PRODUCTION, OPTIMIZATION & APPLICATIONS OF INDUSTRIALLY IMPORTANT ENZYMES FROM *BEAUVERIA* SP. MTCC 5184

A THESIS SUBMITTED TO

SAVITRIBAI PHULE PUNE UNIVERSITY

DOCTOR OF PHILOSOPHY (PH.D)

IN BIOTECHNOLOGY

BY

SNEHAL VIJAY MORE

UNDER THE GUIDANCE OF

Dr. (Mrs.) ASMITA PRABHUNE

DIVISION OF BIOCHEMICAL SCIENCES

NATIONAL CHEMICAL LABORATORY,

PUNE - 411 008, INDIA

JUNE 2017

..... Dedicated To My Parents and

Father-in-Law

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

The work presented in this thesis would not have been possible without my close association with many people. I take this opportunity to extend my sincere gratitude and appreciation to all those who made this Ph. D. thesis possible.

First, I would like to extend my sincere gratitude to my research guide Dr. (Mrs.) Asmita Prabhune for her dedicated help, advice, inspiration, encouragement and continuous support, throughout my Ph.D. Her enthusiasm, integral view on research and her mission for providing high-quality work, has made a deep impression on me. I am really glad to be associated with a person like Dr. Asmita in my life because her friendly nature has always made me feel at ease with her.

I am also grateful to my DAC members Dr. Archana Pundle, Dr. Sushma Gaikwad, and Dr. Nitin Patil for their invaluable help and suggestions. I am thankful to Dr. Bhushan Chaudhari and Dr. Kalpna Joshi for their useful suggestions.

I express my heart-felt gratitude to Dr. (Mrs.) R. Seeta Laxman for teaching me the basic things in professional and personal life. She is the one with whom I started my research carrier and my work on industrially impotant enzymes.

My thanks are due to Dr. Sachin Agawane for the animal study exteriments. His moral support and motivation was a driving force throughout my Ph. D work. My thanks are also due to Mrs. Shalaka Ghaikewari and Mrs. Shivani Chaudhary for useful discussions during course work.

My special thanks to fellow lab mates Dr. Madhura Rale- Singh, Dr. Animesh Deval, Dr. Kasturi, Joshi- Navre, Dr.Vrushali, Dr. Pradeep Singh, Dr.Avinash, Dr. Ruchira, Parul, Pushpa, Priti, Amrita, Swarali, Sahana, Palna, Pooja, Hrishikesh, Sakalya, Navnath, Komal, Pavitra,

and Mihir, Navnath,Pavitra, Komal for sharing a great relationship. I will always cherish the warmth shown by them.

A special mention of thanks to my friends in NCL, Sunita Thombre, Gracy, Dr. Nutan, Mr. Kalal, Ejaj, Sandhya, Ekata, Sanskruti and many more for their constant support and cooperation.

I must thank the office staff, Indira Mohandasan for her kind support. I acknowledge Giriji, Jagtapji, Ramakant and Trehanji who were always ready to give their timely help whenever required.

I owe my deepest gratitude towards my husband ***Vijay*** *for his eternal support and understanding of my goals and aspirations. Without his help, I would not have been able to complete my Ph. D. work. I am thankful to my sons* ***Swapnil and Swatej****, my daughter-in-law* ***Komal*** *and specially my grand-daughter* ***Siya*** *for adding happiness and joy to my life.*

*Words fall short if I try to thank my parents Mr.****Nagnath*** *and Mrs.* ***Subhadra*** *and my mother-in-law* ***Vatsala*** *and late father-in-law* ***Ramchandra*** *to whom I woe my deepest gratitude for their infallible love and support.*

A special thanks to my brothers ***Shirish, Sudhir, Sanjay****, sister-in-laws* ***Priyanka, Sadhana*** *and all my family members for their constant support and love.*

I gratefully acknowledge ***Chair, Division Biochemical Sciences*** *and* ***Director, CSIR-NCL*** *for allowing me to submit my research work in the form of thesis.*

SNEHAL MORE

CERTIFICATE

This is to certify that the work presented in the thesis entitled: "Production, Optimization & Applications of industrially useful enzymes from *Beauveria* sp. MTCC 5284", submitted by Mrs. Snehal More, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411008, Maharashtra, India. All the material obtained from other sources has been duly acknowledged in the thesis.

Date: Dr. (Mrs.) Asmita Prabhune

Place: Pune **(Research Guide)**

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Production, Optimization & applications of industrially Important Enzymes From *Beauveria* sp. MTCC 5184**", submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. Asmita Prabhune. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Date: **Snehal Vijay More**

Place:Pune (Research Scholar)

ABBREVIATIONS

Abs	Absorbance
°C	Degree Celsius
CF	Culture filtrate
Da	Dalton
g	Gram
h	Hour
kDa	Kilo Dalton
L	Liter
M	Molar
min	Minute
ml	Milliliter
mM	Millimolar
MTCC	Microbial Type Culture Collection
N-source	Nitrogen source
NCIM	National Collection of Industrial Microorganisms
OD	Optical density
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
UV/Vis	Ultraviolet/visible spectroscopy
V_{max}	Maximum velocity
ME	Malt extract
YE	Yeast extract
SBM	Soya bean meal
MSC	Mustard Seed Cake

WB Wheat bran

ABSTRACT

Introduction:

Enzymes are the catalytic cornerstones of metabolic activities of living being and catalyze most of the reactions in living organisms. Enzymes are also important in reducing both energy consumption and environmental pollution and now modern biotechnology is opening doors that will further expand the use of enzymes into exciting new areas. Microbial enzymes have tremendous potential for different applications. Industrially important enzymes are required in large amount and need to be synthesized by cost effective methods. Fungi are known to secrete variety of hydrolytic enzymes. Enzymes of fungal origin are advantageous due to the ease of biomass removal during downstream processing.

Proteases are metabolically important and inseparable part of entire living beings. They are believed to be the most primitive enzymes in the biological evolution. Proteases belong to the class of hydrolase, which degrade proteins into small peptides and amino acids by catalyzing the reaction involved addition of water to cleave the peptide bond. Proteases are produced by variety of bacteria and fungi (Lazim *et al.,* 2009; Azeredo *et al.,* 2004; Laxman *et al.,* 2005; Bania *et al.,* 2006; Ueda *et al.,* 2007; Devi *et al.,* 2011).The catalytic property of protease tends to make it attractive for wide range of applications in various processes of milk, meat, baking, brewing, detergent, textile, leather and pharmaceutical industry (Gupta *et al.,* 2002; Bhaskar *et al.,* 2007; Rao *et al.,* 1998). Among them alkaline proteases, dominate the worldwide industrial applications.

Xylan is the major renewable hemicellulosic polysaccharide of plant cell wall. It occurs as a heteropolysaccharide with a backbone of 1, 4-linked xylose monomers that may be branched with arabinose, glucuronic acid, and acetate substituents at the 2- and 3-position of xylose. Xylanase catalyzes the random hydrolysis of xylan to xylooligosaccharides. From a commercial point of view, xylanases are an important group of hydrolases, with a worldwide market of US $200 million (Katapodis et al 2007). Xylan degrading enzymes are reported from bacteria, actinomycetes and fungi (Sunna & Antranikian 1997). The use of cost-effective agro-residues as substrates in fermentation media may reduce the cost of enzyme production substantially.

The importance of xylanase has tremendously increased due to its biotechnological applications in the food, pulp, paper and textile industries, agri-industrial residues utilization, and ethanol and animal feed production (Poonam Singh Nigam 2013)

Sophorolipids are extracellular glycolipids produced by *Candida species* when grown on mixtures of carbohydrates and fatty acids. Typically sophoroplipid consists of a dimeric sophorose connected by a glycosidic bond to the penultimate hydroxyl group of 15-18 carbon fatty acids. Surfactants are known to help extracellular enzyme secretion in fungi however there are very few reports on use of sophorolipid for increased enzyme production.

The work described in the thesis emphasizes on screening and optimization of protease and xylanase production from a fungal strain isolated in our laboratory and identified as a new strain of *Beauveria.* A study on overproduction of both the enzymes was carried out with addition of sophorolipids in the fermentation media. Application of protease in silk processing as an eco-friendly alternative was explored. Using sericin a by-product of silk processing and sophorolipid a novel

wound healing formulation was prepared. Wound healing activity of the formulation was tested on rat model and validated with histopathology. Finally metal nanopartical formation by *Beauveria* sp. during growth and with culture filtrate was studied.

- **Outline of the thesis**
- **The thesis is divided into four chapters**
- **Chapter 1: General Introduction**

The ***first chapter*** is an introduction to the thesis. It includes brief account on enzymes viz proteases and xylanases. It provides concise review of literature on proteases and xylanases pertaining to sources of these enzymes, microbial production and optimization for maximum production. This chapter also describes importance and industrial applications of proteases and xylanases.

Chapter 2: Screening and optimization of protease and xylanase production by *Beauveria* sp.

The ***second chapter*** is divided into two sections. This chapter gives details of standardization for enzyme production. Initial screening for protease and xylanase was done with plate assay method. Coproduction of protease and xylanase was carried out with solid state as well as submerged fermentation.

Section A: Screening and optimization of protease production by *Beauveria* sp.

Optimization of protease production was done in shake flasks where effects of various physicochemical and nutritional factors were studied. This includes pH, temperature, effect of media, carbon & nitrogen sources, surfactants and inducers. Protease production was highest in Glucose, 1% and yeast extract 0.3% (GYE) medium with mustard seed cake (MSC). Among various agricultural residues, MSC was found to be the best inducer. Addition of nitrogen sources did not show beneficial effect on production except urea. Chemical surfactants such as Tween20, Tween80 or Triton X100 did not show any increase

in the production. Approximately 2 fold increase was archived with the optimization studies.

Section B: Screening and optimization of xylanase production by *Beauveria* sp.

Optimization of xylanase production was studied in shake flasks where effects of various physicochemical and nutritional factors were studied. This includes pH, temperature, effect of media, carbon & nitrogen sources, surfactants, inducers. Xylanase production was highest in the medium containing malt extract 03%, yeast extract 03%, peptone 0.5% and glucose 1% (MGYP) medium with wheat bran (WB). Among various agricultural residues, WB was found to be the best inducer. Out of various sugars checked, glucose showed highest xylanase production. Chemical surfactants such as tween20, tween80 or triton X100 did not show any increase in the production. Approximately 1.2 fold increase was acchived with the optimization studies.

Chapter 3: Fatty acids and sophorolipids as enhancers for enzyme production and properties of enzyme preparations

The third chapter deals with further enhancement in production of protease and xylanase from *Beauveria* sp. by addition of biodegradable fatty acids like oleic acid, lenoleic acid and biosurfactant sophorolipids. This chapter is also divided into two sections. Properties of crude enzyme preprations like optimum pH, optimum temperature, pH stability, temperature stability and effect of metal ions on activity were studied.

Section A: Effect of addition of fatty acids and sophorolipids on protease production by *Beauveria* sp.

sophorolipids are a structurally diverse group of surface-active molecules synthesized by microorganisms. They are synthesized by employing non pathogenic yeast strains.

Effect of addition of oleic acid sophorolipid and lenoleic acid sophorolipid was studied on protease production. It was observed that when 1mg/ml of sophorolipid was added to the medium the protease production enhancesed. The increased production was 231.2 and 169.8% in case of oleic and lenoleic sophorolipid respectively. Since both the sophorolipids showed increased production various concentrations of sophorolipids were tested for protease production. It was observed that 1mg/ml was the best concentration which showed maximum increase in protease production. Protease activity which was 3.94 IU/ml before optimization was found to be 32 to 36 IU/ml after media optimization. Optimum pH and temperature for protease was pH 9.0 and 50°C. Protease was stable in a broad pH range of 3.0-11.0. Protease was stable upto 45°C showing approximately 50% residual activity after 1h incubation.

Section B: Effect of addition of fatty acids and sophorolipids on xylanase production by *Beauveria* sp.

Effect of addition of oleic acid sophorolipid and lenoleic acid sophorolipid was studied on xylanase production. It was observed that when 1mg/ml of sophorolipid was added to the medium the xylanase production enhancesed. The increased production was 148.9 and 168.5% in case of oleic and lenoleic sophorolipid respectively. Since both the sophorolipids showed increased production various concentrations of sophorolipids were tested for xylanase production. It was observed that 0.5mg/ml was the best concentration which showed maximum increase in xylanase production with150.3 and 250.6% with oleic and lenoleic sophorolipid respectively. Xylanase activity after optimization was found to be 17 to 19 IU/ml after media optimization which was 7.0 IU/ml before optimization. Optimum pH and temperature for xylanase was pH 6.0 and 50°C. Xylanase was stable in the pH range of 4.0- 8.0. Xylanase was stable upto 50°C showing approximately 50% residual activity after 1h incubation.

Chapter 4. Applications of protease from *Beauveria* sp.

The ***fourth chapter*** describes degumming of raw silk by protease and hydrolysis of silk sericin using protease. By using sericin obtained by protease treatment from cocoons and sophorolipid, a wound healing formulation was developed and animal studies are also described in this chapter.

Degumming of raw silk using protease and applications

Conventionally degumming is carried out at 90 to 110°C temperature by boiling the raw silk with Marseilles soap and sodium bicarbonate which eventually requires lot of water and energy. In this study, degumming of Chinese bivoltine raw silk fibres with alkaline protease produced by *Beauveria* sp. (MTCC 5184) was studied. Complete degumming was obtained in 45 minutes with 75 units of enzyme per gram of silk. Degumming was found to be optimal at 50°C and pH 9.00. Scanning electron micrographs showed that the sericin deposits were removed and the obtained fibres were clean, separated, had smooth feel with shine as compared to untreated fibres. Sericin isolated from silk cocoon (by-product which goes waste) was hydrolysed with the same alkaline protease obtained from *Beauveria sp.* to get small molecular weight peptides. These peptides can be utilized further for cosmetic, pharmaceutical and various industrial applications.

The by-product of silk processing, sericin is useful as it is antioxidant, UV resistant, absorbs and releases moisture easily. Sericin has a lot of biological activities such as antibacterial, antioxidant, tyrosinase inhibition, anti-cancer activities, cryoprotection and promotion of digestion. Sophorolipids show excellent skin compatibility and also have antibacterial property. An unusual formulation consisting of sericin and sophorolipid with sodium alginate as a binding agent was developed. Both the ingredients used are biocompatible and biodegradable. This cream/gel was tested for wound healing in vistar

rats. A commercial ointment providine was used as control. The animal group, treated with Sericin and sophorolipid gel, showed fast contraction and healing when compared with control and standard compounds. These observations were validated with histopathological studies where more fibroblast proliferation, angiogenesis, keratinisation was observed. Antioxidant activity of the formulation was checked using 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

Conclusion and future prospects

This part of the thesis summarizes the work presented in the thesis and emphasizes on possible further research in this area.

CHAPTER 1

GENARAL INTRODUCTION

Introduction

Proteases

Enzymes are crucially significant to the survival of life. Over the past, quite a few generations, science has wide open the anonymity of enzymes and has applied this understanding to create better use of these incredible substances in an ever-growing number of applications. The worldwide market for enzymes useful in various industries has reached about US $4.5 billion in 2012, with a projection of US $7.1 billion for 2018. (http://www.companiesandmarkets.com/Market/Healthcareand-). Enzymes are moreover important in reducing energy utilization and environmental pollution. Modern biotechnology has opened the doors which will additionally increase the use of enzymes into exciting novel areas. In recent years magnificent advancement as well as life improvement is experienced due to the the increased use of enzyme technologies. This betterment in life is because of science and fast transformation of new findings into realistic technologies and industrialized processes.

Enzymes, proteins in nature with eceptions of RNAse and synzymes with very much specific catalytic functions produced by each and every living organism. Enzymes are vital for the entire metabolic processes and are accountable for numerous essential biochemical reactions in all living beings (Khandelwal 2013). Enzymes vary in function for the reason that they have the inimitable ability to facilitate biochemical reactions without altering themselves. Speed of the rate of the reactions is increased spectacularly by enzymes as they provide different pathway of lower activation energy. The catalytic potential of enzymes make them distinctive. Enzymes not only work competently and speedily but are also are recyclable. Enzymes catalyze reactions that form only a single stereoisomer. The binding site of an enzyme is chiral therefore reagents are deliverd to one side of the functional group of the reactant. Enzyme-catalyzed reactions are

also stereo specific that is reaction of only one stereoisomer is typically catalyzesed by the enzyme. They have turned out to be very important biocatalysts these days owing to requirement for eco- friendly approach to industrial processes.

Proteases

Proteases (EC.3.4.21) are metabolically significant and supposed to be the mainly primitive enzymes in the biological progression and inseparable element of entire living beings. They are, as most of the digestive proteases of higher organisms have a common lineage with microbial origin. Their physiological functions are versatile and varied, both at cellular and organelle level. Proteases belong to the class of hydrolase that cut down proteins to small peptides as well as amino acids by catalyzing the reaction involved water addition to cleave the peptide bond. They show wide diversity with respect to their properties like substrate specificity, mechanism of action, attachment of amino acids at the active site, temperature and pH for activity and stability. Proteases are involved in protein turn over and digestion hense are significant for physiological reactions also. They also play important role in fungal morphogenesis, spore formation and spore germination. The catalytic property of protease tends to make it attractive for wide range of applications in various processes of milk, meat, baking, brewing, detergent, textile and leather industry (Bhaskar *et al.,* 2007; Sareen and Mishra, 2008; Jellouli *et al.,* 2009; Shankar et al. 2011; Khandelwal et al 2014). They are widely exploited in pharmaceutical industry and in basic research as analytical tools (Rao *et al.,* 1998). Among them alkaline proteases, dominate the worldwide industrial applications.

Physiological significance of proteases

Proteases are involved in numerous multifaceted physiological functions. They are extremely significant for conducting the important metabolic and regulatory functions in all living forms. Proteases play vital role in lots of

physiological processes such as maintenance of protein turn over, cell proliferation along with migration, fertilization of egg by sperm, morphogenesis in development, release of hormones as well as peptides from precursor proteins, activation of zymogens, coagulation of blood, transport of proteins across cellular membranes, germination of seed, sporulation and discharge of conidia in fungi (Rao *et al.,* 1998).

Sources of protease

Plant and Animal

Proteases are omnipresent and extensively distributed in plants, animals in addition to microbes. Plant proteases are occupied in diverse aspects of plant physiology along with development like seed germination, defense mechanism, programmed cell death, protein turnover and post translation modification (Rabade *et al.,* 2011). However, the number of industrially important proteases from plant origin is limited. The major plant proteases studied and presently in use, with an vital role in food in addition to pharmaceutical industry are papain, bromelain as well as ficin, extracted from papaya (*carica papaya),* pineapple *(Ananus comosus)* and fig (*Ficus carica*) respectively. They are highly consumed in industry as a proofing agent during beer finishing operations in the brewing process, facilitates meat tenderization and used as biocatalyst for amino acid and peptide synthesis (Rabade *et al.,* 2011). Despite of applications in food processing along with brewing, bromelin has several medicinally important qualities, which include antiinflammatory, ani-thrombotic, fibrinoytic and anticancer functions (Chobotova *et al.,* 2010). Trypsin from pancreas, chymotrypsin, pepsin, as well as rennins are the well known examples of animal proteases and are usually released from their zymogens by autolysis or due to the proteolytic action of other enzyme (Rao *et al.,* 1998). Trypsin, which hydrolyses food proteins in all invertebrates and vertebrates is the most important intestinal enzyme. Because of its well defined specificity, trypsin

is widely used in proteomics. It is also used in the production of microbial media ingredients, animal cell culture. Trypsin has also found applications to cure inflammation and to dissolve blood clots. In recent years, trypsin is used as target for controlling the insect pests by using trypsin inhibitors. Chymotrypsin is found in animal pancreatic extract and functionally activated by the action of trypsin from its zymogenic form. It is extensively used for diagnostic and analytical purposes. Rennin is a pepsin-like protease produced from the precursor 'prorennin' present in the stomach of every nursing mammal. This enzyme is used in stable curd and cheese making which has good flavor (Nirmal *et al.*, 2011).

Microbial proteases

Despite of the importance of plant as well as animal proteases from industrial point of view, microorganisms stand for an outstanding resource of proteases. They are easy to culture at large scale and produced using renewable sources in fairly short time duration by using well-known fermentation method for abundant and expected supply. Being extra-cellular in character, secretion of enzymes in fermentation broth takes place directly and thus the downstream processing of enzyme extraction becomes simpler than plant and animal proteases. In addition, microbial proteases have longer shelf life and storage stability (Gupta *et al.,* 2002a). Further, microorganisms offer an advantage of strain improvement by mutagenesis and genetic manipulation (Kumar and Takagi 1999). A major portion of worldwide sale of proteases is occupied by microbial origin and alkaline serine proteases be the most significant cluster of commercial enzymes. Despite of widespread Studies on proteases, still there is scope intended for new proteases having discrete properties, may show innovative industrial applications. With emphasis on environmental friendly technologies, enzymes especially proteases are expected to replace chemicals used in the conventional methods thus increasing their demand. Hence, in recent years,

search is directed for proteases from microbial cultures isolated from diverse habitats or newer proteases with novel and unusual properties.

Bacteria

Although, protease production by number of bacteria like *Pseudomonas aeruginosa* (Oh et al., 2000), *Flavobacterium, Staphylococcus aureus*, *Achromobacter* and species belonging to *Streptomyces* (Lazim *et al.,* 2009; Azeredo *et al.,* 2004) is known , bulk of the commercial alkaline proteases are created by strains belonging to *Bacillus* sp (Deng *et al.,* 2010). Alkaline proteases from *Bacillus* strains are mainly exploited as detergent additives for the reason that they posses high pH and temperature stability and broad substrate specificity (Gupta *et al.,* 2002b). In addition, they are also useful in dehairing operations in leather industry (Madhavi *et al.,* 2011). Furthermore, neutral bacterial proteases are useful in food industry intended for preparation of protein hydrolysates as well as in brewing industry.

Fungi

A variety of hydrolytic enzymes are secreted by fungi, among them protease is important group of enzymes. Proteinase K, is an alkaline enzyme produced by *Tritirachium album,* is one of the most primitive representatives of fungal proteases (Kotlova *et al.,* 2007). *Aspergillus* is the most studied fungal strain for the production of protease (Anadan *et al.,* 2007; Hajji *et al.,* 2007; Chellapandi 2010; Sharma and De 2011; Kranthi *et al.,* 2012). Other fungal proteases which have appeared in literature are *Penicillium* (Dahot 1993; 2006;), *Fusarium* (Ueda *et al.,* 2007; Sharma et al 2016), *Trichoderma* (Kredics *et al.,* 2005), *Rhizopus* (Devi *et al.,*2011), and *Conidiobolus*

(Phadatare *et al.,* 1993; Bhosale *et al.,* 1995; Laxman *et al.,* 2005; Bania *et al.,* 2006; Khandelwal 2013). Due to their hyphal nature which penetrates the substrate, fungi be most appropriate for production by solid state fermentation (SSF). There are plenty of reports available on fungal protease production by solid-state fermentation (Kranthi *et al.*, 2012; Chutmanop *et al.,* 2008; Haq and Mukhtar, 2004). Besides solid-state fermentation, fungal proteases are moreover produced by submerged fermentation in shake flasks (Devi *et al.*, 2011; Ire *et al.*, 2011; Tunga *et al.,* 2003; Germano *et al.,* 2003; Chellappan *et al.,* 2006) as well as in fermentor (Rao *et al.*, 2006; Laxman *et al.*, 2005). Another advantage is that the downstream processing is easy for fungal enzymes because cell free enzyme can be obtained by simple filtration. Fungal proteases are active over broad pH range (4-11) and exhibit broad substrate specificity, consequently may find applications in diverse industrial processes. Protease production is very much influenced with nutritional factors (carbon as well as nitrogen source, inducers and metal ions) in addition to physical factors (pH, temperature, aeration). Table 1.1a summarizes protease production and properties of some fungal proteases.

Effect of Carbon and Nitrogen Sources

Enzyme production is extremely affected by nitrogen and also carbon sources. Organic nitrogen containg sources such as to soyabean meal, cornstep liquor, soya oil, corn glutan and inorganic nitrogen sources such as nitrates, ammonium salts, amino acids are reported to be utilized for the production of protease. Generally complex nitrogen source are favored in the fermentation media for protease production because they are gradually degraded and slow release of amino acids and peptides takes place in the medium. Thase released amino acid/peptides work as inducers for production of protease. In speedily hydrolysed synthetic media the enzyme production was observed to be repressed (Larchar *et al.,* 1996).

Table 1.1a Properties of some fungal proteases.

Fungi	Fermentation	pH optimal / stability	Temp optimal / stability	Type of substrate	Reference
Aspergillus sp. 13.33	SSF	-	45 °C	Wheat bran	Macchione *et al.*, 2008
Aspergillus sp.	SSF	-	30 °C	Soybean	(Rajmalwar and Dabholkar, 2009)
Aspergillus awamori	SmF	5.0	55 °C	Wheat bran	Negi and Banerjee, 2009
Aspergillus clavatus ES1	SmF	8.5	50 °C	Wheat bran, fish flour	(Hajji *et al.*, 2007)
Aspergillus flavus 1.2	SSF	-	45 °C	Wheat bran	(Macchione *et al.*, 2008)
Aspergillus flavus	SSF	7.5–9.5	32 °C	Wheat bran	(Malathi and Chakraborty, 1991)
Aspergillus flavus	SSF	7.5	45 °C	Wheat bran	(Kranthi *et al.*, 2012)
Aspergillus flavus	SSF	7.0	36 °C	Wheat bran, soy protein	(Agrawal *et al.*, 2005)
Aspergillus	SSF	8.0	50 °C	Wheat	(Silva *et al.*,

fumigates				bran	2010)
Penicillium sp. LCJ228	SmF	7.0	30 °C	Casein	(Benluvankar et al 2016
Aspergillus oryzae NCIM 1212	SSF	7.0	36 °C	Wheat bran, soy protein	Agrawal *et al.*, 2005
Aspergillus oryzae NCIM 1032	SSF	7.0	36 °C	Wheat bran, soy protein	Agrawal *et al.*, 2005
Beauveria bassiana	SmF	7.0	26 °C	Shrimp shell, soy powder	Agrawal *et al.*, 2005
Beauveria felina	SSF	7.0	28 °C	Wheat bran, soy protein	Abidi *et al.*, 2011
Trichoderma harzianum	-	8.0	60 °C	Soya bean meal	(Savitha *et al.*, 2011)
Engyodontium album	SSF	10.0 – 11.0	45–60 °C	Wheat bran	(Chellappan *et al.*, 2006)
Fusarium oxisporum	SmF	6.5	35 °C	casein	Shrma *et al.*, 2016
Thermoascus aurantiacus	SSF	-	45 °C	Wheat bran	(Macchione *et al.*, 2008
Thermomucor indicae-seudaticae N31	SSF	5.7	70 °C	Wheat bran	Merheb-Dini *et al.*, 2010)

Trichoderma harzianum	-	8.0	60 °C	Soya bean meal	(Savitha *et al.*, 2011)
Trichoderma reesei QM9414	-	8.0	50 °C	Glucose	(Dienes *et al.*, 2007)

Applications of proteases

Proteases find applications in various industries such as food, detergent, leather, textile, pharmaceutical industries etc. Proteases are one of the three significant marketable enzymes, followed by amyloglucosidases and glucose isomerases. For the application in food, leather and detergents industry crude protease preparation is generally used while for pharmaceutical and medicinal applications proteases are needed in pure forms. Applications of protease in diverse fields are described below.

Detergent

Detergents represent the main end use sector for industrial enzymes. Approximately 25% of the entire global trade for enzymes accounts for proteases is in detergent industry. The worldwide trend has been to decrease wash temperatures and prohibit phosphates. To pay compensation for the reduced cleaning capability at lower temperatures, detergent manufacturers have bowed to enzymes for be of assistance and have introduced quite a few classes of enzymes into their products. Lower wash temperatures appreciably decrease the energy required for a load of laundry. The detergent enzyme should be proficient in acting on variety of things like blood, sweat and food etc. (Rao *et al.,* 1998; Kumar and Takagi, 1999).

Novo Industry has come up with an alkaline protease produced by *Bacillus licheniformis* named as Alcalase. Serine proteases from mostly *Bacillus*

strains are used in current market. The alkaline proteas eproduced by *B. clausii* I-52 is noteworthy and suitable in industrial viewpoint. It works in a wide pH and temperature range. It is also stable in presence of an anionic surfactant like SDS or oxidants like peroxides and perborates. All these properties consequently propose its appropriate nature as additive in detergent industry (Joo *et al.,* 2003). Some of the proteases from fungal sources are also reported to be suitable for their use detergent industry (Phadatare *et al.,* 1993; Tanksale *et al.,* 2001; Hajji *et al.,* 2007).

Food

In food industry, cheese making, baking and meat tenderization has been carried out with the help of proteases (Rao *et al.,* 1998). Protein hydrolysates that are produced by hydrolyzing plant and animal proteases comprise well defined peptide profiles and extensively used in therapeutic nutritional goods and infant food formulations. Blood pressure can be regulated by these protein hydrolysates. In latest years, considerable attention has been there in inventing new enzyme assisted methods for the hydrolysis of proteins like soya protein, gelatin, casein, whey and other proteins, to get hydrolysates from thase proteins with more nutritional value (Sumantha *et al.,* 2006). 'Takabate' an alkaline protease from *Bacillus liqueniformis* was usedfor the hydrolysis of fish proteins (Carreno 1991). Kumar and Takagi, (1999) used alkaline protease from *B. amyloliquefaceins* for producing methionine loaded hydrolysate from chickpea protein. Proteases are supplemented in dough while preparing the bread, to alter wheat gluten along with milk proteins. Both exo and endo proteinases from *Aspergillus oryzae* are used in bread making. Proteases hydrolyse wheat gluten which helps in enhancement of dough elasticity that in turn facilitates easy machine process and increase in loaf volume, superior grain, symmetry and texture. Proteases can moreover be used for the enrichment of fruit juices or soft drink and in manufacturing of protein loaded therapeutic diets (Kumar

and Takagi, 1999). Proteases are also used to remove the unwanted haziness in beers.

Leather processing

Leather manufacture involve sequence of steps together termed as 'tanning' and dehairing is the most important step of tanning in which the hair, noncollagenous proteins, epidermis and extra cementing materials are removed from the skin. Conventional process of dehairing involve the use of elevated proportions of lime and sulfide Khandelwal *et al.,* (2014). Conventional dehairing is measured to be one the main polluting procedure and has harsh impact on water source and soil. Lime, sodium sulphide, and caustic soda aside from common salt and degreasing chemicals used in pre-tanning are largely accountable for pollution load. In actuality, one third of the pollution ganarated by the leather industries is outcome from the waste generated through dehairing operations (Kamini *et al.,* 1999). Other than this, there are additional harmful factors connected with conventional dehairing method like damage of skin owing to overexposure to sulfide, complexity in precise method control, loss of hair and wool because of sulfide damage and expensive effluent management.Taking into consideration theses issues, eco-friendly move towards leather processing by means of enzymes, mostly alkaline proteases is favored. Proteases locate their use in the soaking, dehairing and bating steps of leather manufacture process (Gupta *et al.,* 2002a). Bating is especially significant step where enzymes have been effectively used for ages. Bating process in leather manufacturing cannot be replaced by chemical method. In the earlier method of bating infusion of animal dung was used for softening dehaired skins, which was too unpleasant and had severe impact on the health of workers. Use of proteases soaking and dehairing is more recent though they have been used for bating for about a century. The eventual aim of soaking is swelling of the skin structure and can be achieved by addition of little quantity of protease to soaking liquor and reduce period of soaking. For

soaking and dehairing, in general crude protease preparation is favored. Presently many commercial enzyme preparations as well as the majority of the reported proteases used for dehairing require presence of little amount of sulfide with or without lime and termed as enzyme assisted dehairing (Thanikaivelan 2004; Kandasamy *et al.,* 2012). Though, a small number of reports appeared recently on lime and sulfide free dehairing using alkaline proteases (Rajkumar *et al.,* 2011; Sundarrajan *et al.,* 2011; Verma *et al.,* 2011 Khandelwal *et al.,* 2014). Most of the proteases considered for dehairing are from bacterial origin and are produced by *Bacillus* strains whereas reports on fungal proteases for dehairing are limited (Madhavi *et al.,* 2011; Archana and Pillai, 2012; Khandelwal *et al.,* 2014).

Degumming of silk

Though proteases are explored for various applications, one of the least studied areas is to make their use in silk industry (Laxman, 2012). Raw silk is mainly composed of fibroin, a basic fibrous part of the silk and accounts for about 78%–80% of the total silk and sericin, a gluey material that gives harsh and stiff feeling to the silk fiber, contributes 22%–25% in the silk. Fibroin is highly insoluble protein due to its characteristic amino acid composition, including non polar amino acids glycine, alanine and valine. In sericin polar amino acids like serine and aspartic acid are present predominantly and make it tend to be soluble in hot and soap water. Degumming is a process in which sericin is completely removed from the fibroin wall to acquire shine and smoothness with other properties (More *et al.*, 2013). In the conventional method of degumming, silk fibers are treated with alkali and soap at 98-100°C or are boiled at elevated temperature and pressure for long duration to solubilize sericin and its subsequent removal from fibroin. Conventional degumming methods are high resources consuming processes with respect to water and energy. Moreover, the effluent generated by these methods has several environmental impacts. In contrast, use of enzyme in degumming process is advantageous. Enzymatic

degumming involves hydrolysis of sericin at milder condition of pH and temperature. It also requires low input energy, less time with no hazardous chemical pollution. Although the first report on degumming dates back to more than ninety years, the use of proteases in silk industry is relatively new and has generated a lot of interest only in last twenty years (Laxman, 2012). One of the major advantages of enzymatic degumming is the easier recovery of sericin form degumming liquid as compared to alkali soap degumming method. Sericin is useful as it resists oxidation, antibacterial, UV resistant, and absorbs and releases moisture easily. Sericin protein and its hydrolysates have numerous applications in cosmetics, medicine and for preparation of medical biomaterials, compound polymer and hydrogels (Padamwar and Pawar, 2004: Laxman, 2012).

Pharmaceutical and therapeutic

Acidic proteases from *Aspergillus oryaze* are used in digestive aids. Elastase from *B. subtilis* 316M immobilized on bandage is in use for healing wound, abscesses, carbuncle and burns (Gupta *et al.,* 2002). Proteases are as well used as possible bacteriocidal agents and for elimination of protein contaminants from antibiotic preparation. Serratiopeptidase an enzyme isolated from the non-pathogenic enterobacteria *Serratia and* often prescribed in various specialties like surgery, orthopedics, otorhinolaryngology, gynecology and dentistry for its anti-inflammatory, anti-endemic and analgesic effects .In the research in recent years, exploration illustrated enzyme also plays a vital role in the management of atherosclerosis as it possess fibrinolytic and caseinolytic properties Like most enzymes, serratiopeptidase also possesses broad substrate affinity and has been to be reported therapeutically useful in the management of pain and inflammation (Tiwari 2017).

Waste management

Alkaline proteases group has emerged as a prospective factor in favor of the management of industrial and domestic waste, generated by a variety of food-processing industries like poultry and meat. Major constituent of feather is a fibrous and insoluble keratin protein. Worldwide poultry-processing industries generated numerous million tons of feathers per annum as waste. Since there is a high protein content in feathers they can be hydrolysed and can be used in animal feed, biofertilizer etc. (Jeong *et al.,* 2010). Hydrolysis of feather to soluble proteins and amino acids by microbial and enzymatic method is exceptionally attractive, as it is a inexpensive reaction and can be acarried out at gentle conditions with priceless products. Feather possibly will also discover significant application in the fermentation industry for the manufacture of marketable enzymes (Gessesse *et al.,* 2003).

Other applications

Apart from above stated applications, proteases emerged as important tools in many scientific research, especially in biotechnological studies and are being used routinely. 'Proteinase K', is routinely used in DNA extraction procedure. In animal cell culture, cells are dissociated from monolayer by giving trypsin treatment. Alkaline protease from *Conidiobolus coronatus* was in succession used for cell dissociation in animal cell culture (Chiplonkar *et al.,* 1985). A neutral protease, isolated from *Bacillus sp* was used for cleaning contact lenses (Pawar *et al.,* 2009). Protease from *Conidiobolus coronatus* was used in favor of silver recovery from waste photographic films (Shankar *et al.,* 2010). Peptide mass finger printing of proteins has become an important identification tool in proteomics, in which protein is fragmented in several peptides using trypsin and the hydrolysates are subjected to matrix-assisted laser-desorption ionization (MALDI) to identify

their masses. The peptide masses thus obtained are compared with known peptide masses from database using computational program.

Market scenario of proteases

In recent years, there is increased demand of enzymes as catalyst at industrial level. Most of the commercial enzymes being used today are hydrolases and proteases constitute about 65 % of the whole enzyme market (Rao *et al.,* 1998). The international protein hydrolysis enzymes market profit is estimated to be valued at $2,767 million by 2019 (http://www.marketsandmarkets.com/Market-Reports/protein-hydrolysis-enzymes-market-12636477.html) and India is an striking market among high growth rates in the past years. Key factors motivating market development comprise new enzyme technologies endeavoring to improve cost efficiencies and productivity, and rising interest amongst customers in substituting chemical products with new organic compounds for instance enzymes. Additional feature boosting market enlargement include rolling demand from industries like textile, animal feed manufacturers, detergent manufacturers, pharmaceutical companies as well as cosmetics vendors, their application range is steadily increasing with newer applications being explored, and there is anticipated to be an uphill development in the use of proteases. The major producers of proteases are Novo Industries (Denmark), Gist – Brocades (Netherlands), Genecor International and Miles Laboratories (United States). In last few years several new companies has been arise in Indian Bioindustrial Scenario and their production and export is succeeding day by day. M/s Biocon, M/s Advance Biochemicals and M/s Maps India are some of the leading producers of proteases in India. In general detergent and tannery proteases are produced by bacterial stains for commercial purpose and to best of our knowledge there is no commercial proteases is available from fungal origin which is being used for these operations. Majority of the commercial fungal proteases are used in food and related process.

Xylanases

Xylanases (EC 3.2.1.8) are the enzymes which degrade xylan and these are produced by insects, yeasts, bacteria, actinomycetes, fungi and algae (Sunna & Antranikian 1997, Beg *et al.,* 2001, Subramaniyan & Prema 2002, Howard *et al.,* 2003, Polizeli *et al.,* 2005). Hydrolysis of the xylan backbone is performed by the action of endo-β (1→4)-xylanases (EC 3.2.1.8). These enzymes catalyze the arbitrary hydrolysis of xylan to xylooligosaccharides, while β-xylosidases act on nonreducing ends of xylooligosaccharides (Anthony *et al.,* 2003, Aro *et al.,* 2005). Endoxylanases or xylanases are classified as glycosil-hydrolases (endo-β-1,4- xylanase; EC 3.2.1.8). They can hydrolyze xylo-oligomers of diverse degrees of polymerization (being more active at higher degrees of polymerization) but cannot hydrolyze xylobiose, allowing them to be undoubtedly distinguished from β-xylosidases. In xylanolytic microorganisms, there is a diversity of xylanases because of several genes and/or post-transcriptional and post-translational processing. Hence they are different in their specificity concerning xylan

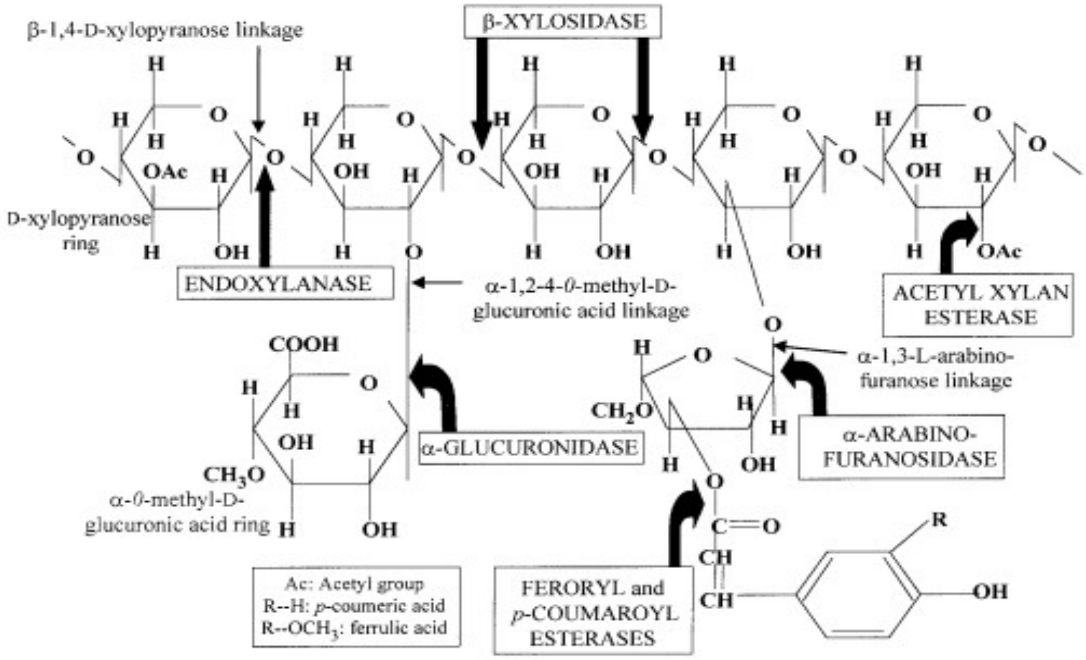

Fig 1.1: A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases

(Wong *et al.,* 1988). Multiplicity of xylanases is due to their high specificity, and a lot of of these enzymes can merely act on regions that are not substituted, whereas others need a particular type of branching adjoining to the cleavage site (Kulkarni *et al.,* 1999, Polizeli *et al.,* 2005, Juturu & Wu 2012). Other enzymes function synergistically, acting on oligomers or dimers, resulting in the total degradation of the polymer. In general, polysaccharide degrading enzymes are subject to regulation mechanism synthesis. Xyalanases are inducible enzymes and not constitutively produced. Their synthesis is induced by the suitable substrate and is repressed readily by assimilated sugars, particularly glucose. The majority proficient inducers are the polymer-substrate intended for which the enzymes will be synthesized, on the other hand, due to their high molecular

weight, these complex substrates cannot go through microbial cells and exert their effect (Kumar *et al.,* 2008).

The hydrolysis reaction catalysed by xylanases proceeds through an acid – base mechanism linking two residues. The first residue acts as a common catalyst and protonates the oxygen of the glycosidic bond. The second residue acts as a nucleophile which, in the case of retaining enzymes interacts with the oxocarbonium intermediate or promotes the creation of an OH ion from a water molecule. Xylanases mostly show a double displacement mechanism involving a glycosyl enzyme intermediate which is produced and hydrolysed via. oxocarbonium ion resembling transition state.

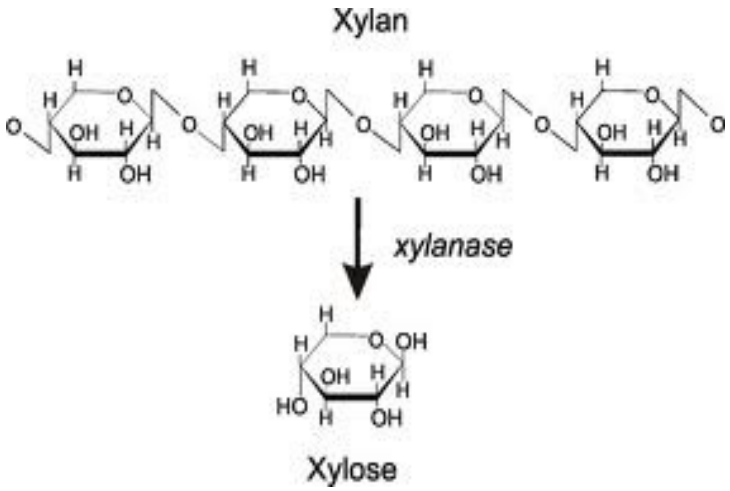

Fig 1.2: Action of Xylanase

Occurrence of Xylanases

Xylanase is the enzyme that degrade the linear polysaccharide beta -1, 4-xylan into xylose, thus breaking down hemicelluloses, one of the key components of plant cell walls. It plays a major role in microorganisms thriving on plant sources. Xylanase are ubiquitous in nature, they occur both in prokaryotes and eukaryotes and have been reported from marine

and terrestrial bacteria, rumen bacteria, protozoa, fungi, marine algae, snails, crustaceans, insects and seeds of terrestrial plants and germinating seeds (Walia *et al.,* 2013). Amongst the prokaryotes, xylanase is produced by bacteria and cyanobac- teria from marine environments (Anna- malai *et al.,* 2009). Several bacteria, fungi and actinomycetes isolated from unusual ecosystems have been studied for xylanase production ((Biely 1993, Beg *et al.,* 2001, Goswami & Pathak 2013). Fungi are mainly attractive producers of this enzyme from an industrial point of view. Fungi excrete xylananses into the medium hence easy for downstream processing. In addition, fungal cultures are normally produce much higher xylanases than those from yeasts or bacteria. In bacteria xylanases are not only produced at lesser levels than in fungi, but are also limited to the intracellular or periplasmic fractions (Polizeli *et al.,* 2005). Enzymes produced by fungi display a variety of physicochemical properties, structures, specific activities and yields. These xylanases have increased hydrolytic efficiencies and extents of xylan hydrolysis.

Xylan

Lignocellulose wastes are generated from agricultural practices and industrialized processes, particularly from agro related industries for example breweries, paper-pulp and textile industries all over the globe. Lignocellulose wastes refer to plant biomass wastes that are mostly composed of lignin, cellulose and hemicelluloses. These wastes normally collect in the surroundings, thereby causing pollution (Okafor *et al.,* 2007). Nevertheless, these wastes are eco-friendly plus can be transformed to biofuels, expensive products like chemicals. They also can be converted to cheap energy sources for fermentation and animal feed (*et al.,* 2003).

The term 'Hemicellulose' was foremost coined by Schulze (1891) to symbolize the segregate obtained from plants that is diluted with alkali .These are the hetero polymers containing the Xylan moreover consist

of D-xylose monomeric units as well as traces of L-arabinose. Xylan is a cluster of hemicelluloses that are originate in plant cell walls and some algae. Xylans are polysaccharides made up of units of xylose, a pentose sugar. Xylan is a hetero-polysaccharide containing substituent groups of acetyl, 4-O-methyl-D-glucouronosyl and α-arabinofuranosyl residues linked to the β-1,4, linked xylose units. Based on the natural substituents found on the backbone, xylans are categorized as linear homoxylan, arabinoxylan, glucuronoxylan or glucuronoarabinoxylan. Homoxylans consisting entirely of xylosyl residues are not prevalent in surroundings; they have been isolated from inadequate sources, like esparto grass, tobacco stalks and guar seed husks (Sunna and Antranikian 1997).

Xylans being renewable resources and effortlessly subjected to hydrolysis either by acid or by microbial enzymes, have established global consideration for probable utilization in the creation of useful products. Enzymatic hydrolysis of xylan compared to chemical hydrolysis is beneficial by virtue of high specificity, mild reaction circumstances and no substrate loss due to chemical modification. Xylan structure is heterogens and complex in plant hence its complete breakdown requires the cluster of hydrolytic enzymes with varied specificity and modes of action. The xylanolytic enzyme system carrying out the xylan hydrolysis is typically composed of these hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase (Fig. 1.1b).

All these enzymes react together to convert xylan into its constituent sugars (Beg *et al.,* 2001). Products of xylan hydrolysis are mostly xylooligosaccharides and the sugar xylose is proficient of microbial bioconversion into liquid fuels, solvents, single cell proteins or even chemical intermediates in the course of the application of specific microorganisms (Biely, 1985). The plentiful agricultural wastes that are

generated through crop cultivation possibly will thus be efficiently and inexpensively utilized during bioconversion.

Microbial Xylanases

Xylanase is an impotant enzymewhich is degrades hemicelluloses backbone of xylose and has been reported extensively from a number of microorganisms. Microbial xylanases have got more attention thses years because they are structurally more stable as well of their ease in manipulations over plant or animal xylanases.Xxylanase are used in various industries extensively for past few decads (Techapun *et al.,* 2003). The enzyme from diverse sources work in a different way at diverse temperatures and pH values. A number of studies have been reported on xylanase producing microorganisms in nature which include yeast, bacteria and fungi (Simoes *et al.,* 2009). Filamentous fungi, such as *Aspergillus* spp (Haq *et al.,* 2004), *Penicillium spp* (Fadel and Fouda, 1993), *Streptomyces* spp (Kansoh and Gammel, 2001), *Bacillus* spp (Rashid, 1999) and *Trichoderma* spp (Azin *et al.,* 2007; Seyis and Aksoz, 2005) have been mainly comprehensively studied and manipulated in the production of xylanase. Table 1.2 summarizes the xylanase producing orgamisms. Additionally, production of xylanase from fungi has higher enzyme activity compared to those of bacterial strains (Subramaniyan and Prema, 2002). As a result, fungi have been proved as the organisms with the potential for biosynthesis of xylanase. However, *Aspergillus niger* remains the fungi of choice for xylanase biosynthesis (Wong *et al.,* 1998; Sorgotto *et al.,* 2012; Guimaraes *et al.,* 2013). Bactrrial xylanases mostly are intracellular and moreover they produce lower levels of xylanases than fungal sources (Knob *et al.* 2010). Fungal sources for xylanase production like, *Aspergillus*, *Trichoderma,* and *Penicillium*. *Aspergillus niger* have been extensively studied. Thermophilic xylanases are also very important for industrial use hence organisms like

Thermomyces lanuginosus, *Thermoascus aurantiacus, Talaromyces thermophiles,* and *Myceliophthora thermophila* are also widely investigated for xylanase production (Maalej *et al.,* 2009; Milagres *et al.,* 2004; Moretti *et al.,* 2012). Xylanase producing fungi studied in recent years are *Colletotrichum graminicola* (Zimbardi *et al.,* 2013), *Aspergillus flavus* MTCC 9390 (Bhushan *et al.,* 2012), *Aspergillus flavus* DFR-6 (Pal et al., 2010), *Aspergillus fumigatus* (Kanagasabai et al., 2013), *Melanocarpus albomyces* (Gupta *et al.,* 2013), *Aspergillus Niger* (Vimalashanmugam and Viruthagiri, 2013a), *Aspergillus terreus* (Vimalashanmugam and Viruthagiri, 2013b) *Rhizopus stolonifer* CECT 2344 (Pérez-Rodríguez *et al.,* 2014).

Table 2: Xylanase production by microorganisms

Microorganism	**Molecular weight (kDa)**	**Optimal pH**	**Optimal Tempratur e**	**Reference**
Acrophialophora nainiana	22	7	55	Salles et al. 2000
Aspergillus terreus BCC129	33	5	60	Chantasingh *et al.,* 2006
Paecilomyces thermophila	28	7	75	Zhang *et al.,* 2010
Sporotrichum thermophile ATCC 34628	24	5	60	Vafiadi *et al.,* 2010
Humicola insolens	21	6-6.5	55-60	Dusterhoft *et*

				al., 1997
Myrotecium verrucaria	16	5.5	45	Loera-Corral & Villaseñor-Ortega 2006
Sporisorium reilianum	42	5	70	Álvarez-Cervantes *et al.*, 2013
Fusarium graminearum	27	8	35	Beliën *et al.*, 2005
Fusarium oxysporum	21.6	5.5	55	Jorge *et al.*, 2005
Streptomyces olivaceoviridis A1	26	5.2	60	Wang *et al.*, 2007
Aspergillus nidulans KK-99	ND	55	8.0	Taneja *et al.*, 2002
Myceliophthora sp. 53	53	75	6.0	Chadha *et al.*, 2004
Penicillium brasilianum	31	ND	ND	Jorgensen *et al.*, 2003
Penicillium capsulatum	22	48	3.8	Ryan *et al.*, 2003
Penicillium sp.	. 25	50	2.0	Kimura *et al.*, 2000
Thermomyces lanuginosus	24.7	70	6.0–6.5	Singh *et al.*,

				2000
Aspergillus fumigatus 19	8.5	55	5.5	Silva *et al.,* 1999

Xylanase producing fungi studied in recent years are *Colletotrichum graminicola* (Zimbardi *et al.,* 2013), *Aspergillus flavus* MTCC 9390 (Bhushan *et al.,* 2012), *Aspergillus flavus* DFR-6 (Pal *et al.,* 2010), *Aspergillus fumigatus* (Kanagasabai *et al.,* 2013), *Melanocarpus albomyces* (Gupta et al., 2013), *Aspergillus Niger* (Vimalashanmugam and Viruthagiri, 2013a), *Aspergillus terreus* (Vimalashanmugam and Viruthagiri, 2013b) *Rhizopus stolonifer* CECT 2344 (Pérez-Rodríguez *et al.,* 2014).

Industrial applications of xylanases

Xylanase plays a key role in the breakdown of xylan into xylose in industry. There are a range of vital applications of xylanase (Bhat 2000, Aristidou and Pentilla 2000, Subramaniyan and Prema, 2002, Beg *et al.,* 2001, Techapun *et al.,* 2003). Xylanases have been used since 1980s, initially it was used in animal feed digestion, then food, textile and paper industries. At present, xylanase and cellulase, collectively with pectinases, account for 20% of the global enzyme market (Polizeli *et al.,* 2005). Xylanases are used to speed up the baking process of cookies, cakes, crackers, and other foods by serving to break down polysaccharides in the doughi in food industry, in animal feeds, xylanase is used for increasing the digestibility of wheat by poultry and swine, by diminishing the viscosity of the feed (Godfrey *et al.,* 1996).

Paper and Pulp Industry

The most promising and extensively spread use of xylanase is in the prebleaching of kraft pulps (Bajpai 1999). During the Kraft process, *i.e.*, the

chemical conversion of wood into wood pulp (lignocellulosic fibrous material), the lignin-carbohydrate complex is hydrolysed. Thus, endo-1,4-β-xylanase can be used in this process to increase the extraction of lignin. In that way it becomes more accessible for bleaching. For bleaching of kraft pulp in paper industry,a number of chlorine-based chemicals and sodium hydrosulfite are used that cause smany effluent-based problems. The use of these chemicals generates chlorinated organic substances, some of which are toxic, mutagenic, persistent, and highly resistant to biodegradation, in addition to causing numerous harmful disturbances in biological systems and forming one of the major sources of environmental pollution (Beg *et al.,* 2001). By enzymatic approach the consumption of chlorine chemicals for bleaching is reduced. In addition it was reported that, beside the positive effect on the environment, enzymatically treated pulp is brighter and has improved fibre quality (Kalim *et al.,* 2015).

Madlala *et al.,* (2001) showed that the use of enzymes increases the pulp brightness (over 5 brightness points over the control) and reduces the amount of bleaching chemicals used (up to 30% for chlorine dioxide). They evaluated commercial Xylanase P and crude xylanase from *Thermomyces lanuginosus* to demonstrate the bleaching process of paper pulp. Crude xylanase preparations from *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 was tested for pulp bleaching and found that at a charge of 10 U per gram of pulp it was possible to reduce the usage of chlorine dioxide up to 30% without compromising the pulp brightness Chipeta *et al.,* (2005).

Animal feedstock

Animal feed, including cereals like wheat, triticale and soy-based diet, are rich in lignocelluosic biomass. However, the viscous properties of lignocelluloses hinder their digestion. By enzyme supplementation to feedstock, nutrients entrapped in the macromolecules are released and

thereby the digestion enzymes have better access to their substrate (Kalim *et al.,* 2015). Xylanase may improves the nutritional properties of agricultural silage and grain feed. Positive effects of xylanases on animal growth were reported for fishery, piggery and poultry (Huichang 2006, Malagutti *et al.,* 2010, O'nil *et al.,* 2012), as well as for cattle. Supplementation of fibrolytic enzymes like xylanases and cellulases significantly increased the average daily milk yield in Murrah buffaloes (Shekhar et al 2010). Similar results were obtained for goats (Bala et al 2009).

Baking and brewing Industry

Xylans play an important role in bread making due to their water absorption capability and interaction with gluten. Utilization of xylanases improves bread qualities, *e.g.* good volume rise, smooth texture and appearance, and dry, balanced dough with prolonged shelf time. In 2012, xylanases, produced by a *Bacillus licheniformes* strain, were made commercially available by Novozyme as an additive for baking industry under the brand name "Panzea". Beside that one, different microbial sources for xylanases, which putatively can be applied in baking industry, were reported. This effect of xylanase is further enhanced when amylase is used in combination with xylanase (Maat *et al.,*. 1992). According to Collins *et al.*, 2006, psychrophilic enzymes may be suitable for use in the baking industry as they are generally optimally active at the temperatures most frequently used for dough preparation (at or below 35 °C). These enzymes could also be used as more efficient baking additives than the currently used commercial mesophilic enzymes, which are optimally active at higher temperatures. In biscuit-making, xylanase is recommended for making cream crackers lighter and improving the texture, palatability and uniformity of the wafers (Polizeli *et al.,* 2005).

Xylanases, in conjunction with cellulases, amylases and pectinases, lead to an improved yield of juice by means of liquefaction of fruit and vegetables; stabilization of the fruit pulp; increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc., reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate (Polizeli *et al.,* 2005). Xylanase, in combination with endoglucanase, takes part in the hydrolysis of arabinoxylan and starch, separating and isolating the gluten from the starch in the wheat flour. This enzyme is also used in coffeebean mucilage (Harris and Ramlingam 2010).During the manufacture of beer, the cellular wall of the barley is hydrolyzed releasing long chains of arabinoxylans which increase the beer's viscosity rendering it "muddy" in appearance. Thus, xylanasesare used to hydrolyze arabinoxylans to lower oligosaccharides diminishing the beer's viscosity and consequently eliminating its muddy aspect (Harris and Ramlingam 2010).

Production of Xylo-Oligosaccharides as Food Additive

Endoxylanase catalysed xylan hydrolysis results in xylo-oligosaccharides (XOS). The latter showed pre-biotic effects, and can be used in functional foods. They enhance the growth of potentially health promoting bifidobacteria in colon and thereby restrict the growth and proliferation of other harmful bacteria (Aachary and Prapulla 2011). Therefore, XOS are used in beverages like soy milk, tea and coffee, dairy products, desserts like pastries, cakes, biscuits, puddings, jellies and jams, and special preparations for elderly people and children, or as active component of symbiotic preparations (Aachary and Prapulla 2011, Kumar *et al.,* 2012). Further, XOS are reported to inhibit the growth of sarcoma-180 and other tumours, probably by indirectly stimulating the non-specific immunological host defence In addition to the immune-stimulating effects, anti-inflammatory activity was reported too (Kalim *et al.,* 2015).

Bioethanol Production

Using plant biomass as starting material for the production of ethanol is a complex process which involves several conversions. First, the lignocellulosic biomass has to be delignified; yielding xylan and cellulose, which then can be hydrolysed to xylo-oligosaccharides and cellobiose, respectively. These oligosaccharides can subsequently be converted into simple sugars, *i.e.* glucose. The latter represent the compounds of interest for bioethanol production (Kalim *et al.,* 2015). This may help to make the saccharification process of lignocellulosic biomass more efficient and cheaper. A pretreatment of straw, using xylanases and cellulases can effect downstream saccharification and fermentation, important for bioethanol production. Even though the biofuel technology continues to advance, the demand is stalling since the yields have to be increased and the costs decreased to compete with conventional fuels.

Degumming of fibers

In combination with a mild chemical pretreatment, pectinases and xylanases play a vital role in the degumming and better separation of bast fibres. Enzymatic hydrolysis of bamboo materials make them suitable for textile processing (Wong and Saddler 1992).Xylanases from *Bacillus subtilis* B10 were successfully used for degumming of ramie bast fibres(Huang *et al.,* 2006). Further, xylanases and pectinases are also used in the debarking of wood (Wong and Saddler 1992).

References

- Aachary AA, Prapulla SG. (2011) Compr Rev Food Sci F. 10:1-15.
- Abidi F, Chobert J-M, Haertlé T *et al.* (2011) Process Biochem 46:2301-2310.
- Agrawal D, Patidar P, Banerjee T (2005) Process Biochem 40:1131-1136.

- Anandan, D., Marmer, W.N. and Dudley, R.L. (2007). *J Ind Microbiol Biotechnol,*34:339-347.
- Annamalai N, Thavasi R, Jayalakshmi S, Balasubramanian T (2009) Ind J Biotechnol 8: 291–297
- Anthony T, Raj KC, Rajendran A, Gunasekaran P. (2003) Enzyme and Microbial Technology 32: 647–654.
- Archana G, Pillai P (2012) Nova Science Publishers ISBN: 978-1-62100-131-7 **.**pp.189-211
- Aristidou A, Pentillä M (2000) Curr Opin Biotechnol 11: 187–198
- Aro N, Pakula T, Pentitla M. (2005) FEMS Microbiology Reviews 29: 719–739
- Azeredo, L.A.I., Freire, D.M.G., Soares, R.M.A., Leite, S.G.F. and Coelho, R.R.R. (2004). *Enzyme Microb Technol,* 34: 354-358.
- Azin, M., Moravej, R. and Zareh, D. (2007) *Enzyme and Microbiology Technology,* 40: 801-805.
- Bajpai, P. (1999) Biotechnol Progress, 15: 147–157. doi:10.1021/bp990013k
- Bala P, Malik R, Srinivas B. (2009) Anim Sci J. 80:265-72.
- Bania, J., Samborski, J., Bogus, M. and Polanowski, A. (2006). *Arc Insect Biochem Physiol*, 62: 186-196.
- Barata RA, Andrade MHG, Rodrigues RD *et al.* (2002) J Biosci Bioeng 94:304-308.
- Beg Q K, Kapoor M, Mahajan L, Hoondal G S. (2001) 56: 326–338.
- Benluvankar V., Evelyne Priya S., Joel Gnanadoss J. (2016) Journal of Applied Biol & Biotech 4 (01),: 20-26
- Bhaskar, N., Modi, V.K., Govindaraju,K.,Radha,C. and Lalitha, R.G. (2007). *Biores Technol,* 98: 388–394.
- Bhat MK (2000) Biotechnol Adv 18:355–383

- Bhosale, S. H., Rao, M. B., Deshpande, V. V. and Srinivasan, M. C. (1995). *Enzyme Microb Technol,* 17:136–139.
- Bhushan B, Pal A, Jain V (2012) Enzyme Engg 1: 103.
- Carreno, F.L.C. (1991). *Biotechnol Edu*, 2: 150-153.
- Chakrabarti, S. K., Matsumura, N. and Ranu, R.S. (2000). *Curr Microbiol,* 239-244.
- Chellapandi, P. (2010). *E-J of Chem,* 7: 479-482.
- Chellappan, S., Jasmin, C., Basheer, S.M., Elyas, K.K., Bhat, S.G. and Chandrasekaran, M.(2006) Process Biochem 41:956-961.
- Chipeta Z A, Du Preez J C, Szakacs G, Christopher L. (2005) 69: 71–78.
- Chiplonkar, J. M., Gangodkar, S. V., Wagh, U. V., Ghadge, G. D., Rele, M. V. and Srinivasan, M. C. (1985). *Biotechnol Lett,* 7: 665-668.
- Chobotova, K., Vernallis, A.B. and Majid, F.A.A. (2010). *Cancer Lett,* 290: 148–156.
- Chopra, S. and M. L. Gulrajani., (1994). *Indian J. Fibre and Textile Res*, 19, 76-83.
- Chutmanop, J., Chuichulcherm, S., Chisti Y. and Srinophakun P. (2008). *J Chem*
- Collins T, Hoyoux A, Dutron A, Georis J, Genot B, Dauvrin T. (2006) Journal of Cereal Science 43: 79–84
- Crispim, A. and Mota, (2003). *J Soc Leather Technol Chem*, 87: 203-207.
- Dahot, M.U. (1993). *J Islamic Acad Sci,* 6:193-196.
- Deng, A., Wu,J., Zhang, Y., Zhang, G. And Wen T. (2010). *Bioresource Technol*, 101:7100–7106.
- Devi, P.R., Raghavan, P.V., Vasudheven, I., Joshua, L. and Vijaykumar, M. (2011). *Int J Biol Technol,* 2:46-49.
- Dienes D, Börjesson J, Hägglund P (2007) Enzym Microb Technol 40:1087-1094.

- Fadel, M. and Fouda, M. S. (1993) *Microbiology*, 148: 304-312.
- Germano, S., Pandey, A., Osaku, C. A., Rocha, S. N. and Soccol, C. R. (2003). *Enz Microb Technol*, 32: 246-251.
- Gessesse, A., Kaul, R.H., Gashe, B.A. and Mattiasson, B. (2003). *Enzyme Microb Technol*, 32: 519–524.
- Global Markets for Enzymes in Industrial Applications, (2014), http://www.companiesandmarkets.com/Market/Healthcareand-Medical/Market-Research/Global-Markets-for-Enzymesin- Industrial-Applications/RPT1072821
- Goswami GK, Pathak RR. (2013) International Journal of Basic & Clinical Pharmacology 2, 237–246.
- Guimaraes, N. C. A. Sorgotto, M. Peixoto -Nogueinra, S. C. Betini, J. H. A. Zanoelo. F. Marques, M.
- Gupta, R., Beg, Q.K., Khan, S. and Chauhan, B. (2002b). *Appl Microb Biotech*, 60: 381-395.
- Hajji, M., Kanoun, S., Nasri, M. and Gharsallah , N. (2007). *Process Biochem* , 42:791-797.
- Haq, I. and Mukhtar, H. (2004). *J Basic Microbiol,* 44: 280-287.
- Haq, I., Tasneem, M. , Raana, K., Khan, A., Mukhtar, H. and Javed, M. (2004) *International Journal of Agriculture and Biotechnology,* 6: 1115-1118.
- Harris A. D. and Ramalingam C. (2010) J Exp Sci Vol. 1(7) 01-11
- Huang J, Wang G, Xiao L. (2006) Bioresour Technol. 97:802-8.
- Huichang C. (2006) J Anhui Agricult Sci. 34:3077.
- Ire, F.S., Okolo, B.N. and Moneker, A.A. (2011). *African J Food Sci,* 5: 695-709.
- ISBN 1682-296X.
- Jellouli, K., Bougatef, A., Manni, L., Agrebi, R., Siala, R., Younes, I.and Nasri, M.(2009). *J Ind Microbiol. Biotechnol,* 36:939–948.

- Jeong, J.H., Jeon, Y.D., Lee, O.M., Kim, J.D., Lee, N.A., Park, G.T. and Son, H.J.(2010). *Biodegrad*, 21:1029–1040.
- Joo H.-S, Kumar C.G, Park G.-C, Paik S.R, Chang C.S. *J Appl Microbiol*, (2003).95: 267-172.
- Juturu V, Wu JC. (2012) Biotechnology Advances 30: 1219–1227.
- Kalim, B. Bohringer, N. Ali, n. schaberle, T.F. British Biotech Journal, (2015) 7(1): 1-20
- Kamath, P., Subrahmanyam, V.M., Rao J.V. and Raj P.V. (2010). *Indian J PharmSci,* 161-166.
- Kanagaraj, J., Vellapan, K.C., Chandra Babu, N.K. and Sadulla, S. (2006). *J Sc IndRes*, 65: 541-548.
- Kanagasabai, V. and Thangavelu, V. (2013) J. of Adv. Scient Research 4(2): 13-20.
- Kandasamy N, Velmurugan P, Sundarvel A, Jonnalagadda RR, Bangaru C, Palanisamy T (2012) J Clean Prod 25:27-33
- Kansoh, A. L. and Gammel, A. (2001) *Acta Microbiologica et Immunologica Hungarica*, 48: 39-52.
- Khandelwal, H. B. (2013) Ph. D. Thesis submitted to Savitribai Phule Pune University.
- Knob, A., Terrasan, C. R. F., and Carmona, E. C. (2010) *World J. Microbiol. Biotechnol.* 26(3), 389-407.
- Kranthi, V.S., Rao, M. and Jaganmohan, P. (2012). *Int J Microbiol Res*, 3: 12-15.
- Kredics, L., Antal, Z., Szekeres, A., Hatvani, L., Manczinger, L.Vagvolgyi, C. andNagy, E. (2005) *Acta Microbiologica et Immunologica Hungarica,* 52: 169-184.
- Kumar GP, Pushpa A, Prabha H. (2012) IRJP. 3(7) 1- 4.
- Kumar J, Singh S, Singh OV. (2008) Journal of Industrial Microbiology and Biotechnology 35: 377-391.
- Kumar, C. G. and Takagi, H. (1999). *Biotechnol Adv.* 17: 561–594.

- Laxman, R. S., (2012). In "Biotechnology of Microbial Enzymes" Ed. Vijai Kumar Gupta and Manimaran Ayyachamy, Nova Science Publishers, 277-295.

- Laxman, R. S., More, S. V., Rele, M. V., Rao, B. S., Jogdand, V. V., Rao, M. B., Deshpande, V. V., Naidu, R. B., Manikandan, P., Kumar, D. A. and Kanagaraj (2007). Process for the preparation of alkaline protease. USP 7186546.

- Laxman,R.S., Sonawane, A.P., More, S.V., Rao, B.S., Rele, V.V., Jogdand,V.V., DeshpandeV.V. & Rao, M.B. (2005). *Process Biochem*, 40: 3152-3158.

- Lazim, H., Mankai, H., Slama, N., Barkallah, I. and Limam, F. (2009). *J Ind Microbiol Biotechnol,* 36:531-537.

- M'hir., S., Mejri, A., Sifaoui, I., Slama, M.B., Mejri, M., Thonart, P. and Hamdi, M. (2012). *Arc Appl Sci Res,*4:1110-1116.

- Maalej, I., Belhaj, I., and Belghith, H. (2009 *Appl. Biochem. Biotechnol.* 158(1), 200-212.

- Maat J, Roza M, Verbakel J, Stam H, DaSilra M, Egmond M. (1992) in: Xylans and Xylanases, Elsevier, Amsterdam.

- Macchione MM, Merheb CW, Gomes E (2008) Appl Biochem Biotechnol 146:223-230.

- Madhavi, J., Srilakshmi, J., Rao, M.V.R. and Rao, K.R.S. (2011). *Int J Biosci Biotechnol,* 3: 11-26.

- Madlala A, Bissoon S, Singh S, Christov L. Biotechnology Letters (2001) 23: 345–351.

- Malagutti L, Colombini S, Rapetti L, Pirondini M, Magistrelli D, Galassi G. (2010) Energy and Protein Metabolism and Nutrition. 403-4.

- Malathi, S. and Chakraborty, R. (1991). *Appl Enviorn Microbiol,* 57: 712-716.

- Merheb CW, Cabral H, Gomes E *et al.* (2007) Food Chem 104:127-131

- Merheb-Dini C, Gomes E, Boscolo M *et al.* (2010) Food Chem 120:87-93.

- Milagres, A. M. F., Santos, E., Piovan, T., and Roberto, I. C. (2004). *Process Biochem*. 39(11), 1387-1391.

- More, S. V. Khandelwal, H. B. Joseph, M. A. Laxman, R. S. (2013) *J. nat. fiber*. 10(2): 98-111

- Moretti, M. M. S., Bocchini-Martins, D. A., Silva, R., Rodrigues, A., Sette, L. D., and Gomes, E. (2012). *Braz. J. Microbiol*. 43(3), 1062-1071.

- Negi S, Banerjee R (2009) Food Res Int 42:443-448.

- Nirmal, N.P., Shankar, S. and Laxman, R.S. (2011). *Int JBiotech Biosci*, 1: 1-40.

- O'Neill HV, Mathis G, Lumpkins BS, Bedford MR. (2012) Poult Sci. 91:1356-60

- Oh, Y.S., Shihb, I.L., Tzeng,Y.M. and Wanga, S.L. (2000). *Enzyme Microb Technol,* 17:3-10.

- Padamwar, M.N. and Pawar, A.P. (2004). *J Sci Ind Res,* 63: 323-329.

- Pawar, R., Zambare, V., Barve, S. and Paratkar, G. (2009). *Biotechnol,* 8: 276-280.

- Pérez-Rodríguez N. Oliveira F. Pérez-Bibbins B. Belo I. Torrado Agrasar A. Domínguez J. M.

- Phadatare, S. U., Rao, M.B. and Deshpande, V. V. (1997). Enzyme Microb

- Phadatare, S. U., Srinivasan, M. C. and Deshpande, V. V. (1993). *Enzyme Microb*

- Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. (2005) Applied Microbiology and Biotechnology 67: 577–591.

- Rabade, N.G., Corona, J.A.B, Barradas, J.S.A. and Salvador, M.C.O. (2011). *Biotechnol Adv,* 29:983-996.

- Rajkumar R, Jayappriyanon KR, Rengasamy R (2011) J Basic Microbiol 51:614-624

- Rajmalwar S, Dabholkar PS (2009) Afr J Biotechnol 8:4197-4198
- Rao, Y.K., Lu, S.C., Liu, B.L. and Tzwng, Y.M. (2006). *Biochem Eng J*, 28: 57-66.
- Rashid, A. N. (1999) *Journal of Microbial Biotechnology*, 9(2): 157-162.
- Sareen, R. and Mishra, P. (2008). *Appl Microbiol Biotechnol,* 79: 399–405.
- Savitha S, Sadhasivam S, Swaminathan K *et al.* (2011) J Taiwan Inst Chem Eng 42:298-304
- Schulze E. Ber Dtsch Chem Ges (1891) 24:2277–2287.
- Seyis, I. and Aksoz, N. (2005) *International Biodeterioration & Biodegradation,* 55: 115-119
- Shankar, S., More, S.V. Laxman, R.S. (2010). *Kathmandu Univ J Sci Engg Technol*, 6: 60-69.
- Shankar, S., Rao, M. and Laxman, R.S. (2011). *Process Biochem* 46: 579-585.
- Sharma D, Saraf A, Shukla K and Roy V (2016) world J OF Pharmacy and Pharma Sciences 5(2): 1043-1054
- Sharma, D. Saraf, A. Kamlesh Shukla, Roy, V. (2016) World J. Pharm. Pharmaceu. Sci. 5 (2) 1043-1054
- Sharma, N. and De, K. (2011). *Agric Biol J N Am*, 2:1135-1142.
- Shekhar C, Thakur SS, Shelke SK. (2010) Trop Anim Health Prod. 42:1465-70.
- Silva RR, Rodrigues A, Cabral H (2010) J Biotechnol 150:419 - 576.
- Simoes, M. L. G., Tauk-Tornisielo, S. M. and Tapia, D. M. P. (2009) 8(22): 6317-6326
- Sogotto, M. Guimaraes, N. C. A. Zanoelo, F. F. Margues, M. R. Peixoto-Nogueinra, S. C. Giannesi, G. C. (2012) Afr. J. Biotechnol. 11(32): 8076-8084

- Subramaniyan S, Prema P. (2002) Critical Reviews in Biotechnology 22: 33–64.
- Sumantha, A., Larroche, C. and Pandey, A. (2006). *Food Technol Biotechnol,*44:211-220.
- Sundararajan, S., Chandrababu, N.K. and Chittibabu, S. (2011). *J Biosci Bioeng,*111: 128-133.
- Sunna A, Antranikian G. (1997) Critical Reviews in Biotechnology 17: 39–67.
- Sutar, I. I., Srinivasan, M.C. and Vartak, H.G. (1991). *Biotechnol Lett,* 13: 119-124.
- Sutar, I.I., Srinivasan, M.C. and Vartak, H.G. (1992). *World J MicrobiolBiotechnol*, 8:254-258.
- Tanksale, P., Chandra, P.M., Rao, M. and Deshpande, V. (2001). *Biotech Lett*, 23:51-54.
- Techapun, C., Poosaran, N., Watanabe, M. and Sasaki, K. (2003) *Process Biochem,* 38: 1327-1340.
- Thanikaivelan P, Rao JR, Nair UB, Ramasami T (2005) Crit Rev Environ Sci Technol 35: 37-79
- Tiwari M. (2017)Asian J. Pharma. Sci.12 (3): 209–215
- Tunga, R., Shrivastava, B., Banerjee, R. (2003). *Proc Biochem*, 38: 1553-1558.
- Ueda, M., Kubo, T., Miyatake, K. and Nakamura, T. (2007). *Appl MicrobiolBiotechnol,* 74:331-338.
- Verma A, Pal HS, Singh R, Agrwal S (2011) Int J Agri Envir Biotechnol 4: 173-178.
- Vimalashanmugam, K. and Viruthagiri, T. (2013b) International Journal of ChemTech Research 5(5): 2365-74.
- Vimalashanmugam, K. and Viruthagiri, T. 2013a. Asian J. of Biochem. and Pharm.l Research 3(2) : 44-62.
- Walia A, Mehta P, Chauhan A, Shirkot CK (2013) Ann Microbiol 63:187–198

- Wong KKY, Saddler JN. (1992) Crit Rev Biotechnol. 12:413-35.
- Wong KKY, Tan LUL, Saddler JN. (1988) Microbiological Reviews 52: 305–317

CHAPTER 2

Screening and optimization of protease and xylanase production by *Beauveria* sp.

SECTION A

Section A: Screening and optimization of protease production by *Beauveria* sp.

Abstract

The newly isolated fungal strain was identified as a new strain of *Beauveria* sp. on the basis of morphological features and the dendrogram. Initially, there was a white fungal growth which on further incubation at 28° C shows small protrusions which developed into erect white stalks containing spores. Microscopically thin and long fungal mycelium was observed with tiny circular spores after incubation at 28° C for a week. This strain was characterized and distinguished from *Beauveria felina* using the 18S rDNA & ITS sequences and morphological characteristics.

Screening of protease production was carried out by plate assay method. Optimization of protease production was studied in shake flasks where effects of various physicochemical and nutritional factors such as pH, temperature, carbon &nitrogen sources, inducers, surfactants, age of stock etc. were investigated. Highest protease production was observed in GYE medium followed by MGYP. Among various agricultural residues tested for protease production, MSC was found to be the best inducer. Protease production was optimum at pH 8.0 and 28°C. Mustard seed cake at 2% concentration produced optimum levels of protease. Among the carbon sources tested glucose was the best carbon source. Yeast extract was the best nitrogen source followed by urea. Chemical surfactants like tweens and Triton X-100 showed no effect on protease production.

Introduction

Protease market has increased notably, due to their characteristic degradative and synthetic functions and growing steadily due to heavy demand from various industries for instance detergent, food, leather, pharmaceutical, diagnostics in addition to waste management. Regardless of the extensive research carried out on proteases, there is a requirement for proteases with novel properties that may have innovative biotechnological applications. Therefore in modern years, the hunt has been focused for newer proteases by means of novel and extraordinary properties. Looking into the strength of microbial diversity, there is a chance of discovery of microorganisms producing novel enzymes with improved properties and appropriate for commercial utilization.

Microbial proteases are extracellular in nature and hence they are generally produced by submerdged fermentations. (Savitha *et al.*, 2011). Bacteria are the main prevailing group of alkaline protease producers and genus *Bacillus* being the most important source exploited for industrially important proteases (Gupta *et al.,* 2002). However, in recent years there are large numbers of publications on fungal proteases. Moreover, enzymes of fungal source are beneficial easier downstream prosessing by removal of cell mass. Other advantages of fungal enzymes are they can be grown on renewable inexpenssive material , produce high levels of titres, and production of enzymes in less time.. Additionally, the enzymes, from fungal sources are extracellular, recovery becomes easy from the fermentation broth (Vishwanatha *et al.*, 2010b). The utilization of fungi as enzyme producer is safer than the use of bacteria, because they are normally recognised as GRAS (generally regarded as safe) (Germano *et al.*, 2003).

Though it is beneficial to use fungal source, fermentation system is a incredibly complicated multi-phase, multi-component system. Growth and production are influenced by a range of parameters, like medium used for cultivation, inoculum, pH, temperature, aeration, etc. The secretion of an enzyme depends on the growth phase of a microorganism. Most of the proteases are generally produced by means of submerged fermentation

because of advantages in consistent enzyme production characteristics with defined medium and process conditions. Downstream process in submerged fermentation is easier in spite of the cost-intensiveness for medium components.

Media composition and environmental factors like temperature, pH, incubation time etc. play a significant role in enzyme production by microorganisms. These factors are significant to promote, stimulate, boost and optimize the production of proteases. To facilitate elevated and commercially feasible yields of protease, it is necessary to optimize fermentation media for the growth and production of protease (Sharma *et al.,* 2015).A defined medium for the best production of alkaline proteases from different microbial sources is not available in literature because organism or strain have their individual unique conditionsfor maximum enzyme production. Some of the major factors influencing protease production are discussed below.

Carbon sources

Protease production is mainly influenced by the presence of some easily metabolizable sugars, such as glucose, in the fermentation media (Gupta *et al.*, 2002).). Glucose is the most common carbon source used for protease production (Laxman *et al.*, 2005; Chellapandi, 2010; Ire *et al.*, 2011).Sucrose and fructose were found to be equally good as carbon sources for protease production by *C. coronatus* NCIM 1238 (Sutar *et al.*, 1992) while sucrose was better than glucose for protease production by *C. coronatus* NCL 86.8.20 (Phadatare *et al.,* 1993). An improved level of protease production by *Bacillus pseudofirmus* AL-89 has been observed by addition of glucose while for *Nesterenkonia sp.* AL-20 protease production was suppressed in the glucose containing medium. In the latter case, the glucose may act as catabolic inhibitor as described by Gessesse *et al.,* in 2003. Glucose at elevated concentration repressed the enzyme production by *Streptomyces* sp. and 0.5% (w/v) concentration was most favorable for

enzyme production whereas growth was optimum at 1% (w/v) concentration (Mehta *et al.,* 2006). This is perhaps owing to the catabolic repression mechanism. The highest protease production by S*. roseiscleroticus* was attained when starch was used as the carbon source and it was least with dextrose (Vonothini *et al.,* 2008), while, Jaiswal *et al.,* in 2008 reported that maltose and glucose were comparable and considerably better than starch and fructose for production of protease by *Bacillus circulans*. Corn starch at a concentration of 0.5% (w/v) gave the highest productivity of protease, followed by wheat flour and wheat bran. However, supplementation of potato starch caused a decrease in protease titre, which possibly may be due to the presence of protease inhibitors in potato Kumar *et al.,* (2004). The highest protease production by *S. roseiscleroticus* was observed when starch was used as the carbon source whereas protease production was lowest with dextrose (Vonothini *et al.,* 2008). Sucrose at 5% (w/v) was found optimal for protease production by actinomycete *Nocardiopsis prasina* HA4 which is reported by Ningthoujam *et al.,* in 2009. Starch was found to be a better source of carbon for protease production by *B. cereus* and *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004: Nilegaonkar *et al.,* 2007).

Nitrogen sources

Organic nitrogen sources such as soybean meal, corn steep liquor, soya oil, corn gluten, skim milk, casein, peptone, fishmeal, and variety of oil seed, cakes as well as inorganic nitrogen like nitrates or ammonium salts, amino acids etc have been studied for protease production. Different researchers have used different organic nitrogen sources (simple or complex), inorganic nitrogen sources and amino acids for enhancing production of protease. Srinubabu *et al.,* in 2007 reported $(NH4)_2HPO_4$ as the best nitrogen source for protease production by *A.oryzae* 637. Synthetic media repressed enzyme production as synthetic media was rapidly hydrolysed (Larcher *et al.,* 1996). Although researchers have investigated the effect of amino acids on protease production, it becomes impractical to use amino acids for large scale

production. Moreover, some microorganisms prefer proteins and peptides to amino acids as nitrogen sources for growth as well as protease production (Phadatare, 1991**)**. Among inorganic nitrogen sources, nitrates and ammonium salts are generally used for protease production (Kumar and Takagi, 1999). Replacement of sodium nitrate in the basal medium by ammonium nitrate improved enzyme production by *C. coronatus* NCIM 86.8.20 (Phadatare *et al.,* 1993). Among various organic nitrogen sources, skim milk produced maximum protease in case of *Bacillus caseinilyticus* followed by malt extract, peptone and yeast extract. Ammonium chloride as inorganic nitrogen source was shown to reduce the production of protease while inorganic salts like MgCl2 and CaCl2 were shown to induce.

Inducers

Production of protease is an intrinsic property of all organisms and these enzymes are in general constitutive produced in very small amounts and most of times they are partially inducible (Gupta *et al.*, 2002). For industrial use of the enzyme, it is necessary to attain high yields and therefore production medium generally supplied with protein rich materials as an inducers. The choice of specific inducer is entirely depend on the microbial strain used for production. An inducer for large scale protease production in submerged fermentation should follow certain criteria such as easy availability, good storage stability, low cost and forms a homogenous mixture in the medium etc. In addition, it is advantageous to have an inducer which is suitable/ stable during fermentation operations like sterilization, mixing and downstream processing (Khandelwal 2013).

There are quite a few reports unfolding the use of agro-industrial residues as an inducer for the production of alkaline protease, by bacteria (Darani *et al.*,2008, Dasalva *et al.*, 2016, Benuvankar *et al.*, 2016) and fungus (Macchione *et al.*, 2008, Sindhu *et al.*, 2009, Murthy and Naidu 2010). Commonly used inducers for protease production cited in the literature include: casein (Sutar *et al.*, 1992; Phadatare *et al.*, 1993; Nilegaonkar *et al.,*

2007; Abidi *et al.,* 2008; Vijayanand *et al.,* 2010), peptone (Vijayanand *et al.,* 2010; Ire *et al.,* 2011; Vadlamani and Parcha, 2012), skimmed milk (Vijayanand *et al.,* 2010), soybean meal (Phadatare *et al.,* 1993; Laxman *et al.,* 2005; Nilegaonkar *et al.,* 2007; Srinubabu *et al.,* 2007; Abidi *et al.,* 2008) and variety of oil seed cakes (Shikha *et al.*, 2007; Ramachandran *et al.*,2007; Shankar *et al.,* 2010; Vijayanand *et al.,* 2010).

Incubation temperature

Temperature is an additional significant parameter that needs to be controlled and varied from organism to organism for both maximum cell growth and enzyme production. Majority fungi were found to produce protease in the mesophilic temperature range of 28- 30°C with the exception of few thermophilic organisms which need higher temperatures. (Sutar *et al.*, 1992; Kumar and Takagi, 1999; Laxman *et al.*, 2005; Srinubabu *et al.*, 2007; Nirmal *et al.*, 2011; Ire *et al.*, 2011).

Medium pH

pH of the fermentation medium is very important as the pH of culture affects every enzymatic processes and transportation of various components across the cell membrane. In view of the fact that proton motive force during chemiosmosis is affected by the medium pH value, it is probable that under optimum pH range, the relative metabolic effectiveness is high hence pH is an important parameter to optimize Singh *et al.*, (2010). Media with neutral initial pH has been cited for alkaline protease production by *P. chrysogenum* IHH5 (Haq *et al.*, 2006), *S. roseiscleroticus* (Vonothini *et al.*, 2008), *Bacillus aquimaris* VITP4 (Shivanand and Jayraman; 2009) and *P. aeruginosa* MCM B-327 (Zambre *et al.*, 2011). While slightly acidic pH (pH 6.3–6.5) has been reported as optimum for protease production by *B. cereus* SIU1 by Singh *et al.*, (2010).

Materials and Methods

Malt extract, yeast extract and peptone were obtained from M/s Hi-Media Chemicals, India. Hammerstein casein was obtained from M/s Sisco Research Laboratories, Soyatose, soyapeptone were purchased from M/s Warkem Biotech Pvt. Ltd, India. Soybean meal (SBM) was purchased from M/s Ruchi Soya Industries Ltd, India. Mustard seed cake (MSC), cotton seed cake (CSC), groundnut seed cake (GNC), mung flour (MF), gram flour (GF), skim milk (SM) and baker's yeast were obtained from local market. All other chemicals used were of analytical grade.

Microorganism

Beauveria sp MTCC 5184 was isolated from rabbit dung and was deposited in the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, (IMTECH), Chandigarh, Pune, India. It was grown over a pH range of 5.0 to 9.0 with an optimum at 6.5-7.5 and the temperature range of 15 to 35˚C with an optimum at 28°C. After 7 days of growth sporulation was observed on agar plates.The fungus was maintained on MGYP agar (malt extract, 0.3%; glucose, 1%; yeast extract, 0.3%; peptone, 0.5% and agar, 2%) slants and sub-cultured once in a month and preserved at 4°C after growth and sporulation.

Media

Table 2.1a: Composition of media used in the present study

Media	**Composition (g/L)**
GYE	Glucose-10; yeast extact-3.0
MGYP	Glucose-10; yeast extact-3; peptone-5; malt extract-3
Mikami	Glucose-1.5; yeast extract-1.5; peptone-5.0; beef extract-5.0
MF	Glycerol- 10; K_2HPO_4- 3.5; $MgSO_4$- 0.6; Yeast Extract- 1.0

Inoculum preparation

Agar piece (2x2 cm) with spores from 5-8 days old MGYP plate was inoculated in 250 ml Erlenmeyer flask containing 50 ml GYE medium unless otherwise mentioned. The inoculum was incubated at 28°C and 180 rpm for 24-48 h on a rotary shaker. After growth 10% (v/v) inoculums was used for inoculation.

Screening of protease production

Screening of protease production was carried out by plate assay method.Mikami medium containing 2.5 % agar was used for screening. 2% skim milk was autoclaved separately at 15 lb for 10 and was mixed in the above medium so as to attain a final concentration of 1% (w/v) skim milk. Vegetative culture pregrown for 24-48 h in the same medium was spot inoculated on the plate. The plate was observed after 72 hours for clearance zone around the culture.

Co-production of protease and xylanase in submerged fermentation

Co-production of protease and xylanase was carried out in Mikami medium + 2% wheat bran medium by submerged fermentation. Spore suspension from a 5-8 day old slant was used for preparing the Mikami inoculum for 48 hours. 10% (v/v) inoculums was used to inoculate experimental flasks and incubated at 28°C and 180-200 rpm for 96h on a rotary shaker. Samples were aseptically removed at regular intervals and cell free broth was checked for protease and xylanse activities.

Protease Production

Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 50 ml GYE (in gms /L glucose, 10; yeast extract, 3) medium with 2% mustard seed cake (w/v) as an inducer unless otherwise mentioned. 24-48 h old vegetative inoculum (10% v/v) grown in GYE medium was used to

inoculate the experimental flasks unless otherwise mentioned.. Flasks were incubated at 28°C, 180-200 rpm for 3-5 days. Samples were removed periodically, centrifuged at 10,000 rpm for 10 min and the clear supernatant was used as the source of crude enzyme.

Effect of inducers

Effects of protein rich organic nitrogen sources was carried out in Mikami medium as described above. Inducers used were soyatose (ST), soyapeptone (SP), gram flour (GF) and mung flour (MF), and agricultural residues such as soybean meal (SBM) and mustard seed cake (MSC) at 2%(w/v) concentration.

Effect of different media

Effect of media was studied in MGYP, Mikami, GYE and MF. 2% mustard seed cake was used as an inducer. (Media composition described in Table 2.1a)

Effect of various carbon and nitrogen sources

Effect of various carbon and nitrogen sources was studied in GYE + 2% MSC medium. Carbon sources used were glucose, starch, fructose, sucrose lactose and sugar alcohols like glycerol and mannitol. All the carbon sources were used at 1% (w/v) concentration. Various inorganic nitrogen sources namely urea, ammonium sulphate, ammonium chloride, diammonium hydrogen phosphate, sodium nitrate and potassium nitrate were added at equivalent nitrogen concentration that of the yeast extract.

Effect of pH of the medium

Effect of pH of the medium was studied in GYE + 2% MSC medium in the pH range 5.0 to 9.0. Medium was adjusted to required pH with sterile 0.1 N HCl or 0.1 N NaOH before inoculation. The pH of the medium during the fermentation was not controlled. Irrespective of the initial pH of the medium, final pH at the end of the fermentation reached 7.5 to 8.0.

Effect of temperature

Effect of temperature was studied on protease production by *Beauveria* sp. at temperatures ranging from 20 to 45°C. Experimental flasks after inoculations were incubated to the said temperatures.

Determination of Alkaline Protease Activity

Protease as caseinolytic activity was estimated at 50°C, pH 9.0 according to Laxman *et al.,* (2005). The reaction mixture contained an aliquot of suitably diluted enzyme and 10 mg Hammerstein casein in 0.1 M sodium carbonate buffer pH 9.0 in a total volume of 2 ml. After incubation at 50°C for 10 min, the reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid (acidified with concentrated hydrochloric acid). The precipitate formed was filtered through Whatman No.1 filter paper after standing at room temperature for 30 min. The absorbance of the trichloroacetic acid-soluble fraction was measured at 280 nm. The amount of Tyrosine produced is calculated from a pre-calibrated graph of absorbance at 280 nm against tyrosine concentration. One unit of activity is defined as the amount of enzyme required to release 1 μM of Tyr/min under the assay conditions.

Results and Discussion

Description of the fungus *Beauveria sp.*

The fungal strain, *Beauveria* species was isolated from rabbit dung and bears accession number MTCC-5184 (Microbial Type Culture Collection, Chandigarh, India). Initially, there was a white fungal growth which on further incubation at 28° C shows small protrusions which developed into erect white stalks containing spores (Fig.1.1a). The wet mount of well-grown fungus in submerged fermentation was prepared on a glass slide. Microscopic observation (100X) showed that the organism formed a thick network of thin mycelia and older cultures showed numerous spores. Conidia were hyaline, 1-celled, ovoid and not produced in the gelatinous material.

This strain was characterized and distinguished from *Beauveria felina* which is similar to the existing fungus *using* the 18S rDNA & ITS sequences and morphological characteristics (Shankar et al 2014).

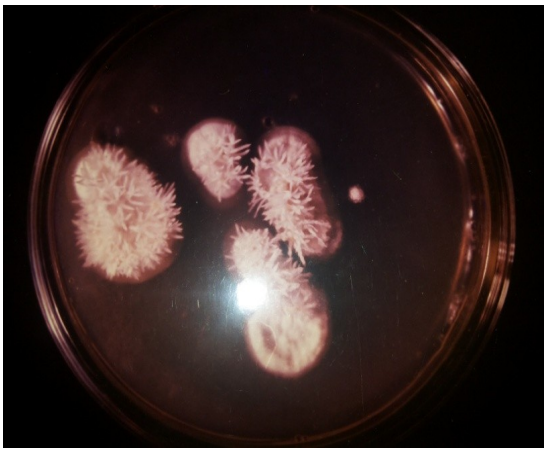

Fig.2.1a: Morphology of *Beauveria sp.*

Fig.2.2a: Microscopic picture of *Beauveria sp.*

Based on the dendrogram it can be seen from fig 1.2 that the new isolate was showing maximum homology with the strain *Beauveria felina***.** There were marked differences in their morphology though 18S rDNA and ITS sequences of MTCC 5184 showed 99% homology with *Beauveria felina*. *B. felina* NCIM 1314 grown as a flat colony with cottony growth and no stalks were formed even after prolonged incubation whereas isolate MTCC 5184

formed white erect stalks within 7-10 days while (Figure 2.2a). Hence the isolate MTCC 5184 was designated as a new strain of *Beauveria* sp.

Screening of protease production

Mikami medium 1% skim milk was spot inoculated on the plate as described in materials and method. The plate was observed after 72 hours for clearance zone around the culture. For more visibility, the plate was flooded with 5%TCA (trichloroacetic acid) for 10 min (Fig 2.5a). A prominent clearance zone was observed around the growing fungal colony after 72-96h.

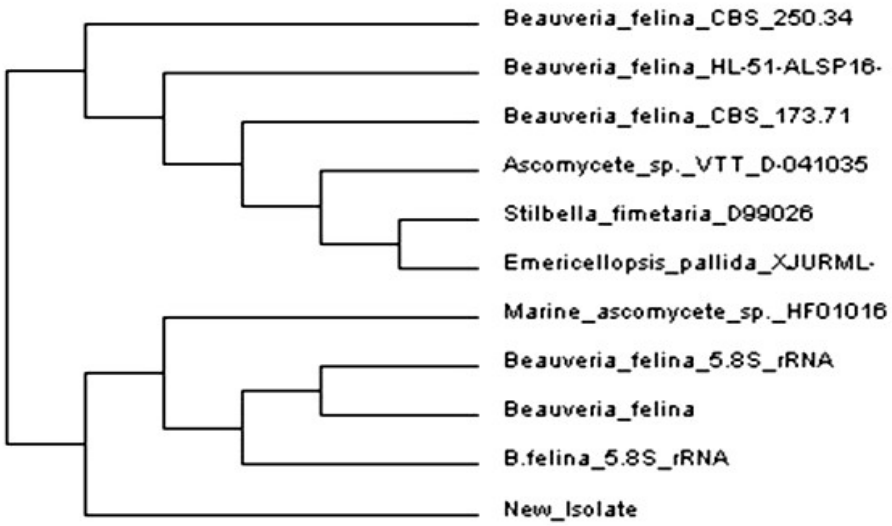

Figure 2.3a: Dendrogram showing the phylogenetic relationship between the ITS sequences of *Beauveria sp.* MTCC 5184 and *B. felina* NCIM 1314.

Figure 2.4a: Morphology of *Beauveria felina* NCIM 1314 and *Beauveria* sp MTCC 5184

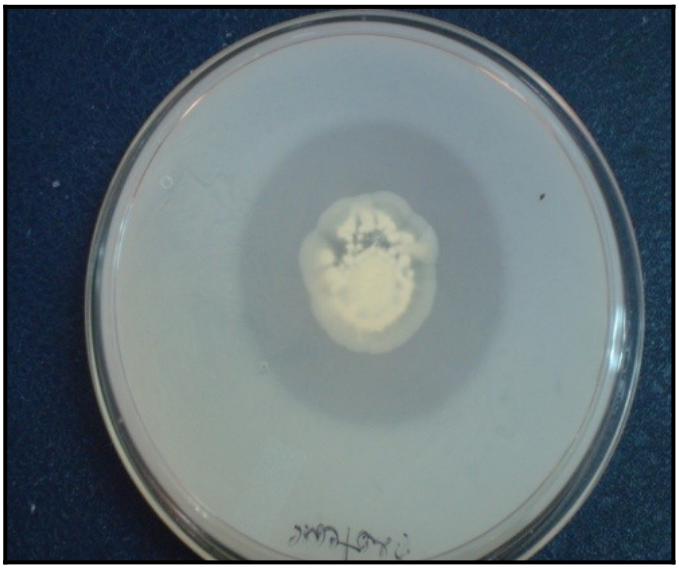

Fig 2.5a: Beauveria sp. showing clearance zone on skim milk plate

Co-production of Xylanase and protease in submerged fermentation

Co-production of Xylanase and protease in submerged fermentation was carried out in Mikami + 2% Wheat bran medium. Both protease and xylanase production was obtained in one single fermentation. As can be

seen from Table 2.2a, maximum protease production was observed at 72h while the xylanase was maximally produced after 96h. To optimize protease and xylanase production, separate fermentations for both the enzymes were carried out.

Table 2. 2a: Co production of Xylanase and protease

Time (h)	Protease (IU/ ml)	Xylanase(IU/ml)
48	3.10	1.933
72	3.94	3.290
96	3.78	7.083
120	1.80	5.100

Optimization of protease production.

Effect of inducers

Among all the inducers tested, mustard seed cake was found to be the best inducer followed by SBM, WB, ST, CSC, GNC and GF (Fig. 2.6a). Our results are in agreement with Bajaj and Sharma (2011) also reported mustard seed cake as the perfect nitrogen source for production of protease by *Streptomyces ambofaciens*. Wheat bran enriched with fish scales and egg shell in a ratio of 1:2:0.005 (w/w) was used to produce protease by *Penicillium sp.* (Hamzah *et al.,* 2009). Broken rice of different varieties i.e. PONNI, IR-20, CR-1009, ADT-36 and ADT-66 was used for protease production by A. niger MTCC 281 and maximum protease production was observed in PONNI (Paranthaman *et al.,* 2009). Defatted soybean cake was used for protease production by a *Penicillium* sp.(Germano *et al.,* 2003). *Botrytis cinerea* produced maximum protease when the medium was supplemented with marine *Spirulina* algae followed by 2% soybean protein (Abidi *et al.,* 2008).

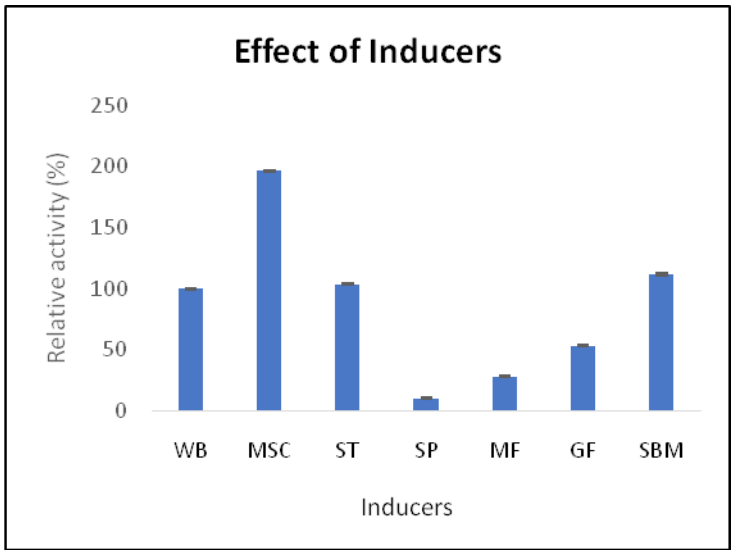

FIG 2.6a: Effect of Inducers on Protease production.

Fish flour and chicken feathers are also reported as inducers for protease production (Ellouz *et al.,* 2001; Gessesse *et al.* 2003). The production of the protease by way of the addition of black gram husk powder as an inducer to the medium was better by *Penicillium* sp. LCJ228 (Benluvankar *et al.,* 2016). Similar results with black gram husk were reported by (Kirankumar et al 2014).

Effect of different media

It was noted that among the media used for protease production, GYE was the most excellent medium for protease production. It was observed that MGYP and MIkami medium showed almost somolar activities while in MF medium the production was considerably less (Table 2.3a). Laxman et al (2005) reported optimum production of alkaline protease from *Conidiobolus coronatus* in MGYP containing 2%SBM medium while slightly lower activities were obtained in medium containing 1% glucose, 0.3% yeast extract and 3% soybean meal.

Table 2.3a: **Effect of different media on protease production**

Time (h)	Activity IU/ml			
	MGYP	Mikami	GYE	MF
48	1.39	1.55	2.54	0.61
72	4.02	3.97	7.41	2.14
96	1.13	1.89	1.58	1.16
120	0.75	0.9	1.16	0.34

Paecilomyces lilacinus formed alkaline protease on medium with glucose and tryptone (Kotlova et al 2007). Srinubabu *et al.,* (2007) investigated different media for production of protease by *A. oryzae* with 1% cotton seed cake and 2% SBM as inducers. Maximum activities were obtained in a modified medium which contents (g/100 ml): malt extract-1; glucose-6; yeast extract-1; peptone-2; K_2HPO_4-0.5; $MgSO_4$-0.5 and $FeSO_4$-0.01. Benluvankar *et al.,* (2016) reported maximum production of protease by *Penicillium* sp. LCJ228

in media containing g/L: Glucose, 10; Peptone, 10; K_2HPO_4, 1; $MgSO_4$, 0.2; Na_2CO_3, 5.

Effect of MSC concentration

Effect of MSC concentration was studied in GYE medium.MSc concentrations used were ranging from 1to 4%. It was observed that 2% concentration was best for protease production (Fig 2.7a)

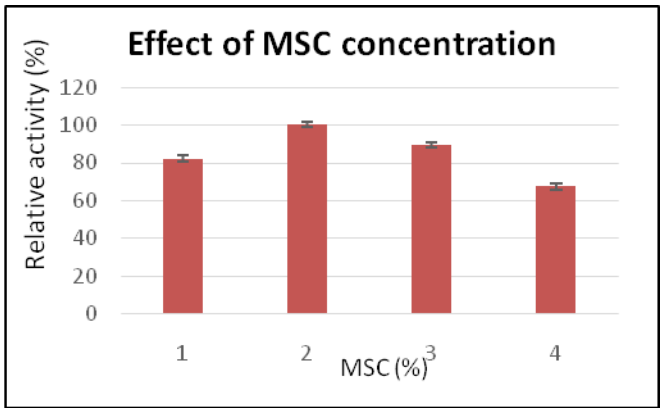

FIG 2.7a: Effect of MSC concentration

Soybean meal at 2–3% concentration was established to be best for protease production by *Conidiobolus coronatus* (Laxman *et al.*, 2005). Poza et al (2001) showed high yields of extra-cellular protease from *Candida caseinolytica*with medium having 2.4% skim milk powder as an inducer. A. oryzae NRRL 2217 was capable of producing maximum protease on mixed inducers coconut oil cake: wheat bran in a mass ratio of 1:3 (Sumantha *et al.*, 2005).

Effect of Glucose Concentration

Effect of glucose concentration on protease production was studied in GYE medium with 2% MSC as an inducer. Glucose concentration used for this study was 0-1.5%. It can be seen from Fig 2.8a. that glucose at 1% concentration was best for protease production. Approximately 50% and

80% production was obtained when glucose was used at 0.5 and 1.5% respectively. Comparable results were reported by Anandan *et al.,* (2007) that glucose at 1% concentration was optimum for alkaline protease production by *A. tamarii* and further increase (1.5–5.0%) appeared to inhibit the production. In similar way effect of glucose concentration was studied on production of protease by *Penicillium* sp. LCJ228 and it was found that 2% glucose concentration was best (Benluvankar *et al.,* 2016). Decreased protease production at elevated concentration of glucose could be due to the repression exerted by an excessive amount of metabolizable sugar in medium. In contrast, the optimum concentration of glucose was found to be 5% for acidic protease production by *A. carbonarius* (Ire *et al.,* 2011).

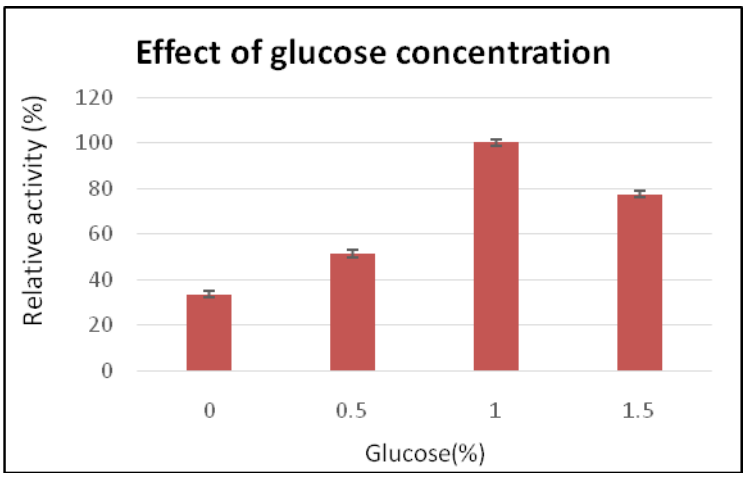

FIG 2.8a: Effect of glucose concentration

Effect of Yeast Extract concentration

Effect of yeast extract concentration was studied in GYE medium with 2% MSC as an inducer. Yeast extract concentrations used were in the range of 0.15 to 0.6%. It can be seen from fig 2.4 that yeast extract at 0.3% concentration was best for protease production. Growth and protease production by *B. cereus* BG1 were highest with 0.2% yeast extract (Frikha *et al.,* 2005). Yeast extract in the range of 0.3 to 0.5% was optimum for protease production by *C. coronatus* PTA-4132

(Laxman *et al.,* 2005). One percent yeast extract was optimum for growth and enzyme production by alkalophilic actinomycete while further increase resulted in repression of the enzyme secretion (Mehta *et al.,* 2006). Benluvankar *et al.,* (2016) reported 1.5% yeast extract concentration to be best for protease production by *Penicillium* sp. LCJ228.

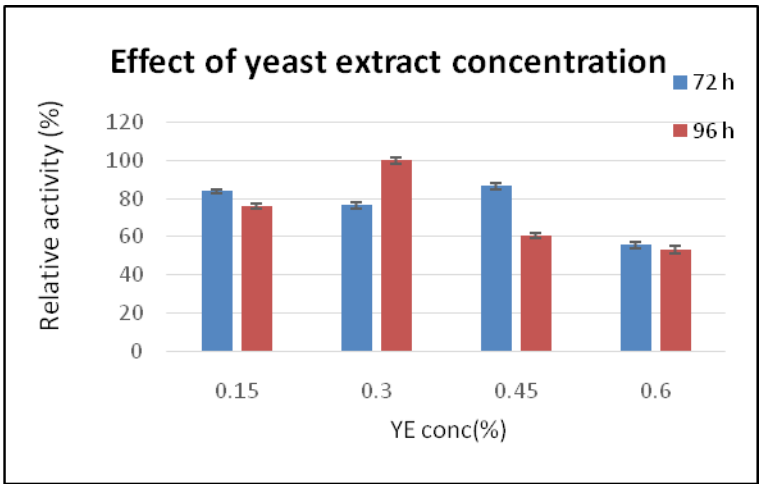

FIG 2.9a: Effect of yeast extract concentration

Effect of various carbon sources

Proteases are mostly produced through stationary phase and thus are usually regulated by carbon and nitrogen sources in the medium. Number of carbon sources such as glucose, sucrose, fructose, glycerol etc. has been studied as carbon sources for productionof protease. Studies have also indicated that a high carbohydrate concentration repressed enzyme production (Nirmal *et al.,* 2011).

Among the various carbon sources tested, glucose and starch were best followed by fructose, sucrose and lactose while sugar alcohols like glycerol and mannitol were poor carbon sources. Glucose as carbon source at 1% and 2% MSC were optimum for Protease production (Fig.2.10a). These results are in concurrence with Mrudula *et al.,* (2012) in the case of *B.*

subtilis. Between the mono-, di- and polysaccharides used by Benluvankar *et al.,* (2016), glucose was the most excellent carbon source for maximum protease production. This may be due to the cause that glucose is a monosaccharide and it is readily accessible for the metabolism of the fungus for protease production. It was concluded that fructose and glucose were the best carbon sources for improvement in the production of protease by *Aspergillus flavus* and *Aspergillus terrus* (Chellapandi, 2010).

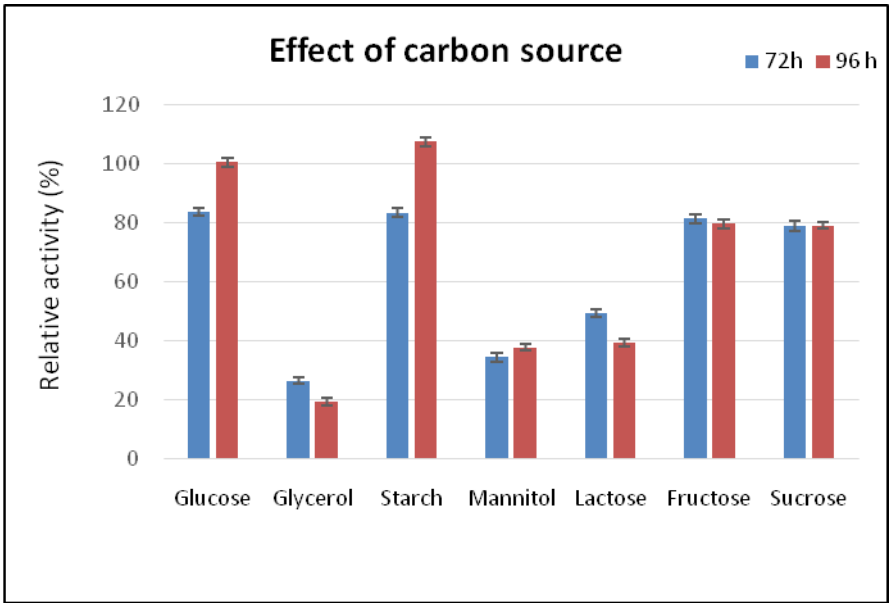

FIG 2.10a: Effect of carbon source on protease production

In contrast with these results highest protease production by *S. roseiscleroticus* was achieved when starch was included as the carbon source while it was minimal with dextrose (Vonithini *et al.*, 2008). Kumar *et al.,* (2014) used various carbon sources like glucose, lactose, galactose and starch for protease production by *Bacillus aryabhattai* K3 and reported maximum protease production with lactose (10 g/l) as a carbon source in the medium. Similarly, Ananthan (2014) reported maximum protease production in case of *Vibrio* Sp. GA CAS2 in medium containing lactose.

Effect of various Nitrogen sources

Effect of inorganic nitrogen sources on protease production was studied in GYE+2%MSC medium. Except for urea, all the nitrogen sources repressed protease production to varying levels. Ammonium salts viz. ammonium sulphate and ammonium nitrate totally inhibited protease production. Nitrate salts like sodium nitrate and potassium nitrate resulted in 30-40% repression and diammonium hydrogen phosphate showed total repression in 24 h but significant activities were obtained on further incubation (Fig 2.11a). $(NH_4)_2HPO_4$ was also found to be the best nitrogen source for protease production by *A. oryzae* 637 (Srinibabu *et al.*, 2007). Ammonium sulphate has been citted to be the top nitrogen source for protease production by *Bacillus* sp. strain AS-S20-I (Mukherjee and Rai 2011). On contrary, ammonium chloride and ammonium sulphate have been reported to suppress alkaline protease production by *Bacillus* sp. 2–5 (Darani *et al.*, 2008).

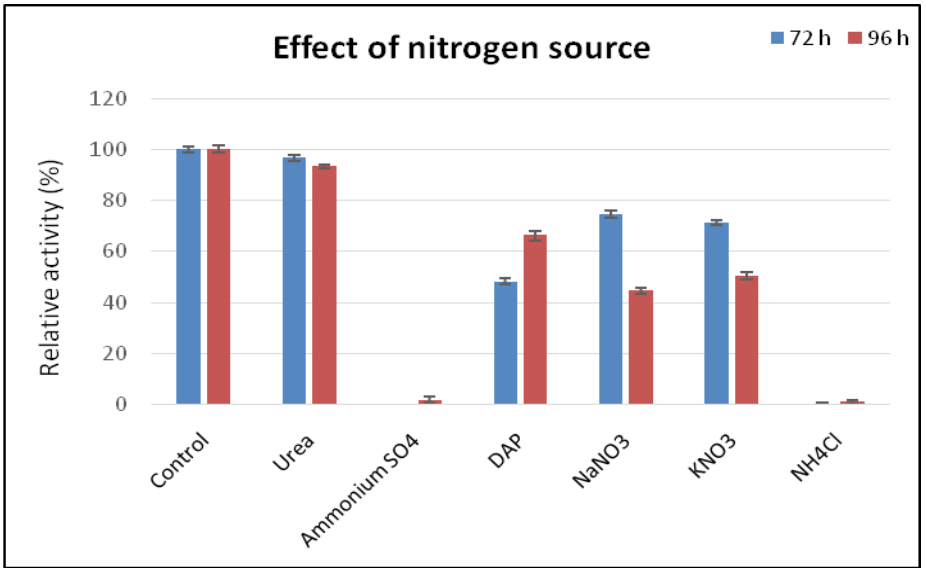

FIG 2.11a: Effect of nitrogen source on protease production

Neither ammonium chloride nor urea was beneficial for biomass or protease production by *L. mylittae*; however, corn steep liquor enhanced the protease production (zhou *et al.*, 2009). Kamath *et al.*, (2010) and Ananthan (2014) found that potassium nitrate leads to maximum protease production by *A.*

niger and *Vibrio* Sp. GA CAS2. Different ammonium salts showed the diverse effect on protease production by *B. cereus* where ammonium phosphate was inhibitory while other ammonium salts supported protease production (Nilegonkar *et al.*, 2007).

Effect of surfactants on protease production

Effect of a variety of chemical surfactants such as Tween 20, Tween 40, Tween 60, Tween 80 and Triton X-100 was studied at 1% concentration in GYE medium with 2% MSC. It was observed that None of the surfactants had an effect on protease production (Fig 2.12a). Tween20 and Triton X-100 inhibited the protease production. In contrast, highest protease yield of was obtained by *Streptomyces Pseudogrisiolus* NRC-15 by the addition of Tween 20 in the medium as compared to control (Mostafa *et al.*, 2012). The protease production by *Vibrio* Sp. GA CAS2 was observed to be high with surfactant Tween 80 (Ananthan 2014). Similar results with tween 20 and Tween 80 were reported for *B. subtilis* by Mrudula *et al.*, (2012).

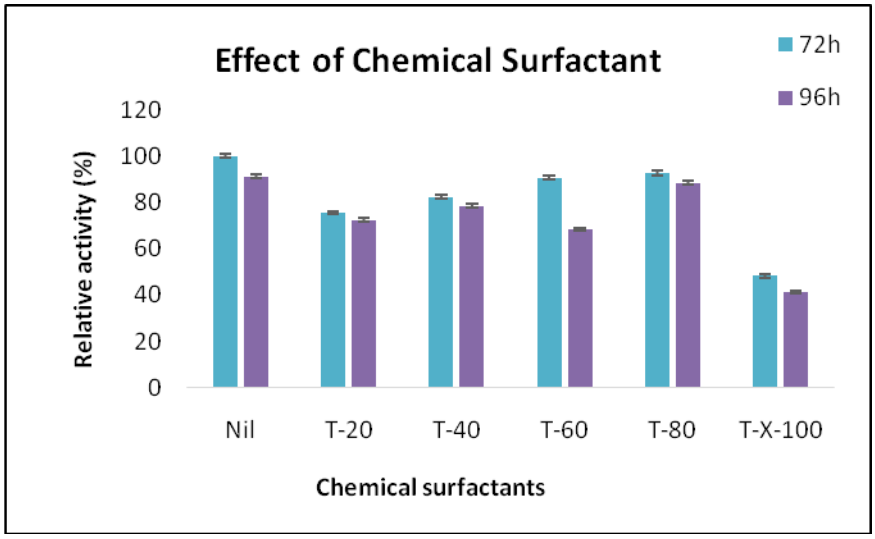

FIG 2.12a: Effect of chemical surfactants

Effect of inoculum size

Inoculum size also plays a vital role in protease production. In the present investigation, the effect of inoculum size on protease production of *Beauveria* sp. was studied in GYE medium with 2% MSC. Inoculum (v/v) used for this experiment was in the range of 5 to 20%. As can be seen from Fig (2.13a) 10% inoculums was optimum for maximum protease production. Benluvankar *et al.,* (2015) reported 2 g/L inoculum size appreciably improved protease production by *Penicillium sp.* LCJ228.

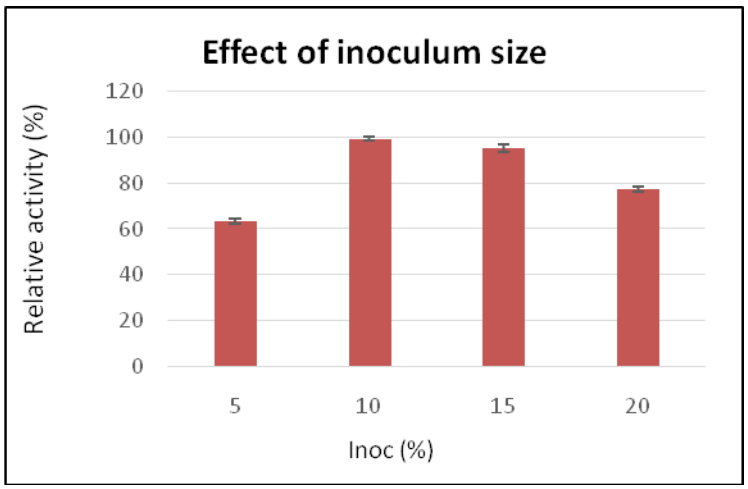

FIG 2.13a: Effect of inoculum size on protease production

Effect of pH of the medium

The pH of culture alters all enzymatic processes as well as transportation of various components acrossthe cell membrane. Hence it is an important parameter to optimize. Effect of initial pH on the medium was studied in GYE medium with 2% MSC with the range of pH 5.0 to 9.0 (Fig 2.14a). It is observed that at pH 8.0 maximum production of the protease was produced by *Beauveria* sp. similar results were reported for *Trichoderma viridae* VPG-12 by Kirankumar *et al.,* (2014). A medium with neutral initial pH has been reported for alkaline protease production by *P. chrysogenum* IHH5 (Haq *et al.,* 2006), *S. roseiscleroticus* (Vonothini *et al.,* 2008), *Bacillus aquimaris* VITP4 (Shivanand and Jayraman 2009) and *P. aeruginosa* MCM B-327

(Zambre *et al.*, 2011). While slightly acidic medium (pH 6.3–6.5) have been reported as optimum for protease production by *B. cereus* SIU1 (Singh *et al.*, 2010). Higher initial pH of 10.0 for maximum protease production is reported by *A. oryzae* 637 (Srinubabu *et al.*, 2007) and *Streptomyces pseudogrisiolus* NRC-15 (Mostafa *et al.*, 2012).

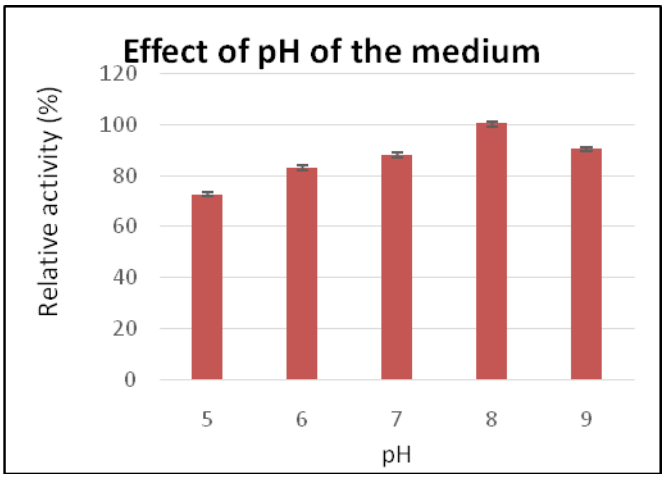

FIG 2.14a: Effect of pH of the medium on protease production

Effect of temperature

Temperature is also a crucial parameter that needs to be controlled and varied from organism to organism for maximum cell growth as well as enzyme production. Effect of temperature was studied on protease production by *Beauveria* sp. at temperatures 20°C, 28°C, 37°C, and 45°C. Maximum production was found at 28°C while approximately 80% production was observed at 20°C (Fig. 2.15a) *P. fluorescens* was able to produce protease in the range of 27–57°C with the maximum production at 37°C (Kalaiarasi and Sunitha 2006).

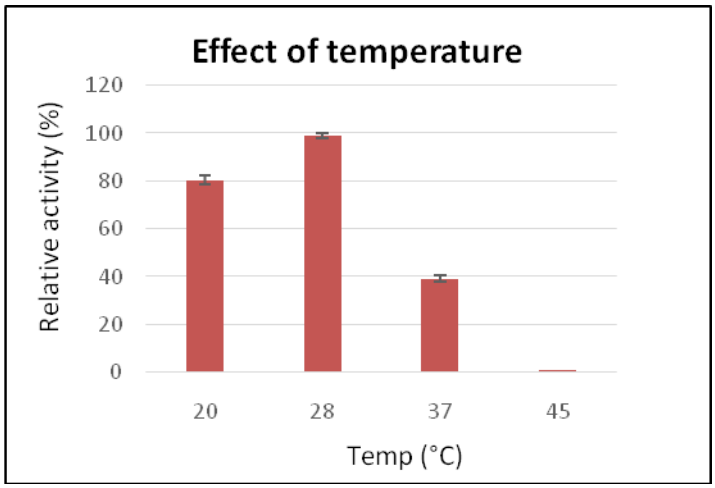

FIG 2.15a: Effect of temperature

A temperature of 37°C has been reported as optimal temperature for protease production by a Bacillus species *B. aquimaris* VITP4 (Shivanand and Jayraman 2009). In contrast to that, a temperature of 40°C has been reported to be best for the production of protease by Bacillus sp. 2–5 (Darani et al 2008).

Conclusion

A new fungal strain was isolated from rabbit dung in our laboratory. The fungus was identified as a new strain of *Beauveria.* This fungus was screened for protease production and showed a very good clearance zone on MGYP plate with 1% skim milk. Protease production was tested in different media and it was found that GYE medium was best for the production of protease. Different agricultural residues were tested as an inducer for protease production and mustard seed cake at 2% (w/v) concentration was proved to be the excellent inducer. Among various carbon sources used glucose was best utilized by the organism for protease production. Among nitrogen sources yeast extract was found to be the best nitrogen source for maximum production of protease followed by urea. Optimum protease production was observed when initial media pH was 8.0 and temperature for incubation was 28°C. 10% (v/v) inoculum was found to be optimal for the

production of protease. Chemical surfactants like Tween 20 or Tween 80 had no beneficial effect on protease production. Optimization studies revealed that a cost effective medium containing 1% glucose, 0.3% yeast extract and 2% mustard seed cake gave highest protease production. Approximately 2 fold increase was archived with the optimization studies.

References

- Abidi, F., Limam, F. and Nejib, M.M. (2008). *Process Biochem,* 43: 1202-1208.
- Anandan, D., Marmer, W.N. and Dudley, R.L. (2007). *J Ind Microbiol Biotechnol,* 34: 339-347.
- Ananthan G. (2014) World J of Pharma and Pharma Sci 3 (12): 756-770.
- Bajaj, B.K. Sharma, P. (2011), New Biotechnol. http://dx.doi. org/10.1016/ j.nbt.2011.01.001
- Benluvankar, V. Jebapriya, G.R. Gnanadoss, J.J. (2015) Int. J. Life Sci. Pharm. Res. 5(1): 12–19.
- Chellapandi, P. (2010). *E-J of Chem,* 7: 479-482.
- da Silva, O.S. de Oliveira, Porto R.L. Motta A.L.F. Porto, T.S. (2016) Adv. Enzyme Res. 4: 125–143.
- Darani, K.K. Falahatpishe, H.R. Jalali, M. (2008) Afr. J. Biotechnol. 7: 1536–1542
- Devi, M.K. Banu, A.R. Gnanaprabhal, G.R. Pradeep, B.V. Palaniswamy, M. (2008) Indian J. Sci. Technol. 1: 1–6.
- Ellouz, Y., Bayoudh, A., Kammoun, S., Gharsallah, N. and Nasri, M. (2001). *Bioresour Technol,* 80: 49-51.
- Frikha, G.B., Kamoun, A.S., Fakhfakh, N., Haddar, A., Manni, L. and Nasari, M. (2005). *Ind Microbiol Biotechnol,* 32: 186 -194.

- Germano, S. Pandey, A. Osaku, C.A.. Rocha, S.N Soccol, C.R. (2003) Enzyme Microb. Technol. 32: 246–251.
- Gessesse, A. Kaul, R.H. Gashe, B.A. Mattiasson, B. (2003) Enzyme Microb. Technol. 32 519–524.
- Hamzah, H.M. Ali, A.H.L. Hassan, H.G. (2009) J. Eng. Sci. Technol. 4: 81–89
- Haq, I.U. Mukhtar, H. Umber, H. (2006) J. Agric. Soc. Sci. 2: 23–25.
- Ire, F.S., Okolo, B.N., Moneke, A.N. and Odibo, F.J.C. (2011). *Afr J Food Sc,* 5: 353-365
- Jaswal, R.K. Kocher, G.S. Virk, M.S. (2008) Indian J. Biotechnol. 7: 356–360.
- K. Kalaiarasi, P.U. Sunitha, (2009) Afr. J. Biotechnol. 8: 7035–7041.
- Kamath, P., Subrahmanyam, V.M., Rao J.V. and Raj P.V. (2010). *Indian J Pharm Sci,* 161-166
- Kirankumar, S. Hanchinalmath J. V. Sai Sundeep, Y. Borah, D. Talluri, VSSL (2014) *Int. Lett. of Nat Sci* 9: 77-84
- Kotlova E K, Ivanova N M, Yusupova M P, T L, Ivanushkina NE & Chestukhina G G (2007) *Biochem (Moscow*), 72: 117-123.
- Kumar, C.G. Joo, H.S. Koo, Y.M. Paik, S.R. Chang, C.S. (2004) World J. Microbiol. Biotechnol. 20: 351–357.
- Kumar, R. Sharma, K.M. Vats, S. Gupta, A. (2014) Int. J. Adv. Pharm. Biol. Chem. 3(2): 290–298.
- Laxman, R.S., Sonawane, A.P., More, S.V., Rao, B.S., Rele, M.V., Jogdand, V.V., DeshpandeV.V., and Rao, M.B.,(2005). *Process Biochem*, 40: 3152-3158.
- Macchione, M.M. Merheb, C.W. Gomes, E. Silva, R.D. (2008) Appl. Biochem. Biotechnol. 146: 223–230.
- Mehta, V.J. Thumar, J.T. Singh, S.P. (2006) Bioresour. Technol. 97 1650–1654
- Mostafa, S. E. Moataza, M. Awad, H. M. Selim M. H. Hassan H. M. (2012) E-J of Chem 9(2): 949-961

- Mothe, T. Sultanpuram, V.R. (2016) Biotechnology 6 (53): 2–10.
- Mrudula, S. Apsana Begum, A. Ashwitha K. and Pavan Kumar P. (2012) Int J Pharm Bio Sci 3(3): 619 – 631
- Mukherjee, A.K. Rai, S.K. (2011) New Biotechnol. 28(2): 181– 189.
- Murthy, P.S. Naidu, M.M. (2010) World Appl. Sci. J. 8: 199– 205.
- Nilegaonkar, S.S., Zambare, V.P., Kanekar, P.P., Dhakephalkar, P.K. and Sarnaik, S.S. (2007). *Bioresour Technol*, 98: 1238-1245.
- Ningthoujam, D.S. Kshetri, P. Sanasam, S. Nimaichand, S. (2009) World Appl. Sci. J. 7: 907–916.
- Nirmal N.P., Shankar, S., Laxman, R. S. (2011) *Int. J.Biotech & Biosci.*, 1 (1) : pp 1-40
- Paranthaman, R. Alagusundaram, K. Indhumathi, J. (2009) World J. Agric. Sci. 5: 308–312.
- Poza M, de Miguel T, Sieiro C & Villa T G (2001) *J Appl Microbiol*, 91: 916- 921.
- Sharma, A.K., Sharma, V., Saxena, J., Yadav, B., Alam, A., Prakash, A. (2015) Appl Res J 1 (7): 388-394
- Shivanand, P. Jayaraman, G. (2009) Process Biochem. 44: 1088–1094.
- Sindhu, R. Suprabha, G.N. Shashidhar, S. (2009) Afr. J. Microbiol. Res. 3: 515–522.
- Singh, S.K. Tripathi, V.K. Jain, R.K. Vikram, S. Garg, S.K. (2010) Microb. Cell Fact. 9: 59.
- Srinubabu, G. Lokeswari, N. Jayaraju, K.E. (2007) J. Chem. 4: 208–215
- Sumantha, A. Szakacs.G. Sandhya C, C.R. Soccol, A. Pandey, (2005) Food Technol. Biotechnol. 43: 313–319.
- Vonothini, G. Murugan, M. Sivakumar, K. Sudha, S. (2008) Afr. J. Biotechnol. 7: 3225–3230.
- Vonothini, G. Murugan, M. Sivakumar, K. Sudha, S. (2008) Afr. J. Biotechnol. 7: 3225–3230.

- Zambare, V. Nilegaonkar, S. Anovel, P.K. New Biotechnol. (2011), http://dx.doi.org/10.1016/j.nbt.2010.10.002.
- Zhou, L.H. Zhang, Y.Q. Wang, R.J. Shen, X.L. Li, Y.Q. Guan, (2009) W.J. Afr. J. Biotechnol. 8: 1591–1601

SECTION B

Section B: Screening and optimization of xylanase production by *Beauveria* sp.

Abstract

Screening of xylanase production was carried out by plate assay method on MGYP plate containing 1% xylan. Optimization of xylanase production was studied in shake flasks where an effect of various physicochemical parameters and nutritional factors were studied. This includes pH, temperature, the effect of media, the effect of inoculum, carbon & nitrogen sources, surfactants, inducers. Xylanase production was highest in the medium with malt extract 03%, yeast extract 03%, peptone 0.5% and glucose 1% (MGYP) medium with wheat bran (WB). Among various agricultural residues, WB was found to be the best inducer at 2% concentration. Out of various sugars checked glucose showedmaximum xylanase production. Among the various nitrogen sources tested yeast extract was the best followed by urea. 10% inoculums showed maximum production of xylanase. Chemical surfactants such as tween20, tween80 or Triton X100 did not show any increase in the production. Xylanase production by *Beauveria* sp. was 8.290 IU/ml by submerged fermentation.

Introduction

In recent years, the use of many microorganisms in industrial fermentation processes has been focused, particularly for xylanase production. Use of xylanase in the industry has increased appreciably over the years (Techapun *et al.,* 2003). Xylanase is the most important enzyme and is involved in the degradation of the hemicelluloses backbone of xylose. Xylanases has been produced widely from various microorganisms such as

bacteria, actinomycetes and various fungi. The enzyme from various sources work at unique temperatures and pH values (Ho, 2014a). Filamentous fungi, such as *Aspergillus* spp (Haq *et al.,* 2004), *Penicillium spp* (Fadel and Fouda, 1993), *Streptomyces* spp (Kansoh and Gammel, 2001), *Bacillus subtilis* (Sanghi *et al.,* 2008) and *Trichoderma* spp (Azin *et al.,* 2007) have been mainly studied and extensively manipulated in the production of xylanase. Though there are bacterial sources producing xylanases, fungal sources are preferred for several reasons such as, due to their robost nature, growth and fermentations with renewable sources, ease in downstream processing etc. (Subramaniyan and Prema, 2002). As a result, fungi have been proved as the organisms with the capability for biosynthesis of xylanase. In the recent years, xylanase has mainly been produced by using fungal strains such as, *Colletotrichum graminicola* (Zimbardi *et al.,* 2013), *Aspergillus flavus* MTCC 9390 (Bhushan *et al.,* 2012), *Aspergillus flavus* DFR-6 (Pal *et al.,* 2010), *Aspergillus fumigatus* (Kanagasabai *et al.,* 2013), *Melanocarpus albomyces* (Gupta *et al.,* 2013), *Aspergillus Niger* (Vimalashanmugam and Viruthagiri, 2013), *Aspergillus terreus* (Vimalashanmugam and Viruthagiri, 2013) *Rhizopus stolonifer* CECT 2344 (Pérez-Rodríguez *et al.,* 2014). In spite of different sources for xylanase production by above stated fungi, *Aspergillus niger* remains the fungi of choice for xylanase biosynthesis (Wong *et al.,* 1998). Considering the probable applications of xylanases in a range of industries plus environment, it is essential to reduce the cost of production by optimizing medium formulation and growth parameters of microorganism in submerged fermentation (SmF). Submerdged fermentation is the preferred culture technique of microorganisms in the liquid medium with obvious reason of producing higher enzyme yields, productivity which involves lesser cost of maintenance and lower risk of contamination (Ho and Hood 2014). Presently, 80-90% of commercial xylanases are produced in submerged culture for the reason that it has a higher degree of escalation, better level of automation and greater flexibility of scaling up (Ho, 2014a). Submerged

fermentations are used for the production of xylanase due to the better understanding of fungal metabolism and their positive responses.
As like other industrially impotant enzymes, xylanase also is generally affected by the medium composition, carbon and nitrogen sources used, inducers, and physical parameters like pH, Temperature etc. Some of them are discussed below.

Carbon sources

The production of microbial xylanase on submerged fermentation is robustly affected by the type of carbon source used. Different carbon sources for optimizing medium formulation for xylanase production by a variety of microorganisms have been employed such as starch, sucrose, maltose, glucose, lactose, fructose, mannose, galactose, xylose, sorbitol and glycerol (Ho, 2014a). The roll of maltose has been reported for fungal biomass production for the growth of diverse fungi (Simões and Tauk-Tornisielo, 2006; Peixoto *et al.,* 2003; Fadel 2000), and glucose is one of the majority used carbon sources for biomass growth since it is easily metabolized (Andrade *et al.,* 2002). In another report by Simoes *et al., (*2009) reported lactose, sorbitol and maltose as the preferred carbon sources for xylanase production from *Penicillium implicatum, Trichoderma viride* and *Aspergillus niger* respectively. When Seyis and Aksoz (2005), considered the effect of various carbon sources on xylanase activity by *Trichoderma harzianum,* sucrose and glucose were found to produce xylanase and out of these, sucrose showed higher xylanase biosynthesis as compared to glucose. Similar results with sucrose were reported for *A. brasiliensis* and *Streptomyces* species by Ho and Hood 2014 and Rifaat *et al.,* 2005. Galactose was the succeeding best carbon source for *U. esculenta* as reported by Chung and Tzeng (2004), whereas Gupta *et al.,* 2009 reported that addition of xylose resulted in an increase in production of xylanase by *Fusarium solani* F7.

Nitrogen sources

Different organic and inorganic nitrogen sources put forth a significant influential impact on their capability to improve the growth of microorganisms as well as enzyme production. Optimization of nitrogen source in the fermentation is also essential as that of carbon source (Ho and Hood 2014). Effect of organic as well as inorganic nitrogen sources on xylanase production has been extensively studied. Generally it is observed and reported that organic nitrogen sources are preferred for xylanase production (Katapodis *et al.*, 2007) Aqeel and Umar (2010) observed that organic nitrogen sources like peptone and yeast extract generally had the stimulating result on the production of the enzyme. Among the nitrogen sources tested yeast extract was reported to be the best for xylanase production by *A.brasiliensis* (Ho and Hood 2014). In case of *A. flavus* yeast extract and peptone were observed to increase xylanase production (Bhushan *et al.*, 2012). Similar results have been reported by Kumar et al. (2012) but in case of *Bacillus pumilus*. Pal and Kaushik (2012) considered the effect of different nitrogen sources on the growth of *Rhizoctonia solani* and the maximum xylanase activity was obtained from the organic nitrogen sources such as yeast extract and peptone. Li *et al.*, (2011) also reported similar results with *Streptomyces chartreusis.* In contrast to these results, Shah and Madamwar (2005) reported that *Aspergillus foetidus* MTCC 4898 could show considerably good activity even in the absence of any organic nitrogen source. Fattah *et al.*, (*2011*) reported xylanase activity with inorganic nitrogen source sodium nitrate by *Cladosporium macrocarpum* NRC 15. The results obtained did not demonstrate any noteworthy difference between the organic and inorganic nitrogen sources on the production of xylanase by *Fusarium solani* F7 Gupta *et al.*, (2009).

Inducers

Generally, lignocellulosic resources are utilised in culture medium formulation as an inducers since they are abundant in nature, low in cost and have a high level of carbohydrate content which is appropriate to generate fermentable sugars. Agricultural residues like wheat bran, corn cob, rice bran, palm kernel cake, sugarcane bagasse, maize, barley husk, soybean hulls and sawdust are normally used as inducers/carbon sources in the xylanase production (Ho, 2014b). Tallapragada and Venkatesh (2011) reported the use of oatspelts xylan for xylanase production by *Aspergillus niger*. Use of commercial/purified xylan to induce xylanase synthesis increases the cost of production therefore for commercial applications; there have been attempts to use low-cost agricultural wastes which are rich in hemicellulose for xylanase production. Li *et al.,* (2011) and Ahmad *et al.,* (2012) reported the highest xylanase activity by *Streptomyces chartreusis* and *Aspergillus niger* using corn cobs as an inducer. Wheat bran is considered as one of the widely utilised inducers for the growth of many organisms in the production of xylanase. It has been a preferred inducer for fungi like *Aspergillus* (Li. *et al.,* 2006; Xu et al 2008; Kavya and Padmavati 2009; Dhillon *et al.,* 2011; Sorgotto *et al.,* 2012; Guimaraes *et al.,* 2013), *Penicillium chrysogenum* (Okafore *et al.,* 2007), *Sclerotinia sclerocium* (Ellouze *et al.,* 2008), *Fusarium solani* (Bakri *et al.,* 2013) and *Simplicillium obclavatum* (Roy *et al.,* 2013). Other inducers used for xylanase production reported in literature are rice bran (Kavya and Padmavati 2009), sugarcane bagasse (Milagres *et al.,* 2004), Palm kernel cake (Pang and Ibrahim 2005), Soybean (Kavya and Padmavati 2009), barley (Soliman *et al.,* 2012), sawdust (Bakir *et al.,* 2001), maize (Goyal *et al.,* 2008).

Incubation temperature

The incubation temperature is one of the significant parameters to decide the performance of xylanase fermentation. A good number filamentous fungi are mesophilic which call for the optimal growth temperatures between 25 and 35°C (Simoes *et al.,* 2009; Gupta *et al.,* 2009; Tallapragada and

Venkatesh, 2011). Shah and Madamwar (2005) stated that 30°C was the optimum temperature for xylanase production by *A. foetidus.* Kavya and Padmavathi (2009) reported that *A. niger was* grown well at 28°C with the xylanase production. Gupta *et al.,* (2009) reported that maximum xylanase production was obtained at 30°C by free and immobilised cells of *Fusarium solani* F7. Nevertheless, while growth temperature increased to 35°C, a considerable decrease in xylanase production was observed. Thus most of the fungi grow at ambient temperature and their optimum temperature for xylanase production is also near to ambient temperature. Conversely, some reports indicated that the optimal temperatures for the production of some of the fungal xylanases occurred at the range between 30°C and 50°C (Singh *et al.,* 2009; Bajaj *et al.,* 2010; Tallapragada and Venkatesh, 2011).

pH of the medium

The pH of the medium has a great authority on the performance of xylanase activity where it plays an important part in initiating the excretion of xylanase enzyme. The initial pH affects several enzymatic systems in addition to their transport across the cell membrane (Shah and Madamwar 2005). Previous studies investigated a variety of initial pH media for xylanase production by diverse strains of fungi. Majority of the fungi are reported to produce xylanase at initial pH lower than 7.0 (Shah and Madamwar 2005; Gupta *et al.,* 2009). Ahmad *et al.,* (2012) reported that the initial pH of the medium of 5.5 produced the optimum xylanase activity of 60.03 U/mL by *A. niger.* Conversely when the initial pH of the medium was adjusted to 8.0, Tallapragada and Venkatesh (2011) obtained the optimum xylanase production. Simoes *et al.,* (2009) observed initial pH of 5.0 optimum for xylanase production by *Penicillium implicatum*, while initial pH of 5.5 was reported to be optimum for *Fusarium solani* by Gupta *et al.,* (2009). Maximum xylanase production at pH 6.5 by *A. brasiliensis* was observed by Ho and Hood (2014).

Materials and Methods

Malt extract, yeast extract and peptone were obtained from M/s Hi-Media Chemicals, India. Hammerstein casein was obtained from M/s Sisco Research Laboratories, India. All other chemicals were of analytical grade. Wheat bran and various agricultural residues were obtained from local market.

Microorganism

Beauveria sp MTCC 5184 was isolated from rabbit dung and was deposited in the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, (IMTECH), Chandigarh, Pune, India. It was maintained on MGYP agar (g/L malt extract, 3; glucose, 10; yeast extract, 3; peptone, 5 and agar, 20) slants and sub-cultured once in a month and preserved at 4°C after growth and sporulation

Screening of xylanase production

Screening for xylanase production by Beauveria sp. was performed by plate assay method. Mikami medium containing 1% xylan was used for this study. Vegetative culture pregrown for 24-48h in the same medium was spot inoculated on the plate. The plate was observed after 72 hours for clearance zone around the colony by flooding the plate with 0.1% Congo red stain. The stain was than drained off and 1% NaCl was added for 10 minutes to observe the clearance zone.

Inoculum preparation

Agar piece (2x2 cm) with spores from 5-8 days old MGYP plate was inoculated in 250 ml Erlenmeyer flask containing 50 ml MGYP medium unless otherwise mentioned. The inoculum was incubated at 28°C and 180 rpm for 24-48 h on a rotary shaker. After growth 10% (v/v) inoculum was used for inoculation.

Xylanase Production

Spores from 2-3 weeks old MGYP slant was used for inoculum development. 48 h old vegetative inoculum (10% v/v) grown in MGYP was used to inoculate the experimental flasks. Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 50 ml MGYP (glucose, 1%; yeast extract, 0.3%; malt extract, 0.3%; Peptone 0.5%) medium with 2% wheat bran (w/v) as inducer. Flasks were incubated at 28°C, 200 rpm for 3-5 days. Samples were removed periodically, centrifuged at 10000 rpm for 10 min and the clear supernatant was used as the source of crude enzyme.

Effect of Inducers

Effect of inducers on xylanase production was studied in MGYP medium. Various agricultural residues such as corn cob, corn stover, wheat bran and xylan as inducer at 2% (w/v) concentration were used for this study.

Effect of Medium

Effect of three different media was studied on xylanase production with 2% wheat bran as inducer.Three media used were MGYP, Mikami and GYE (composition described in chapter 2 section A). Inoculums for the different media were prepared in respective media without wheat bran.

Effect of various carbon and nitrogen sources

Effect of various carbon and nitrogen sources was studied in MGYP + 2% WB medium. Carbon sources used were glucose, starch, maltose, sucrose lactose and sugar alcohols like glycerol and mannitol. All the

carbon sources were used at 1% concentration. Various inorganic nitrogen sources namely urea, ammonium sulphate, ammonium chloride, diammonium hydrogen phosphate, sodium nitrate and potassium nitrate were added at equivalent nitrogen concentration to that of yeast extract.

Effect of pH of the medium

Effect of pH of the medium was studied in MGYP + 2% WB medium in the pH range 5.0 to 9.0. Medium was adjusted to required pH with sterile 0.1 N HCl or 0.1 N NaOH before inoculation. The pH of the medium during the fermentation was not controlled. Irrespective of the initial pH of the medium, final pH at the end of the fermentation reached 7.5 to 8.0.

Effect of temperature

Effect of temperature was studied on xylanase production by *Beauveria* sp. at temperatures 20°C, 28°C, 37°C, and 45°C. Experimental flasks after inoculations were incubated to the said temperatures.

Substrate Preparation

Two grams of oatspelts xylan was suspended in 100 ml of 50mM phosphate buffer of required pH and stirred for 16-18h in 5-10°C. The insoluble material was removed by centrifugation (10000rpm, 20 min) and soluble fraction approximately (0.9 -1%) was used as the substrate.

Determination of Xylanase Activity

The total reaction mixture of one ml contained 0.5 ml of the suitably diluted enzyme in phosphate buffer (50 mM pH 7.0 unless otherwise mentioned) and 0.5 ml of xylan solution. The reaction mixture was incubated at 50°C for 30min followed by the addition of 1ml DNSA to terminate the reaction.

The resultant reaction mixture is kept in a boiling water bath for 5 min and colour intensity was read at 540nm.after diluting with 10ml distilled water (Bernfeld, 1955). One unit of xylanase activity is defined as the amount of enzyme that produced 1 umol of xylose equivalent/min under standard assay conditions.

Results and Discussion

Screening of xylanase production

Screening for xylanase production by Beauveria sp. was performed on Mikami medium containing 1% xylan. The vegetative inoculums was spot inoculated on the palte and was incubated at 28°C for 96 h. A prominent clearance zone was observed on the plate indicating the xylanase production (Fig 2.16b).

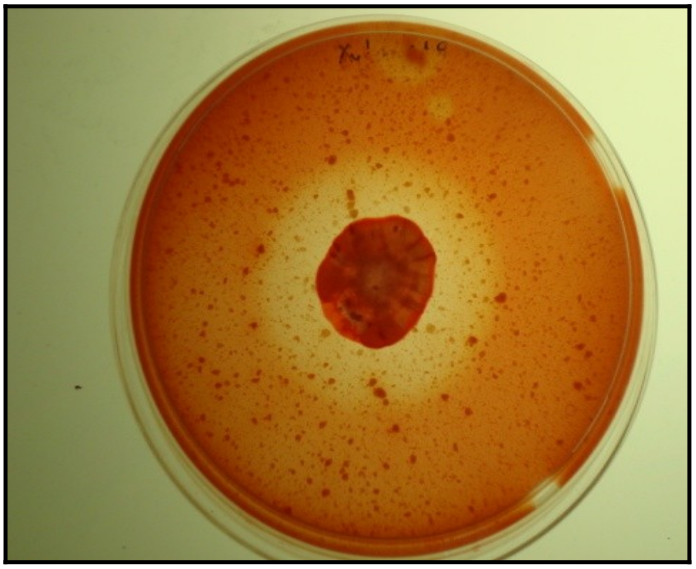

FIG 2.16b: Clearance zone on xylan agar plate

Xylanase production was confirmed by submerged fermentation. Optimization of xylanase production was carried out with respect to various media constituents and physical parameters which are described below.

Effect of Inducers on Xylanase production

Effect of inducers on xylanase production was studied in MGYP medium. It can be seen from Fig 2.17b that wheat bran was the best inducer of xylanase production by *Beauveria* sp. 75 to 80% xylanase production was obtained with Corn cob and roasted wheat bran; corn stover produced approximately 50% xylanase as compared to wheat bran. It was observed that with all the inducers the xylanase production was maximum is 72 hours. Similar results for *Aspergills* were reported in the literature (Lli. *et al.,* 2006; Xu *et al.,* 2008; Kavya and Padmavati 2009; Dhillon *et al.,* 2011; Sorgotto *et al.,* 2012; Guimaraes *et al.,* 2013). Similar results for xylanase production by *A. niger* LCBT-14 with wheat bran was reported by Abdullah *et al.,* (2014).Wheat bran is a derivative of wheat milling industry in which it is originated from the surface layer of the wheat kernel. In general, wheat bran is defined as the outer hard layer of the grain (Ho, 2014b). Gupta *et al.,* (2009) reported 52.81 U/ml of xylanase by *Fusarium solani* using wheat bran as an inducer. *A. niger* CECT 2700 produced the maximum amount of xylanases using dry corncob as inducer (Pérez-Rodríguez *et al.,* 2014). Joshi and Khare (2012) reported xylan to be the best inducer for xylanase production by *Scytalidium thermophilum.* The highest xylanase production was observed in sugarcane bagasse followed by wheat bran by Chromohalobacter sp. TPSV 101 (Prakash *et al.,* 2009).

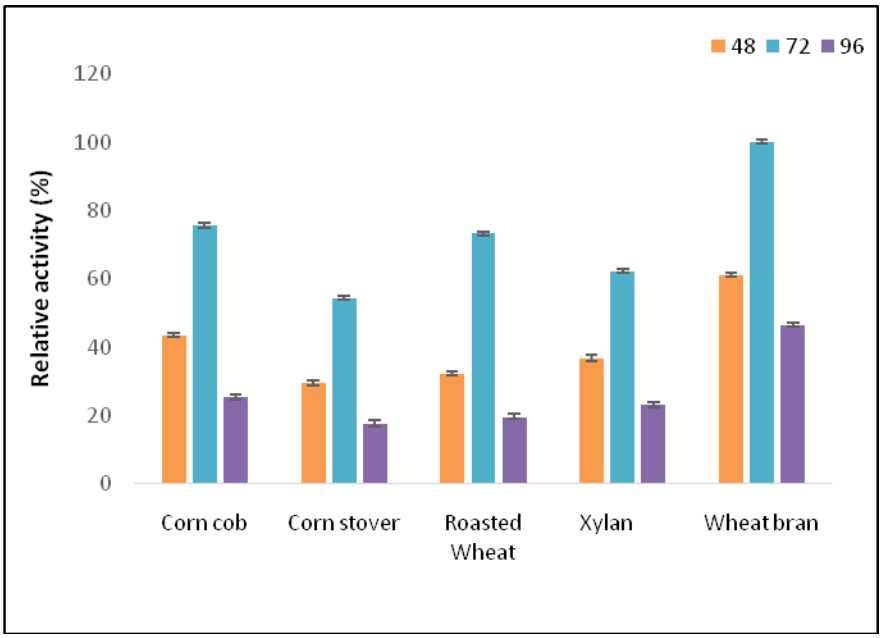

FIG 2.17b: Effect of inducer on xylanase production

Effect of Medium on Xylanase production

Effect of three different media was studied on xylanase production by *Beauveria* sp. It was noted that MGYP was the best medium for xylanase production with 2% wheat bran (Fig 2.18b) Mikami medium showed approximately 90% while GYE medium showed 59% of xylanase production as compared to MGYP medium. In GYE medium maximum xylanase production was observed after 96h whereas in the other two media production was maximum in 72h. Shah and Madamwar (2005) used MS basal medium for xylanase production with 1% birch wood xylan by *Aspergillus foetidus*. Gupta *et al.*, (2009) used Mandel's medium with wheat straw for xylanase production by *Fusarium solani* F7. *Trichoderma viride* was reported to produce optimum xylanase in Vogel's medium (Fortkamp and Knob 2014).

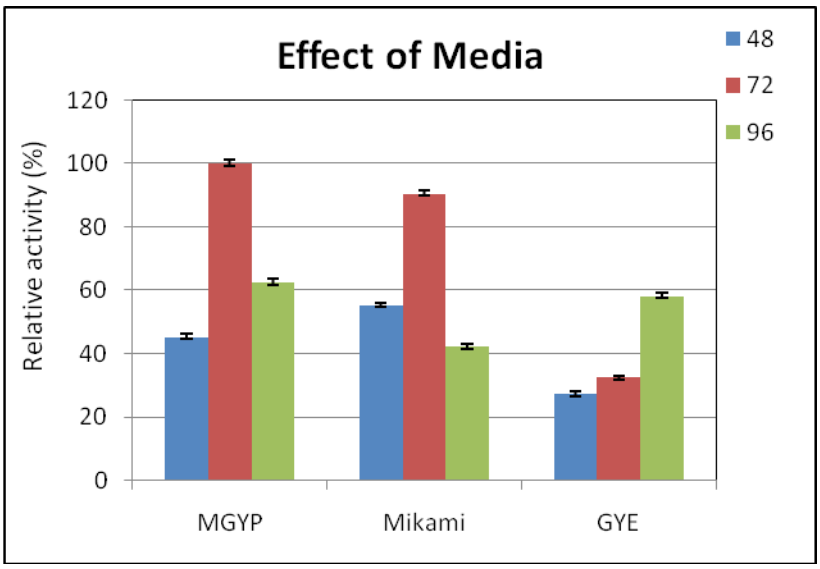

FIG 2.18b: Effect of Media on xylanase production

Effect of Wheat bran concentration on Xylanase production

Effect of wheat bran concentration ranging fron 1-4% was studied in MGYP medium. It was concluded that 2% wheat bran concentration was most excellent for optimum xylanase production with 80% production with 1% wheat bran. The increase in wheat bran concentration did not help to increasethe production (Fig 2.19b). Fortkamp and Knob (2014) reported for first-time use of pineapple peel at 2% concentration for xylanase production by *Trichoderma viride*.

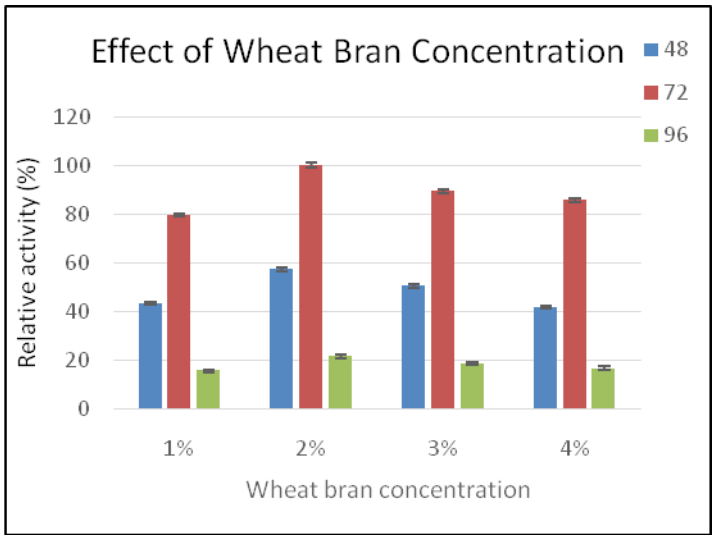

FIG 2.19b: Effect of WB Concentration

Effect of carbon sources

Effect of simple and complex carbon sources were studied on xylanase production by *Beauveria sp*. Table 2.4b shows that glucose was the best-utilized carbon source for xylanase production by *Beauveria* sp. This is followed by sucrose which showed 66% increase in production as compared to control. However, with lactose, it was 58% which are in agreement with Abdullah *et al.,* (2014) who evaluated the effect of a variety of carbon sources such as lactose, glucose, maltose, xylose and sucrose on xylanase production from Aspergillus niger LCBT-14. (Among all tested carbon sources, glucose gave the maximuml enzyme production. Sati and Bisht, (2006) also reported similar results with glucose as a carbon source for xylanase production from *Tetracladium marchalianum*. In contrast with these results, when Seyis and Aksoz, (2005) studied the effect of different carbon sources on xylanase production by *Trichoderma harzianum.* They reportred sucrose and glucose as better carbon sources to induce xylanase and out of these, sucrose showed higher xylanase production than glucose. Simoes *et al., (*2009) reported lactose, sorbitol and maltose as the preferred carbon sources for xylanase production from *Penicillium implicatum* , *Trichoderma viride* and *Aspergillus niger* respectively.

Table 2.4b: Effect of carbon source on xylanase production

MGYP+ carbon Source	Xylanase activity (%)		
	72 hours	96 hours	120 hours
Glucose	21.78	100	72.00
Glycerol	5.9	29.40	21.17
Starch	14.23	48.46	36.70

Maltose	17.60	34.34	25.17
Lactose	25.31	58.11	39.98
Mannitol	28.27	56.93	42.11
Sucrose	16.60	66.11	39.53

Effect of nitrogen sources on xylanase production

Effect of various nitrogen sources was studied on xylanase production by *Beauveria* sp. in MGYP medium with 2% wheat bran. Various inorganic nitrogen sources namely urea, ammonium sulphate, ammonium chloride, diammonium hydrogen phosphate, sodium nitrate and potassium nitrate were added at equivalent nitrogen concentration. It can be seen from Fig 2.20b yeast extract was the best nitrogen source for xylanase production followed by urea.Similar results for Xylanase Production by *Aspergillus brasiliensis* was reported by Ho and Hood (2014). Aqeel and Umar (2010) concluded that organic nitrogen sources like peptone and yeast extract typically had the stimulating outcome on the production of the enzyme. Pal and Kaushik (2012) studied the consequence of a variety of nitrogen sources on the growth of *Rhizoctonia solani* and the maximum xylanase activity was observed from the organic nitrogen sources of yeast extract and peptone. According to Li *et al.,* (2011), considerable xylanase activity was obtained by *Streptomyces chartreusis* when cultured in medium containing yeast extract as a nitrogen source. Goyal *et al.,* (2008) studied xylanase production in presence of inorganic nitrogen sources where urea wasfound to produce the least xylanase activity. Highest xylanase production by was obtained when ammonium sulphate was supplemented to the fermentation medium by Aspergillus niger LCBT-14. (Abdullah et al 2014)

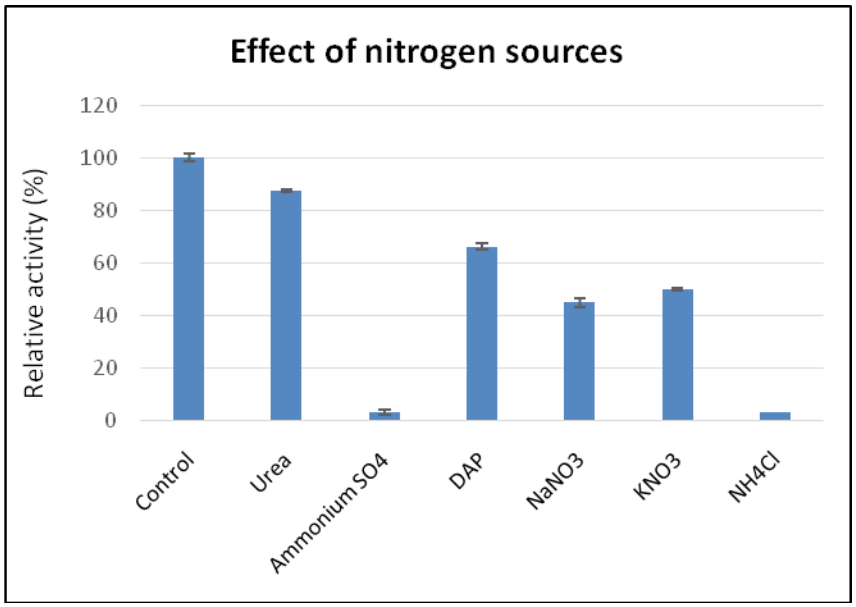

FIG 2.20b: Effect of Nitrogen Sources on xylanase production

Effect of surfactants

Surfactants are surface active agents which help in enzyme secretion. Effect of various surfactants was studied in MGYP medium with 2% wheat bran. Surfactants like Tween 20, Tween80, TritonX-100 and sodium dodecyl sulphate (SDS) were used at 0.1% concentration. It is observed from Fig 2.21b that none of the surfactants had any positive effect on xylanase production.

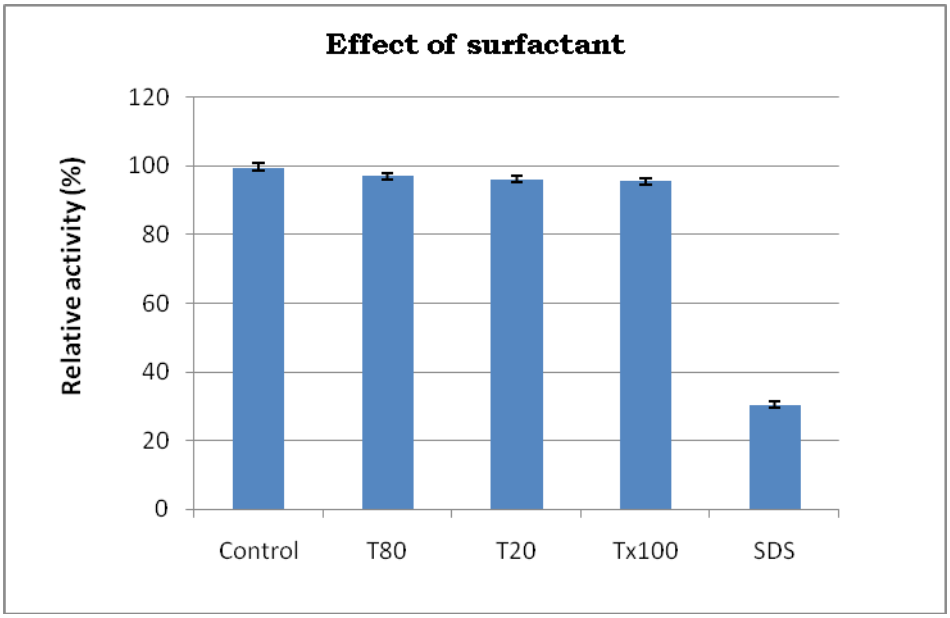

FIG 2.21b: Effect of Surfactants on xylanase production

Effect of initial pH on medium

Effect of initial pH of the medium was studied in MGYP medium with 2% wheat bran. Highest xylanase production was found to be at pH 7.0 with almost 90% production at pH 8.0 (Fig 2.22b). Maximum production of xylanase by T. viride was reported to be 7.0 to 7.5 (Fortkamp and Knob 2014). Alternatively the optimum pH value for xylanase production by *Trichoderma* sp. T-1 and T-2 were found to be 5.5 and 5.7 respectively (Mohan *et al.,* 2011) Effect of different pH (4–9) of fermentation medium on xylanase production by *A. niger* LCBT-14 was evaluated and maximum xylanase production was obtained at pH 4.0 (Abdullah *et al.,* 2014). Prakash *et al.,* (2009) reported highest xylanase production by *Chromohalobacter sp.* TPSV 101 at pH 9.0. Simoes *et al.,* (2009) observed initial pH of 5.0 optimum for xylanase production by *Penicillium implicatum*, while initial pH of 5.5 was reported to be optimum for *Fusarium solani* by Gupta *et al.,* (2009). Maximum xylanase production at pH 6.5 by *A. brasiliensis* was observed by Ho and Hood (2014).

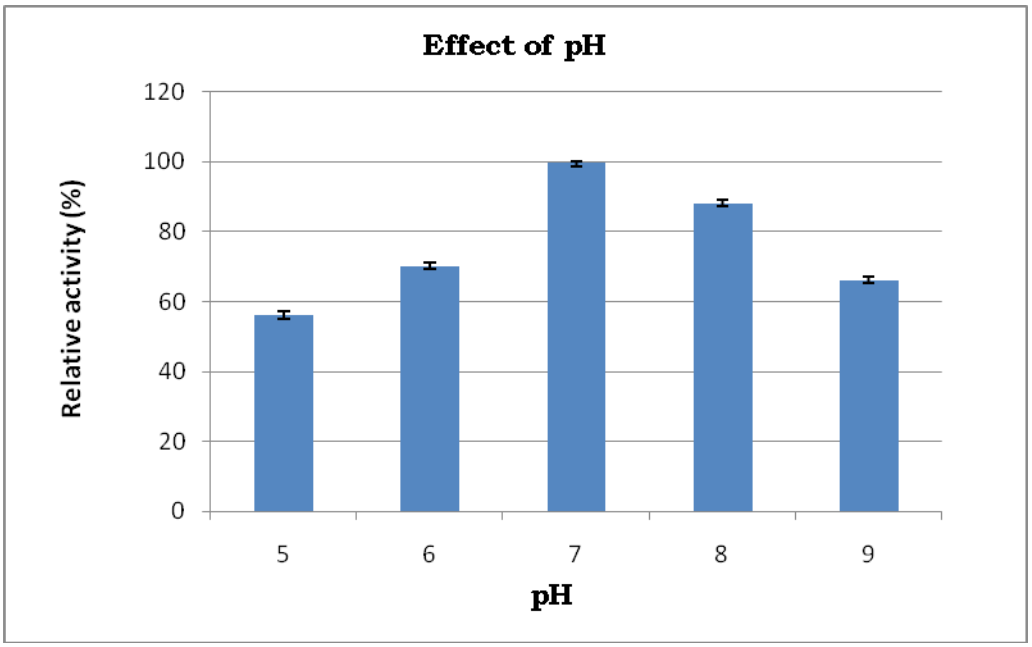

FIG 2.22b: Effect of pH on xylanase production

Effect of incubation temperature

Effect of incubation temperature was studied by carrying out the fermentation at various temperatures ranging from 20-45°C. Optimum production was at 28°C while 78% xylanase production was observed at 20°C Fig (2.23b). The increase in temperature resulted in a decrease in xylanase production this is due to inhibition of growth of the fungus. This result coincides with the report from Tallapragada and Venkatesh, (2011). Kavya and Padmavathi (2009) showed that *A. niger was* grown well at 28°C with the xylanase production. Influence of varying incubation temperature (25–50°C) on xylanase production by *A. niger* LCBT-14 was studied and optimum production was obtained at 30°C (Abdullah *et al.,* 2014). Gupta *et al.,* (2009) reported similar results for *Fusarium solani* F7.

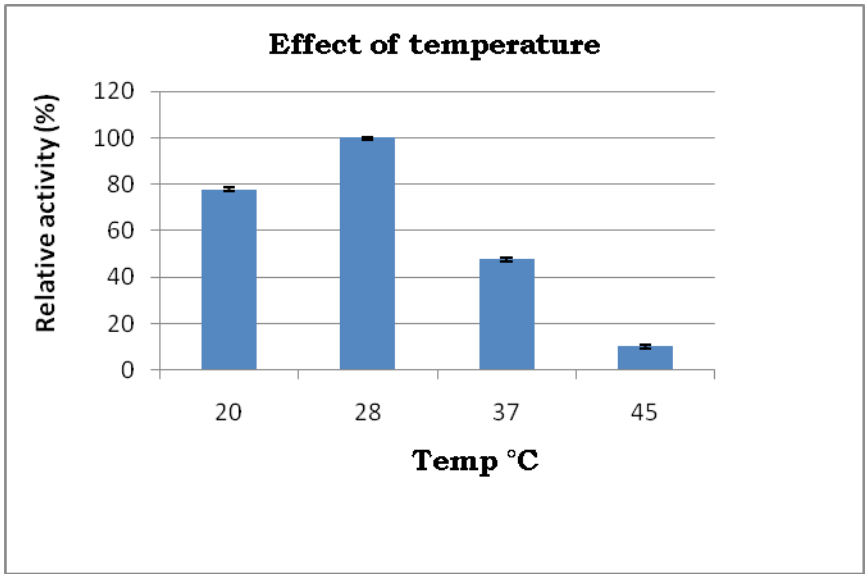

FIG 2.23b: Effect of temperature on xylanase production

Conclusion

Beauveria sp. was screened for xylanase production and showed a very good clearance zone on MGYP plate with 1% xylan. Xylanase production was tested in different media and it was found that MGYP medium was best for the production of xylanase. Different agricultural residues were tested as an inducer for protease production and wheat bran at 2% concentration was proved to be the best inducer. Among various carbon sources used glucose was best utilized by the organism for xylanase production. Yeast extract was the best nitrogen source for maximum production of protease followed by urea. Optimum xylanase production was observed when initial media pH was 7.0 and temperature for incubation was 28°C. Chemical surfactants like Tween 20 or tween 80 had no beneficial effect on xylanase production. Optimization studies revealed that a medium containing 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 2% wheat bran gave highest xylanase. production. Approximately 1.2 fold increase was acchived with the optimization studies.

References

- Abdullah, R. Nisar, K. Aslam, A. Iqtedar, M. Naz, S. (2014) Nat. Prod. Res. http://dx.doi.org/10.1080/14786419.2014.968157
- Afr. J. Biochem. Res.1(4) : 48-531
- Ahmad, Z., Butt, M. S., Anjum, F. M., Awan, M. S., Rathore, H. A., Nadeem, M. T., Ahmad, A. and Khaliq, A. (2012) *Afr. J. of Biotechnol*, 11(7): 1674-1682.
- Andrade VS, Sarubbo LA, Fukushima K (2002) Braz. J. Microbiol. 33(2): 106
- Aqeel B. M, Umar D. M (2010) World Appl Sci J 8: 85-90.
- Azin, M., Moravej, R. and Zareh, D. (2007) *Enzyme and Microbiol Techno,l* 40: 801-805.
- Bakir U, Yavascaoglu S, Guvenc F, Ersayin A (2001) Enzyme and Microbial Technology 29: 328- 334.
- Bakri, Y. Jawhar, M. Arabi, M. I. E. (2013) Res. Biotechnol. 4(1): 31-37
- Bhushan B, Pal A, Jain V (2012) Enzyme Engg 1: 103.
- Chung K, Tzeng D (2004). J. Biol.Sci. 4(2): 246-252.
- Dhillon G, S. Oberoi, H. S. Kaur S. Bansal S Brar, S. K (2011) Ind. Crops. Prod. 34(1): 1160-1167
- Ellouz, O. Faltouch, M. Mestiri, F. Aniba, M. R. Marzouki, M. N. (2008) Ind. J. Biochem. Biophys. 45(6): 404-409
- Fadel M (2000) J. Biol. Sci., 1(5): 401-411
- Fadel, M. and Fouda, M. S. (1993) *Microbiol*ogy, 148: 304-312.
- Fattah A. F. A., Doaa, A. R. Mahmoud, S. M. Shash, F. M. P. and Sherien, M. M. A.(2011) *World Appl. Sci. J,* 15(10): 1376-1381.
- Fortkamp, D., Knob, A. (2014) Afr. J. Bioechnol 13(22): 2248-2259.
- Goyal M1, Kalra KL, Sareen VK, Soni G (2008) Braz J Microbiol 39: 535-541.
- Guimaraes, N. C. A. Sorgotto, M. Peixoto -Nogueinra, S. C. Betini, J. H. A. Zanoelo. F. Marques, M. R. Polizeli, M.L.T. H. Giannesi, G. C.(2013) J. Biocat. Biotransformation 2(1): 1-6

- Gupta, G., Sahai, V., Gupta, R. K. (2013) Ind. J. of Chem. Technol. 20: 282-9.
- Gupta, V. K. Gaur, P. Yadava, S. K. Darmwal, N. S. (2009) BioResource. 4(3): 932-945
- Haq, I., Tasneem, M. , Raana, K., Khan, A., Mukhtar, H. and Javed, M. (2004) *Int J of Agric and Biotechnol,* 6: 1115-1118.
- Ho H. L and Hood, J. S. (2014) J Biodivers Biopros Dev 1:1-13.
- Ho. H. L. (2014a) *Int. J. of Food Ferment. Technol.* 4(1): 1-11.
- Ho. H. L. (2014b) J Biodivers Biopros Dev 1:3
- Kanagasabai, V. and Thangavelu, V. (2013) J. of Adv. Scient Research 4(2): 13-20.
- Kansoh, A. L. and Gammel, A. (2001) *Acta Microbiologica et Immunologica Hungarica*, 48: 39-52.
- Katapodis, P. Christakopoulou, V. Kekos, D. Chris takopoulos P. (2007) *Biochem EngJ. 35: 136–141*
- Kavya V, Padmavathi T (2009) Pol JMicrobiol 58: 125-130.
- Kumar D, Verma R, Sharma P, Rana A, Sharma R, et al. (2012) Biological Forum-An Int J 2: 83-87.
- Li X, Sun B, Zhao J, Lv Y, Li HSE, Zhu Y (2011) Afr J Biotechnol 10: 14132-14142
- Milagres AMF, Santos E, Piovan T, Roberto IC (2004) Process Biochemistry 39: 1387-1391. Tallapragada, P. and Venkatesh, K.(2011) *Journal of Microbiology and Biotechnology Research,* 1(4): 137-147.
- Mohan, G. Guhankumarand, P. Balakrishnan V. (2011) Res. Plant Biol. 1(3): 15-20.
- Okafore, U. A. Emozue, T. N. Okochi, V. I. Onyegeme- Okerenta B. M. Nurodo- Chinedu, S. (2007)
- Pal P, Kaushik P (2012) Int JLife Sci 1: 16-18.
- Pang PK, Ibrahim CO (2005) Songklanakarin J. of Sci. Technol. 27: 325-336.

- Peixoto SC, Jorge JA, Terenzi HF, Polizeli MLT (2003) Int. Microbiol. 6: 269-273.
- Pérez-Rodríguez N. Oliveira F. Pérez-Bibbins B. Belo I. Torrado Agrasar A. Domínguez J. M. Appl Biochem Biotechnol (2014) 173:803–825
- Prakash S. Veeranagouda Y. Kyoung L. Sreeramulu K. (2009) World J Microbiol Biotechnol 25:197–204
- Rifaat H. M, Nagieb Z. A, Ahmed Y. M (2005) Appl Eco Environ Res 4: 151-160.
- Roy, S. Durra, T. Sarkar, T. S. Ghosh, S. (2013) springerPlus 2(1): 1-10
- Sanghi, A. Garg , N. Sharma, J. Kuhar K. Kuhad, R. C. Gupta, V. K. (2008) World J Microbiol Biotechnol 24: 633–640
- Seyis, I. and Aksoz, N. (2005*Int. Biodeterioration & Biodegradation,* 55: 115-119
- Shah AR, Madamwar D (2005) World J. Microbiol Biotechnol 21: 233-243.
- Simões M.L.G., Tornisielo S.M.T. (2006) Afr. J. Biotechnol. 5 (11): 1135-1141.
- Simoes, M. L. G., Tauk-Tornisielo, S. M. and Tapia, D. M. P. (2009) *Afr, J. of Biotechnol* 8(22): 6317-6326.
- Sogotto, M. Guimaraes, N. C. A. Zanoelo, F. F. Margues, M. R. Peixoto-Nogueinra, S. C. Giannesi, G. C. (2012) Afr. J. Biotechnol. 11(32): 8076-8084
- Soliman HM, Abdel-Dayem AS, El-Tanash AB (2012) Int. J. Agri. Res 7: 46-57.
- Subramaniyan, S. and Prema, P. (2002) *Critical Reviews in Biotechnol,* 22: 33-64.
- Techapun, C., Poosaran, N., Watanabe, M. and Sasaki, K. (2003.)*Process Biochem,* 38: 1327-1340.
- Vimalashanmugam, K. and Viruthagiri, T. (2013b) International Journal of ChemTech Research 5(5): 2365-74.

- Vimalashanmugam, K. and Viruthagiri, T. 2013a. Asian J. of Biochem. and Pharm.l Research 3(2) : 44-62.
- Wong, K. K. Y., Tan, L. U. L. and Saddler, J. N. (1988) *Microbiology Reviews*, 52: 305-317.
- Xu YM, Li YL, Xu SC, Liu Y, Wang X, et al. (2008) J. Zhejiang University Science 9: 558-566.

CHAPTER 3

Sophorolipids as Enhancers for Enzyme Production and Properties of Enzyme Preparations

SECTION A

Effect of addition of sophorolipids on protease production by *Beauveria sp.* and properties of protease

Abstract

Sophorolipids are a structurally diverse group of surface-active molecules synthesized by microorganisms. They are synthesized by employing non-pathogenic yeast strains and have been approved by Food and Drug Authority and generally regarded as safe (GRAS).

Effect of addition of oleic acid sophorolipid and linoleic acid sophorolipid was studied on protease production. It was observed that when both the sophorolipids were added to the medium the protease production enhanced. The increased production was 231.2 and 169.8% in case of oleic and linoleic sophorolipid respectively. It was observed that 1mg/ml was the best concentration which showed a maximum increase in protease production. Enzyme obtained was further checked for its physical properties such as optimum pH and stability and optimum temperature and stability were studied. It was observed that optimum pH and temperature for protease was pH 9.0 and 50°C. Protease was stable in a broad pH range of 3.0-11.0. Protease was stable upto 45°C showing approximately 50% residual activities after 1h incubation.

Introduction

Surfactants, in general,, are surface-active agents that lower surface and interfacial tension. Structurally they are amphiphilic in nature so are

soluble in both aqueous and organic solvents.Due to these properties surfactants are used in cosmetics, detergents, cosmetics, concrete additives and pharmaceuticals. They are chemically synthesized hence they leave a negative impact on the environment.

Biosurfactants

Biosurfactants are the surfactants produced by microbial conversion. They are amphiphilic molecules and have unconventional properties like antimicrobial, biocompatibility, biodegradability, low toxicity etc. which make them superior for use than chemically derived surfactants (Develter and Lauryssen 2010). As they have both hydrophobic and hydrophilic moieties that give the ability to accumulate at or adjust themselves to orient on interfaces, consequently reducing surface tension and interfacial tension of liquids. They have the capability to form molecular aggregates as well as micelles. Therefore, attention in biosurfactants has amplified in various fields as multifunctional materials for the new century.

Sophorolipids

Gorin *et al.,* (1961) first described that sophorolipids are produced by various yeast species, out of which *Candida bombicola* is the most extensively studied. They are generally considered as the most promising glycolipids, as the production organism is non-pathogenic, and because of the ease of product recovery, high productivity, and substrate conversion (Lang and Hubbard 2003). Sophorolipids, have extraordinary surface activity and skin compatibility thus goes well with personal care and cosmetic applications. They also have dispersibility and emulsification properties in addition to several advantageous biological properties such as anti-microbial, anti-viral etc and have been approved by FDA. For the production of sophorolipid (acidic and lactonic), non-pathogenic yeast such as *Candida bombicola* is supplemented with glucose as a hydrophilic carbon source and fatty acid as lipophilic feed.

Effect of chemical surfactants on enzyme production

Surfactants are soluble amphiphiles that are surface acting. They reduce surface tension or free energy of the reaction medium. Surfactants disrupt the lipid bilayer of the cell membranes to solubilise them or they can form mixed micelles with the membrane component to increase the extraction of the protein from cells in the medium (Evans and Abdullahi, 2012). Rees and Manguire (1969) reported that the addition of Tween 80 and sucrose monopalmitate, nonionic surfactants, to fungal cultures showed a prominent increase in yields of the enzymes cellulase, amylase, sucrase, glucanase, xylanase, purine nucleosidase, and benzoyl esterase. They proposed that, this accomplishment was an effect of the surfactant on cell permeability.

Effect of chemical surfactants on protease production

There are many reports available on the effect of nonionic and ionic surfactants as an additive in fermentation medium for protease production by microorganisms in literature.

Effect of surfactants on protease production by *C. coronatus* NCL 86.8.20 had no inhibition or increase with Tween 80 at 0.1 and 0.3% concentrations and activities similar to control were observed devoid of surfactant indicating that surfactant had no stimulatory effect (Phadatare, 1991). While with Tween 20 no inhibition was detected at 0.1%, there was 63% drop in activity when increased to 0.3% concentration.Evans and Abdullahi (2012) studied the effect of tween 80 and acetonitrile on protease production by *Bacillus subtilis*. They reported that the addition of Tween-80 increased the protease yield by 5 folds. Similar reports with increased protease production were reported by other researchers for various bacteria, actinomycetes and fungi in the literature. Ananthan 2014 studied the effect of various surfactants

like Tween20, Tween40, Tween80, SDS and PEG on protease production. The protease production by *Vibrio* Sp. GA CAS2 was found to be high with surfactant Tween 80 while others inhibited the protease production (Ananthan 2014). Mrudula *et al.,* (2012) observed that Tween 20 and tween80 enhanced the protease production while Triton-100 did not show any effect in case of *Bacillus subtilis*. In contrast to this report, Motasfa *et al.,* (2012) reported that the highest protease yield of was obtained by *Streptomyces pseudogrisiolus* NRC-15 by the addition of Tween 20 in the medium as compared to control. Whereas on the other hand there are certain reports which state that these surfactants have no effect or inhibitory effects on protease production.Effect of different surfactants on protease production by *Conidiobolus brefeldianus* was studied in the medium supplemented with 0.1% surfactant. Control without the addition of any surfactant was included for comparison. Tween 20 and Triton X-100 inhibited growth as well as protease production while Tween 40, Tween 60 and Tween 80 slightly inhibited protease production (Khandelwal 2013)

Effect of sophorolipids on enzyme production production

Effect of sophorolipids on enzyme production by microorganisms' is hardly investigated. There is just one report by Gross *et al.,* (2015) where they have prepared a natural mixture of sophorolipids by fermentation of *Candida bombicola*. The sophorolipid was constituted by a disaccharide sugar viz. sophorose and a fatty acid or an ester group. They observed that when *Bacillus subtilis* was grown in presence of sophorolipids, increased production of amylase was observed. The increase in amylase production was noted to be 1.39 fold. Similarly, they grew *Pleurotus ostreatus* 473 strain supplemented with sophorolipid at 1mM concentration in DMSO, which produces two enzymes viz. laccase and manganese peroxidase. They

obsereved increased production of laccase was observed whereas the production of manganese peroxidase decreased in presence of sophorolipid. The increase in laccase production was found to be 4.53 fold while decrease in manganese peroxidase was 27%. Hence they concluded that sophorolipids could be used as protein inducers and/ or repressors by adding them in the fermentation medium. Another report but not with neat sophorolipid, wherein only sophorose induced cellulase production (Hrmova *et al.,* 1991). A similar report on sophorose as a potent inducer of cellulose production was reported by Chi-Ming and Lu-kwang (2009). In this report, they investigated the feasibility of using sophorolipids that contain sophorose, for cellulase induction by the co-cultures of *Hypocrea jecorina* Rut C30 and *Candida bombicola.* The results of the study indicated that the sophorolipids produced by *C. bombicola* can be degraded by *H. jecorina* Rut C30 and the sophorose generated from the degradation can effectively induce the fungal cellulase synthesis.

As of now, there are no such reports on the effect of sophorolipid on protease production. That provoked us to study the effect of sophorolipids on protease production by *Beauveria sp*. Effect of oleic acid sophorolipid and linoleic acid sophorolipid on protease production by *Beauveria* sp. is described in this section.

Enzymes are presently used in a number of different industrial products and processes and new areas of application are continuously being added. Microorganisms account for a two-thirds share of marketable protease production globally. Natural microorganisms have over the years been an enormous resource of enzyme diversity. With the increasing apprehension in relation to environmental issues, microbial enzymes have emerged as alternative biocatalyst in quite a lot of industrial processes. Their usefulness is escalating day by day, as new enzymes with novel properties are being explored. The majority of the presently used industrial enzymes are hydrolytic in actionand are being used for the degradation of a range of natural substances. Most of the

processes in food, pharmaceutical, leather, textile and detergent industries make use of crude enzyme preparations.

Proteases also have been studied for their various properties so as to decide their use in the suitable industries. Properties of the enzyme decide its use. Hence it is important to study the properties. Generally important properties are optimum pH, optimum temperature, stability of the protease towards pH and temperature, effect of metal ions etc.

Optimum pH and pH stability

It is observed that most of the times pH optima for alkaline proteases is between 8.0-11.0 and for acidic proteases it ranges between 2.0-6.0 (Nirmal *et al.,* 2011). There are reports with alkaline pH optima. Jellouli *et al.,* (2011) and Srinivas and Naik (2011) reported pH optima of 10.0 and 11.0 for protease from *Bacillus licheniformis* MP1 and *Bacillus halodurans* JB 99 respectively. A optimum pH for *Conidiobolus brefeldianus* was reported to be 9.0 by Khandelwal (2013) and similarly Chi *et al.,* (2007) reported optimum pH of 9.0 for *Aureobasidium pullulans.* Extracellular alkaline protease from *Aspergillus clavatus* was reported to be optimally active at pH 9.5 (Tremacoldi and Carmona 2005). It is also repoted that proteases active in alkaline pH have great industrial importance especially in formulation of detergents (Gupta *et al.,* 2002). Many of the fungal proteases show stablity in the pH range of .05 to 10.0, with especially good stability in neutral pH.

Optimum temperature and temperature stability

In general proteases from mesophilic fungi have their optimum temperature in the range of 37 to 60°C. In the industries where the operations have to be carried out at ambient temperatures or slightly above proteases active in the temperature range of 30-50°C are suitable. Protease from *Conidiobolus* strains were reported to active in temperature range of 40 to 50°C (Khandelwal 2013, Laxman *et al.,* 2005). Temprature stability is also one

the major factor for commercial uses of proteases. The protease from *Aspergillus tamari* was stable for more than ten hours up to 45 °C (Boer & Peralta 2000).

Effect of metal ions

Generally metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at higher temperatures. The presence of Ca^{+2} is known to activate the protease and increase the thermostability as mentioned by Kotlova *et al.,* (2007).
Beauveria sp secretes protease in short periods on simple and inexpensive medium and finds application in leather processing for dehairing, textile industry. This protease is able to separate the endothelial cells and can be used in animal cell culture, it is compatible with many of the commercial detergents preparations (Shankar *et al.,* 2011) Therefore, it was of interest to analyze the crude protease with respect to optimum conditions for activity, stability to pH, temperature, and metal ions etc. and is helpful in evaluating its prospective for other applications.

Materials and methods

Materials

Malt extract, yeast extract, peptone, oliec acid and linoleic acid were obtained from Hi-media chemicals, India. All other chemicals were of analytical grade. Xylan from oat spelts was obtained from Sigma CO. USA.Starch was obtained from AnalaR (AR). Wheat bran used was purchased from local market.

Methods

Fermentations

Fermentations were carried out in GYE + 2% MSC medium as described in Chapter 2 (Section A). Sophorolipids were added in the fermentation media aseptically just before inoculations.

Synthesis and extraction of Sophorolipid

For SL production, seed culture was prepared by inoculating 10mL of fresh MGYP nutrient medium with *C. bombicola* ATCC 22214 followed by incubation at 30℃, 180 rpm for 24 h. This inoculum was added to 90mL MGYP nutrient medium in a 500mL Erlenmeyer flask and incubated further for 48 h. Cells were harvested and washed twice with sterile distilled water. The
cell pellets (biomass ~1.5 g dry weight in 100mL medium) were redispersed in 100mL of 10% glucose solution supplemented with 1mL of Oleic acid (dispersed in 1mL ethanol), and again incubation was continued for 96 h when a brown and viscous SL mass was seen settled at the bottom of the flask. It was separated using a pipette tip cut at the nozzle and subjected to ethyl acetate extraction. Culture medium was centrifuged at 5,000 rpm, at 10℃ for 20min.The supernatant was extracted twice with equal volumes of ethyl acetate, the organic layer was dried over anhydrous Na2SO4, and the solvent was removed by rotary vacuum evaporation. The yellowish brown semicrystalline product was washed twice with n-hexane to remove unconverted fatty acid (Joshi-Navre *et al.,* 2011).Linoleic acid sophorolipid was prepared by using linoleic acid instead of oleic acid

Protease production with sophorolipid

Protease production was carried out in GYE + 2% MSC medium with oleic and linoleic sophorolipid. Sophorolipids were dispersed in an appropriate quantity of absolute alcohol (99%) prior to addition into the experimental media. For different concentrations of sophorolipids 0 to 1.5 mg/ml sophorolipid concentrations were used in case of oleic as well as linoleic sophorolipid.

Protease Estimation

Protease estimations were carried out with casein as a substrate essentially as per described in Chapter 2 (Section A)

Scanning electron microscopy of the mycelium of Beauveria sp.

Beauveria sp. was grown media containing GYE + 2% MSC with and without sophorolipid for 96h at 28°C in shaking condition. Oleic sophorolipid was added at 1 mg/ml concentration. At the end of 96h both control and with sophorolipid sample was scanned on scanning electron microscope Stereoscan-440 Model from LEICA– Cambridge, U.K.

Effect of pH on Protease Activity and Stability

Optimum pH was determined by estimating the protease activity (Enzyme taken was 1.5 IU) at 50°C and pH values ranging from 5.0 to 12.0 (acetate buffer, pH 5.0; citrate buffer, pH 6.0; phosphate buffer, pH 7.0; Tris-HCl buffer, pH 8.0; carbonate buffer, pH 9.0. and 10.0; and sodium phosphate–NaOH, pH 11.0). Stability of protease was examined by incubating the enzyme at 28°C in buffers at pH values ranging from 3.0 to 11.0 for 1h. Residual activity was estimated as described earlier and expressed as percentage of the initial activity which was considered as 100%.

Effect of Temperature on Protease Activity and Stability

Optimum temperature was determined by estimating the protease activity at pH 9.0 and temperatures ranging from 30 to 70°C for 10 min (Enzyme taken was 1.5 IU). Thermal stability was examined by incubating the enzyme at temperatures ranging from 4 to 70°C for 1 h. Thermal stability was also examined by incubating the enzyme at 50°C, pH 9.0 up to 2 h. Residual activity was measured at 50°C, pH 9.0 and expressed as percentage of initial activity which was considered as 100%.

Effect of metal ions

Effect of metal ions was studied on protease activity during estimation. While estimating the protease activity metal ions were added to the reaction mixture at 10mM final concentration in the reaction mixture. Contol reaction was carried out without any metal ions and the activities are calculated taking control activity as 100%.

Results and Discussion

Industrially important proteins, like enzymes, are required in huge quantity and should be synthesized by cost effective methods. Due to the constant requirement of such enzymes, simple media containing renewable sources are required to be used for over-production. To study the possible enhancement in protease production sophorolipid which is produced in our laboratory was used in the fermentation media.

Protease production was carried out in GYE + 2% MSC medium with the addition of 1mg/ml oleic sophorolipid. It can be seen from Table 3.1a that maximum increase in protease production was 2.31 fold after 120h with 1.38 fold increase after 96h.

Table 3.1a: Fold increase in protease production by addition of oleic sophorolipid

	Activity (%)		
	72h	96h	120h
Without oleic sophorolipid	100	100	100
With oleic sophorolipid	101.4	138.5	231.2

Similarly, protease production was carried out in GYE + 2% MSC medium with the addition of 1mg/ml linoleic sophorolipid. It can be seen from Table 3.2a that maximum increase in protease production was 1.69 fold after 120h with 1.51 fold increase after 96h.

Table 3.2a: Fold increase in protease production by addition of linoleic sophorolipid

	Activity %		
	72h	96h	120h
Without linoleic sophorolipid	100	100	100
With linoleic sophorolipid	100	151.5	169.8

Since there was an increase observed by addition of both the sophorolipid effect different concentrations of both the sophorolipids was studied on protease production. The concentrations used for this study were in the range of 0.5 to 1.5mg/ml. This study was carried out from 72h to 144h. It can be concluded from Table no 3.3a and 3.4a that with oleic acid sophorolipid maximum increase in protease production was observed to be 2.48 fold with 1mg/ml concentration after 120h. It was also noted that

sophorolipid concentration above that was inhibitory for protease production. Whereas in the case of linoleic acid sophorolipid maximum increase in protease production was observed to be 1.65fold with 1mg/ml concentration after 120h.

Protease production in optimized medium with sophorolipid

Protease production in was concluded to be maximum in GYE+ 2% MSC + Oleic acid Sophorolipid (1mg/ml). Effect of oleic acid sophorolipid was better for protease production than linoleic acid sophorolipid. Protease activity which was 3.94 IU/ml before optimization was found to be 32 to 36 IU/ml after media optimization.

Table 3.3a: Effect of concentration of oleic sophorolipid on protease production

SL conc (mg/ ml)	**Relative activity (%)**			
	72h	**96h**	**120h**	**144h**
nil	100	100	100	100
0.25	52.67	109.5	165.68	149.5
0.50	68.76	117.6	181.89	153.40
1.0	97.43	106.6	247.98	142.4
1.50	41.22	48.07	67.44	39.66

Table 3.4a: Effect of concentration of linoleic sophorolipid on protease production

SL conc (mg/ ml)	**Relative activity (%)**			
	72h	**96h**	**120h**	**144h**
nil	100	100	100	100
0.25	38.07	79.50	116.86	113.61

0.50	46.42	87.30	131.93	107.21
1.0	73.43	129.60	163.44	132.3
1.50	51.82	55.03	67.65	59.23

Scanning electron microscopy of the mycelium of *Beauveria* sp.

Scanning electron microscopy of *Beauveria* sp. was carried out after growth of 96h in media containing GYE + 2% MSC with and without sophorolipid. It is seen from Fig 3.1a control experiment (without sophorolipid in the medium) showed intact cells with contents where as the sample with addition of sophorolipid, the mycelia were found smoother and showed weakening of cell wall and contents have secreted out. This may be due to the ability of sophorolipid to destabilize cell membranes or permeabilized by disturbing their integrity and permeability. Due to this, the maximum enzyme produced by the fungal cells was leached out in the medium effectively and the production was increased.

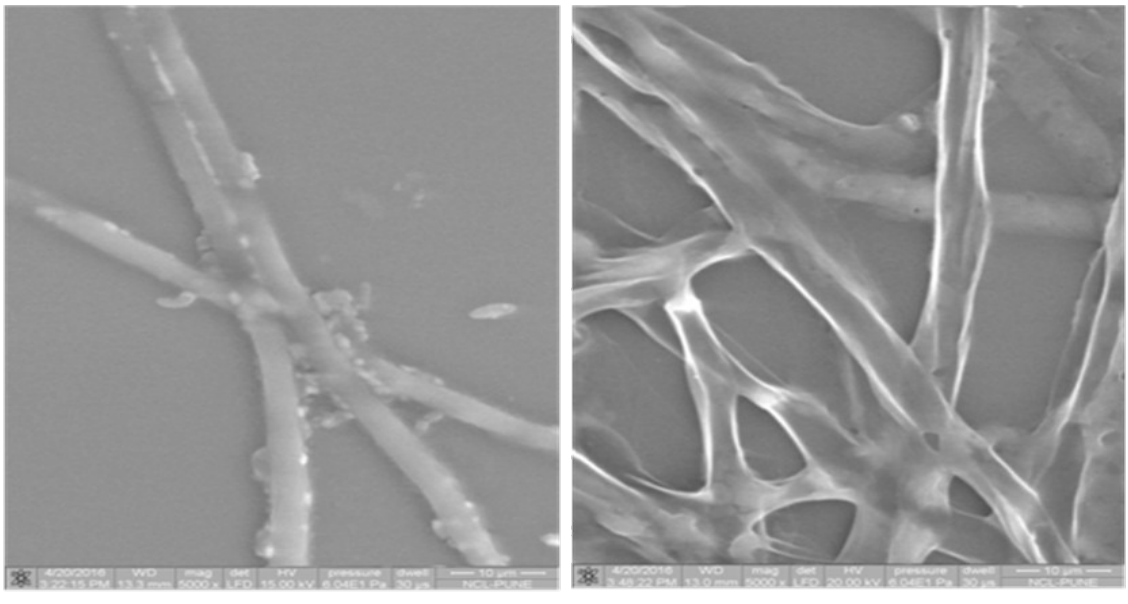

Fig 3.1a: Effect of sophorolipid on the growth of Beauveria sp.

Properties of protease from *Beauveria sp.*

For industrial applications, it is very important to know the most favourable conditions at which the enzyme acts optimally. Proteases must have activity and stability under relatively hostile conditions, often comprising extremes in temperature, pH etc. Hence properties like optimum pH and stability, optimum temperature and stability and stability in presence of metal ions were studied for the protease.

Optimum pH

Effect of various pH ranging from 5.0 to 12.0 was checked on protease activity. It is observed that maximum activity was at pH 9.0 and 90% activity was at pH 10.0. At pH 8.0 activity was 60 % wherein pH 11.0, activity of the enzyme dropped to 30 % These results are similar with the report from Chi et al (2007) who reported that the *Aureobasidium pullulans* protease had an optimum pH of 9.0. Also in another report extracellular alkaline protease from *Aspergillus clavatus* was seen *to* be optimally active at pH 9.5 (Tremacoldi and Carmona 2005). A number of proteases from bacteria and fungi like *B. subtilis* DM-04 (Mukarjee *et al.,* 2008), *A. oryzae* (Sandhya *et al.,* 2005), *B. felina* (Agarwal *et al.,* 2005), A. *niger* MTCC 218 (Prathaman et al 2009), and *A. oryzae* CFR305 (Murthy *et al.,* 2010) are reported to have pH optima between 7.0 to 7.5. On the other hand, Jellouli *et al.,* (2011) and Srinivas and Naik et al (2011) observed pH optima of 10.0 and 11.0 for protease from *Bacillus licheniformis* MP1 and *Bacillus halodurans* JB 99 respectively.

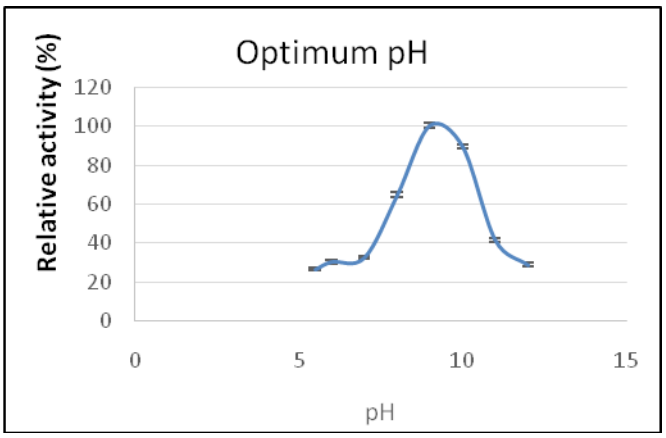

Fig 3.2a: Optimum pH of protease from *Beauveria* sp.

Optimum Temperature

Effect of temperature on protease activity was studied in the temperature range of 30 to 70°C. It can be observed from Fig 3.3a that optimum temperature for protease from *Beauveria* sp. was 50°C. The protease activity at 40 and 60°C was found to be 60%. At 30°C and 70°C approximately 30% residual activity was observed. Similarly, Hajji *et al.,* (2007) reported that protease from *Aspergillus clavatus* ES1 was optimally active at 50°C. For some of the fungi like *Penicillium* sp. (Agrwal *et al.,* 2004), *B. felina* (Agrwal *et al.,* 2005) and *A. oryzae* CFR305 (Murthy *et al.,* 2010) the mesophilic temperature optima was reported. Another report from Prathaman et al in 2009 mentioned optimum temperature 35°C for A .niger protease. Srinivas and Naik *et al.,* (2011) and Jellouli *et al.,* (2011) observed temperature optima of 70°C for protease from *Bacillus halodurans* JB 99 and *Bacillus licheniformis* MP1 respectively.

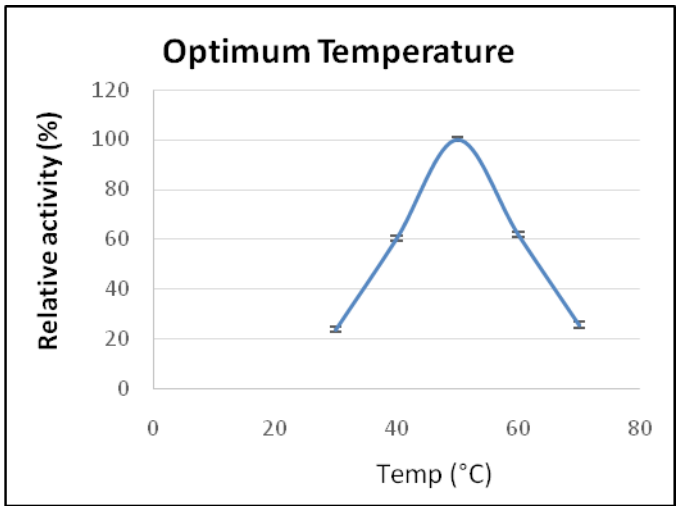

Fig 3.3a: Optimum Temperature of protease from *Beauveria* sp.

pH stability

pH stability of protease from *Beauveria* sp. was investigated in the pH range of 4.0 to 11.0. It is concluded from Fig 3.4a that the protease was stable in a wide pH range. At pH 4.0 and 11.0 the residual activity was above 80% whereas at pH 8.0 to 10.0 it was almost 100%. Similar observations were made in alkaline protease from *Paecilomyces lilacinus which* was stable over a broader pH range 5.0 -11.5 (Kotlova et al 2007). Hajji *et al.*, (2007) reported alkaline protease from *Aspergillus clavatus* ES1 to be stable in the pH range of 7.0-9.0. Crude protease from *Penicillium* sp. was stable in the pH range of 6.0-9.0 (Germano *et al.*, 2003). Ammonium sulphate precipitated alkaline protease from *A. clavatus* was stable in the pH range of 6.0-12.0 but was unstable below pH 5.0 (Tremacoldi and Carmona, 2005).

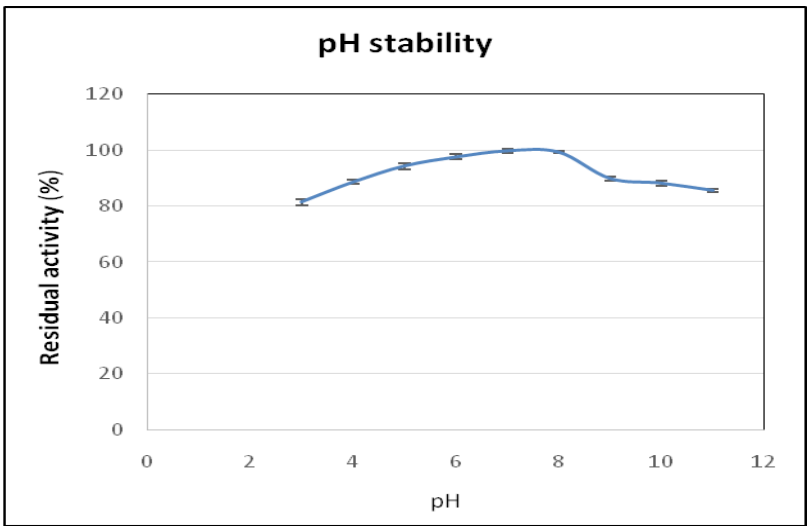

Fig 3.4a: pH stability of protease from *Beauveria* sp.

Temperature Stability

To study the temperature stability of protease from *Beauveria* sp. the enzyme was incubated in the temperature range of 4 to70°C for 1h and residual protease activity was estimated. It is seen from Fig 3.5a that the protease was stable at partially 45°C for 1h retaining above 60% of its activity. At 30°C 100% activity was noted and at 40°C above 80% activity was retained after 1h. *Protease* from *Thermoascus aurantiacus* (Merheb *et al.*, 2007) was stable up to 1 h at 60°C while the acid protease from *Rhizopus oryzae* was stable in the temperature range of 30–45°C (Kumar *et al.*, 2005).

Effect of metal ions on protease activity

Metal ions like Ca^{2+} and $Mg^{2+}$are known to activate while $Hg^{2+}$and Cu^{2+}etc are known to inhibit the proteases(Nirmal *et al.*, 2011). Effect of metal ions on protease activity was studied at 10mM final concentration in the reaction mixture. It can be concluded from fig 3.6a that Ag and Hg completely inhibited the activity while there was 20% inhibition by Cu^{2+} and Ni^{2+}. There was no enhancement in activity be any of the metal ions tested. The

addition of 5 mM CaCl2 enhanced the activity 105.3% of alkaline protease enzyme produced by *A. niger* (Devi *et al.*, 2008). Basu *et al.*, (2008) reported activation of protease from *A.niger* AB100 by metal ions such as Ca^{2+}, Fe^{2+}, Zn^{2+}andMg^{2+}. $Ca^{2+}$and Mg^{2+} also activated bleach stable alkaline serine protease from *Aspergillus clavatus* ES1 (Hajji *et al.*, 2007).

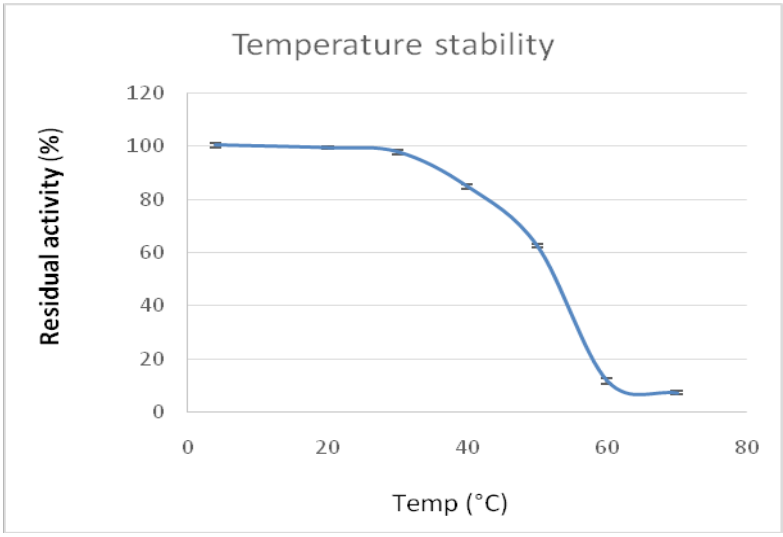

Fig 3.5a: Temperature stability of protease from *Beauveria* sp.

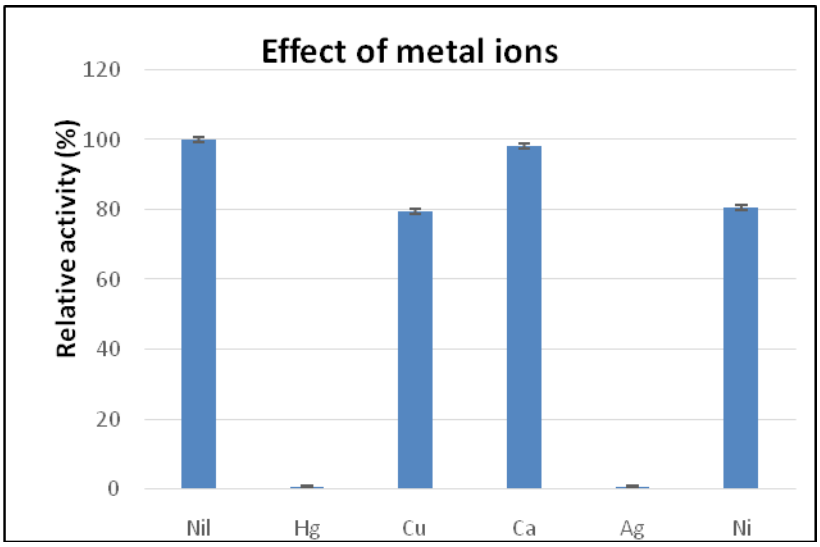

Fig 3.6a: Effect of metal ions on protease production

Conclusions

To enhance the protease production further oleic and linoleic sophorolipids were added to the fermentation medium. It was observed that when both the sophorolipids were added respectively to the medium the protease production was enhanced. The increased production was 231.2 and 169.8% in case of oleic and linoleic sophorolipid respectively. It was observed that 1mg/ml was the best concentration which showed a maximum increase in protease production. It was noted that oleic sophorolipid gave better enhancement than linoleic sophorolipid. Optimum pH was found to be at pH 9.0. Optimum temperature for protease was 50°C. Protease was stable in a broad pH range of 3.0-11.0. Protease was stable upto 45°C showing approximately 50% residual activities after 1h incubation. Effect of metal ions on protease activity was studied at 10mM final concentration in the reaction mixture Ag and Hg completely inhibited the activity while there was 20% inhibition by Cu and Ni.

References

- Agrawal, D. Patidar, P. Banerjee, T. Patil, S. (2004) Process Biochem.39: 977–981.
- Agrawal, D. Patidar, P. Banerjee, T. Patil, S. (2005) Process Biochem. 40: 1131–1136.
- Ananthan G. (2014) World J of Pharma and Pharma Sci 3 (12): 756-770.
- Basu, B.R. Banik, A.K. Das, M. (2008) World J. Microbiol. Biotechnol. 24 449–455.
- Bernfeld P. (1955). Methods in Enzymology 1:149-158.
- Boe,r C. G. Peralta, R. M. (2000)., *J Basic Microbiol,* 40: 75–81.
- Chi Z, Ma C, Wang P & Li H F (2007) *Bioresour Technol,* 98:534-538.
- Chi-Ming L.and Lu-kwang J. (2009) Enyzme microbial technol. 44: 107-111.
- Develter, D. W. G. Lauryssen, L. M. L. (2010) *Eur. J. Lipid Sci. Technol.* 112: 628–638.

- Ellaiah, P. Srinivasulu,B. Adinarayna, K. (2004) Process Biochem. 30:529-534.
- Evans E. C. Abdullahi C. (2012) Proc. Rom. Acad., Series B *2*: 108–112
- Gorin, P. A. J. Spencer J. F. T. Tulloch, A. P. (1961) *Can. J.of Chem.* 39: 846– 855.
- Gross, R.A. Shah, V. Nerud, F. Madamwars, D. (2015) US patent No. US9012194 B2
- Gupta, R., Beg, Q. K., Khan, S. and Chauhan, B. (2002). *Appl Microbiol Biotechnol*, 60: 381-395.
- Hajji M, Kanoun S Nasri M & Gharshallah N (2007). *Proc Biochem,* 42:791-797.
- Hrmova, M., Petrakova, E., Biely, P., (1991) Journal of General Microbiology 137, 541-547.
- Jellouli, K. O.G. Bellaaj, O.G. H.B. Ayed, H.B. Manni, L. Agrebi, R. Nasri, M. (2011) Process Biochem. http://dx.doi.org/10.1016/j.procbio.2011.02.012
- Joshi-Navare, K. Shiras, A. Prabhune, A. (2011) *Biotechnology Journal*, 6(5): 509– 512.
- Khandelwal, H. B. (2013) Ph. D. Thesis Savitribai Phule Pune University.
- Kotlova E K, Ivanova N M, Yusupova M P, T L, Ivanushkina NE & Chestukhina G G (2007) *Biochem (Moscow*), 72: 117-123.
- Kumar S, Sharma N S, Saharanb M R & Singh R (2005 *Proc Biochem,* 40: 1701–1705.
- Lang, S. Hubbard, A. T. in *Novel surfactants: preparation, applications and biodegradability*, 2003, vol. 114, pp. 279–315.
- Laxman, R. S. Sonawane, A. P. More, S. V. Rao, B. S. Rele, M. V. Jogdand, V. V. Deshpande, V. V. Rao, M. B. (2005). *Proc Biochem,* 40: 3152–3158.

- Merheb C W, Cabral H, Gomes E & Da-Silva R (2007) *Food Chem*, 104: 127–131.
- Mostafa, S. E. Moataza, M. Awad, H. M. Selim M. H. Hassan H. M. (2012) E-J of Chem 9(2): 949-961
- Mrudula, S. Apsana Begum, A. Ashwitha K. and Pavan Kumar P. (2012) Int J Pharm Bio Sci 3(3): 619 – 631
- Mukherjee, A.K. Adhikari, H. Rai, S.K. (2008) Biochem. Eng. J. 39: 353–361.
- Murthy, P.S. Naidu, M.M. (2010) World Appl. Sci. J. 8: 199–205
- Nirmal, N. P. Shankar, S. Laxman, R. S. (2011) *Int. J.Biotech & Biosci.*, 1 (1) : 1-40
- Paranthaman, R. Alagusundaram, K. Indhumathi, J. (2009) World J. Agric. Sci. 5: 308–312.
- Reese, E.T. and Maguire A.(1969). *Appl Microbio,* 17: 242- 245.
- S. Shankar, M. Rao, R.S. Laxman, (2011) Process Biochem. 46: 579–585.
- Sandhya, C. A. Sumantha, A. Szakacs, G. Pandey, A. (2005) Proc. Biochem. 40: 2689–2694.
- Shrinivas, D. Naik, G.R. (2011) Int. Biodeterior. Biodegrad. 65: 29–35.
- Tremacoldi C R & Carmona E C (2005) *World J Microbiol Biotechnol,* 21: 169-172.

SECTION B

Effect of addition of sophorolipids on xylanase production by *Beauveria sp.* and properties of xylanase

Abstract

Effect of addition of oleic acid sophorolipid and linoleic acid sophorolipid was studied on xylanase production. It was observed that when 1mg/ml of sophorolipid was added to the medium the xylanase production was enhanced. The increased production was 148.9 and 168.5% in case of oleic and linoleic sophorolipid respectively. Since both the sophorolipids showed increased production, various concentrations of sophorolipids were tested for xylanase production. It was observed that 0.5mg/ml was the best concentration which showed a maximum increase in xylanase production with150.3 and 250.6% with oleic and linoleic sophorolipid respectively. Physical properties of xylanase like optimum pH and pH stability and optimum temperature and temperature stability were studied.It was concluded that optimum pH and temperature for xylanase was pH 6.0 and 50°C. Xylanase was stable in the pH range of 4.0- 8.0. Xylanase was stable upto 50°C showing approximately 50% residual activities after 1h incubation.

Introduction

Effect of chemical surfactants on enzyme production

Effect of chemical surfactants on general enzyme production has been described in the section A of this chapter.

Effect of chemical surfactants on xylanase production

There are many reports available on the effect of nonionic and ionic surfactants in fermentation medium for xylanase production by microorganisms in literature. Various surfactants like Tween-80 are commonly added to the medium to increase the yields of xylanases (Haltrich *et al.,* 1996; Kuhad *et al.,* 1998; Ding *et al.,* 2004). It is believed that Tween-80 enhances the permeability of cell membranes and thus affects the secretion of certain proteins, even though the precise mechanisms of action have not been determined (Haltrich *et al.,* 1996). Saleem, Akhtar, and Jamil (2002) reported that supplementation of 0.2% concentration of tween-80 had a positive effect on the production of xylanase by Bacillus subtilis. Tween-80 stimulated the xylanase synthesis in *T. lanuginosus* CAU44 and enhanced xylanase production by 1.6-fold (Jiang *et al.,* 2005). They also studied the effect of Triton X-100 and fatty acid (olive oil) on the xylanase production by *T. lanuginosus* CAU44 and found that both of them did not have a significant effect on xylanase production. Xylanase production by *Trichoderma viride*-IR05 was enhanced by the addition of various enhancers such as tween-80, Triton X-100 and sodium dodecyl sulfate (SDS). There was an increase in xylanase production by 1.45, 1.30 and 1.06fold by Tween 80, TritonX-100 and SDS respectively (Irfan et al 2014). The effect of different surfactants (Tween 80, Triton X 100 and SDS) on the xylanase production by *T. harzianum* MTCC 4358 was investigated. Among these materials, only Tween80 exerted a marked effect on the production (Sakthiselvan *et al.,* 2012).

Effect of sophorolipids on enzyme production production

Effect of sophorolipids on enzyme production by microorganisms' has not been investigated. There are one or two reports that are discussed in the section A of this chapter.

As of now, there are no such reports on the effect of sophorolipid on xylanase production. That provoked us to study the effect of sophorolipids on xylanase production by *Beauveria sp*. Effect of oleic acid sophorolipid

and linoleic acid sophorolipid on xylanase production by *Beauveria* sp. is described in this section.

Natural microorganisms have over the years been an enormous resource of enzyme diversity. With the increasing apprehension in relation to environmental issues, microbial enzymes have emerged as alternative biocatalyst in quite a lot of industrial processes. Their usefulness is escalating day by day, as new enzymes with novel properties are being explored. Properties like optimum pH and optimum temperature and stability of enzymes is very important for any commercial applications.

Optimum pH and pH stability

Optimum pH and pH stability remains to be a critical parameter for biotechnological applications of xylanases. Majority of the xylanases have optimum pH in the range of 5.0 to 7.0. Ahmad *et al.,* (2012) reported pH 5.0 as optimum for *T. harzianum* whereas *T. viride* xylanase exhibited optimal activity at pH 6- 6.5 (Fortkamp and Knob 2014). Xylanases ehibit pH stability over a wide pH range. Xylanase from T. reesei displayed stability at pH values of 3.0 -7.5 (Huang *et al.,* 2013) whereas Irfan and Syed (2012) showed that xylanase produced from *T. viride* was stable in the pH range of 4.0 to 7.0.

Optimum temperature and temperature stability

Optimum temperature and stability is also one of the important parameter for xylanases for their use in industries. Ali *et al.,* (2016) reported temperature optima at 40°C for xylanase from *F. fomentarius.* The optimal temperature for xylanases from *T. harzianum* xylanases was 60°C.

Xylanase from *T. viride* was stable at 40°C upto 90 min (Fortkamp and Knob 2012). Adesina *et al.,* (2017) reported that the xylanase from

Fusarium Spp was stable up to 55°C retaining up to 75% of its activity after one hour exposure

Effect of metal ions

Some metal ions protect the enzymes from denaturation and addition of metal ions sometimes is known to stimulate the xylanase activities (Irfan and Sayed 2012). Sathiyavathi and Parvatham (2013) also reports similar results in the case of *Trichoderma* sp. whereas Sathiyavathi and Parvatham (2013) in the case of *Trichoderma* sp. whereas Soroor *et al.,* (2013) reported xylanases from *T. reesei* to be inhibited by Cu and Hg^{2+}.

Materials and methods

Materials

Malt extract, yeast extract, peptone, Oleic acid and linoleic acid were obtained from Hi-media chemicals, India. All other chemicals were of analytical grade. Xylan from oat spelts was obtained from Sigma CO. USA. Wheat bran and rice bran used were purchased from local market.

Methods

Fermentations

Fermentations were carried out in MGYP + 2% WB medium. Inoculum was used at 10% (V/V) as described in Chapter 2 (Section B).

Synthesis and extraction of Sophorolipid

Synthesis and extraction of oleic and linoleic acid sophorolipids were carried out as described in Chapter 3 Section A.

Xylanase production with sophorolipid

Protease production was carried out in MGYP + 2% wheat bran medium with oleic acid and linoleic acid sophorolipid. Sophorolipids were dispersed in an appropriate quantity of absolute alcohol (99%) prior to addition into the experimental media. For different concentrations of sophorolipids 0 to 1.5 mg/ml sophorolipid concentrations were used in case of oleic as well as linoleic sophorolipid. This study was carried out from 72h to 120 h.

Xylanase estimation

Xylanase estimations were carried out by DNSA method as per described in Chapter 2 (Section B)

Effect of pH on Protease Activity and Stability

Optimum pH was determined by estimating the protease activity at 50°C and pH values ranging from 5.0 to 12.0 (acetate buffer, pH 5.0; citrate buffer, pH 6.0; phosphate buffer, pH 7.0; Tris-HCl buffer, pH 8.0 and carbonate buffer, pH 9.0). Stability of protease was examined by incubating the enzyme at 28°C in buffers at pH values ranging from 3.0 to 11.0 for 1 h. Residual activity was estimated as described earlier and expressed as percentage of the initial activity taken as 100%. (Enzyme taken was 1.5 IU).

Effect of Temperature on Protease Activity and Stability

Optimum temperature was determined by estimating the protease activity at pH 9.0 and temperatures ranging from 30 to 70°C for 10 min(Enzyme taken was 1.5 IU). Thermal stability was examined by incubating the enzyme at temperatures ranging from 4 to 70°C for 1 h. Thermal stability was also examined by incubating the enzyme at 50°C, pH 9.0 up to 2 h. Residual activity was measured at 50°C, pH 9.0 and expressed as percentage of initial activity taken as 100%.

Effect of metal ions

Effect of metal ions was studied on protease activity during estimation. While estimating the protease activity metal ions were added to the reaction mixture at 10 mM final concentration in the reaction mixture. Contol reaction was carried out without any metal ions and the activities are calculated taking control activity as 100%.

Results and discussion

Xylanase production was carried out in MGYP + 2% WB medium with the addition of 1mg/ml oleic and linoleic sophorolipid. It can be seen from Table 3.5b that maximum increase in protease production was 1.68 fold and 1.41 fold increase after 96h with oleic and linoleic acid sophorolipid respectively.

Table 3.5b: Fold increase in Xylanase production by addition of Oleic and Linoleic sophorolipid.

	Activity %		
	72h	96h	120h
Without sophorolipid	100	100	100
Linoleic sophorolipid	123.4	168.5	152.2
Oleic sophorolipid	101.8	140.9	110.6

Since there was an increase observed by addition of both the sophorolipids on xylanase production, effect different concentrations of both the sophorolipids was studied on xylanase production. The concentrations used for this study were in the range of 0.5 to 1.5mg/ml. It can be concluded that with oleic acid sophorolipid maximum increase in xylanSase production was observed to be 1.50 fold with 0.5 mg/ml concentration after 96h (Table

3.6b). It was also noted that sophorolipid concentration above that was inhibitory for xylanase production

Table 3.6b: Effect of concentration of oleic sophorolipid on xylanase production

SL conc (mg/ ml)	Relative activity (%)		
	72h	96h	120h
nil	100	100	100
0.25	77.40	139.90	88.63
0.50	83.63	150.30	119.1
1.0	98.21	135.90	89.28
1.50	18.1	38.30	74.7

Similarly, effect of linoleic acid sophorolipid was studied in the range 0.5 to 1.5 mg/ml concentration. It was also noted that sophorolipid concentration above that was inhibitory for protease production observed to be 1.65fold with 1mg/ml concentration after 120h. It can be concluded from Table 3.7b that with linoleic acid sophorolipid maximum increase in xylanase production was observed to be 2.50 fold with 0.5 mg/ml concentration after 96 h. Concentrations of sophorolipid beyond that inhibited xylanase production.

Table 3.6b: Effect of concentration of lenoleic sophorolipid on xylanase production

SL conc (mg/ ml)	Relative activity (%)		
	72h	96h	120h
nil	100	100	100
0.25	97.21	210.7	145.7
0.50	100.60	250.60	136.0

1.0	93.61	168.40	140.20
1.50	13.4	17.4	30.7

Xylanase production in optimized medium

Xyalanase production in was concluded to be maximum in MGYP+ 2% Wheat bran + linoleic Sophorolipid (0.5 mg/ml). Xylanase activity after optimization was found to be 17 to 19 IU/ml after media optimization which was 7.0 IU/ml before optimization.

Properties of xylanase from *Beauveria* sp.

Optimum pH

To investigate the optimum pH of xylanase from Beauveria sp., xylanase activity was studied at various pH ranging from 3.0 to 9.0. It was observed that the optimum pH for xylanase was at pH 6.0. At pH 5.0, 90% activity was found and at pH 7.0 approximately 70% activities was observed (Fig 3.7b). In agreement with our result *T. viride* xylanase also exhibited optimal activity at pH 6- 6.5 (Fortkamp and Knob 2014). Ali *et al.*, (2016) reported similar pH optima at pH 6.0 for xylanase from *F. fomentarius.*While Irfan and Syed (2012) and Ahamad *et al.,* (2012) reported pH optima at pH 5.0 for xylanases from *T. viride* and *T. harzianum* respectively. Xylanase (Xyn628) 01from *Streptomyces* sp. CS628 was reported to have optimum pH of 11.0 (Rehman *et al.,* 2014). Pithadiya *et al.,* (2016) reported optimum pH for *Bacillus circulans* at pH 8.0.

Optimum Temperature

Effect of temperature on xylanase activity was studied in the temperature range of 30 to 70°C. It can be observed from Fig 3.8b that optimum

temperature for xylanase from *Beauveria* sp. was 50°C. The xylanase activity at 40, 45 and 55°C was found to be above 80% and 60°C only 50 % activity was retained which dropped further to 30% at 70°C.

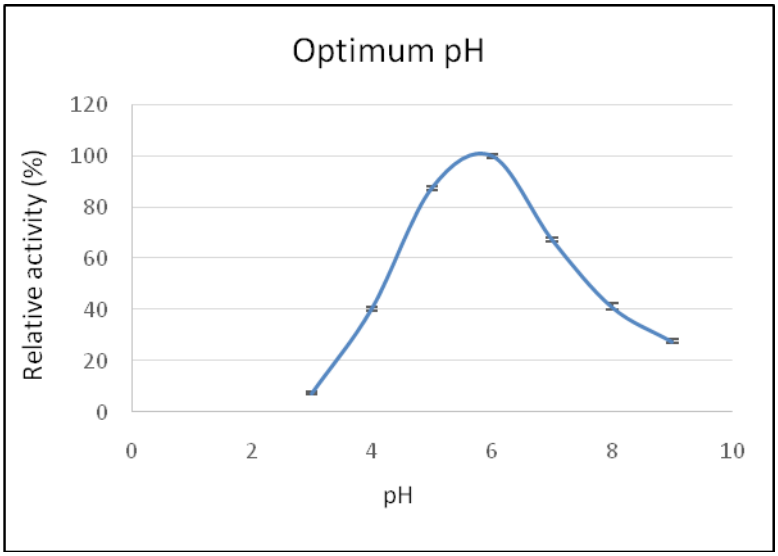

Fig 3.7b: Optimum pH of xylanase from *Beauveria* sp.

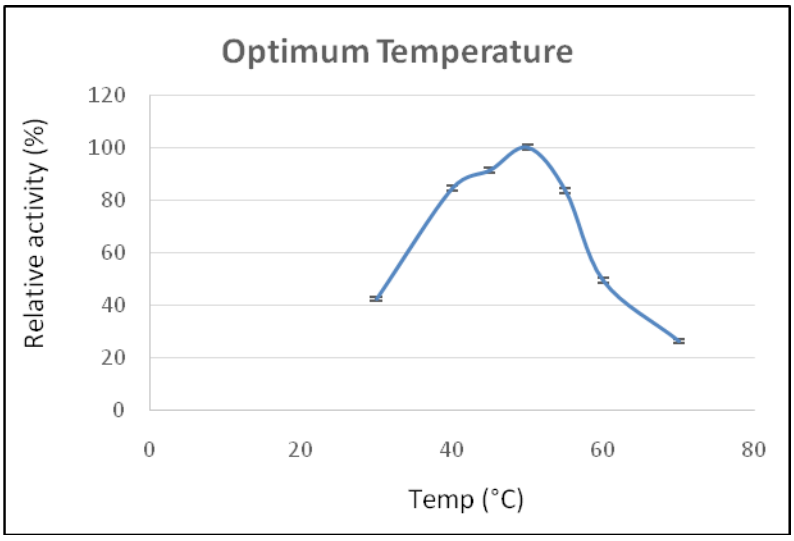

Fig 3.8b: Optimum Temperature of xylanase from *Beauveria* sp.

This temperature has been reported as optimal from *Trichoderma* spp. (Irfan and sayed 2012; Lopes *et al.*, 2013), *T. viride* (Fortkamp and Knob 2014). Ali

et al., (2016) reported temperature optima at 40°C for xylanase from *F. fomentarius.* The optimal temperature for xylanases from *T. harzianum* xylanases was 60°C. Similarly, xylanase (Xyn628) from *Streptomyces* sp. CS628 was reported to have an optimum temperature of 60°C (Rehman *et al.,* 2014). On the other hand, Pithadiya *et al.,* (2016) reported optimum temperature for *Bacillus circulans* at 30°C

The enzyme stability remains the significant characteristic for its biotechnological aspects. Hence the pH and thermal stability of xylanase from *Beauveria* sp. were investigated.

pH stability

pH stability of xylanase from *Beauveria* sp. was investigated in the pH range of 3.0 to 11.0. The enzyme was stable from pH 4.0 to pH 8.0. Enzyme started losing its activity at pH 9.0 and also at pH 3.0 only 50% residual activity was observed (Fig 3.9b). Irfan and Syed (2012) studied that xylanase produced from *T. viride* showed stability in the pH range of 4.0 to 7.0. Whereas xylanase from T. reesei displayed stability at pH values of 3.0 -7.5 (Huang *et al.,* 2013). Xylanase (Xyn628) from *Streptomyces* sp. CS628 was found to be stable in a broad range of pH 5.0–13.0 (Rehman *et al.,* 2014). Adesina *et al.,* (2017) reported that the xylanase from *Fusarium* Spp was stable up to pH range of 4.0 to 8.0. It was able to retain up to 80% activity after exposure for one hour to pH 9.0.

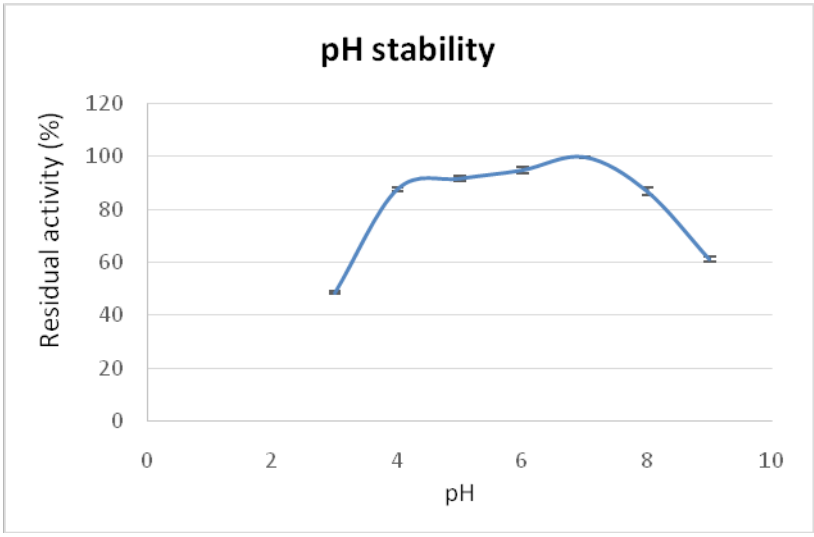

Fig 3.9b: pH stability of xylanase from *Beauveria* sp.

Temperature stability

To study the temperature stability of xylanase from *Beauveria* sp. the enzyme was incubated in the temperature range of 4 to70°C for 1h and residual xylanase activity was estimated. As seen in Fig 3.10b that the protease was stable upto 40°C for 1h and there was gradual drop in the activity. At 50°C only 50% activity was retained which was further dropped below 40% at 60°C and only 10% activity was retained at 70°C (Fig 3.10b). Xylanase from *T. viride* was stable at 40°C upto 90 min (Fortkamp and Knob 2012). Xylanase (Xyn628) from *Streptomyces* sp. CS628 was found to be stable upto 60°C (Rehman *et al.*, 2014). Adesina *et al.*, (2017) reported that the xylanase from *Fusarium* Spp was stable up to 55°C retaining up to 75% of its activity after one hour exposure.

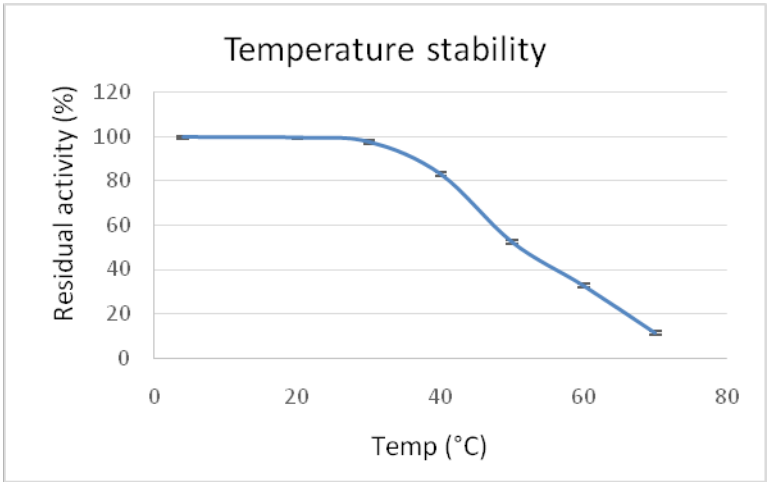

Fig 3.10b: Temperature stability of xylanase from *Beauveria* sp.

Effect of metal ions on Xylanase activity

Effect of metal ions on xylanase activity was studied at 10mM final concentration in the reaction mixture. None of the metals had a stimulative effect on xylanase activity while Cu^{2+}, Fe^{2+}, Hg^{2+} and pb^{2+} inhibited the activity. In contrast to these results, Mg ions activated the xylanase activity from *T. viride* (Irfan and syed 2012). Similar results were also reported by Sathiyavathi and Parvatham (2013) in the case of *Trichoderma* sp. whereas Soroor *et al.*, (2013) reported xylanases from *T. reesei* to be inhibited by Cu and Hg^{2+}.

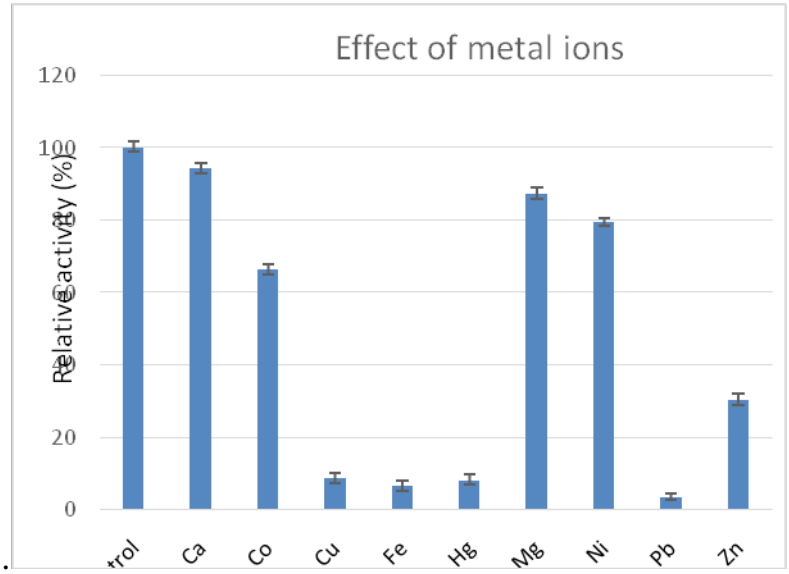

Fig 3.11b: Effect of metal ions

Conclusions

To enhance the xylanase production further oleic and linoleic sophorolipids were added to the fermentation medium. It was observed that when 1mg/ml of sophorolipid was added to the medium the protease production enhanced. The increased production was 148.9 and 168.5% in case of oleic and linoleic sophorolipid respectively. Since both the sophorolipids showed increased production various concentrations of sophorolipids from 0.5 to 1.5 mg/ml were tested for xylanase production. It was observed that 0.5mg/ml was the best concentration which showed a maximum increase in xylanase production. It was observed that 0.5mg/ml was the best concentration which showed a maximum increase in xylanase production with 150.3 and 250.6% with oleic and linoleic sophorolipid respectively. It was noted that linoleic sophorolipid gave better enhancement than oleic sophorolipid for xylanase production. Effect of various pH ranging from 5.0 to 12.0 was checked on xylanase activity. Optimum pH was found to be at pH 6.0. Effect of temperature on xylanase activity was studied in the temperature range of 30 to 70°C. Optimum temperature for xylanase was 50°C. Xylanase was stable in a broad pH range of 4.0-8.0. xylanase was stable upto 45°C

showing approximately 50% residual activity after 1h incubation. Effect of metal ions on xylanase activity was studied at 10mM final concentration in the reaction mixture. It can be concluded that Ag and Hg completely inhibited the activity.

References

- Fortkamp, D., Knob, A. (2014) *Afr. J. Bioechnol* 13(22): 2248-2259.
- Ahmad, Z., Butt, M. S., Anjum, F. M., Awan, M. S., Rathore, H. A., Nadeem, M. T., Ahmad, A. and Khaliq, A. (2012) *Afr. J. of Biotechnol*, 11(7): 1674-1682.
- Seyis, I. and Aksoz, N. (2005*Int. Biodeterioration & Biodegradation,* 55: 115-119
- Irfan, M. Syed, Q. (2012) *Int. J. Appl.Res.Nat. Prod*.5(1): 7-11.
- Haung, X. Ge, J. Fan, J. Chen, X. Xu, X. Li, J. Zhang, Y. Zhon, D. (2013) Afr. J. Microbiol.Res.7(36): 4521-4532.
- Lopes, A. M. Valeri, D. Pessoa-Junior A. P. (2013) *Afr.J. Biotechnol.* 12(19): 2640-2645.
- Rahman M. A. Choi Y. H. Pradeep G. C. Choi Y. S. Choi E. J. Cho S. S. Yoo J. C. (2014) *Appl. Biochem. Biotechnol.* 173:1469–1480
- Ali, M. B. Irshad M. Anwar Z. Zafar M. Imran M. (2016) *Adv. Enz. Res.* 4: 20-33
- Ding, C.H., Jiang, Z.Q., Li, X.T., Li, L.T. & Kusakabe, I.(2004) World J. Microbiol. Biotechnol. 20: 7–10.
- Haltrich, D., Nidetzky, B., Kulbe, K.D., Steiner, W. & Zupancic, S. (1996) Bioresource Technol. 58:
- 137–161.
- Saleem, M., Akhtar, M. S., & Jamil, S. (2002). J. Agric. Biol. 4.
- Gross, R.A. Shah, V. Nerud, F. Madamwars, D. (2015) US patent No. US9012194 B2
- Hrmova, M., Petrakova, E., Biely, P., (1991) *J. Gen. Microbiol.* 137, 541-547

- Sakthiselvan, P. Balakrishnan N. Nagarajan P. (2012) *Afr. J. Biotechnol.* 11(57): 12067-12077
- El-Gendy, M.M.A. El-Bondkly A.M.A. (2014) *J. Microb. Biochem. Technol.* 6(3): 54-166
- Adesina, F. C. Onilude, A. A. Oluboyede, O. A. Olajide, A. Bamkefa, B. Abiola-Olagunju, A. (2017) *EC Microbiol.* 6.1: 04-13.
- Pithadiya, D. Nandha, D. Thakkar A. (2016) *Arch. Appl. Sci. Res.* 8 (2):1-10

,

CHAPTER 4

Applications of Protease from *Beuveria* sp.

Abstract

Conventionally degumming is carried out at 90 to 110°C temperature by boiling the raw silk with Marseilles soap and sodium bicarbonate which eventually requires a lot of water and energy. In this study, degumming of

Chinese bivoltine raw silk fibres with alkaline protease produced by *Beauveria* sp. (MTCC 5184) was studied. Complete degumming was obtained in 45 minutes with 75 units of enzyme per gram of silk. Degumming was found to be optimal at 50°C and pH 9.00. Scanning electron micrographs showed that the sericin deposits were removed and the obtained fibres were clean, separated, had smooth feel with shine as compared to untreated fibres. Sericin isolated from silk cocoon (by-product which goes waste) was hydrolysed with the same alkaline protease obtained from *Beauveria sp.* to get small molecular weight peptides. These peptides can be utilized further for cosmetic, pharmaceutical and various industrial applications.

The by-product of silk processing, sericin is useful as it is antioxidant, UV resistant, absorbs and releases moisture easily. Sericin has a lot of biological activities such as antibacterial, antioxidant, tyrosinase inhibition, anti-cancer activities, cryoprotection and promotion of digestion. Sophorolipids show excellent skin compatibility and also have antibacterial property. An unusual formulation consisting of sericin and sophorolipid with sodium alginate as a binding agent was developed. Both the ingredients used are biocompatible and biodegradable. This cream/gel was tested for wound healing in Wistar rats. A commercial ointment providine was used as a control. The animal group, treated with Sericin and sophorolipid gel, showed fast contraction and healing when compared with control and standard compounds. These observations were validated by histopathological studies where more fibroblast proliferation, angiogenesis, keratinisation was observed. Antioxidant activity of the formulation was checked using 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

Introduction

Silk

Silk has been used as a textile fiber for over 5000 years. Silk carries desirable physical characteristics such as good mechanical properties, brightness, drape ability, comfort, softness, and dye ability. The convenience of reeling long (300–1200 m) continuous fibers from cocoons ha certainly contributed to its success as a specific fiber (Arami *et al.,* 2007). It is recognized for its characters such as luster, water absorption, heat retention, smooth feeling etc. Because of its sheen and luster it is known as the Queen of all fibers.

Silk fiber consists of the two fibrous proteins, fibroin and a gummed amorphous protein named sericin, which cements the fibroin fibers together. Fibroin and sericin proteins are present in about 75 and 25% of total weight respectively. Fibroin protein is a high molecular weight polypeptide (~ 350KDa), insoluble in hot water and made up of glycine, alanine and serine in molecular ratio 3:2:1, with a six residue repetition of -(Gly-Ser-Gly-Ala-Gly-Ala)n (Zhou *et al.,* 2000). This very much regular sequence is implicated in the formation of an antiparallel β-pleated sheet structure that characterizes the crystalline regions in the spun filament. Sericin is primarily amorphous and acts as a gum binder to maintain the structural integrity of the cocoon, so sericin is more water-soluble than fibroin. It is a yellow, brittle and inelastic substance. It conceals the unique luster of fibroin and acts as an adhesive for the twin fibroin filaments. Also, it prevents the penetration of dye liquor and other solutions during wet processing, so sericin needs to be removed before any further steps in silk processing. Removal of Sericin from fibroin is known as silk degumming and is an essential process to obtain an ideal fiber for the textile industry (More *et al.,* 2013).

Silk degumming

Degumming is a process where sericin is totally removed from the fibroin wall to obtain shine, smoothness and other properties (Freddi *et al.*, 2003). Removal of natural wax, some colouring components and mineral matter is also achieved during degumming process. Degumming of silk involves the cleavage of peptide bonds of sericin, either by hydrolytic or enzymatic methods, and the subsequent removal of sericin from the silk fibroin. The principle of silk degumming process is increasing the silk gum solubility by breaking the peptide linkage of sericin structure into small molecules such as amino acid and its oligomer with hydrolysis reaction.It is an important step and the glue around the fibers should be totally removed in order to prepare the fiber to successive mechanical process and attain a proper dyeing and boost the softness, absorbency, and luster. Degumming is a cost incurring process and it causes about 20-25% weight loss, decreases the yellowness and strength. As a result, the whiteness of the fibers can be increased due to the sericin removal. To preventing the fiber damages during the treatment, a number of parameters such as temperature, time and pH should be controlled (Talebpour *et al.,* 2013). Textile industries are under significant environmental pressure owing to its huge energy and water consumption and subsequent environmental pollution.

Conventional degumming

Conventionally, removal of sericin is achieved by boiling at high temperatures with a degumming solution containing soap or addition of chemical agents like acid, alkali or detergent. A weakly alkaline solution like sodium bicarbonate, ammonia or an acidic solution like tartaric, citric acid, succinic acid, dichloro, trichloro acetic acids have been used for degumming of silk Freddi et al 1996; Khan et al 2010). In the traditional methods of degumming, the raw silk fibers are treated with alkali and soap at 95°C–110°C or are boiled at elevated temperature and/or pressure for 1–2 h. Disadvantages associated with these methods are uneven degumming, loss

of strength in fibers and high resources consumption with respect to water and energy as well as the high output of effluents with polluting substances (Freddi *et al.*, 2003).

Arami *et al.,* (2007) reported conventional degumming with Marseille soap (5g/L) keeping the ratio of solid to liquid as 1:30 for 60 min. They observed that Marseilles soap treatment results in the complete removal of sericin, but the quantity of soap needed is high, and this makes the method expensive and nonecofriendly. Also, the higher temperature (100°C) most likely will cause partial degradation of fibroin. These are considered as the main disadvantages of the soap treatment method (the conventional method).

Enzymatic degumming

Enzymatic degumming involves the proteolytic degradation of sericin, using the specific proteins with minimum effect on fibroin. Enzymes are selective and biodegradable, and there is no soap required in the enzymatic degumming process; therefore, uneven dyeing problems caused by metallic soap can be avoided. Silk's affinity to dyes, especially to reactive dyes, is significantly improved by the enzymatic treatment. Enzymes are ecofriendly products, operate under mild conditions and low temperatures, and so consume less energy than other methods (Gulrajani *et al.,* 1990). Proteolytic enzymes do not readily attack fibroin in a fibrous form apparently because the protein chains in silk are densely packed without bulky side chains. It has a lesser risk of over degumming than alkaline soap degumming moreover weight loss can be easily modified by adjusting the concentration of enzyme, the reaction time and the use of optimum pH and temperature. Biodegumming with enzymes proved to be a prospective substitute to the chemical degumming process, which offers energy savings and environmental profit and does not damage the fibres. Several enzymes have been reported to be useful in degumming. However, the low degumming

efficiency of the enzymatic process and the high processing costs have hindered its application on an industrial scale (Shahid *et al.,* 2016).

Enzymatic degumming involves hydrolysis of sericin at the milder condition of pH and temperature. It also requires low input energy, less time with no hazardous chemical pollution (Rajasekhar *et al.*, 2011). However, the use of enzymes in the silk industry is relatively unexplored and it has generated a lot of interest only in the last twenty years (Arami *et al.,* 2007; Chopra and Gulrajani 1994; Fan *et al.,* 2010; Freddi *et al.,* 2003; Gulrajani 1992; Gulrajani *et al.,* 2000). Animal, plant and microbial proteases have been reported for degumming of silk (Gulrajani, 1992; Nakpathom *et al.,* 2009) Anis *et al.,* (2016) carried out degumming with Savinase at 500 C at pH 8.5 and weight loss obtained with 8% enzyme concentrations were comparable to the efficiency of the conventional method.

Microbial proteases used for degumming are mainly from *Bacillus* species, though a few fungal proteases are also used (Anghileri *et al.,* 2007; Arami *et al.,* 2007; Freddi *et al.,* 2003; Gulrajani *et al.,* 1996, 2000). Use of fungal proteases has the additional advantage of ease of downstream processing over bacterial proteases. However, most of these studies are carried out with commercial enzymes mainly proteases such as alcalase, savinase from Novozymes and degummase. Kim *et al.,* (2016) studied the effect of NaOH, Na_2CO_3, Alcalase and Savinase from Novozymes on degumming and they observed that enzymes were more competent degumming agents than alkalis when they were used in low concentrations, in the case of alkali treatments a substantial quantity of fabric weight loss was seen. They also reported that the amount of alkalis essential for the degumming process was 10-40 folds higher than enzyme concentration and enzymes presented higher efficiency in terms of weight loss. However, reports of degumming with fungal proteases are very few (More *et al.,* 2013). One report from our own group showed enzymatic degumming using two alkaline proteases from *C. brefeldianus* and an actinomycete (BOA-2). Complete degumming was

obtained with low enzyme concentrations in shorter durations of time (30–45 min). The fibers were comparable in tensile strength or elongation at break by enzymatic degumming demonstrating no strength loss (More *et al.,* 2013). Devi and Priyadarshini (2017) carried out degumming raw silk fabric using *Carica Papaya* skin and concluded that enzymatic degummed and dyed fabric showed better results when compared with commercial degumming in physical, comfort and mechanical properties.

Sericin

The byproduct of silk processing which is sericin is useful as it resists oxidation, antibacterial, UV resistant, and absorbs and releases moisture easily. Sericin protein can be cross-linked, copolymerized, and blended with other macromolecular materials, especially artificial polymers, to produce materials with improved properties. Sericin has a lot of biological activities such as antioxidant, tyrosinase inhibition and pharmacological functions such as anticoagulation, anti-cancer activities, cryoprotection and promotion of digestion. However, sericin as such is indigestible due to absorbability, protease digested small peptides obtained from Sericin show better bioavailability in the mucous membrane as compared to whole protein (Wu *et al.*, 2008).Therefore, to improve the functional and nutritional properties of native sericin, recent efforts are directed towards the production of bioactive peptides obtained from enzymatic hydrolysis. This could be optimally applied for functional food manufacture for human nutrition and health (Wu *et al.*, 2008).Lower molecular weight sericin peptides (≤ 20 kDa) are used in cosmetics like skin care and hair care products, health products and medications (Zhang, 2002).

Wound Healing

Wound healing is a complicated process in which the skin repairs itself after injury. The normal wound healing process can be broadly classified into three stages namely the haemostasis, inflammatory, proliferative and

maturation phases (Chen *et al.,* 2014). In the early stage of wound healing, a fibrin clot is produced at the site of the wound in a moment following injury, Fibrinogen is cleaved into fibrin monomers by thrombin, and the peptide monomers are polymerized by Factor XIII. The resulting fibrin acts as a scaffold for different cells to move about in and out of the wound bed. Five to seven days later than the initial injury, fibroblasts migrate to the wound site, secreting new collagen, and keratinocytes migrate from the wound edge and shape a thin epithelial cell layer to secure the wound (Tawil and Wu 2012). The inflammatory phase that lasts up to two days involves an orderly recruitment of cells to the wound area, which is followed by proliferative phase lasting up to 6 days. In this phase, the fibroblasts, keratinocytes and other cells in the wound bed begin to actively proliferate to close the wound. The maturation phase follows the proliferative phase, will take about two weeks, by which time the wound will be completely healed by restructuring the initial scar tissue. However, certain wounds are problematic and do not follow the normal time table for the healing process and take a longer time to heal. Re-epithelialisation is a critical step in wound healing; in which epidermal keratinocytes laterally migrate to close a wound. However, in chronic wounds, keratinocytes migration is blocked and the wounds remain open. Further, most wounds heal rapidly and efficiently but the results are not perfect, as the healing process leaves a scar on the skin. Scar tissue is less flexible than normal skin and can be cosmetically disfiguring affected area. As is evident from the above, the two major goals of wound healing (tissue repair) which includes rapid healing and complete reconstruction of the damaged area without leaving a scar.

Use of Sericin in wound healing

Sericin protein is useful because of its special properties viz., antioxidant, antibacterial, UV resistant, absorbs and release moisture easily and inhibits the activity of tyrosine kinase (Gulrajani 2005). Sericin is also biocompatible and biodegradable in nature. The most important feature of the silk protein

is that the dipeptides and tripeptides can easily permeate into the bloodstream through the dermis layer of skin (Komatsu *et al.,* 1975). Aramwit and Sangcakul (2007) evaluated the effects of sericin on wound healing and wound size reduction using rats. Along with 8% Sericin cream they used Betadine as a control. They found that sericin-treated wound-size reduction was much greater than in the control. Aramwit *et al.,* (2013) claimed the first report of demonstrating that sericin is safe and useful in the treatment of burn wounds and confirmed that sericin can promote the healing of open wound as shown by the scratch assay. Efforts are being made all over the world to discover agents that can promote fast wound healing and thereby reduce the cost of hospitalization and save the patient from other severe complications. The need for new therapeutics for wound healing has encouraged the drive to examine the nature and value of silk products. (Padol *et al.,* 2012)

Sophorolipid

Sophorolipids are glycolipid type of bio surfactant. Different biosurfactants possess antimicrobial property. Various biosurfactants, for example, from *Bacillus circulans*, *B. subtilis*, *B. licheniformis*, *Candida antarctica*, and *Pseudomonas aeruginosa*, have been reported to have potent antimicrobial activity. By its structure, biosurfactant is supposed to exert its toxicity on the cell membrane permeability as a detergent-like effect (Gharaei-Fathabad., 2011). Biosurfactants are coming up as emerging class of biomedical compounds. They are a suitable alternative to synthetic medicines and antimicrobial agents and could be used as safe and effective therapeutic agents (Singh and Cameotra, 2004)

The present Chapter deals with degumming of Chinese bivoltine silk using an alkaline protease from *Beauveria* sp. Degumming of silk at varying enzyme concentrations was studied in order to arrive at the minimum concentration of enzyme required for complete removal of sericin. Optimization with respect to temperature, time and pH was studied. The

efficiency of degumming was assessed in terms of weight loss, feel tensile strength, elongation at break as well as visible and scanning electron microscopic observations. Sericin obtained from degumming was hydrolyzed with the protease and small molecular weight peptides were analyzed with MALDI.

There are no reports on the use of sophorolipids in wound healing preparations so far, therefore, here we have attempted to find out whether sophorolipids can improve wound efficiency of sericin.

Materials and Methods

Materials

Chinese bivoltine silk twists (2 ply) from Central Silk Board, Bangalore, were used. Alkaline protease from *Beauveria sp.* (MTCC 5184) was produced by fermentation. Malt extract, yeast extract, beef extract, and peptone were obtained from M/s HiMedia Chemicals, India. Soybean and mustard seed cakes were purchased from M/S Ruchi Soya Industries Pvt Ltd., India and local market, respectively. Casein was procured from Sisco Research Laboratory, India. All other chemicals used were of analytical grade. Sodium alginate obtained from Sigma-Aldrich. Cipladin (Povidone-iodine) was purchased from local market.

Micro-organism and Protease production and protease assay

Micro-organism and Protease production and protease assay were carried out as described in Chapter 2 Section A.

Degumming Method

Chinese bivoltine silk twists were dried in hot air oven at 95°C –100°C for 3–4 h to remove traces of moisture. Enzymatic degumming was performed in a glass beaker with solid to liquid ratio of 1:30; protease concentrations varying from 10 to 100 U/g of silk yarn and in 100 mM buffer of required pH. Phosphate buffer for pH 7.0, Tris-HCl buffer for pH 8.0, and carbonate-

bicarbonate for pH 9.0 and 10.0 were used. The degumming process was carried out in a Julabo water bath set to temperatures ranging from 40°C – 65°C with intermittent rotation of the silk fiber.

Degumming of silk by conventional alkali-soap method was carried out with Marseilles soap and sodium bicarbonate at 95°C for 1 h with solid to liquid ratio of 1:30. All the degumming experiments were carried out in duplicate. Weight loss comparable to the conventional method is taken as complete degumming as it is considered to remove sericin completely.

Determination of Weight Loss

After degumming, silk fibers were washed with hot water at 65°C for 20 min followed by washing with cold water (cold wash) with intermittent shaking. Finally, silk fibers were kept in hot air oven for 6 h at 95°C –100°C for drying to reach a constant weight. Weight loss of treated silk denotes the quantitative evaluation of the degumming process. It was measured by calculating the difference in weight of silk fiber before and after degumming and expressed in terms of percentage weight loss. The release of sericin in to the reaction mixture was measured as an increase in turbidity by measuring the increase in absorbance at 660 nm.

Scanning Electron Microscopic Studies

Morphological characterization of silk fiber was performed by means of scanning electron microscope. The filaments of untreated, conventionally degummed and enzymatically degummed silk samples were scanned on scanning electron microscope Stereoscan-440 Model from LEICA–Cambridge, U.K.

Extraction of sericin proteins from degumming liquid

As sericin was visible as insoluble sediment at the bottom, it was separated from degumming liquor following degumming process. The sediment was separated by centrifugation at 1000 rpm for 5-10 minutes. Sericin pellet

(insoluble sericin) was washed with deionized water to remove soluble sericin as well as impurities. The soluble portion and insoluble portion was lyophilized. Soluble portion was used for hydrolysis and insoluble portion was used for the preparation of wound healing formulation.

Hydrolysis of sericin byprotease from Beauveria sp.

Hydrolysis of sericin was carried out with 2% sericin concentration in a total volume of 200 µl at 50°C; pH 9.00 (05 M carbonate-bicarbonate buffer. Hydrolysis was carried out at two different concentration 1 & 2 units for 15 min. At the end of hydrolysis, the enzyme was inactivated by boiling for 5 min. The mixture was centrifuged at 10000 rpm for 10 min. The supernatant was subjected to SDS–PAGE (15%) and protein bands were visualized by silver staining method as well as MADLI-TOF analysis.

MALDI-TOF of sericin hydrolysates

The lower weight molecular masses of sericin peptides were analyzed by MALDI–TOF mass spectrometry using Absciex (Applied Biosystems) unit. Four micro litres of hydrolysates was mixed with 16 µl of sinapinic acid and spotted on MALDI target plate, allowed to air dry and analyzed.

Formulation of wound healing gel

Sericin protein was obtained which was further subjected to lyophilisation to get a powder form. Sophorolipid from oleic acid was prepared as described in materials and methods in Chapter 3 section A. In order to optimize the concentration of gelling agent to achieve proper consistency of the gel formulations were prepared with different gelling or thickening agents, various gums, Carboxy methyl cellulose sodium, sodium alginate, Hydroxypropyl Methylcellulose (HPMC) and different concentration of viscosity enhancer as 1to 8% were tried and finally gel that showed good spreadability and consistency was selected.

Gelling agent sodium alginate was slowly added to the sericin gel with continuous stirring and heating on a water bath (Temp. 60-70°C). Sophorolipid was added to it and stirred continuously till a uniform gel was formed after 15-20 minute. Finally, the gel was placed in UV light for 20-25 min and stored in a plastic container at room temperature.

Table 4.1: Ingredients used for making formulation

	F1	F2
Ingredients	Quantity	Quantity
Sericin	8-10%	8-10%
Sophorolipid	1mg/ mL	--------
Sodium alginate	2-3%	2-3%

F1= Test gel, F2= Sericin control gel

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was carried out according to Tayori takechi *et al.*, 2014.

In-Vivo Wound Healing Model

In vivo experiments were performed at Symbiosis school of biomedical sciences, Lavale, Pune. Wister rats (Male) weighing 250 ±20 gm. used and all the study was performed as per CPCSEA guidelines (CPCSEA Reg. No. SSBS/ AH/04-2015).The animals were housed in a standard individual metal cages and room maintained at temperature 22±1^{O}C and relative humidity 55±5% with an alternating 12 h light–dark cycle. Food and water were provided *ad libitum*. All the experiments on animals were conducted after obtaining permission from Institutional Animal Ethical Committee of the Institute.

Pharmacological evaluation

The pharmacological evaluation was carried out with Incision wound model. Animals were divided into four groups (six animals each) viz, A- providine (market drug), B- Sericin and sophorolipid formulation, C- Only Sericin and D- untreated. Body weights of the animals were in the range of 240 to 270. All animals of four groups were anaesthetized with anaesthetic ether, and a paravertebral long incision of 4.4cm length was made through the skin and

cutaneous muscle at a distance about 1.5 cm from the middle on right side of the depilated back.

All group of the animal were received sufficient amount of preparation applied externally (Refer Table No). All the test formulations were applied once a day for 10 days starting from the day of the incision. The wound-healing property was evaluated by wound length and wound closure time. The wound area was measured immediately by placing a transparent paper over the wound and tracing it out on every alternate day.

Histopathological studies

Skin specimens from treated and untreated rats were collected in 10% buffered formalin and after the usual processing, 5 mm-thick sections were cut and stained with haematoxylin and eosin (McManus and Mowry, 1965). Sections were qualitatively assessed under the light microscope and graded with respect to fibroblast proliferation, collagen formation, epithelization, keratinization and scar formation.

Results and Discussion

Enzyme degumming involves the proteolytic degradation of sericin, using specific proteins with minimum effect on fibroin. Since enzymes are selective and biodegradable, there is no soap required in the enzymatic degumming process; therefore, uneven dyeing problems caused by metallic soap are avoided. Silk's affinity to dyes, especially to reactive dyes, is significantly improved by the enzymatic treatment.

Enzymatic degumming has a lesser risk of over alkaline soap degumming and weight loss can be easily modified by adjusting the concentration of enzyme, and the reaction time with optimum pH and temperature. In the enzymatic method, silk is treated at a low temperature which not only reduces energy cost but also prevents fiber weakness.

Effect of Enzyme Concentration on Degumming

Effect of enzyme concentration on silk degumming was studied using 25 to 100 units of enzyme per gram of silk. Degumming was also carried out with the conventional method as described in materials and method section. Enzymatic degumming was carried out at fixed time of 1h at pH 9 and 50°C. Comparison of the performance of *Beauveria sp.* protease at a fixed time the protease was able to degum the silk fibers completely since the weight loss was comparable to that of obtained by the conventional method. Conventional methods of degumming parameter were taken as control.

The increase in enzyme concentration resulted in an increase in weight loss (Fig 4.1). 50 U/g enzyme was optimum for the degumming comparable to conventional degumming with 20.8% weight loss. These results are in agreement with earlier reports where the different concentration of other enzyme cocktails was used for degumming of various silk verities such as crepe, tasar etc.

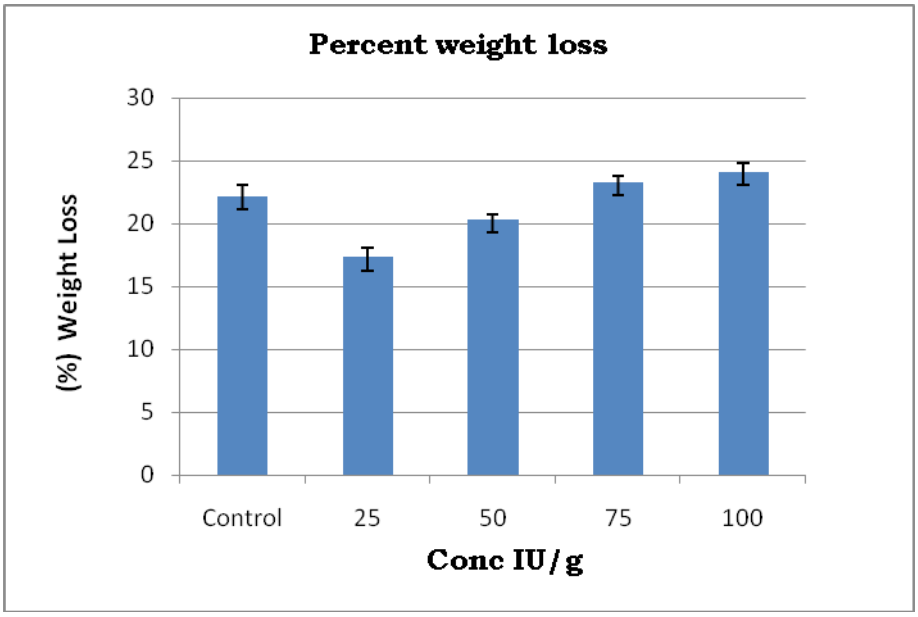

Figure 4.1: Effect of enzyme concentration on weight loss of silk after degumming

Gulrajani *et al.* reported degumming of tasar silk with degummase with 7.69% weight loss. Degumming of plain and crepe silks with 15% alcalase

for 3 h gave weight loss of 18.5 and 16.3% respectively (Gulrajani *et al.*, 2000). Arami *et al.* reported an increase in weight loss with an increase in enzyme concentration from 0.25 to 1g/L beyond which there was no further increase in weight loss. Krishnaveni and Rajkumar studied the effect of enzymatic degumming with biodegummase, papain, trypsin, and pepsin on dyeing of silk and reported higher weight loss (21%) compared with soap (16%).

Effect of Time on Degumming

Time kinetics experiment was carried out by enzymatic method. Effect of time on degumming was studied with time range 30-75 min to optimize time, required for complete degumming at 50°C, pH9.0 with 100u/g of the enzyme. It can be seen from Fig (4.2) that weight loss after degumming increased with incubation time. Almost 100% degumming was complete within first 30 min and thereafter the increase was slow. Weight loss after 1 h with proteases was 24.41%, while the weight loss after 30 min was 22.66%. Gulrajani *et al,* (1996) studied degumming with several proteases and found that most of the degumming was complete in first one hour and a further increase in treatment time from 1h to 3h did not bring about significant weight loss. Arami *et al.,* (2007) studied degumming of Persian silk and reported sericin removal of 21.52% with alcalase in 30 min, 20.08% with savinase in 60 min while it was 22.58% with soap in 120 min. Johnny and

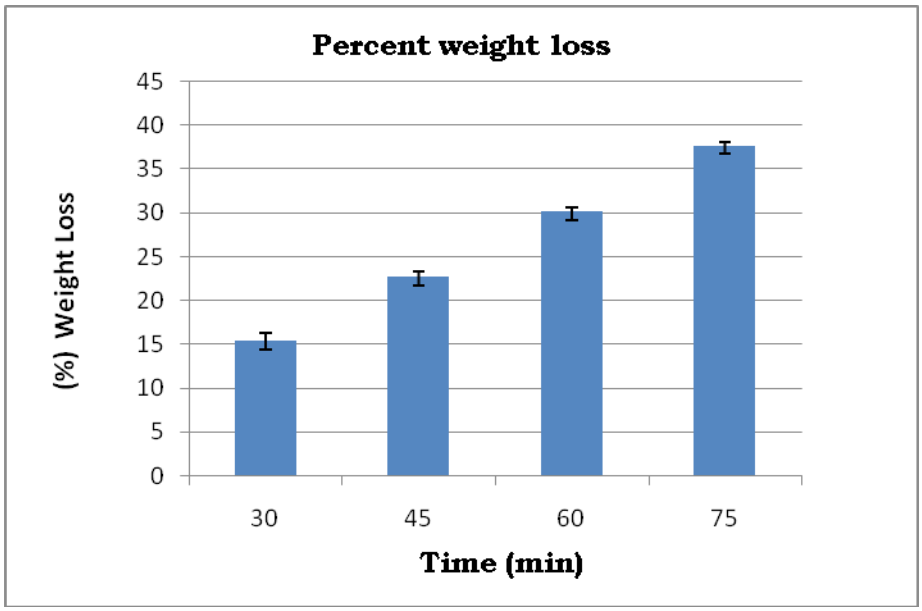

Figure 2: Effect of time on weight loss of silk after degumming

Chinnammal, (2010) performed degumming of Mulberry silk yarn with *Bacillus* protease for 6 h and resultant weight loss was found to be 25.68%. Puri, (2001) studied the silk-degumming efficiency of an alkaline protease from *Bacillus* sp. RGR-14 which showed a weight loss of 7.5% after 5 h incubation. Thus all the reports suggest that the time required for complete degumming is between 1h to 5h and our enzyme shows better results than many of these reports.

Effect of Temperature

Effect of temperature on degumming was studied in the temperature range of 30-70°C to optimize required temperature for complete degumming. Degumming was carried out at pH 9.0 with 100u/g of the enzyme. Highest weight loss with protease was observed at 50°C since the protease was optimally active at that temperature (Fig.4.3). Weight loss (%) of 16.37, 19.8, 17.67 and 15.54 was observed at 30, 40, 60 and 70°C respectively. Enzymatic degumming with proteases is reported in the temperature range of 37°C to 60°C (Arami *et al.*, 2007; Freddi *et al.*, 2003; Chopra and Gulrajani, 1994). In our earlier report More et al (2013) we reported higher

weight loss with *C. brefeldianus* and Alkaline streptomyces (BOA-2) proteases at 50◦C and between 60°C and 65°C respectively since the proteases were optimally active at these temperatures. Generally, temperature optima of enzymes vary from enzyme to enzyme. Most of the enzymes are active in the range of 28-50°C beyond which they are inactivated and lose their activity due to denaturation. Therefore the study of temperature range for any application of enzymes is very important.

Effect of pH

pH is a very important parameter as the activity of enzyme depends on optimum pH value. Different proteases are active at different pH values. Therefore pH of degumming reaction depends on the type of protease to be used. As pH affects the conformation of the protein and causes low or no activity, the effect of pH on degumming was studied in the pH range of 6-10. As shown in (Fig.4.4) increase in weight loss with an increase in pH and maximum weight loss of 24.04% was obtained at pH 9. The

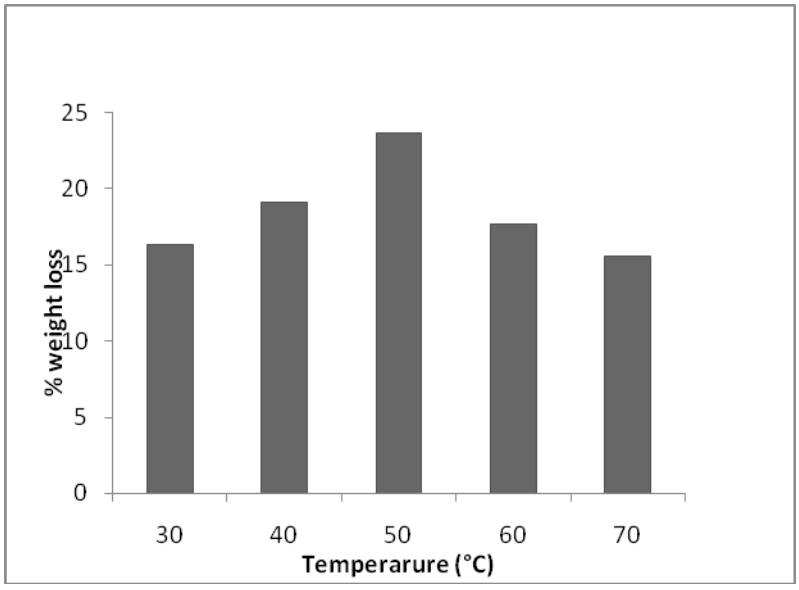

Figure 3: Effect of temperature on weight loss of silk after degumming

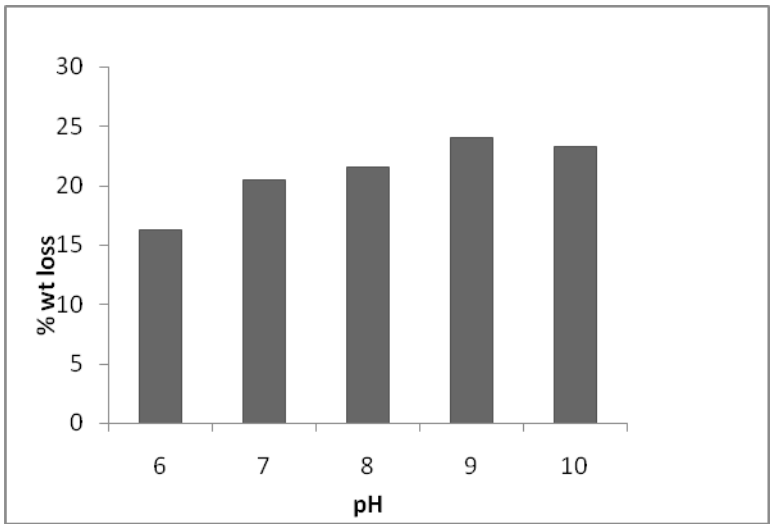

Figure 4: Effect of pH on weight loss of silk after degumming

Optimum pH for activity of the enzyme is 9.0 hence maximum degumming is obtained at that pH. There was a decrease in weight loss with a further increase in pH value. At pH 6.0 and 7.0 % weight loss of 16.31 and 20.45 was observed respectively. Thus the activity of enzyme increases with increase in pH up to a certain value beyond which they are inactivated and lose activity due to denaturation. Alkaline proteases are reported to be more effective than neutral and acidic proteases (Freddi *et al.,* 2003).

Scanning Electron Microscopy Studies

Scanning electron micrographs of untreated and degummed silk fibers with conventional method and protease are shown in (Fig.5.5). It is observed that the fibers are clubbed together in a bundle by means of sericin in the case of untreated silk yarn whereas fibers have fallen apart after degumming with protease. Untreated yarn contains sericin, which is seen as deposits on the surface of filaments holding them together. Degumming with optimum conditions resulted in the removal of the sericin deposits and separation of fibers. Similar observations of no uniform sericin deposits in untreated and undegummed silk and their removal after enzymatic degumming, as observed under scanning electron microscopic studies, are reported by other others (Arami *et al.,* 2007; Nakpathom *et al.*, 2009; More *et al.,* 2013). The

cleaning of the surface by the proteases is responsible for the improvement in the luster of the treated samples.

In our earlier report on degumming of raw silk with microbial proteases, we have studied the tensile strength of fibers degummed with *Beauveria* protease. It was shown that the tensile strength or elongation at break values was almost similar with the untreated fiber indicating no strength loss (More *et al.*, 2013).

Sericin Hydrolysis

Sericin was extracted from silk cocoons as per given in materials and methods. Hydrolysis of sericin was carried out with 1 and 2 Units of the enzyme at 50°C for 15min in shaking water bath. Hydrolysates obtained were subjected to MALDI analysis.

MALDI analysis of sericin hydrolysates

The molecular weights of small peptides derived by enzymatic hydrolysis were too low and could not be identified by SDS-PAGE. Therefore, the molecular weights of the oligopeptides were analyzed by MALDI-TOF. Initially, the effect of protease concentration on the molecular weight distribution was investigated. The MALDI-TOF profiles of the hydrolysate obtained with 1 and 2 units of protease are presented in Figure 6. Control without enzyme showed a sharp peak corresponding to the molecular mass of 4169 Dalton (Fig 4.6a). With minimum protease concentration of 1 unit, the oligopeptides were distributed in the molecular range of 1124.5 to 1345.8 Dalton with a major peak at 1139.7 Dalton (Fig 4.6b) while with 2 units protease concentration oligopeptides were distributed in low molecular range 525 to 616.25 Dalton with a major peak at 616.2 Dalton (Fig 4.6c). Fan *et al.*, (2010) prepared silk sericin hydrolysates (SSH) using six different proteases originated from the plant, animal and microorganism. Hydrolysis was performed for 11 h between 37 to 65°C and pH 7.0 to 8.5 and the molecular size distribution profile of the hydrolysates was determined by

high-performance liquid chromatography (HPLC) on a gel permeation chromatography column. They evaluated the antioxidative and free radical-scavenging activities of the peptides and found that different protease hydrolysates possessed different antioxidative activity and free radical-scavenging activities.

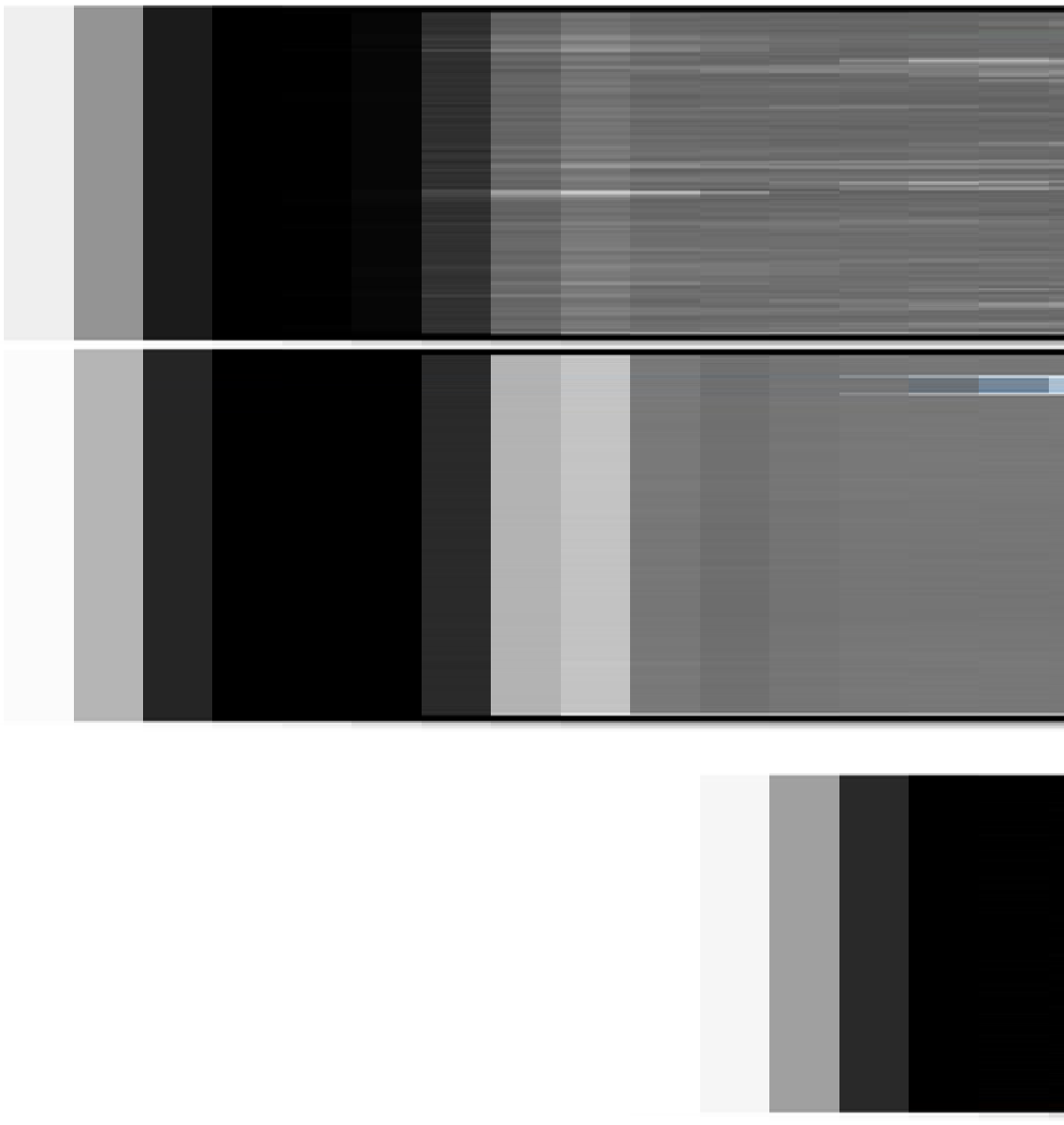

Figure 5: Scanning electron micrographs of silk fibers**.** A1 to A3- Untreated;

B1 to B3 protease treated; C- Conventional

Their results revealed that highest antioxidant activity was exerted by alcalase hydrolysates. Further, they stated that peptide fraction with a molecular weight ranging from 200 to 3000 Dalton was probably associated with higher antioxidant activity and the SSH can be used as a food supplement or used in the pharmaceutical and medical industries. Therefore, the hydrolysates of sericin hydrolysed with *Beauveria sp* protease may have some biological activities which need to be further investigated in detail.

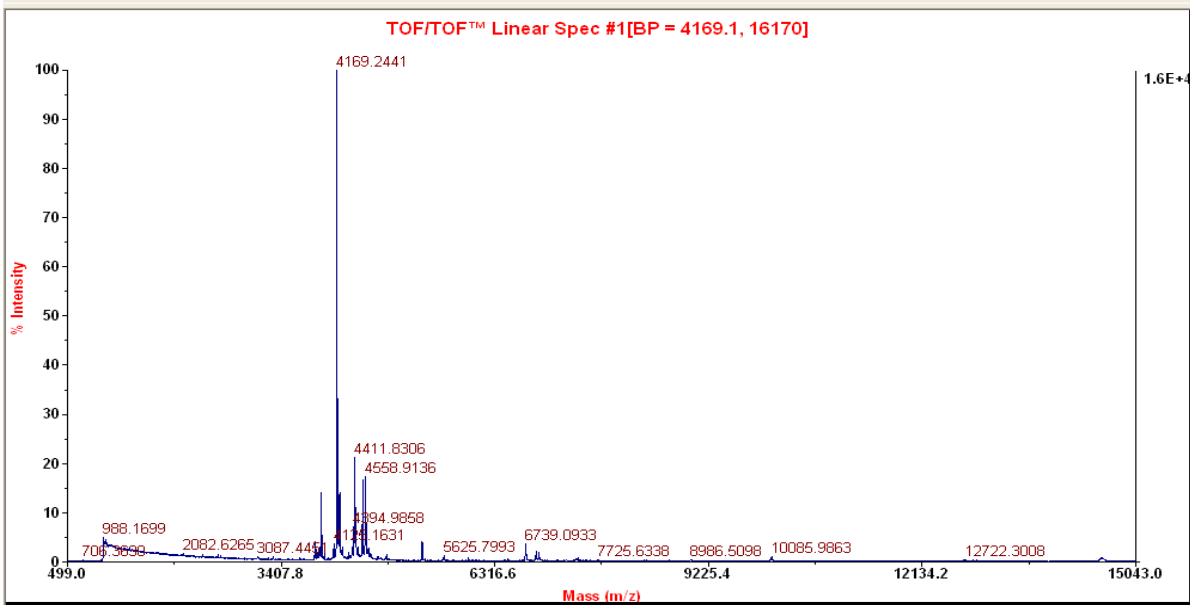

Figure 6a: MALDI analysis of sericin hydrolysates (Control)

Formulation of the wound healing cream

In order to optimize the concentration of the gelling agent to achieve proper consistency of the gel formulations were prepared with different gels or thickening agents, various gums, Carboxy methylcellulose sodium, sodium alginate, Hydroxypropyl Methylcellulose (HPMC). Different concentration of above stated ingredients was checked for their property of gelling and viscosity builder. Concentrations used were ranging from 1 to 10 gm %.

spreadability and consistency. For further studies formulation with 8% sericin, 1% sophorolipid and 3% sodium alginate were used.

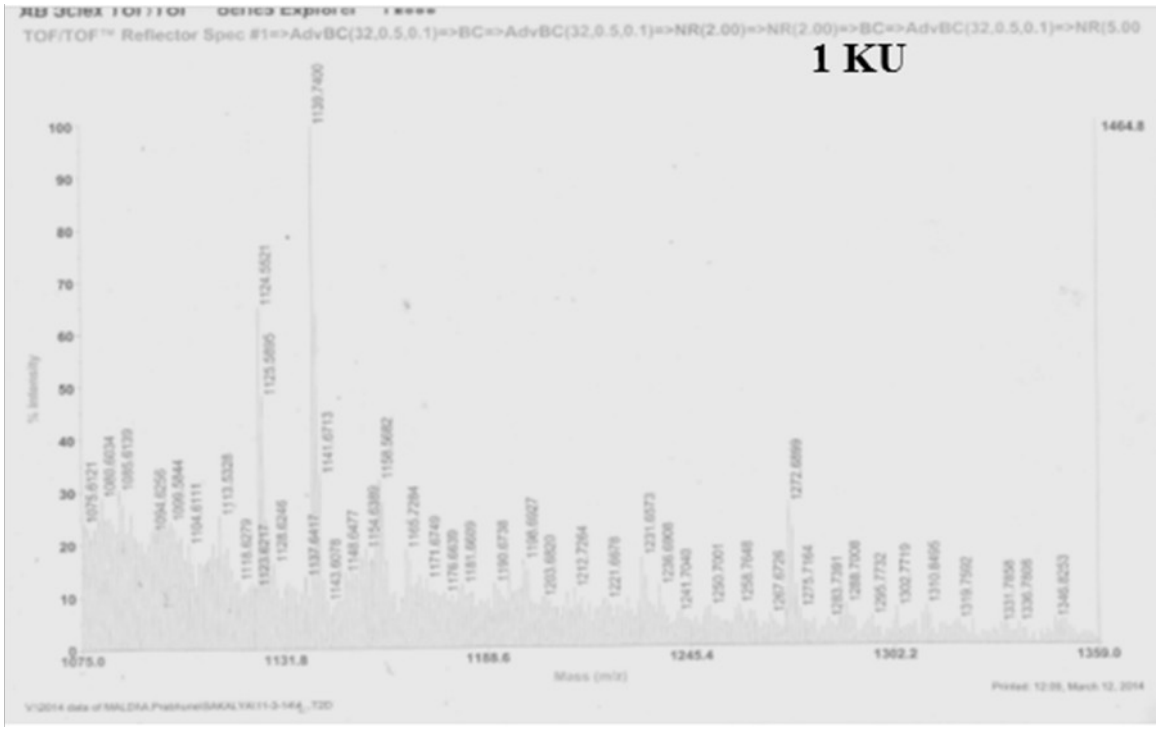

Figure 6b: MALDI analysis of sericin hydrolysates (1 Unit)

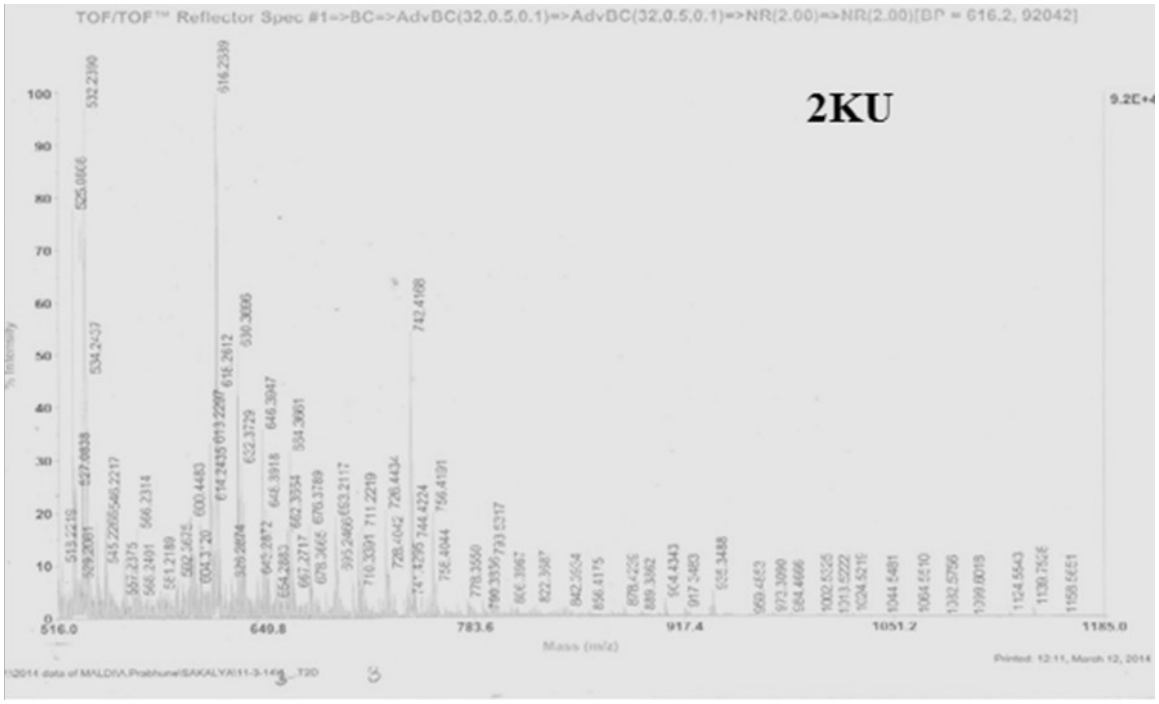

Figure 6c: MALDI analysis of sericin hydrolysates (2 units)

Physical Characteristics of the formulation

The formulations containing Sericin and Sericin+sophorolipid were brown in colour. pH of only Sericin containing formulation and Sericin+sophorolipid was 6.1 and 5.6 respectively which are compatible with our skin. Formulation containing Sericin and sophorolipid containing cream showed better viscosity and spreading diameter then that of only Sericin containing cream

Antioxidant Activity

DPPH is a stable free radical that shows maximum absorbance at 517 nm in methanol. When DPPH radicals encounter a proton-donating substance such as an antioxidant, the DPPH radicals would be scavenged and the absorbance is reduced (Amarowicz *et al.* 2004). Based on this principle, the antioxidant activity of the substance can be expressed as its ability in scavenging the DPPH radicals. Figure show that different concentrations of the formulation (Silk Sericin + sophorolipid) possessed different abilities to quench the DPPH radicals. It was observed that 50µg to 500µg/ml of formulation scavenged 8.2 to 76.1% of DPPH radicals, respectively

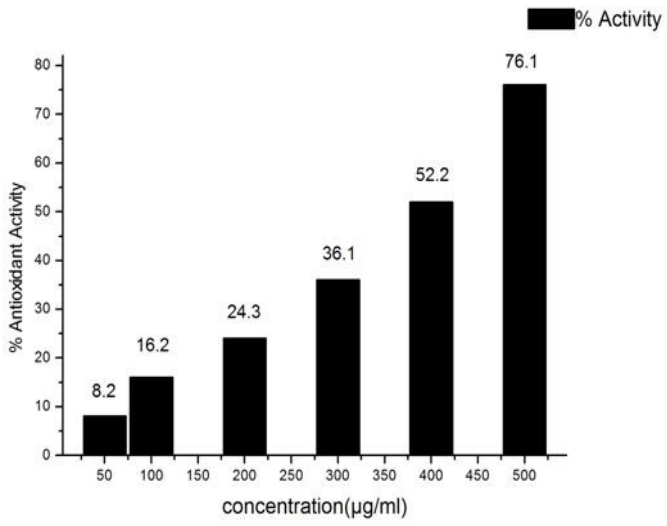

Figure 4.7: Percentage of anti-oxidant activity at a different concentration of formulation.

(Fig 4.7).The concentration of wound healing formulation for scavenging 50% DPPH radicals was 400 µg/ml. Antioxidant activities of silk sericin from silkworm *Bombyx mori was* also evaluated and reported by (Jin Bo Fan *et al.*, 2009). They observed that 50µg to 500µg of formulation scavenged 8.2 to 76.1% of DPPH radicals, respectively.

In Vivo Wound Healing Model

Incision wound healing model followed for study the wound healing potential of the sericin and silver nanoparticles as main ingredients. It took 8 to 10 days for complete healing of wounds of the test group. Photographic comparison and histopathological evaluation of the tissues are shown in following points. Monitoring of inflammatory mediators induced by silk sericin evaluated on incision wound model by (Pornanong Aramwit *et al.*, 2007).

Photographic Comparison

After creating wound according to the guidelines of the CPCSEA, photographs of the each group of animal's wounded part took for the visual comparison and same is shown in figure 3. A- providine, B- Sericin and sophorolipid formulation, C- Only Sericin and D- untreated. Figure 4.8 shows that formulation containing sericin and sophorolipid that is B group shows a faster rate of wound contraction and healing process as compared to commercial preparation used in this study. It is also clearly seen that addition of sophorolipid to sericin has rapid and faster healing. After day 7 approximately 90% wound healing in group B and the complete wound was observed and on day 10. Padol et al (2012b) used commercially available silk proteins with modifications for wound healing and reported wound healing in 15 to 20 days.

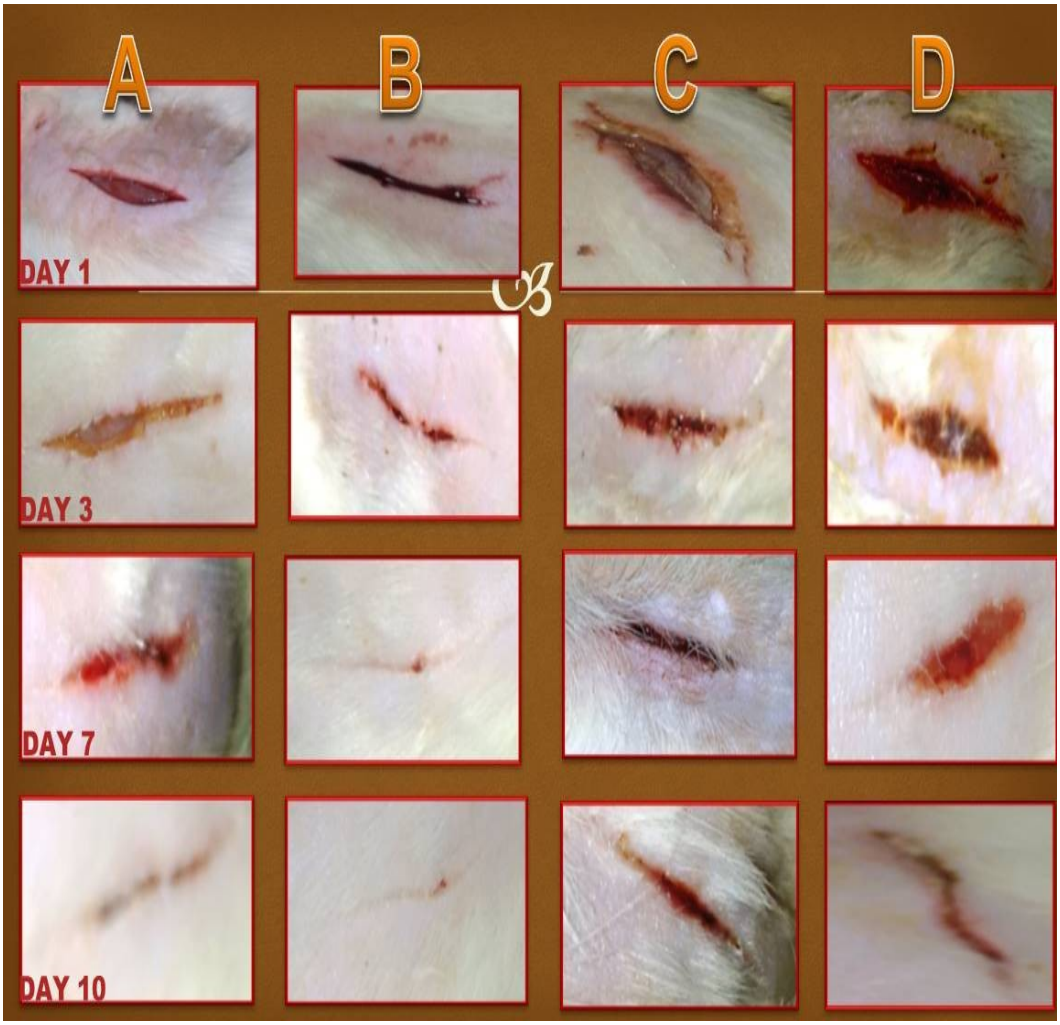

Figure 4.8: Photographic comparison of wounds (A- providine, B- Sericin and sophorolipid formulation, C- Only Sericin and D- untreated.)

Histopathological Evaluation

Histological examination was performed on skin art of wound that has been fixed after the sacrifice of the exposed animal and changes in the tissue and cell morphology were assessed using a light microscopy. The treatment of rat wounds with sericin + sophorolipid formulation led to reducing scar formation and enhanced fibroblast proliferation, angiogenesis, keratinization and epithelisation as compared others groups. The rapid healing and contraction of the wound in the treatment group animals may possibly be attributed to the increase in total protein and collagen content in the wound

granulation tissue. Fibroblast proliferation, angiogenesis, keratinization is more and faster in sericin + sophorolipid treated animals as compared to the group treated with the standard compound. 10% formalin fixed Skin tissue of animals microscopically revealed in group D marked exudates with degenerating inflammatory cells, very slight epithelization characterized by few immature epithelial cells along with inflammatory cell infiltration and there was no fibroblasts proliferation, incomplete epithelization, congestion of vessels in the dermis, proliferation of fibroblasts and infiltration of inflammatory cells were noticed but there was no keratinization.

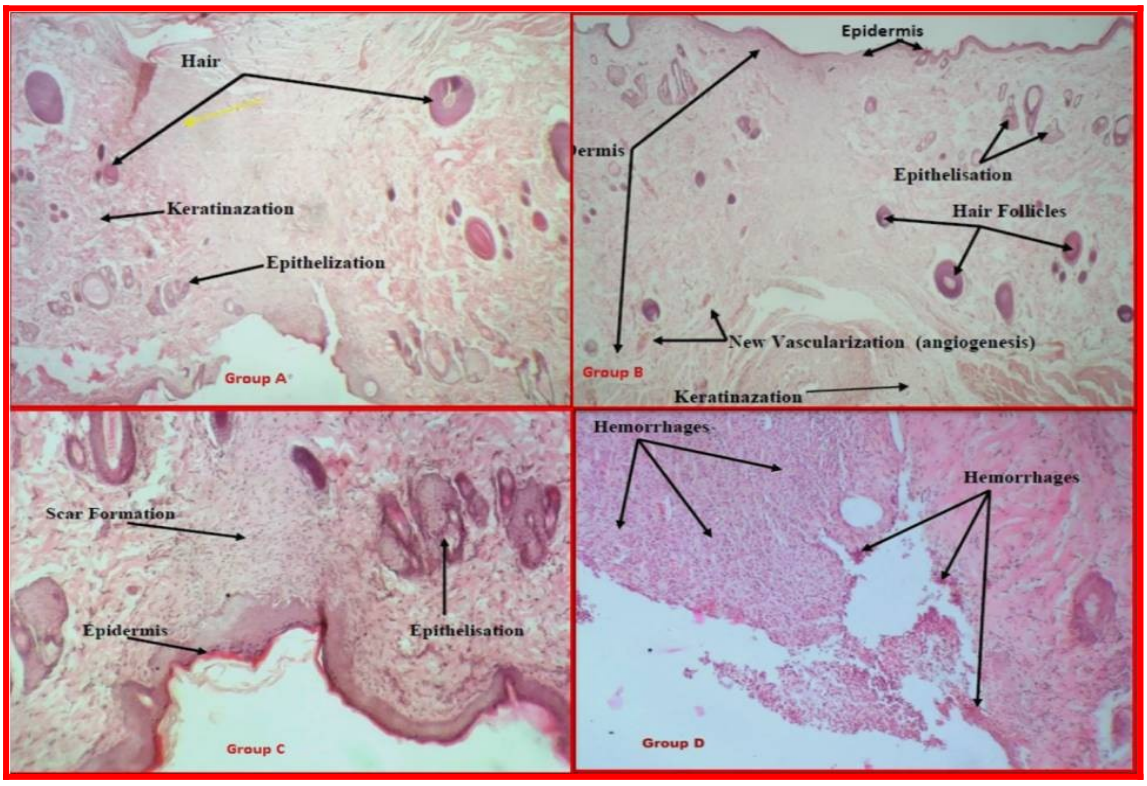

Figure 4.9: Histopathological evaluation (Skin of Rat Group A (treated Povidone Iodine) shows keratinization and epithelization. Skin of Rat Group B (treated with sericin + SL) shows new vascularisation (angiogenesis), keratinization and epithelization. Skin of Rat Group C treated with sericin shows epithelization. It also shows scar formation. Skin of Rat Group C (untreated) shows scar formation and haemorrhages.

The wounds of group B treated with silk sericin with sophorolipid were microscopically revealed it showed epithelization with the formation of the epithelial layer, keratinization was observed, the dermis showed neovascularization, matured fibroblasts increased collagen synthesis and absence of inflammatory cells. In addition group, A treated with povidone iodine showed regular arrangement of fibroblasts in the dermis. severe haemorrhages and marked neovascularisation in the negative control group D (Fig 4.9).

Conclusion

Alkaline protease produced by *Beauveria sp.* was effective in complete degumming within 45 min at low enzyme concentrations, unlike the conventional method requiring 60 min for the same. Degumming was optimum at 50°C which is an added advantage in terms of energy conservation and at pH 7-9. Water requirement for degumming process could be brought down with this method with less effluent generation. Hydrolysis of sericin extracted from cocoons by the protease revealed the presence of small molecular peptides of molecular weight 1139.7Da & 616.2Da as analysed by MALDI-TOF which have several applications in diverse fields. These oligopeptides have many biological potential as wound healing, antioxidant, hydrogel formation etc. As already highlighted, preparation and separation of these peptides and testing their biological activities is essential for several biotechnological applications.

A wound healing formulation was prepared using biocompatible compounds such as Sericin and sophorolipid with sodium alginate as a binding agent. Sericin is a by-product/waste from textile industry which is readily available and cheap source. Since all the ingredients are biocompatible it is a green formulation and has no side effects. The formulation has shown viscosity, pH, spreadability, extrudability in an acceptable range. The formulation showed antioxidant activity. The treatment of rat wounds with sericin + sophorolipid cream has led to reduce scar formation and enhanced fibroblast proliferation, angiogenesis, keratinization and epithelisation as compared others groups. It can be concluded that a new biocompatible wound healing cream will have a potential market in future.

References

- Amarowicz, R. Pegg, R. B. Rahimi-Moghaddam, P. Barl, B. Weil, J.A. (2004) Food Chem. 84:551–562.
- Anghileri, A. Freddi, G. Mossotti, R. Innocenti, R. (2007) *J. Nat. Fibers*. 4(1): 13-23.
- Anis, P. Çapar, G. Toprak, T. Yener, E. (2016) *TEKSTİL ve KONFEKSİYON* 26(4): 368- 374
- Arami, M. S. Rahimi, L. Mivehie, Mazaheri, F. (2007) *J. Appl. Polymer Sci.* 106: 267–27.
- Aramwit, P. Palapinyo, S. Srichana, T. Chottanapund, S. Muangman, P. (2013) *Arch Dermatol Res* 305:585–594 DOI 10.1007/s00403-013-1371-4
- Aramwit, P. Sangcakul, A. (2007) *Biosci. Biotechnol. Biochem*. 71(10): 2473–2477,
- Chen, Z. J. Yang, J. P. Wu, B. M. Tawil, B. (2014) *J. Dev. Biol. 2*(4): 198-209; doi:10.3390/jdb2040198
- Chopra, S. Gulrajani, M. L. (1994) *Ind. J. Fiber Text. Res.*19: 76-83.
- Dandin, S.B. Kumar, S.N. (2007). *Ind. Silk.* 45(9): 5-8.

- Devi, K. L. Priyadarshini, A. (2017) International Journal of Home Science 3(1): 309-311
- Devi, Y. R., Singh, L. R. and Devi, S.K. (2011) *Int. J. Current Res. Rev.* 3(10): 90-92.
- Ehrlich, H. P. Grisliest G. Hunt. T. K. (1972) *Surgery*. 72/ 576–583.
- Fan, J. B. Zheng, L. H. Wang, F. Guo, H. Y. Jiang, L. Ren, F. Z. (2010) *J. Food Biochem.* 34: 382- 398.
- Freddi, G. Allara, G. Candiani, G. (1996) *J. Soc. Dyers Colourists*. 112: 191-195.
- Freddi, G. Mossotti, R. Innocenti., R. (2003) *J. Biotechnol.* 106: 101-112.
- Gharaei-Fathabad, E. (2011) *Ame. J. Drug Discov. Developt*. 1(1): 58–69.
- Gharaei-Fathabad, E. (2011) *American J. Drug Discovery Develop.*. 1(1): 58–69.
- Gulrajani M, L. Agarwal, R. Grover, A. Suri, M. (2000) *Ind. J. Fiber Text. Res*. 25: 69-74.
- Gulrajani, M. L. (1992). *Rev.f Prog Coloration.* 22: 79-89.
- Gulrajani, M. L. Agarwal, S. Agarwal, R. (1999) *Ind. J. Fibre Text. Res.* 24: 226–228.
- Gulrajani, M. L. Gupta, S. V. Gupta, A. Suri, M. (1996) *Ind. J.f Fiber Text. Res.* 21: 270–275.
- Jin Bo Fan, Li-Ping Wu, Li-Shui Chen, Xue-Ying Mao, Fa-Zheng Ren, (2009) *J.Food Biochem*. 33(1):74 - 88.
- Johnny, R. V. A. Chinnammal, S. K. (2012) *Int. J. . Sci. Nat*. 3(1): 51-59.
- Juhea Kim, MiYeon Kwon & Suyeon Kim (2016) J. Nat. Fibers, 1(36): 629-639
- Khan, M.R. Tsukada M. Gotoh Y. Morikawa H. Freddi G. Shiozaki H. (2010) *Bioresource Technol.* 101: 8439-8445.
- Komatsu, K. (1975) *Bull.Sericulture Exp. Sta.* 26, 135- 256.

- Krishnaveni, V. RajKumar. G. (2010) *Colourage*, ISSN 0010-1826. 57: 61–68.
- Laxman, R. S. Sonawane, A. P. More, S. V. Rao, B. S. Rele, M. V. Jogdand, V. V. Deshpande, V. V. Rao. M. B. (2005) *Proc. Biochem.* 40: 3152–3158.
- M.L. Gulrajani, India 15-18th December 2005.pp. 21-29
- More, S. V. Khandelwal, H. B. Joseph, M. A. Laxman, R. S. (2013) *J. nat. fiber*. 10(2): 98-111
- Nakpathom, M. Somboon, B. Narumol, N. (2009) J. Microbiol. Soc. Thailand. 23:142-146.
- Padamwar M.N. Pawar A.P. Daithankar A.V. Mahadik K.R. (2005) *J. Cosmet Dermatol*. 4(4):250-257.
- Padol, A. R. Jayakumar, K. Mohan, K. Manochaya, S. (2012a) Int. J. Materials and Biomaterials Appl. 2(4): 19-24
- Padol, A. R. Jayakumar, K. Shridhar, N.B. Narayana Swamy , H.D. Mohan, K. (2012b) Int. J. Toxicol. Appl. Pharm. 2(3): 31-36
- Puri, S.(2001) MSc thesis, University of Delhi, New Delhi.
- Rajasekhar, A. Vallurupalli R, Reddy M.,N, Sambasiva Rao K.R. S. (2011) *J. Bio-Sci. Bio-Technol.* 3 (4): 43-58
- Shahid, M. Mohammad, F. Chen, G. Tang, R. C. Xing, T. (2016) GreenChem. 18: 2256-2281
- Singh, P. and Cameotra. S. S. (2004) *Trends in Biotechnol*. 22(3): 142–146.
- Singh, P. Cameotra. S. S. (2004) *Trends Biotechnoly*. 22(3): 142–146.
- Takechi, T. Wada, R. Fukuda T., Harada, K. Takamura H. (2014) *Biomed Rep.* 2(3): 364–369.
- Talebpour F, Veysian S.M, Golfazani M.E.H (2013) *J. Textiles Polymers,* 1 (2):*60-64*

- Tawil, B. Wu, B. (2012) *An Introduction to Biomaterials*, 2nd ed.; Hollinger, J.O., Ed.; CRC Taylor & Francis: New York, NY, USA, ; 249–262.
- Wang, Y. Z. Blasioli, D. J. Kim, H. J. Kim, H. S. Kaplan, D. L. (2006) *Biomaterials*. 27(25): 4434–42.
- Wu, J. H. Wang, Z. Xu, S. Y. (2008) *Proc. Biochem*. 43: 480-487.
- Zheng, X, T. Zhao X, H. 2009. *Microbiol.* 36: 193-197.
- Zhou, C. Z. F. Confalonieri, N. Medina, Y. Zivanovic and C. Esnault, C. Yang T, Jacquet M, Janin J, Duguet M, Perasso R, Li ZG. (2000) *Nucl. Acids Res.* 28: 2413-2419.

CHAPTER 5

Research Summary and Future Scope

Thesis Summary

A new fungal strain was isolated from rabbit dung in our laboratory. The fungus was identified as a new strain of *Beauveria.* This fungus was screened for protease production and showed a very good clearance zone on MGYP plate with 1% skim milk. Protease production was tested in different media and it was found that GYE medium was best for the production of protease. Different agricultural residues were tested as an inducer for protease production and mustard seed cake at 2% (w/v) concentration was proved to be the excellent inducer. Among various carbon sources used glucose was best utilized by the organism for protease production. Among nitrogen sources yeast extract was found to be the best nitrogen source for maximum production of protease followed by urea. Optimum protease production was observed when initial media pH was 8.0 and temperature for incubation was 28°C. 10% (v/v) inoculum was found to be optimal for the production of protease. Chemical surfactants like Tween 20 or Tween 80 had no beneficial effect on protease production. Optimization studies revealed that a cost effective medium containing 1% glucose, 0.3% yeast

extract and 2% mustard seed cake gave highest protease production. Approximately 2 fold increase was archived with the optimization studies.

Beauveria sp. was screened for xylanase production and showed a very good clearance zone on MGYP plate with 1% xylan. Xylanase production was tested in different media and it was found that MGYP medium was best for the production of xylanase. Different agricultural residues were tested as an inducer for protease production and wheat bran at 2% concentration was proved to be the best inducer. Among various carbon sources used glucose was best utilized by the organism for xylanase production. Yeast extract was the best nitrogen source for maximum production of protease followed by urea. Optimum xylanase production was observed when initial media pH was 7.0 and temperature for incubation was 28°C. Chemical surfactants like Tween 20 or tween 80 had no beneficial effect on xylanase production. Optimization studies revealed that a medium containing 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 2% wheat bran gave highest xylanase. production. Approximately 1.2 fold increase was acchived with the optimization studies.

To enhance the protease production further oleic and linoleic sophorolipids were added to the fermentation medium. It was observed that when both the sophorolipids were added respectively to the medium the protease production was enhanced. The increased production was 231.2 and 169.8% in case of oleic and linoleic sophorolipid respectively. It was observed that 1mg/ml was the best concentration which showed a maximum increase in protease production. It was noted that oleic sophorolipid gave better enhancement than linoleic sophorolipid. Optimum pH was found to be at pH 9.0. Optimum temperature for protease was 50°C. Protease was stable in a broad pH range of 3.0-11.0. Protease was stable upto 45°C showing approximately 50% residual activities after 1h incubation. Effect of metal ions on protease activity was studied at 10mM final concentration in the reaction mixture Ag and Hg completely inhibited the activity while there was 20% inhibition by Cu and Ni.

To enhance the xylanase production further oleic and linoleic sophorolipids were added to the fermentation medium. It was observed that when 1mg/ml of sophorolipid was added to the medium the protease production enhanced. The increased production was 148.9 and 168.5% in case of oleic and linoleic sophorolipid respectively. Since both the sophorolipids showed increased production various concentrations of sophorolipids from 0.5 to 1.5 mg/ml were tested for xylanase production. It was observed that 0.5mg/ml was the best concentration which showed a maximum increase in xylanase production. It was observed that 0.5mg/ml was the best concentration which showed a maximum increase in xylanase production with 150.3 and 250.6% with oleic and linoleic sophorolipid respectively. It was noted that linoleic sophorolipid gave better enhancement than oleic sophorolipid for xylanase production. Effect of various pH ranging from 5.0 to 12.0 was checked on xylanase activity. Optimum pH was found to be at pH 6.0. Effect of temperature on xylanase activity was studied in the temperature range of 30 to 70°C. Optimum temperature for xylanase was 50°C. Xylanase was stable in a broad pH range of 4.0-8.0. xylanase was stable upto 45°C showing approximately 50% residual activity after 1h incubation. Effect of metal ions on xylanase activity was studied at 10mM final concentration in the reaction mixture. It can be concluded that Ag and Hg completely inhibited the activity.

Alkaline protease produced by *Beauveria sp.* was effective in complete degumming within 45 min at low enzyme concentrations, unlike the conventional method requiring 60 min for the same. Degumming was optimum at 50°C which is an added advantage in terms of energy conservation and at pH 7-9. Water requirement for degumming process could be brought down with this method with less effluent generation. Hydrolysis of sericin extracted from cocoons by the protease revealed the presence of small molecular peptides of molecular weight 1139.7Da & 616.2Da as analysed by MALDI-TOF which have several applications in diverse fields. These oligopeptides have many biological potential as wound

healing, antioxidant, hydrogel formation etc. As already highlighted, preparation and separation of these peptides and testing their biological activities is essential for several biotechnological applications.

A wound healing formulation was prepared using biocompatible compounds such as Sericin and sophorolipid with sodium alginate as a binding agent. Sericin is a by-product/waste from textile industry which is readily available and cheap source. Since all the ingredients are biocompatible it is a green formulation and has no side effects. The formulation has shown viscosity, pH, spreadability, extrudability in an acceptable range. The formulation showed antioxidant activity. The treatment of rat wounds with sericin + sophorolipid cream has led to reduce scar formation and enhanced fibroblast proliferation, angiogenesis, keratinization and epithelisation as compared others groups. It can be concluded that a new biocompatible wound healing cream will have a potential market in future.

Future Scope

Silk The mulberry silkworm pupa is one of the major waste products/by-products of the silk industry. Disposal of the spent silkworm pupae poses a big problem creating nuisance and health hazards. Since pupae are a rich source of proteins, fat, carbohydrates and vitamins, besides it is rich in oil, several workers have investigated the utilization of spent silkworm pupae meal as animal feed (fish and poultry).

Proximate analysis of pupa has shown that it contains 40–60% protein, 20–30% lipid, 4.96% fibre, and other substances like hormones, essential elements like zinc, iron, and vitamins like A,B1, B2, D and E. The total protein and lipid contents of pupae comprise about 55.6% and 32.2% of dry weight, respectively.

These silk pupae can be hydrolysed using protease from *Beauveria* sp. and the hydrolysates can be purified to obtain the essential amino acids and other nutracuticals. These can be further used for food fortification. The formulations may be used as a food supplement for different age groups and special nutritional needs. Powdered form of these isolated small proteins may be used

to fortify a variety of flours such as wheat. The hydrolysates can be used in fruity beverages, jellys, jams candys etc.

Another future scope may be to explore the sericin hydrolysates obtained by hydrolysis using protease from *Beauveria* sp.for possible applications.

List of Publications

Patents Granted

1. US patent no.8765447 B2 granted on July 1, 2014

Seeta Laxman Ryali, Shiv Shankar, **Snehal Vijay More**, Harish Bansilal Khandelwal, Chandra Babu Kannan Narasimhan, Saravanan Palanivel, Padmanabhan Balaram. Fungal strain Beauveria Sp. MTCC 5184 and a process for the preparation of enzymes thereof

Patents Filed

1. Asmita Prabhune and **Snehal More.** Use of Fatty acids and their derivatives as enhancers for enzyme production by Beauveria sp MTCC 5184. **Applied for US patent NCL No. 2015-INV-0013**

2. Asmita Prabhune, **Snehal More** and Sachin Agawane. Silk Sericin and sophorolipids a novel cost effective formulation for wound healing. **Applied for US patent NCL No. 2015-INV-0045**

List of Publications

1. Snehal V. More. (2015) Proteases for degumming: A novel, green and ecofriendly way to quality silk production **International Journal of current Research 7 (11)**, 23032-23038

2. Snehal More, Sakalya Chavan and Asmita Prabhune. Ecofriendly silk degumming and complete utilization of Sericin with alkaline protease from *Beauveria sp.* (MTCC 5184) **Accepted in** J. of Natural Fibers. (2017)

3. Snehal More, Navnath Kadam, Sachin Agawane and Asmita Prabhune . A Novel Formulation for Scar-free Wound Healing Using Silk Sericin and Sophorolipids **communicated** to Natural product research (2017)

4. Snehal More, Pavitra Joshi, Komal Gavas and Asmita Prabhune. Sophorolipids as enhancers for enzyme production by Beauveria sp MTCC 5184. **manuscript under prepration.**

Posters

1. A novel formulation for scar-free wound healing using silk Sericin and sophorolipids
Snehal More, Navnath Kadam, Sachin Agawane and Asmita Prabhune. Poster on science day at NCL February 2016

2. Ecofriendly silk degumming using protease from *Beauveria sp.* MTCC 5184
Snehal More, Saklya Chavan and Asmita Prabhune. Poster during science day celebrations at NCL February 2015.