protein	42	K.KPTATGPSGAANK.K	*A. nidulans*
2	Probable glucan 1,3-beta-glucosidase A	110	K.VIVDLHGAPGSQNGFDNSGR.K K.NGWLFWTWK.T	*A. terreus*, *A. fumigatus* *A. clavatus*
3	Acetolactatae synthase	64	R.KTTPTGPAIPR.I K.GLGGIVQFEILPK.N R.NPDFMGLAGAMHIQHR.R R.NPDFMGLAGAMHIQHRR.V	*A. oryzae* *A.flavus*
4	Spermidine synthase (Putrescine aminopropyl transferase)	71	R.EVVKHETVEEAILCDIDEAVIR.V	*A. nidulans* *A.clavatus*
5	Synaptic vesicle transporter SVOP	76	R.ERGNATVGDVGGTAQR.L R.IMDSTQQDEICSLSAQDR.E R.LIVGLPGFLIAVAGILIFGLTLQYR.T	*A. oryzae* *A. flavus*
6	Uncharacterised protein	70	R.RFVPDSAGGGSNGPR.M	*A. nidulans*
7	Putative uncharacterised protein	92	R.GFDWYHGHSWAK.G	*A. terreus*

Seven proteins from the 2D profile showed similarity to the proteins of *A. nidulans*, *A. oryzae*, *A. clavatus, A. fumigates* and *A.oryzae* proteins (Table 5.1). Among the gel spots, one distinct big spot was present in the secretome of fungus grown in maltose and lactose, while its intensity was less in glucose. This indicated a lower expression of the protein, when glucose was used as carbon source. After MALDI TOF MS/MS the specific protein could not be identified since a matching peptide was not available in the database; and hence the protein remains as uncharacterised protein (Fig.5.4).

One strong lactose inducible protein identified was a probable glucan 1, 3 β glucosidase which was absent in secretome of the fungus grown in glucose and it appeared as multiple spots in lactose induced condition. The intensity of spots was less in maltose as carbon source which confirmed the strong induction by lactose. Both the multiple spots were confirmed as probable 1, 3 β glucosidase (Fig.5.5). β-Glucosidases are key enzymes in

biomass hydrolysis, being the rate-limiting enzyme that is regulated by feedback inhibition from its own product-glucose. These enzymes are therefore of considerable interest as constituents of lignocellulose-degrading systems (Sørensen *et al*., 2011). Lactose is reported as a known inducer of cellulases and is employed in production of cellulases (Sehnem *et al*., 2006; Fang *et al*., 2008). This protein was found to be a better candidate for promoter identification for improving heterologous expression in *A. unguis*.

Three proteins, which include the uncharacterised protein (largest spot), putative synaptic vesicle transporter (Fig. 5.6) and spermidine synthase, were secreted on growth in all the three carbon sources. In fungi, spermidine synthase is reported to be important in cell differentiation processes including sporulation, spore germination and dimorphic transition (Jin *et al*., 2002). Synaptic vesicle transporter protein is a protein involved in vesicular transport. Of these, the most abundant extracellular protein was the uncharacterised protein which showed similarity to *A. nidulans*. Two proteins, one uncharacterised protein which showed similarity to protein of *A. nidulans* and probable β glucosidase was found only in lactose as carbon source. Maltose induction also resulted in minor changes in the expression pattern of proteins but did not lead to the selection of a protein of interest. Therefore, type of the carbon source highly influences the pattern of secreted proteins.

The other proteins identified were acetolactate synthase (Fig.5.7) and a putative uncharacterised protein which showed similarity to *A. nidulans,* the latter found only in the extracellular fluid of fungus grown in glucose as carbon source. In fungi, this protein is mainly involved in amino acid biosynthesis; especially valine, leucine and isoleucine which convert pyruvate into 2 acetolactate as the first step in the pathway. The activity of wild type acetolactate synthase is sensitive to the action of several known toxic compounds like sulphonylurea, imidazolinone compounds (Bower *et al*., 2010). Few proteins were identified as unpredicted proteins in the secretome. For the identification of these proteins, a complete knowledge of the genome and proteome may be essential. The secretome of the fungus revealed more than 100 proteins spots and this confirms the secretion capacity of the fungus.

Fig.5.4. MALDI-TOF MS PMF data of uncharacterized protein

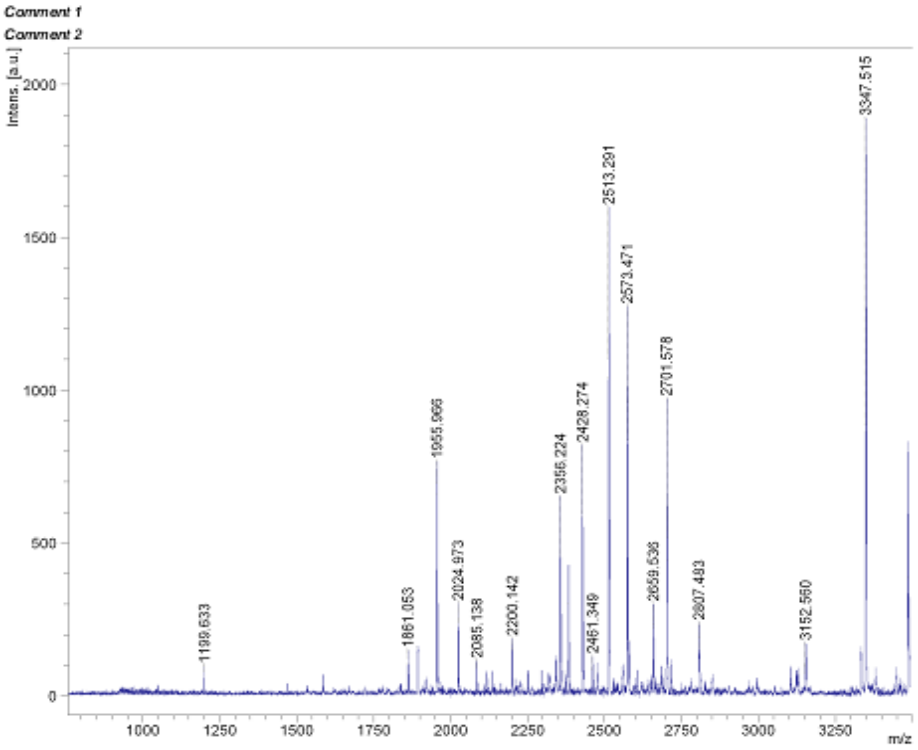


Fig.5.5. MALDI-TOF MS PMF data of probable glucan 1, 3 β glucosidase

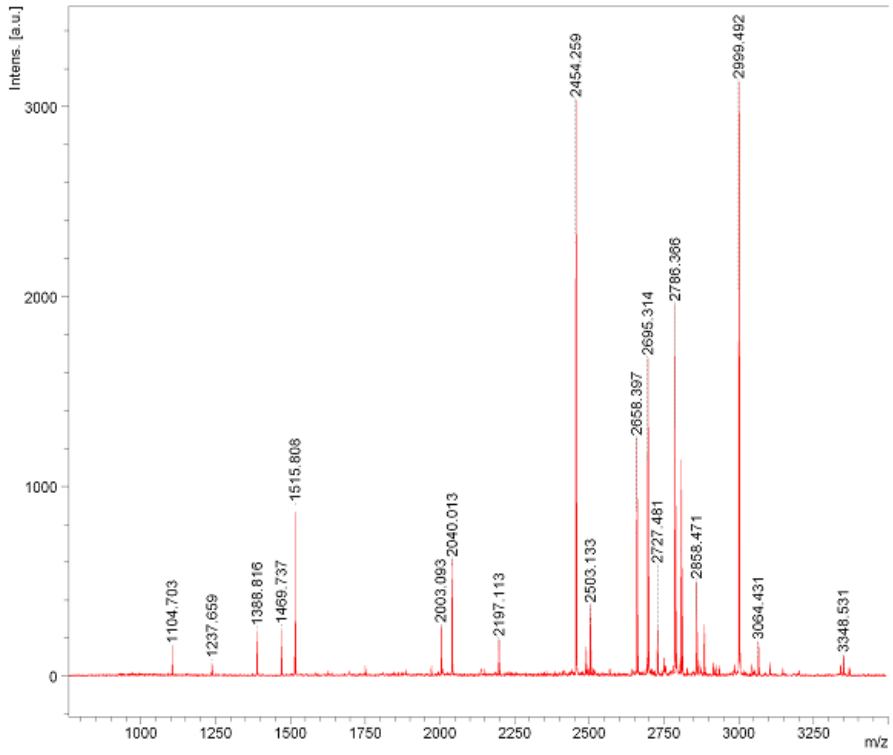


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Fig.5.6. MALDI-TOF MS PMF data of putative synaptic vesicle transporter

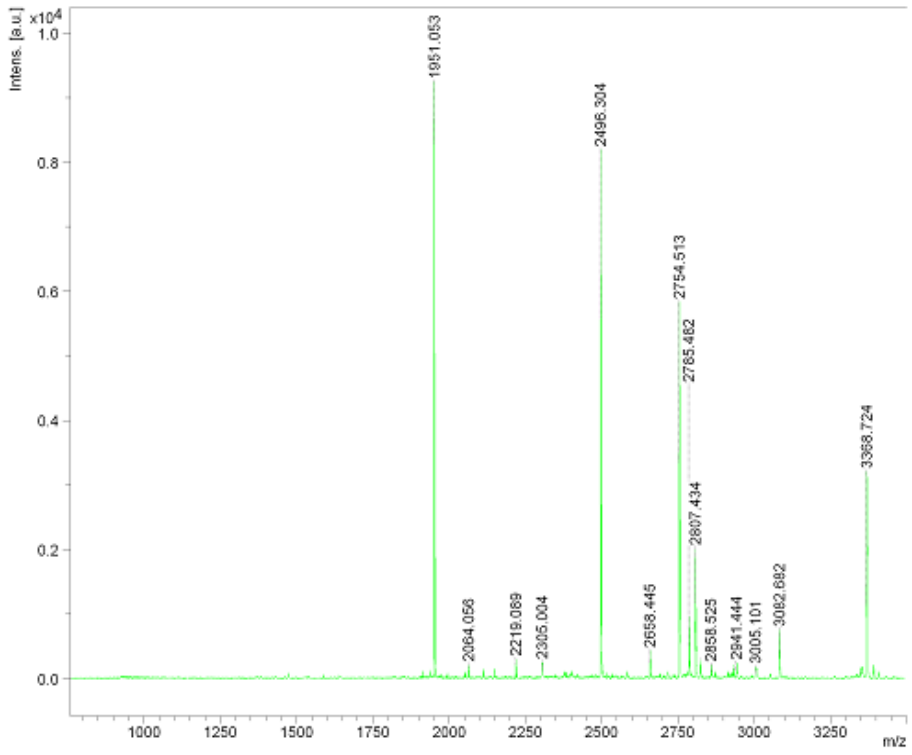
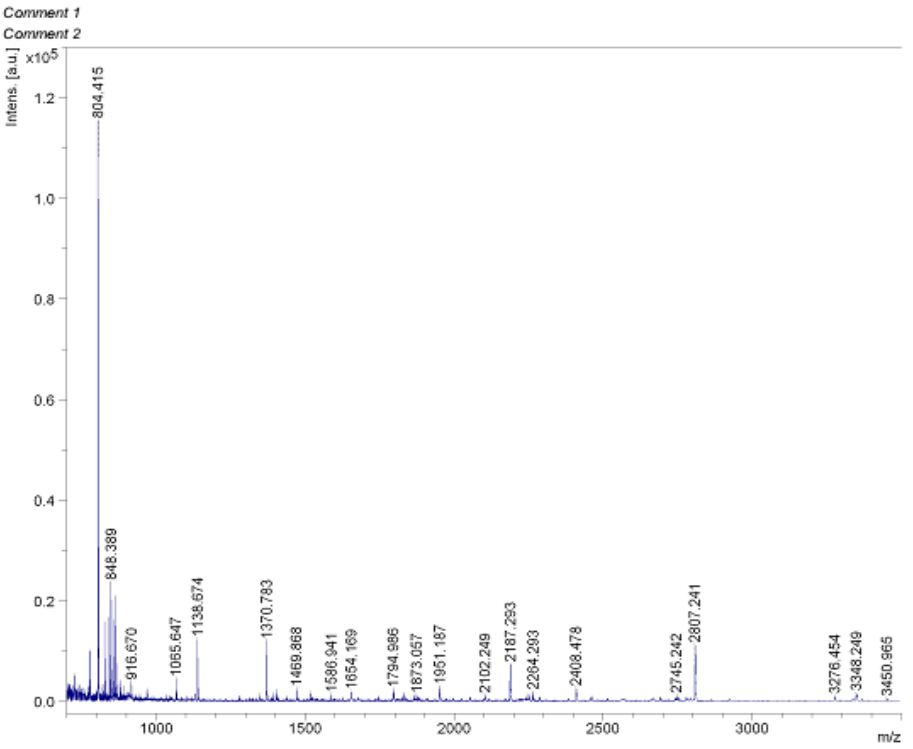


Fig.5.7. MALDI-TOF MS PMF data of acetolactate synthase



5.4. Conclusion

A.unguis secretome in response to different carbon sources was analysed for the identification of potent constitutive and inducible proteins for promoter search. The data and protocol generated can be used as a base for prospecting the native promoters for *A. unguis* which can aid further improvement of the developed protein expression system. Potent lactose inducible protein was identified which holds promise as a source of promoter for efficient inducible expression of heterologous proteins in the fungus. Annotated genome information of the fungus can help in obtaining more comprehensive information of the shortlisted protein. It may be concluded that proteomic analysis is an efficient way to identify strong promoters. Moreover, a strong promoter can further facilitate the metabolic engineering of *A. unguis.*

6. Evaluation of fungal signal peptides for recombinant protein production in *Kluyveromyces lactis*

6.1. Introduction

Secreted proteins from the eukaryotic cells posses a hydrophobic amino terminal extension called signal peptide. A signal peptide at the N-terminal end of a nascent polypeptide directs the molecule into the endoplasmic reticulum (ER) lumen which is the first stage in the secretion pathway

Filamentous fungi are very good produces of extracellular proteins owing a great deal to their highly efficient signal peptides. The use of fungal signal peptides for driving recombinant protein secretion has currently attracted significant attention as the fungal secretion signals are highly efficient compared to any of the currently used signals. Commonly used yeast expression systems contain the *Saccharomyces cerevisiae* αMF pre pro peptide as the secretion signal. Only few fungal signal peptides are used in yeast for recombinant protein production, and most of them are used for driving the secretion of homologous proteins. Among the very common secretion signals of fungal origin used in yeast expression systems are the signal peptide of *Rhizopus oryzae* amylase (Li *et al*., 2011), and hydrophobin signal sequence from *Trichoderma reesei* (Kottmeier *et al*., 2011). The signal peptides that are functional in yeast for heterologous protein production are limited and each protein requires a different signal peptide for effective secretion. The signal should also be compatible with respective protein and do not interfere with its functional folding. The cellobiohydrolase I (CBH1) secretion signal from *Trichoderma reesei* is relatively small compared to other secretion signals, making this a suitable candidate for driving heterologous protein production in yeast (Madhavan and Sukumaran, 2014)

Filamentous fungi are remarkable secretors of hydrolytic enzymes, which have granted them a potential role in biotechnology (Archor *et al*., 2008). They have received attention as hosts for heterologous protein production because of their high secretion capability and eukaryotic post translational modifications (Yoon *et al*., 2010). Since fungal secretion signals are most efficient for driving the secretion of proteins and since yeasts are close relatives of filamentous fungi, it is speculated that fungal secretion signals may be useful for high level secretion of recombinant proteins in yeasts which are used as hosts for expression of heterologous proteins. Therefore, nine signal peptides from different industrially important filamentous fungi namely *Aspergillus niger*, *A. nidulans*, *A. terreus*,

A.awamori and *T. reesei* based on the reported extracellular proteome (Ferreira *et al*., 2011; Saykhedkar *et al*., 2012; Han *et al*., 2010; Herpoel- Glimbert *et al*., 2008) were evaluated in *K.lactis* for secretion of a cloned "Enhanced Green Fluorescent Protein (EGFP)". A comparative study of the efficiency of different secretory signals is presented here.

Generally two approaches have been used to drive the secretion of heterologous proteins in yeast: the use of a heterologous (non yeast) signal peptide; and the use of a homologous (yeast) signal peptide. Even though, there have been some impressive successes in protein secretion with homologous signal peptides, very low secreted production has generally been observed for various heterologous proteins of medical and pharmaceutical relevance (Chung *et al*., 2012; Sreekrishna *et al*., 1997). This might be due to the differences in secretory pathway between yeasts and the higher eukaryotes.

Kluyveromyces lactis has been used for the expression of recombinant proteins for over a decade, and protein expression can be either extracellular or intracellular. *K. lactis* secretes very less amount of endogenous proteins which is an added advantage as it makes the purification of protein easy (Line cereghino *et al*., 2007). High cell densities achievable in submerged fermentation, highly efficient extracellular secretion and easy genetic manipulation make this yeast an attractive cell factory for recombinant protein production (Colombo *et al*., 2014; Krijger *et al*., 2012; Rosa *et al*., 2013; Van ooyen *et al*., 2006). Several signal peptides such as the inulinase from *Kluyveromyces marxianus*, PHAE from *Phaseolus vulgaris* agglutinin (Chung *et al*., 2012; Raemekers *et al*., 1999), viral pre pro toxin signal peptide (Eiden-Plach *et al*., 2004) has been used for secreted expression from yeast. However, the *Saccharomyces cerevisiae* α-mating (α-MF) pre pro-peptide is still the most commonly used signal sequence for recombinant cargo proteins (Brake *et al*., 1984; Gurramkonda *et al*., 2010).

6.2. Materials and Methods

6.2.1. Strains and growth conditions

Escherichia coli DH5α cells were cultivated in Luria Bertani medium (Himedia, India). YPD agar (Himedia, India) plates were used for growth of *K. lactis*. Transformants of *K.lactis* were selected on YCB agar (New England Biolabs, USA) containing 5mM acetamide.

6.2.2. Prediction of signal peptides

The protein sequences of all the nine signal peptides (Table 6.1) were obtained from the genome database of the corresponding organism. The sequences were then examined by the SignalP 3.0 HMM software (Bendtsen *et al*., 2004) to predict the potential signal peptides.

Table 6.1. Signal peptides used in this study

Source Fungus	Source Gene	Sequence of the signal peptide
Apergillus terreus	Oryzin	MQSIKRTLLLLGAVLPAVLA↓GP
Aspergillus niger	β –Glucosidase	MHSISALLSLLGGLALSSA↓AP
	Glucoamylase	MSFRSLLALSGLVCTGLA↓NV
	β Xylosidase	MAHSMSRPVAATAAALLALALPQALA↓QA
	Endoxylanase A	MVQIKVAALAMLFASQVLS↓EP
Aspergillus awamori	α amylase	MRVSTSSLALSVSLFGKLALGLSA↓AE
Aspergillus nidulans	Endoxylanase	MVHLKTLAGSAVFASLATA↓AV
Trichoderma reesei	Cellobiohydrolase 2	MIVGILTTLATLATLAASV↓PL
	Endoglucanase11	MNKSVAPLLLA ASILYGGAVA↓QQ
Apergillus terreus	Oryzin	MQSIKRTLLLLGAVLPAVLA↓GP
Aspergillus niger	β –Glucosidase	MHSISALLSLLGGLALSSA↓AP
	Glucoamylase	MSFRSLLALSGLVCTGLA↓NV
	β Xylosidase	MAHSMSRPVAATAAALLALALPQALA↓QA
	Endoxylanase A	MVQIKVAALAMLFASQVLS↓EP
Aspergillus awamori	α amylase	MRVSTSSLALSVSLFGKLALGLSA↓AE
Aspergillus nidulans	Endoxylanase	MVHLKTLAGSAVFASLATA↓AV
Trichoderma reesei	Cellobiohydrolase 2	MIVGILTTLATLATLAASV↓PL
	Endoglucanase11	MNKSVAPLLLA ASILYGGAVA↓QQ

6.2.3. Construction of fungal signal peptide – EGFP fusion and evaluation of the secretion efficiency of signal peptides

For determination of the secretion signal strength, all the signal peptides were fused in-frame to the 5′ end of the EGFP coding sequence and PCR amplified. The construct was introduced into pKlac1 vector downstream of its native lac4 promoter. EGFP cloned downstream of the native αMF secretion signal (αMFSS) was used as positive control. All DNA manipulations were performed by standard methods (Sambrook *et al*., 1976).

6.2.4. Construction of codon optimized signal peptides for efficient secretion of EGFP

Oryzin and β xylosidase signal peptides were selected for the optimization of codons. Individual codons were analyzed using the codon usage database (http://www.kazusa.or.jp/codon/). Codons with a low percentage usage were replaced by codons that occur at higher frequency. The codon optimized signal peptide was fused to the 5′ end of EGFP coding sequence by PCR using the designed oligonucleotide primers (Table 6.2). The construct was then introduced in to pKlac1 expression vector.

6.2.5. Construction of Kex2 incorporated signal peptide

Kex2 protease cleavage site (AAAAGA) was incorporated into the glucoamylase and cellobiohydrolase 2 signal peptide sequences using engineered oligonucleotide primers for the evaluation of its efficiency in increasing the signal cleavage after entering in to the endoplasmic reticulum.

6.2.6. Construction of human interferon-β fusions with glucoamylase (glaA) or α mating factor (αMF) signal peptide fusion

Human therapeutic protein interferon-beta (HuIFNβ) (501 bp) was fused in-frame to the 3′ end of the glucoamylase signal peptide and PCR amplified. Interferon-β cloned downstream of the native αMF secretion signal (αMFSS) was used as positive control. The construct was introduced into pKlac1 vector downstream of its native lac4 promoter. Individual codons of glucoamylase were analyzed using the codon usage database (http://www.kazusa.or.jp/codon/). Codon optimized glucoamylase signal peptide was fused *in-frame* to the 5′ end of the HuIFNβ coding sequence, PCR amplified and a nucleotide

sequence encoding the KEX2 cleavage site and 6× His-Tag was incorporated upstream of the HuIFNβ gene. The forward and reverse primers used for PCR amplification of IFN were IFNCF and IFNCR (Table 6.2).

Table 6.2. Oligonucleotide primers used for the study

Primer	Primer Sequence (5'-3')
CBH2F	AATTTAAGCTTATGATTGTCGGCATTCTCACCACGCTGGCTACGCTGGCCACA CTCGCAGCTAGTGTGCCTCTAATGGTGAGCAAGGGC
ORZF	AATTTAAGCTTATGCAGTCCATCAAGCGCACTCTCTTGCTCCTGGGAGCTGTC CTTCCCGCGGTCCTAGCTGGTATGGTGAGCAAGGGC
AMYF	AATTTAAGCTTATGAGAGTGTCGACTTCAAGCCTTGCCCTTTCTGTGTTCGGG AAGCTGGCCCTTGGGCTGTCAGCTGCAATGGTGAGCAAGGGC
GLUF	AATTTAAGCTTATGTCGTTCCGATCTCTACTCGCCCTGAGCGGCCTCGTCTGC ACAGGGTTGGCAAATGTGATTATGGTGAGCAAGGGC
BGLF	AATTTAAGCTTATGCACAGCATTAGTGCGCTTCTCAGCTTGCTTGGTGGCCTC GCGCTCTCCAGCGCTGCTCCGATGGTGAGCAAGGGC
XYLF	AATTTAAGCTTATGGCGCACTCAATGTCTCGTCCCGTGGCTGCCACGCCGCTG CTCTGCTGGCTCTGGCTCTTCCTCAAGCTCTTGCCCAGGCCATGGTGAGCAAG GGC
CEL5F	AATTTAAGCTTATGAACAAGTCGTGGCTCCATTGCTGCTTGCAGCGTCCATAC TATATGGCGGCGCCGTCGCACAGATGGTGAGCAAGGGC
ENDXF	AATTTAAGCTTATGGTCCATCTTAAAACCCTCGCCGGCAGCGCTGTCTTTGCC TCGCTGGCCACCGCGGCCGTCATGGTGAGCAAGGGC
ENDNF	AATTTAAGCTTATGGTTCAGATCAAGGTAGCTGCACTGGCGATGCTTTTC GCTAGCCAGGTCTTTCTGAGCCCATGGTGAGCAAGGGC
ESIGR	AAATCTCGAGTTACTTGTACAGCTCGT
EGFPF	AAGCTCGAGATGGTGAGCAAGGGCGAGGAGC
EGFPR	AAGGGTACCTTACTTGTACAGCTCGTCCATGC
AMKF	AATTTAAGCTTATGAGAGTGTCGACTTCAAGCCTTGCCCTTTCTGTGTCC CTTTTCGGGAAGCTGGCCCTTGGGCTGTCAGCTGCAGAGAAGAGAATGGTG AGCAAGGGC
CELKF	AATTTAAGCTTATGAACAAGTCCGTGGCTCCATTGCTGCTTGCAGCGTCC ATACTATATGGCGGCGCCGTCGCAGAGAAGAGAATGGTGAGCAAGGGC
OROPF	AATTTAAGCTTATGCAATCTATTAAACGTACTTTGTTGTTGTTGGGTGCT GTTTTG CCAGCTGTTTTGGCTGGTATGGTGAGCAAGGGC
XYPOF	AATTTAAGCTTATGGCTCATTCTATGTCTCGTCCAGTTGCTGCTACTGCT GCTGCTTTGTTGGCTTTGGCTTTGCCACAAGCTTTGGCTCAAGCTATGGTG AGCAAGGGC

IFNGLUF	AATTTAAGCTTATGTCGTTCCGATCTCTACTCGCCCTGAGCGGCCTCGTCTGC ACAGGGTTGGCAAATGTGATGAGCTACAACTTGCTTG
IFNαMF	AATCTCGAGATGAGCTACAACTTGCTTG
IFNR	AATGGTACCTCAGTTTCGGAGGTAACCT
IFNCF	AATTTAAGCTTATGTCTTTCAGATCTCTATTGGCTTTGTCTGGTTTGGTTTGTACTGG TTTGGCTAATGTTAAAAGAATGAGCTACAACTTGCTTG
IFNCR	AATGGTACCTCAATGGTGATGGTGATGGTGGTTTCGGAGGTAACCT

6.2.7. Transformation of *K. lactis* cells

After linearization with Sac11, the plasmids were transformed into *K.lactis* cells by electroporation using a Biorad Gene Pulser Xcell electroporator. A 2.0 mm cuvette was used and the conditions for electroporation were: a sample volume of 100µl, charging voltage of 1.8 kV, and capacitance of 25µF. Integration of the expression cassette into the genome occurred by homologous recombination into the lac4 locus. Transformed colonies were selected on YPD-acetamide plates and were incubated at 30 °C for 3–4 days. The transformants were analyzed by PCR amplification with the specific primers and sequencing.

6.2.8. Measurement of fluorescence intensity

Supernatant and cells were subjected to fluorescent intensity analyses using a multimode plate reader (Infinite Pro 200, Tecan, Switzerland) with excitation and emission wavelengths of 488 and 520 nm, respectively. Cell-free supernatant (50µl) was mixed with 150µl refolding buffer (0.05 M NaH_2PO_4, 0.1 M NaCl, and 0.5 M Imidazole) and the fluorescence intensity was measured. For fluorescent intensity measurements, 50µl cell suspensions were centrifuged at 13,400 × g for 2 min at room temperature, and the cells washed 3 times with the refolding buffer, were suspended in 200µl of the same buffer.

6.2.9. Poly acryl amide gel electrophoresis (PAGE) and western blot analysis

Cells were harvested after 36 h of growth by centrifugation at 15,450 × g for 2 min. The total protein concentration in the supernatant was determined by Bradford's (1976) method. The protein in the supernatant was precipitated using 10× volumes of 12% trichloroacetic acid (TCA) in acetone, and the precipitate was recovered by centrifugation at 13,400 × g for

20 min at 4 °C. The pellets were washed thrice with acetone; air dried and was resuspended in distilled water. Samples normalized for their protein content were loaded on 12% SDS poly acrylamide gels for electrophoretic separation (Laemmli, 1970).

The EGFP and HuIFNβ protein present in the supernatant was further analyzed by western blotting. The proteins were transferred onto a PVDF membrane by semi-dry blotting and were detected with anti GFP or anti HuIFNβ antibody (Life Technologies, USA) respectively using the chromogenic substrate 3, 3′5, 5′ tetramethyl benzidine (TMB). Digital images of the western blot were captured and densitometric analysis of the signal intensities was performed using ImageJ software (Schneider *et al*., 2012).

6.2.10. Purification

Recombinant HuIFNβ was purified from the culture supernatant through Ni-NTA columns (Qiagen, India) under native conditions according to the manufacturer's protocol. Briefly, the concentrated supernatant was passed through the Ni-NTA columns equilibrated with buffer followed by three washes with wash buffer and eluted with 250mM imidazole. Imidazole was then removed by dialysis and the solution was concentrated by lyophilization. HuIFNβ protein present in the supernatant was further analysed by Western blotting. The proteins were transferred onto a PVDF membrane by semi-dry blotting (Novablot , GE, USA) and was detected with Anti Human Interferon β antibody (Life technologies, USA) using the chromogenic substrate 3, 3'5, 5' Tetramethyl Benzidine (TMB).

6.2.11. Biological activity of the recombinant HuIFNβ (Anti cell proliferation assay)

HeLa cells were cultivated in 96-well plates with DMEM media containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Plates were incubated in a humidified CO_2 incubator at 37 °C. The cells grown to 70-85% confluence were treated with different concentrations of the HuIFNβ for 24h at 37 °C. After incubation, the cell viability was measured by MTT assay. Twenty micro litres of MTT (5 mg/ml) was added to each well and incubated for 4h at 37 °C. Later, the culture medium was removed, 100µl DMSO was added to each well, and the plates were agitated in an orbital shaker for 45 min. The absorbance for each sample was measured at 570 nm using a multi well plate reader (Synergy, Biotek, USA). The percentage of viable cells was calculated based on control cells treated similarly except for HuIFNβ addition.

6.2.12. MALDI-TOF MS analysis

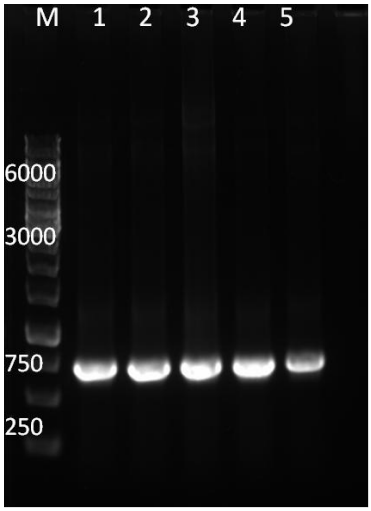
Gel fragments of the desired protein were cut out and incubated in a buffer containing 200mM ammonium hydrogen carbonate and 40% acetonitrile (ACN, 40 % v/v) at 37 °C for 10 min; this was repeated until the gel was completely de-stained. Gel pieces were then vacuum dried and treated with 20 µg/ml Trypsin, 40mM ammonium hydrogen carbonate, 9% ACN (v/v), and 1 mM HCl. After incubation at 37 °C, gel fragments were removed and the supernatant was spotted on the plate for MALDI-TOF MS at a 1:1 ratio with 10–20 mg/ml DAHC. The analysis was performed using ultraflextreme® MALDI-TOF/TOF (Bruker Daltronik, USA). The obtained peptide mass results were then searched against the NCBI database using the MASCOT peptide mass fingerprinting search program (Matrix Science, Boston, MA).

6.3. Results and discussion

6.3.1. Fungal signal peptides induces effective secretion of EGFP in to the culture fluid

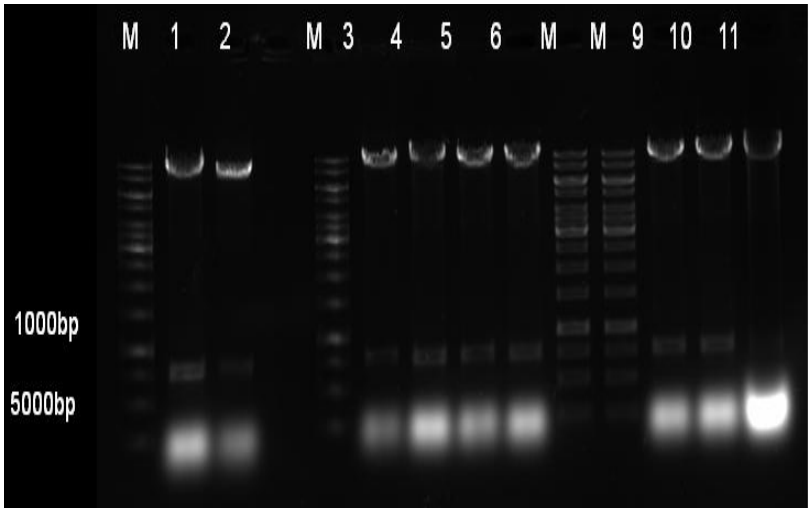
For the identification of novel signal peptides for the secretion of heterologous proteins in *K.lactis*, the secretome data of industrially important filamentous fungi was searched for highly secreted proteins. The shortlisted proteins were further analyzed for putative secretion signal peptides and signal peptidase cleavage sites using the SignalP 3.0 HMM software. The cleavage site for the signal peptidase was predicted (Table 6.1). Signal prediction of all the nine peptides with SignalP revealed the characteristic pattern of signal cleavage with good signal peptide probability. It is very important to have several secretion signal sets to choose from, since signal peptides can differ greatly in their ability for the secretion of a given protein. Hence, to get efficient secretion, testing of many signal peptides is very crucial. To examine the potential of the nine putative signal peptides to support recombinant protein secretion, signal peptides were fused to the N-terminus of green fluorescent protein (GFP) and PCR amplified using oligonucleotide primers (Table 6.2), and the resulting constructs were cloned into the plasmid pKlac1 expression vector. Linearized vector was electroporated into *K.lactis* cells and successful chromosomal integration was confirmed by PCR amplification of a 717bp fragment using specific primers (Fig 6.1A and 6.1B). Sequencing results confirmed the identity of the gene present in the genome of the *K.lactis*.

Fig. 6.1. Construction of expression vectors for evaluation of target Signal peptides fused to EGFP



A: Representative gel profile of PCR amplification of EGFP fused signal peptides

M GeneRuler® 1kb ladder (Fermentas)
1-5 Signal Peptide-EGFP fusion amplified with appropriate primers (Table 6.2)



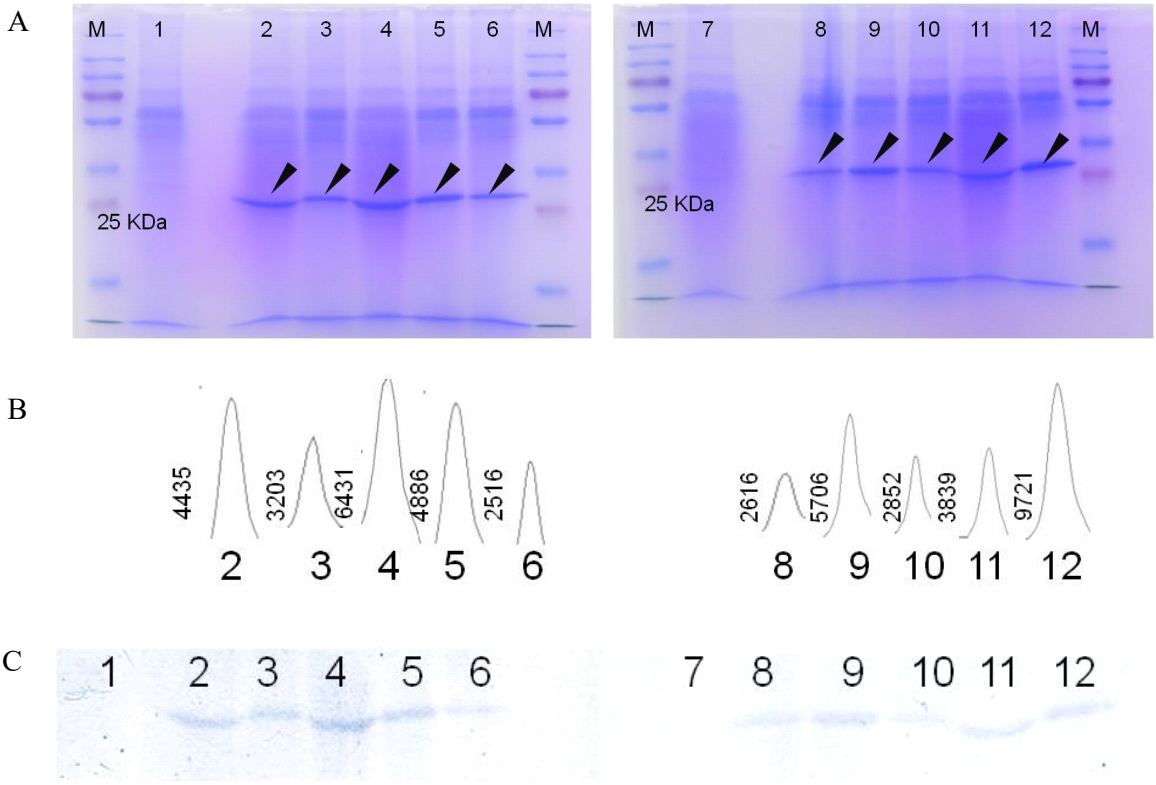
B: Representative profile of restriction digestion of recombinant plasmids

M GeneRuler® 1kb ladder
1-11 Restriction digested plasmids releasing EGFP fused signal peptides

In all the transformants, EGFP could be detected whereas the wild type did not express EGFP. In the fractions originating from the extracellular fluid of the recombinant strains, signals of EGFP with a molecular weight of ~25 kDa were visible in SDS PAGE and western blot (Fig. 6.2A and 6.2B). A substantial amount of recombinant EGFP could be detected in the precipitated proteins of the culture supernatant expressing – EGFP from all the nine signal peptides. The recombinant *K.lactis* cells harboring pKlac1 with fungal signal peptides secreted green fluorescent protein into the culture medium, as evidenced by the fluorescence intensity of the culture supernatants (Fig. 6.3). Among them, β glucosidase and glucoamylase secretion signals (SS) mediated higher secretion of the EGFP proteins and these were higher than that mediated by the αMF signal peptide. Oryzin, cellobiohydrolase 2 and α amylase SS mediated almost equal amount of extracellular protein secretion and were comparable to αMF signal peptide. But in the latter two, fluorescence intensity analysis revealed that a good

amount of proteins were found to be trapped inside the cell. Densitometry of the gel also confirmed the results (Fig. 6.2C).

Fig. 6.2. EGFP secretion from *K. lactis* having filamentous fungal secretion signal (pKlac1-SS-EGFP).

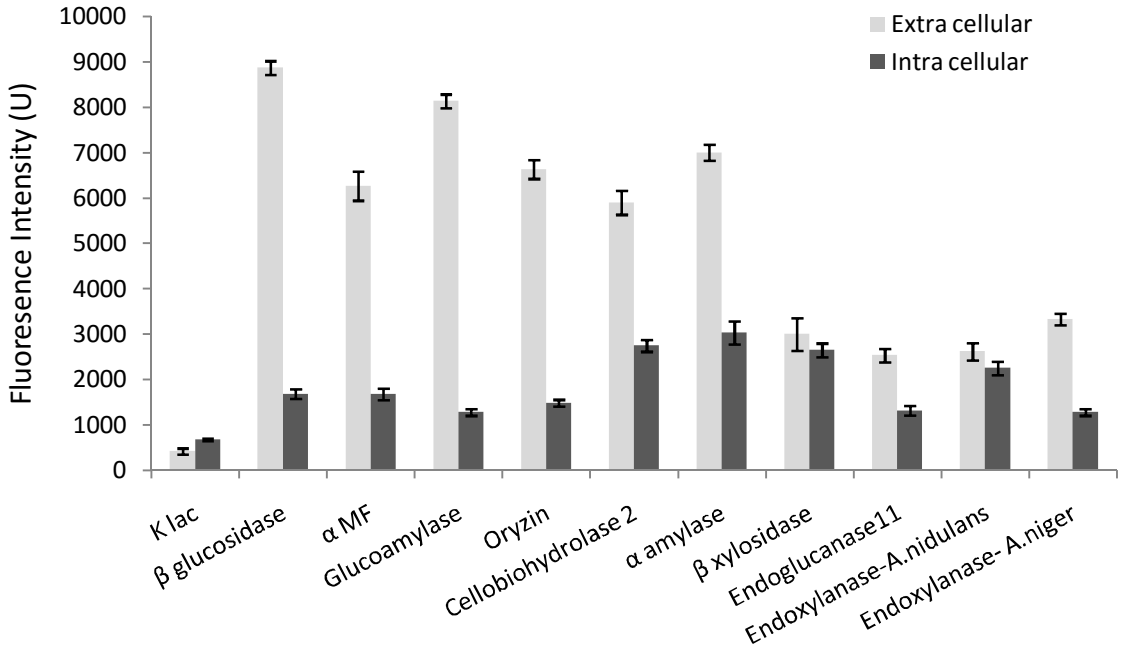


(**A**) SDS-PAGE showing ~25 kDa secreted EGFP protein (indicated by arrow heads) in the culture fluid of *K.lactis* with native αMF secretion signal or filamentous fungal secretion signal. Lanes- M: SDS-PAGE Marker. 1&7: host *K. lactis* culture fluid, 2: culture fluid of *K. lactis* with αMF secretion signal, 3: culture fluid of *K. lactis* with β xylosidase secretion signal, 4: culture fluid of *K. lactis* with β glucosidase secretion signal, 5: culture fluid of *K. lactis* with oryzin secretion signal, 6: culture fluid of *K. lactis* with endoxylanase A (*A. niger*) secretion signal, 8: culture fluid of *K. lactis* with endoxylanase (A. nidulans) secretion signal, 9: culture fluid of *K. lactis* with α amylase secretion signal, 10: culture fluid of *K. lactis* with endoglucanase 11 secretion signal, 11: culture fluid of *K. lactis* with cellobiohydrolase 2 secretion signal, 12: culture fluid of *K. lactis* with glucoamylase secretion signal. (**B**) Peaks showing the band intensities of GFP signals. Lane information same as that for SDS PAGE. (**C**) Western blot of EGFP protein from *K. lactis*. Lane information same as that for SDS PAGE.

Oryzin is reported to be the most abundant protein in the secretome of *A. terreus* (Han *et al*., 2010) and cellobiohydrolase 2 is one of the abundant proteins in the extracellular proteome of *T. reesei* (Herpoel – Glimbert et al., 2008). Other signal peptides- endoxylanase

(*A. nidulans*), endoglucanase11 (*T. reesei*), endoxylanse A (*A. niger*), β xylosidase (*A. niger*) also mediated secretion of EGFP protein to the extracellular fluid, but the fluorescence intensity and SDS-PAGE band intensity were lower than that observed with the strain carrying αMF signal peptide. Proteomic analysis of the secretory proteins in *A. niger* by Ferreira *et al*., (2011) had revealed that glucoamylase and β glucosidase were the major secreted proteins. Secretome diversity is very high in filamentous fungi and this makes them attractive as sources of signal peptides. In all the cases of the signal peptide – EGFP strain, fluorescent intensity analysis showed the presence of EGFP in both intracellular as well as in extracellular fractions and it was higher for α amylase, β xylosidase and cellobiohydrolase 2 signal peptides. Incomplete processing of the αMF signal peptide has been reported in some recombinant proteins which might lead to inefficient secretion of the protein (s) (Hashimoto *et al*., 1998; Koganesawa *et al*., 2001). The efficiency of EGFP secretion by the tested signal peptides varied to a great extent. Secretion capacity based screening was done for all the nine signal peptides because the efficiency of signal peptide is strongly dependent on the protein of interest and it may vary from protein to protein. In addition, secretion out of the cell prevents toxic effects of proteins overexpressed inside the cells. Heterologous fungal signal peptides are strong enough to direct the secretion of heterologous proteins in yeast, and hence, we can possibly bypass one of the current bottle necks in recombinant protein production in yeast. Recent reports showed that the cellular secretory pathway plays a key role than the transcription and translation in protein production event (Love *et al*., 2012). So, the secretion signal optimization is very important in regulating recombinant protein production.

Fig. 6.3. EGFP expression from the native αMF secretion signal or filamentous fungal secretion signal.



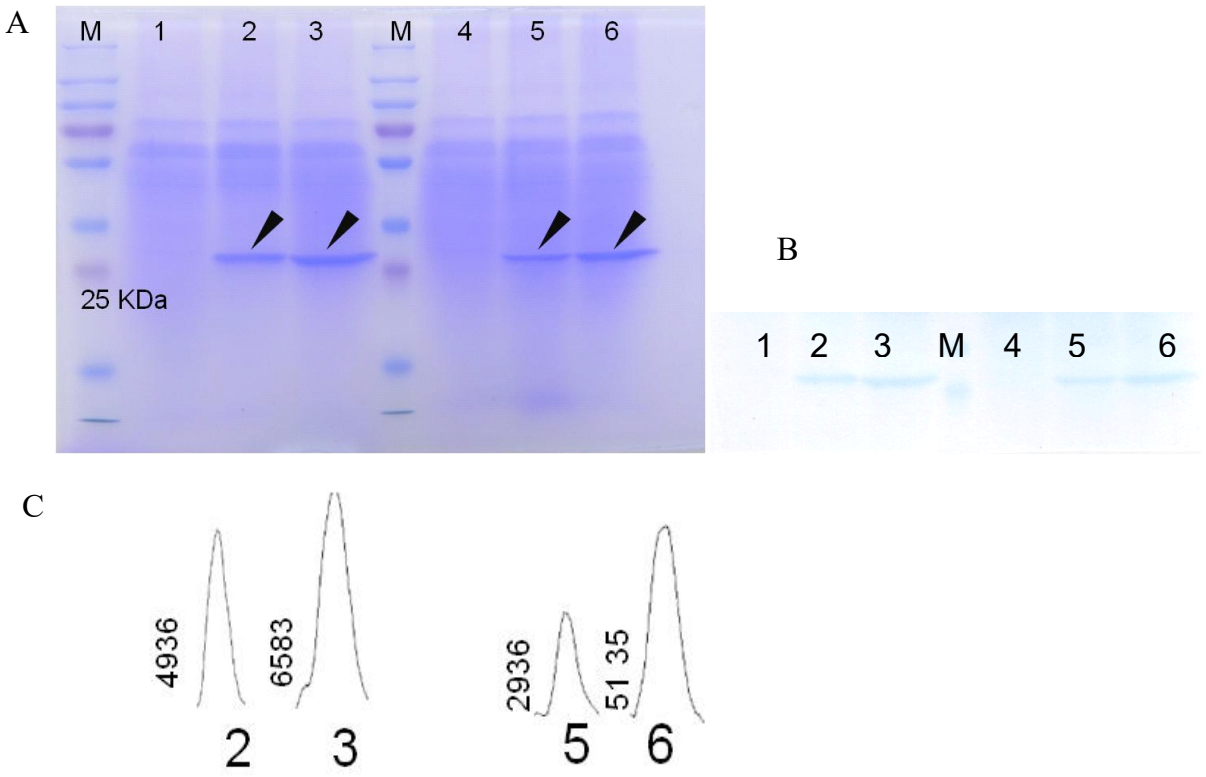
Intracellular and extracellular expression of EGFP in *K. lactis* from the fungal secretion signal and native αMF secretion signal measured as fluorescence intensity. Auto-fluorescence of host *K. lactis* cells and the culture medium was negligible (Klac (host)).

6.3.2. Optimization of signal peptide codons leads to enhanced secretion

Comparison of the codon usage of *K. lactis* with that of filamentous fungi which was used for the present study by use of a codon usage database (http://www.kazusa.or.jp/codon/) showed that the frequencies of individual codons are different from those in *A.nidulans*, *A.niger*, *A.terreus* and *T. reesei* (http://www.kazusa.or.jp/codon/). Based on codon usage, codon-optimized signal peptides were designed. Codons infrequently used in *K. lactis* were replaced by more-frequently used codons. Codon optimization is considered as an efficient strategy for improving the expression levels of heterologous genes that contain codons rarely used in the host organism (Gustaffson *et al*., 2004). The rare codons and high G + C contents can decrease the translational efficiency, or even result in failed expression (Fuller *et al*., 1989; Sinclair *et al*., 2002). We investigated the effect of codon optimization on the expression levels of EGFP proteins in *K. lactis* cells. Oryzin and β xylosidase secretion signals were selected for the codon optimization studies, since oryzin SS mediated protein secretion levels comparable to αMF SS and β xylosidase secreted lesser amount of EGFP compared to αMF

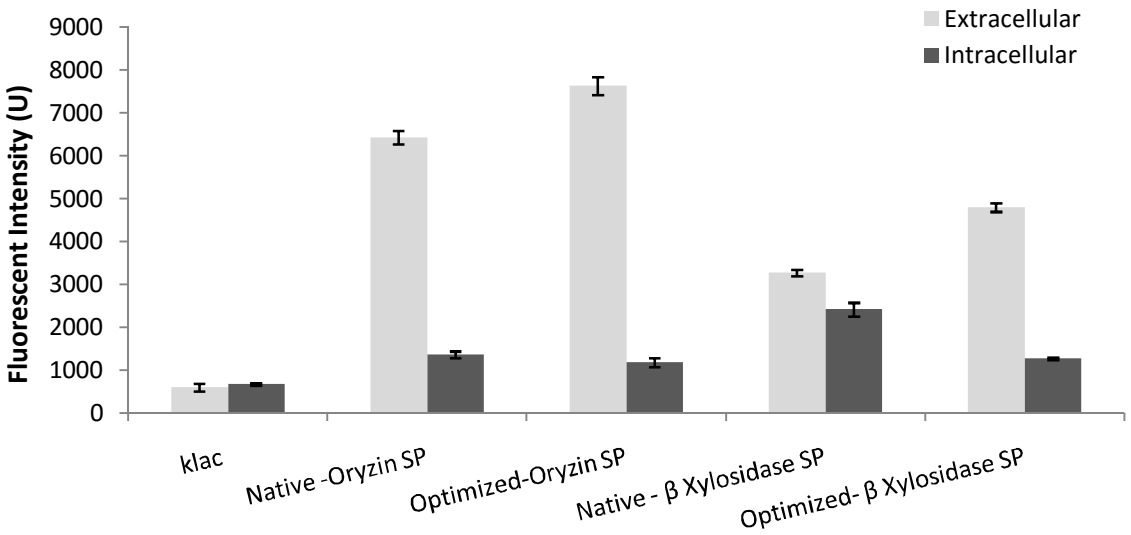
SS. The effectiveness of codon optimized signal peptides in secretion of recombinant proteins from *K. lactis* was analyzed by SDS PAGE, western blot, densitometry and fluorescent intensity analyses. These experiments also confirmed the higher level secretion of EGFP by cells from codon optimized secretion signal (Fig. 6.4 and Fig. 6.5). The findings suggest that codon optimization in the signal peptide aimed at making it more suitable for transcription in *K. lactis* improved the expression and secretion of target heterologous protein.

Fig. 6.4. Effect of codon optimization on EGFP secretion.



(A) SDS-PAGE showing ~25 kDa secreted EGFP protein (indicated by arrow heads) in the culture fluid ofK. lactis with native secretion signal or codon optimized fungal secretion signal. Lanes- M: SDS-PAGE Marker. 1&4: host *K. lactis* culture fluid, 2: culture fluid of *K. lactis* with native oryzin secretion signal, 3: culture fluid of *K. lactis* with codon optimized oryzin secretion signal, 5: culture fluid of *K. lactis* with native β xylosidase secretion signal, 6: culture fluid of *K. lactis* with codon optimized β xylosidase secretion signal. (B) Western blot of EGFP protein from *K. lactis*. Lane information same as that for SDS PAGE. (C) Peaks showing the band intensities of GFP signals. Lane information same as that for SDS PAGE.

Fig. 6.5. EGFP expression from the native and codon optimized secretion signals oryzin and β xylosidase.



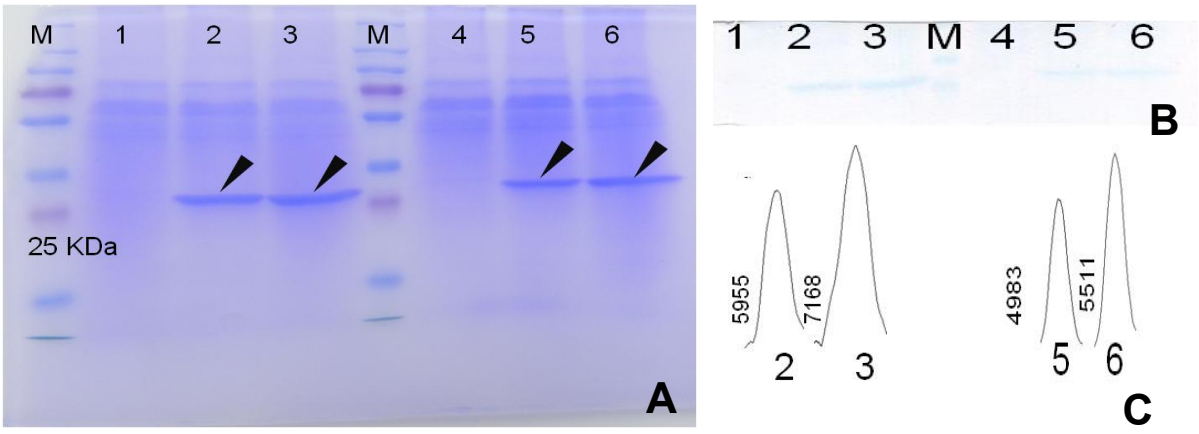
Intracellular and extracellular expression of EGFP in *K. lactis* from the native and codon optimized secretion signal measured as fluorescence intensity. Auto-fluorescence of host *K. lactis* cells and the culture medium was negligible (Klac (host), SP (signal peptide)).

6.3.3. Addition of kex2 protease cleavage site increases the recombinant protein production

The Kex2 endoproteases are encoded by Kex2 genes which cleaves the yeast pre-proteins during protein trafficking in a site specific manner (Fuller *et al*., 1989). Addition of Kex2 cleavage sites to the signal peptides can improve the secretion and hence the effect of Kex2 cleavage site addition in the selected fungal signal peptides was evaluated. To this end, the transformants carrying expression constructs with or without Kex2 cleavage sites were cultivated, sampled and expression levels of EGFP were determined. Kex2 protease cleavage sites were incorporated in α amylase and cellobiohydrolase 2 secretion signals to study the effect of Kex2 site addition on secretion of EGFP. SDS PAGE analysis, western blotting and densitometry analysis showed that introduction of addition of Kex2 significantly increased the secreted EGFP expression in the yeast cells from α amylase and cellobiohydrolase 2 signal peptides (Fig. 6.6A, B and C). The fluorescence intensity in yeast culture media was elevated upon addition of Kex2 cleavage site (Fig. 6.7). However, the intracellular fluorescence of each strain was reduced. This might be due to the better secretion of the Kex2 cleavage site containing signal peptide. The results clearly demonstrated the feasibility of achieving high levels of recombinant protein secretion in *K. lactis* by incorporation of Kex2 cleavage site. Site specific proteolysis is a common feature in protein maturation and plays a crucial role in activation of many enzymes and in the generation of peptide hormones (Bader

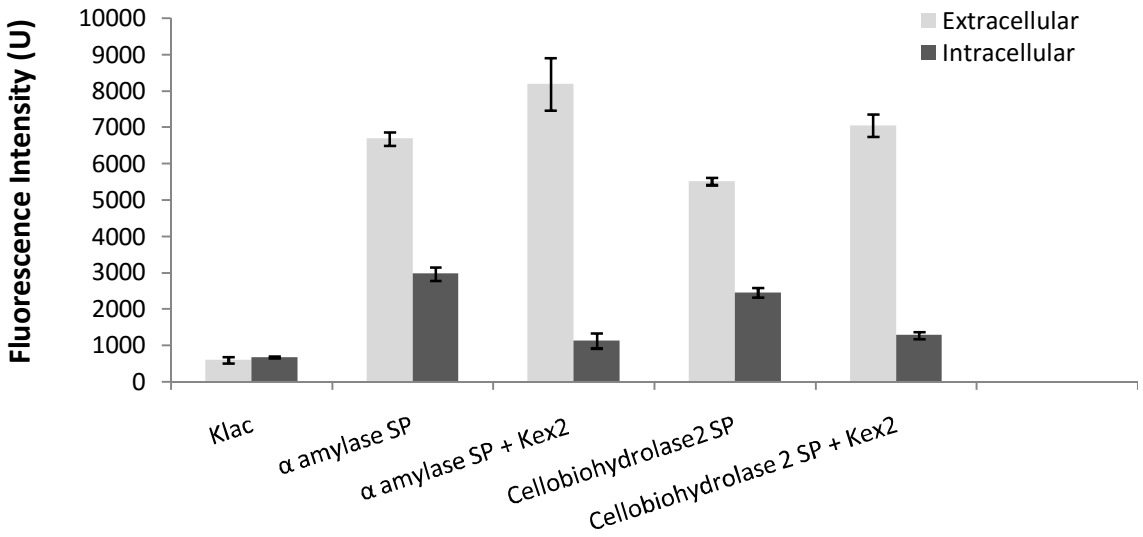
et al., 2008). Recent attempts to improve protein secretion in *Pichia pastoris* expression system include engineering additional copies of Kex2 endoprotease into the genome so as to allow an efficient cleavage of the secretion signal (Yang *et al*., 2013).

Figure: 6.6. Effect of addition of Kex2 cleavage site and EGFP secretion



(**A**) SDS-PAGE showing ~25 kDa secreted EGFP protein (indicated by arrow heads) in the culture fluid ofK. lactis with or without Kex2 cleavage site in the secretion signal. Lanes- M: SDS-PAGE Marker. 1&4: host K. lactis culture fluid, 2: culture fluid of *K. lactis* with α amylase secretion signal, 3: culture fluid of *K. lactis* with Kex2 cleavage site incorporated α amylase secretion signal, 5: culture fluid of *K. lactis* with cellobiohydrolase 2 secretion signal, 6: culture fluid of *K. lactis* with Kex2 cleavage site incorporated cellobiohydrolase 2 secretion signal. (**B**) Western blot of EGFP protein from *K. lactis.* Lane information same as that for SDS PAGE. (**C**) Peaks showing the band intensities of GFP signals. Lane information same as that for SDS PAGE.

Fig. 6.7. EGFP expression from the α amylase and cellobiohydrolase 2 secretion signals with and without Kex2 cleavage site.

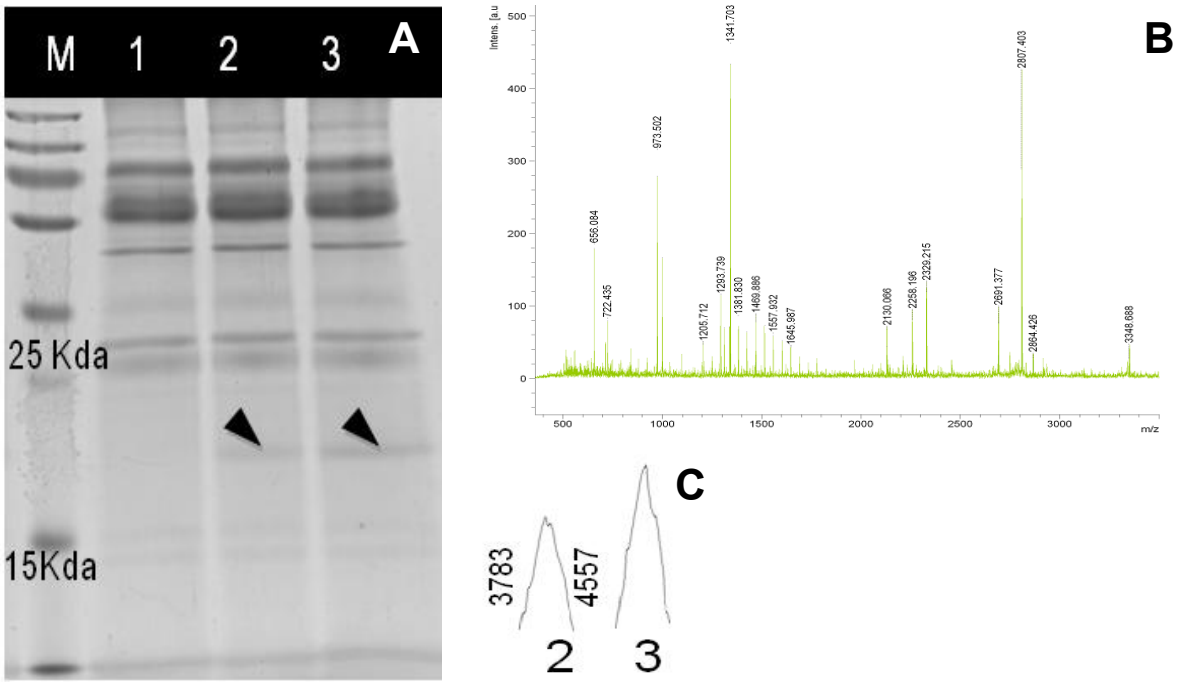


Intracellular and extracellular expression of EGFP in *K. lactis* from the α amylase and cellobiohydrolase 2 secretion signal with and without Kex2 cleavage site measured as fluorescence intensity. Auto-fluorescence of host *K. lactis* cells and the culture medium was negligible (Klac (host), SP (signal peptide)).

6.3.4. Glucoamylase secretion signal mediates the extracellular secretion of HuIFNβ

The efficiency of glucoamylase signal peptide in the secretion of human interferon-beta (HuIFNβ) was tested. The supernatant of *K. lactis* strain harbouring the pKlac 1 vector with HuIFNβ gene fused to glucoamylase or αMF signal peptide; showed the presence of a protein band of approximately 20 KDa (size of HuIFNβ). To confirm the identity of the purified protein, MALDI-TOF MS analysis was performed. The results showed that most of the peaks corresponded to the masses of trypsin-digested fragments of interferon-beta (Fig. 6.8A, B & C). Glucoamylase signal peptide was found to be more efficient in secreting HuIFNβ compared to the αMF signal peptide.

Fig.6.8. HuIFNβ expression from glucoamylase signal peptide.

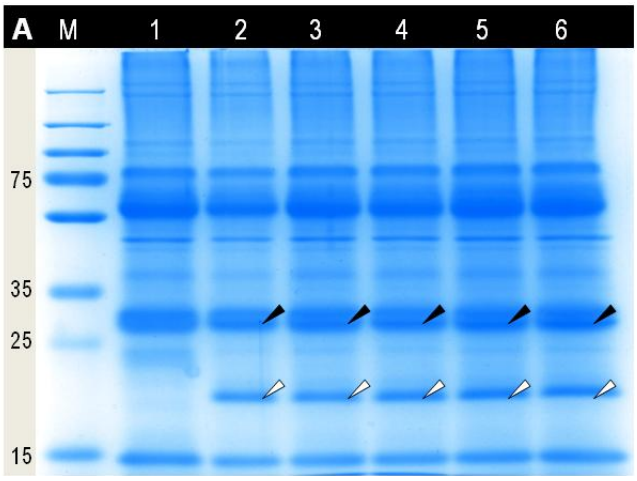


(A) SDS-PAGE showing ~20 kDa secreted interferon-beta protein (indicated by arrow heads) in the culture fluid of *K. lactis* with native αMF secretion signal and glucoamylase secretion signal. Lanes- M: SDS-PAGE Marker. 1: host *K. lactis* culture fluid, 2: culture fluid of *K. lactis* with αMF secretion signal, 3: culture fluid of *K. lactis* with glucoamylase secretion signal. (B) MALDI-TOF MS analysis of purified interferon-beta. (C) Peaks showing the band intensities of HuIFN signals. Lane information same as that for SDS PAGE.

6.3.5. Codon optimized glucoamylsae secretion signal with kex2 protease cleavage site drives the recombinant HuIFNβ into culture fluid

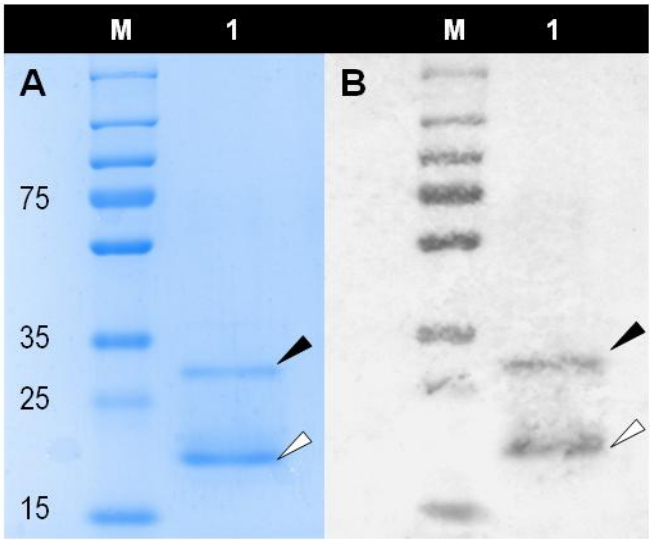
Codon optimized glucoamylase signal peptide was cloned for improved secretion of HuIFNβ. The sequence for signal peptide was fused to those corresponding to the N-terminus of the gene for HuIFNβ and was PCR amplified along with a nucleotide sequence encoding the KEX2 cleavage site, and the resulting constructs were cloned into the pKlac1 expression vector. The secreted recombinant protein was concentrated by precipitation and desalted by dialysis. Recombinant HuIFNβ was eluted from Ni-NTA column at 250mM imidazole concentration. SDS PAGE of the sample detected a ~19 kDa and a ~ 27 kDa secreted protein bands (Fig 6.9) and the 27kDa band was absent in the SDS PAGE profile of the non codon optimized glucoamylase signal peptide. These molecular weights correspond to the reported values for non glycosylated and glycosylated HuIFNβ respectively (Spearman *et al*., 2005). Subsequent Western blot analysis confirmed the purity. Purified HuIFNβ was detected as two bands with molecular weights 19 kDa and 27kDa respectively, matching the same bands detected by SDS PAGE; further indicating that these could be the non-glycosylated and glycosylated HuIFNβ (Fig 6.10A & B). The glycosylated protein was secreted in lower levels compared to the non-glycosylated one, as evidenced from the band intensities of the 19kDa and 27kDa bands detected by SDS PAGE and Western blot analyses. The molecular weight of about 19kDa, for the major band was consistent with the predicted molecular weight of HuIFNβ. HuIFNβ inhibited the growth of HeLa cells in a dose and time dependent manner as indicated by the MTT assay to detect cell proliferation (Fig 6.11). The anti proliferative effect was very evident at higher concentrations of the cytokine and 50% cell growth inhibition was achieved at 40.97 μg/ml of HuIFNβ. This demonstrated that the recombinant HuIFNβ expressed from *K. lactis* is biologically active and the expression system holds promise for large scale production of the protein for potential therapeutic applications.

Fig.6.9. Time course study of HuIFNβ production by codon optimized glucoamylase signal peptide



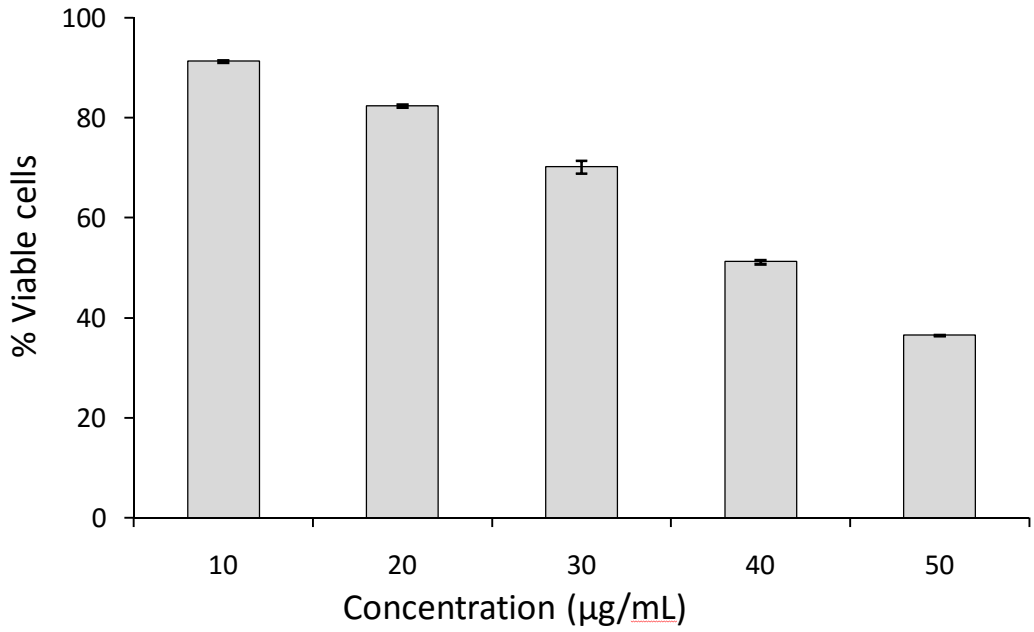
SDS-PAGE of culture supernatants (normalized for total protein) from recombinant *K.lactis* at recovered at different time points. Lanes M-pre-stained protein ladder, 1-culture supernatant from native *K.lactis* recovered after 60h of incubation, 2,3,4, 5 and 6 - culture supernatant from recombinant *K.lactis* recovered after 24, 36, 48, 60 or 72h of incubation. Open and filled arrows represent the 19kDa and 27kDa non glycosylated and glycosylated HuIFNβ respectively.

Fig. 6.10. HuIFNβ secretion from recombinant *K. lactis* clone



A) SDS –PAGE and B) Western blot of Ni-NTA affinity purified HuIFNβ showing ~ 19kDa (open arrow) and ~ 27 KDa (filled arrow) bands corresponding to non-glycosylated and glycosylated proteins respectively. Lanes-M: Pre-stained Protein Ladder (Fermentas, USA), 1: Ni-NTA affinity purified HuIFNβ

Fig.6.11. Anti-proliferative activity of HuIFN-β on HeLa cells by MTT assay



Proliferation of HeLa cells 24h after treatment with HuIFNβ is expressed as percentage of the untreated control. Error bars represents deviation of the median from different experiments

6.4. Conclusion

Through constructing a series of pKlac1 vectors with different signal peptides, yeast transformation and assay of secreted protein expression, it was found that the filamentous fungal signal peptides are efficient in mediating secretion of proteins in the yeast *K. lactis*. All fungal secretion signals were found to be effective for the secretion of heterologous protein in *K. lactis*, and some of these signals were more effective than the native αMF SS used in the yeast. Codon optimization and incorporation of Kex2 protease cleavage site resulted in improved secretion efficiency. Glucoamylase signal peptide supported a higher-level secretion of the recombinant interferon compared to αMF SS. The study indicates the potential to use fungal secretion signals to develop expression systems with efficient extracellular production of heterologous proteins in yeast.

7. Summary and Conclusion

The attraction of filamentous fungi as heterologous hosts is based on their natural ability to secrete large amounts of hydrolytic enzymes into the growth medium and have great potential as microbial cell factories. Their unexplored genetic diversity poses interesting prospects of exploiting them as a source of novel genes, promoters and signal peptides and their use as expression hosts. The main advantages of using filamentous fungi as expression hosts include their natural abilities to secrete a variety of proteins in large quantities and their intensive use in bioprocessing. They can grow on relatively inexpensive substrates, and are able to produce and secrete tremendous amounts of recombinant proteins. Unlike the traditional *E.coli* or Yeast (*Saccharomyces cerevisiae, Pichia pastoris*) based expression systems, filamentous fungi as expression hosts offer several advantages like inexpensive cultivation, easy induction, efficient secretion etc.

Protein synthetic machinery, closer in functioning to the higher order animals makes them attractive production hosts for several proteins of interest in pharma and diagnostic industries. Perhaps, the greatest advantage is in the production of low value high volume proteins which are uneconomical to produce using conventional expression systems. Their highly efficient signal peptides can also be used for improving recombinant protein secretion in other expression systems. While there are expression systems based on filamentous fungi reported previously, there are none available commercially or in the public domain. *Aspergilli* are a large and diverse genus (~250 species) of filamentous fungi including several species with high industrial value (eg. *A .oryzae, A .niger*) and are used in production of several industrially important chemicals (eg. citric acid). Majority of the species are GRAS status and used extensively in commercial scale production. They grow faster and are less susceptible to invasive microbes in culture.

In the present study, a novel filamentous fungus belonging to the taxonomic group *Aspergillus unguis* was used, which showed excellent growth characteristics under submerged fermentation making it attractive host for large-scale production of proteins. Under our culture conditions, the fungus grew as pellets in an even suspension and did not form any large aggregates, allowing better circulation of air and mixing. Since the genetic makeup and complete genome is still unknown, the fungal expression vector was developed by PCR amplification of

efficient glyceraldehyde 3 phosphate dehydrogenase promoter (gapd) and tryptophan synthase (trpC) transcription terminator from *Aspergillus nidulans* and Hygromycin gene from a commercially available vector as selection marker. The GFP gene from *Aequorea victoria* was used as the model test protein for the evaluation of expression system. The constructs were ligated *in-frame* and incorporated into pUC19 backbone for the propagation and maintenance in *E coli*, and sequenced. The genetic transformation of this fungus was studied in detail. After transformation optimization studies, it was found that this strain is easily transformed through electroporation and hygromycin selection makes the selection of recombinant clones easy.

Expression studies with the integrative plasmids in which expression was driven by constitutive glyceraldehyde 3 phosphate dehydrogenase promoter illustrated that the maximum amount of green fluorescent protein was accumulated intracellular. For the extracellular secretion, highly efficient heterologous signal peptides from *Aspergillus niger* were incorporated and tested. *A. unguis* secreted good amount of protein which is comparable to the protein secreted by *Aspergillus niger* from the native signal peptide. These characteristics makes *A. unguis* very promising candidate for heterologous protein production.

To test the capability of the *A. unguis* expression system for the production of human therapeutic proteins, human interferon beta (HuIFNβ) expression was evaluated using the expression system developed. Interferon- beta (IFNβ) is a cytokine involved in the antiviral, anti proliferative and immunomodulatory responses of cells, and a potent drug for multiple sclerosis. Codon optimized human interferon beta (HuIFNβ) gene was fused with the glucoamylase signal sequence in the N-terminus and was incorporated into the *A. unguis* expression vector and the potential of the host to secrete HuIFNβ protein was demonstrated

For efficient gene expression in *A. unguis*, it is essential that the expression of the target gene be enhanced by use of a homologous active promoter from the host. Uncharacterized genome and proteome of this particular species make this task difficult. Hence a study was performed for the isolation of potential native promoters from *A. unguis*, through analysis of the total secretome using two dimensional gel electrophoresis. Over expressed proteins were identified by MALDI TOF MS/MS and these hold the scope for further evaluation to determine their promoters and to test them.

Filamentous fungi are remarkable in their secretion of hydrolytic enzymes, which have granted them a potential role in biotechnology. The high efficiency of fungal signal peptides in

driving secreted expression of recombinant proteins in current yeast based expression systems was demonstrated by expressing GFP and human interferon β in *Kluyveromyces lactis* using fungal signal peptides.

In conclusion, *A. unguis* was demonstrated as a potent heterologous host for recombinant protein production. The strain has all the good qualities of a good expression host- like easiness in transformation, excellent biomass accumulation both in solid state and submerged culture condition, good protein secretion and very good growth characteristics in submerged condition. So it is speculated that the *A. unguis* may have broad utility for recombinant protein production, and a more thorough understanding at the genomic and proteomic level of the organism, and development of native promoter system will allow its optimal industrial use.

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APPENDIX

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µ	Micron/Micrometer
µg	Microgram
µl	Micro liter
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CSIR	Council for Scientific and Industrial Research
DAHC	Diammonium Hydrogen Citrate
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxy Ribonucleotide triphosphate
EDTA	Ethylene Diamin Tetra Acetic acids
Fig	Figure
GAPD	glyceraldehyde 3 phosphate dehydrogenase
L	Liter
mg/L	Milligram per liter
mM	Millimolar
NIIST	National institute for Inter Disciplinary Science and Technology
nm	nanometer
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
rpm	Rotation per minutes
SDS	Sodium Dodecyl Sulphate
sp	Species
UV	Ultra violet
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight