BIOCHEMICAL AND GENETIC CHARACTERIZATION OF *Bacillus* sp. P13 PROTEASE AND ITS APPLICATION IN LEATHER PROCESSING

A THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

BY

PRIYA. S. PILLAI

DEPARTMENT OF MICROBIOLOGY AND BIOTECHNOLOGY CENTRE

FACULTY OF SCIENCE

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

VADODARA-390 002, GUJARAT, INDIA

DECEMBER 2011

DECLARATION

STATEMENT UNDER O. Ph.D. 8/(iii) OF

THE M. S. UNIVERSITY OF BARODA, VADODARA

The work presented in this thesis has been carried out by me under the guidance of Dr. G. Archana, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M.S. Universityof Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

Place: Vadodara **Priya. S. Pillai**

Date: Candidate

This is to certify that the above declaration is true.

Place: Vadodara **Dr. G. Archana**

Date: Research Supervisor

Dedicated to my prized possessions.....

Anant & Jishnu

Acknowledgement

The journey en route for Ph.D was like the course of a wandering river, which winders through valleys and hills, which sings and dances along the countryside echoes, which gobbles the trees like hungry and vexed monster. There were many faces who had sang and danced with me during the happy days, who had meandered around the struggles I underwent and rendered helping hand and moral support to pacify the vexed monster and finally reaching the destination like a river slowly winding down hill, twisting and turning.

I am not at all exaggerating when I acknowledge that I am indebted to my research supervisor Dr. G. Archana (Archana ma'am) for accepting me as a Ph.D. student, when I approached her with the project approved by DST on Proteases for leather, which was never her area of research. Depth and diversity of her scientific knowledge with excellent research guidance are far beyond explanation. Her scientific vision moulded me to traverse into the all the possible aspect of the research problem. I am glad to divulge that I'm bestowed with the efforts she had put up to integrate the right scientific research attitude along with scientific writing skills. I greatly regard her for imbibing in me the ability to plan experiments meticulously with utmost care on smallest of experimental details. I really admire her for her never-ending patience, optimistic approach, powerful words of encouragement and endless zest for science. The faith she had put on me was the best motivation I had to balance my family responsibilities and research carrier and I'm grateful for being understanding and supportive always .I do flatter on her memory power, the swiftness in proof reading (felt embarrassed many times for committing silly mistakes, especially in the manuscripts) , the way she updates herself on vivid areas on all her students working on. I admire her for keeping herself cool, even on the eleventh hour and always wished to imbibe in me. I enjoyed occasional non-scientific chit-chatting and always cherish our trip to Chennai for AMI conference. Words are not enough to thank her for the wonderful five years of association with her, still, thankyou .....Ma'am.

I take this opportunity to thank Prof. G. Naresh Kumar (NK sir), a true scientist, approachable, humble and very down to earth person, whose critical comments and advice especially during lab meetings helped me to see the other facets of the work, which otherwise wouldn't have thought of .

I express my deep sense of gratitude to Prof. T. Bagchi (Present Head, Department of Microbiology) and also to former Heads, Prof. H. S. Chhatpar and Prof. Anjana Desai, for providing me excellent research facilities. Interaction with Dr. Bagchi was very limited, mostly to intimate the status of UV-Spectrometer or to collect his signature, thank you. Sir, for all the support you have extended.

I owe a lot to Dr. Chhatpar for couple of reasons, for permitting me to join in the Department with my independent project, directing me to Dr. Archana and also to allot RL I as my work station.

Prof. Anjana Desai, a true visionary, able administrator and a humble and down –to-earth person. I always marvelled by her swiftness in her action, especially in decision making. We had the freedom (still have) to step in to her cabin to discuss any issues. Dept. Of Microbiology entered into the second golden period(after Dr.V.V.Modi's tenure) under her captaincy and got uplifted tin terms of infrastructure and advanced instrument facility for strengthening research work..

I extend my thanks to Dr. Pranav Vyas, who strengthened me with spiritual knowledge which kept my mind calm, which was coincidental, just before my Ph.D. registration seminar. I also thank. Dr. Sanjay Ingle for allocating his time to analyse the N-terminal sequence data and also for all the other support. Thanks to Dr. Nandita Baxi for readily extending help when required. I would like to acknowledge. Dr. Anuradha Ma'am, a balanced personality, always treated me in a friendly manner. She supported me as when I'am in need. We do enjoy sharing the timely pranks of our sons.

Dr. Mrinalini Nair, for all her words of encouragement to pursue a research career and also cherish the interactions with Dr.Manjarekar related to 'Prions". Thanks to Dr.Bina Chandani, for being a student friendly faculty. Special thanks to Dr. Bony Pilo for the support extended and also for introducing me to Environmental Science Department as a visiting faculty.

I am grateful to Shirish bhai who helped me in all official as well as personal aspects whenever required. He managed my project accounts efficiently and helped me immensely in all university related official works and also in sending the reminders and progress reports to DST.

I also thank Khatri bhai for maintaining the university scholarship account and Talati bhai for issuing chemicals and plastic ware. I thank Praveen bhai for providing glass ware and Seema mam for provoding distilled water during its shortage. My special thanks to Mandvekar bhai, especially for helping me to do BOD analysis. I am greateful to Nairbhai, Varghese bhai and Nitesh bhai who willingly extended their help every time I required. I am thankful to Babukaka and Chiman Bhai for their help as when ever required. I also thank Nilesh bhai, Dileep bhai, Pradeep bhai and Jayesh bhai for their help and cooperation

It is difficult to pen down my feelings towards couple of people, Thomas Ma'am and Aparna Ma'am, their warmth and support was real boon.Thomas Ma'am, is more like a motherly figure to me. The kind of warmth and affection she had extended me was unexplainable. Aparna Ma'am, I treated her like my elder sister, to whom I can discuss anything. We enjoyed shopping, gossiping ... and thanks a lot for imparting many spiritual aspects which I was not aware of and also for all the prayers, advices and support especially during my thesis writing.

From the day I joined to till date, our RS group was like a cricket team, both in terms of quantity and quality. In retrospection, there were all-rounders like Manish, Falguni, specialist like Arif, Geetha, Vipul and Sanket. Dr.Kool, Kuldeep was a role model to everyone to keep cool, even in worst situations. I thank all of them for their support which enabled me to acquainting in the department. Our group of RS's were imbibed the commitments they had shown toward the department and was the motivation we had to work together during departmental renovation.
My work station , RL I, the work culture was cordial, with occasional chit chatting and musical backing of jugalbandi's, Carnatic and Hard-rock was very positive. Our lunch table were always live with scientific discussions and heated non-scientific arguments. Among my lab mates, Nandan and Anoop are like my brothers, I had the freedom to demand also to scold them. Thanks are due to Nandan , especially for helping me take good micrographs of feather. If friend in need is a friend indeed, that is Anil. Thanks to both Anil and Aparna especially for helping me to understand the concept of thermodynamics. I enjoyed all the small talks with Aparna. Anoop (Mr. Dependendable), lots and lots of thanks for formatting the thesis meticulously. Jagu, a very jovial person, great foodie, thanks for all the support, especially for the initial PCR troubleshoots, and I really enjoyed his company along with Subbu, Aaram and Niyati, during AMI conference at Chennai. Sneha, new labmate was also fun to be around, very bubbly and positive....., I wish all the very best for her Ph.D.
Thanks to all the other Rs's for all the support, especially to Darshan and Sanjukta, for being understanding and co-operative teaching partners, Krushi and Harish for their willingness to help, Radhika,, Vimal, Sumant and Subbu for their enthusiasm and support. Cry master, Ketan, deserves special acknowledgement for the help rendered during protein purification. Special thanks to Vihang, for the all the help extended from his perview, Good luck to him. Best wishes for Ruchi Bhate and Sumita for their future endeavours. All the departmental photographers (Anil, Anoop, Jagat, Murli and Vimal) had clicked gel pictures for me, Thank you guys.....
I am thank all the research students from DBL and MNL, especially to Dr. Sandeep for the gel elution tips, Dr. Soshina and Mahima for their warmth and affection.

The ups and downs of my research life were well shared with my two good friends, Murli and Ruchi. Murli the philosopher, always advised to be attached with detachment, thanks a lot..... for all the support.

Being an early bird in the Dept, most of my days started with a chat with him, felt very composed. I go speechless, to acknowledge Ruchi, my association with her grow stronger after I started working with her couple of years back. Very selfless person (mistaken most of the times), the way she helped me do my cloning experiments were commendable. I owe a lot to her and can't be explained by words from the dictionary. I acknowledge the contributions of my dissertation students, Sweta Mandgae (excellent working hand), Arpita, Nimmy, Anil Sharma. Interactions with them were really a learning experience for me. Special thanks to Mousmi Patel (SPU) for helping to do the SEM micrograph of hide. Thanks to Prathik Chandrani and Vaishakhi, for helping me to do the modelling of the protein. I extend my thanks to all the M.Sc.students, especially to Gunjan for providing all the research papers on request, Rucha and Glen, for their support.

Dr.Seetha Laxman, NCL Pune, for accepting me as her student and also for conceiving the basic idea of the project and forwarding the same to DST through NCL.I recollect the words of encouragement from Dr.Sulekha Hazare (NCL) and Dr Rati Rao (ARI Pune) to apply for DST's women scientist Scheme. All my friends from NCL especially Dr. Snehal More, Dr.ShivShankar and Ashwini for all their support., Chennai found a special place in my research carrer, I got back into research track from industrial background through my entry into M.S. Swaminathan research foundation.I cherish the opportunity I had to interact with Prof. Swaminathan and also Thank, Dr.Sudha Nair and Dr.Ajay Pareda for the learning experience. Spic science foundation, Chennai gave me true exposure to research I acknowledge my mentors, Late. Dr. Joseph Thomas, Dr. George Thomas, humble person and a true basic scientist, whom I can approach on all my hurdles and also to Dr.Valli Akella, my supervisior at SPIC , from her I learned how one ably plan their experiments, Dr.Loki and Dr.Waheetha, for all their support. I thank all my friends from SPIC family, especially to Suja and Shivram. Tannery trials would have been impossible without the support of Mr.Shivram, Cheif manager SPIC Bioproducts and extending my gratitude to Dr.Chandrababu, Deputy Director, Tannery Division, CLRI, Tannery staff and the research students for their support during tannery trials.

My Alma-mater's have special place in my life. I acknowledge the role of Rev.Sr.Rosma (Little Flower Convent) and Dr.I.S.Bright Singh and Dr.A.Mohandas, Cochin University for meandering me in different stages of my academic

My wonderful family supported immensely throughout my journey. Excellent support from Nair and Pillai family members, specially to acknowledge, my brother-in-laws , Madhu, Rajeev and Sidhartha, my sister Preetha(for all her prayers on the crucial moments of my journey) and also to sis-in –laws, Aninitha and Maya, chit chatting and gossiping with them was a break from monotony. I specially acknowledge the support and encouragement extended by in-laws. Fantastic vacations with my nephews, Hrishikesh, Varun and nieces, Nivedita, Nandita and Varsha were real stress buster. I thank god for gifting me such wonderful parents, also recollecting the hardships they underwent and also for moulding me what Iam.

I am lucky to have a life companion like Jishnu, his unlimited patience, endless support and compromises made this journey possible, always insisted perfection in whatever Iam doing and supported for that. My wonderful son, the most suffered person of my Ph.D, adjusted with changing baby sitters, accommodated my occasional stay backs, even his little hand had contributed to my research by labelling test-tubes and eppendorfs on the days Idid protein purification. Needless to thank them..... this thesis is their's.

The financial support from Department of science and Technology (DST) to carry out this research work under Women Scientist scheme (WOS-B), DST /SS/005/2004 is deeply acknowledged. Special thanks to Dr. Vineeta Sharma, Dr. Usha Dixit and Mrs. Shobana Bhaskaran, for entertaining my e-mails and phone calls with respect to fund release. Last two years of the research was funded by M.S. University as research scholarship and the same is acknowledged.

Entrance exams and SBC Baroda chapter meets were like family functions. I am really thankful to all the members of department whose contribution made a very positive environment for research and proud to have MSU (Microbiology) alumnus tag.

I am kneeled down to my great grandfathers and family Gods and Goddesses, for making my research journey relatively smooth. The supreme power, Almighty, unravelled different chapters my life beautifully So far, I just accompanied the footprints left by him to walk along and will continue the saga till I will be a part of him.

INDEX

Chapter 2

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BB	Box Behenken
βME	Beta mercapto ethanol
BG	bengal gram powder
BLAST	Basic local alignment search tool
BOD	Biochemical oxygen demand
CCD	Central composite design
CFE	Cell free extract
Cfu	Colony forming unit
COD	Chemical oxygen demand
CRS	Chromeshavings
Cs	Colony size
Cz	Colony zone
DNA	Deoxyribo nucleic acid
DPC	Diphenyl carbizide
E.C.	Enzyme Commission number
ECM	Extra cellular matrix
EtBr	Ethidium bromide
GAGs	Glycosaminoglycans
GG	Green gram powder
IUE	International Union for Environment
kb	kilo-basepair
kDa	kilo-dalton
LB	Luria-Bertanni
MF	milled chicken feathers
OD	Optical density
OM	Optimized medium
PB	Plackett-Burman
PG	Proteoglycans
PM	Production medium

PMSF	Phenyl methyl sulfonyl fluoride
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
REs	Restriction endonucleases
rRNA	Ribosomal ribose nucleic acid
S.D	Standard deviation
SBM	Soyabean meal
SM	Skimmed milk
SDS	Sodium dodecyl sulphate
TDS	Total dissolved solids
TCA	Trichloro acetic acid
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

Note: The full forms of several rarely used abbreviations have been described within the text

LIST OF SYMBOLS

α	Alpha
β	Beta
μ	Micro (10^{-6})
°C	Degree Celsius
%	percentage
3’	3-prime
5’	5-prime
G	Gram
H	Hours
K	Kilo
L	Litre
m	milli (10^{-3})
M	Molar
Min	Minutes
n	nano (10^{-9})
s	Second
U	Unit
V	Voltage
wt	Weight

LIST OF FIGURES

LIST OF TABLES

STORY OF LEATHER MAKING.....

Chapter 1

Review of Literature

If you would be a real seeker after truth, it is necessary that at least once in your life you doubt, as far as possible, all things.
- Rene Descartes

1.1: Introduction

Proteases are envisaged to have extensive applications in leather industry where raw hides are subjected to a series of chemical treatments prior to tanning and finally converted to finished leather. Most important steps in leather-making, which determine the quality of the finished product, involve specific effects on the structural and other proteins of the hide. Early steps of pre-tanning processes aim to remove unwanted globular proteins and hair debris from hides while retaining the collagen layer of the corium which forms the leather. The later stages of pre-tanning aim to modify the collagen bundles so in order that the fibres 'open-up' in preparation for tanning and dyeing agents to penetrate. Proteases are considered valuable in these processes due to their potential to act selectively against specific unwanted proteins without affecting the major leather forming protein.

Hair removal is an essential process in leather manufacture. Current methods use sodium sulphide and lime to remove the hair and swell the hides before other processes such as tanning can be achieved. Sulfide degrades the hair from the terminal ends towards the hair follicles, but frequently leaves behind portions of undegraded hair in the follicles, which can be visible in finished leather. Sulfide also contributes to the high chemical oxygen demand (COD) in tannery effluents. Proteases may play a vital role in these treatments by replacing these hazardous chemicals especially involved in soaking, dehairing and bating. Increased usage of enzymes for dehairing and bating not only prevents pollution problems, but also is effective in saving energy.

Although offering a biotechnological solution for replacing the use of noxious sulfides, enzymatic biocatalysis in leather making has not proved economically viable due to high cost of enzymes and the need of controlled conditions for their action. The present study was deals with the characterization of a novel protease producing bacterium, having an enzyme with good substrate specificity, robust in its stability as well as conditions for action, with potential to offer its candidature for multiple tannery processes in an economically feasible manner.

This Chapter introduces the steps in leather making, emphasizing the biochemical alterations of hides during each step and follows with a review of present status of application of enzymes in the leather making process.

1.1: Structure and composition of hide

Hide (or skin) is usually considered to comprise three layers (**Fig. 1.1)** of which the dermis or corium (which may vary from 0.5 – 3mm in thickness in different parts of the body) is the leather forming region. The dermis, which is closely and tightly associated with epidermis through an amorphous basement membrane, is basically a connective tissue that supports the specialized elements of the skin and is composed of fibroblast cells and a network of collagen fibres embedded in the extra cellular matrix (ECM) (Stanley et al., 1982). Upper dermis, also known as grain layer is rich in soluble proteins, type I collagen, proteoglycans (PG) cementing substances such as hyaluronic acid and dermatan sulphate and elastic fibers and some non-fibrous collagens apart from fibroblasts. The fibroblasts synthesize the components of the ECM including collagen, reticular and elastic fibres and other globular proteins and proteoglycans. The individual unit of corium is collagen fibrils which are woven together to form fibril bundles which are in turn cross-linked to create collagen fibres. The structure of the collagen fibres varies throughout the cross section of the hide, with maximum diameter at the centre and decreases towards the upper and lower end. The network of fibre is referred as weaves, consist of fibres dividing and recombining with other fibres. Fibril bundles of the corium render it strong to withstand the stress and make it stretchable. Subcutaneous adipose (fat tissue) beneath the dermis layer is loose connective tissue contains lipids

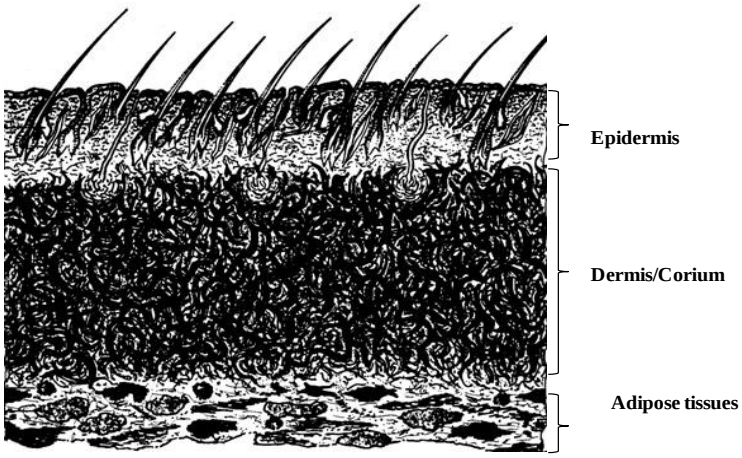


Fig. 1.1: Structure of hide

1.2: Structure of hair follicle

The hair is embedded firmly in the corium by hair follicle, covered by external and internal root sheaths. A cross-section of the hair bulb reveals three layers, an outer cuticle, middle cortex and central medulla (**Fig. 1.2**). The hair growth cycle has three distinctive phases: anagen: the period of active growth; catagen: the period of breakdown and change; telogen: the resting stage before resumption of growth (Paus and Cotsarelis, 1999). The epidermal cells surrounding the dermal papilla form the germinal matrix or root of the hair. During anagen these cells are constantly dividing, and as new cells are formed they push the older ones upwards where they begin to change shape. By the time the cells are about one-third of the way up the follicle they are dead and fully keratinised. Catagen is the end of the active growth period, and is marked by changes occurring in the follicle, resulting in loss of growth and detachment from the base of the follicle forming a club hair which remains in the follicle due to its shape. This period of breakdown or change lasts about three weeks. The shortened follicle rests for about three months known as the telogen phase. On average 85% of follicles are in the anagen stage, 1% of follicles in the catagen stage and about 14% of follicles are in the telogen stage. Thus dehairing of hides is largely concerned in the removal of anagen hairs which are firmly attached to the follicle.

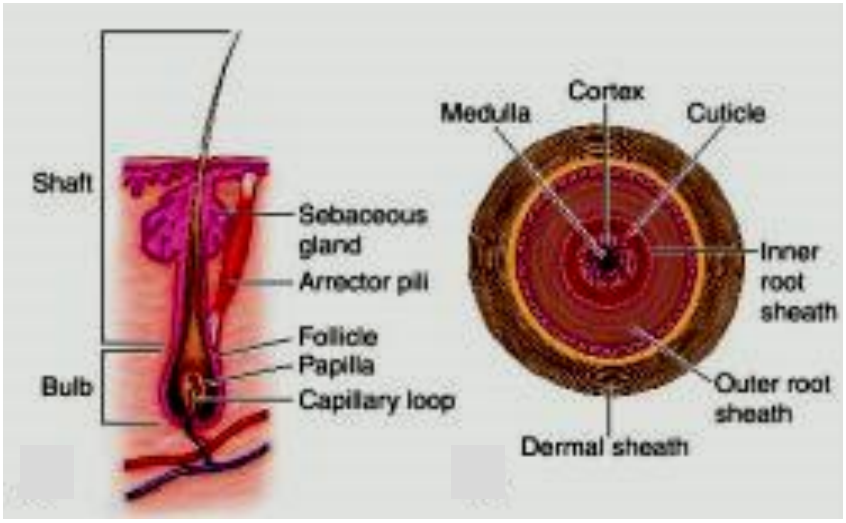


Fig. 1.2: Structure of the hair follicle and cross section of hair root

1.3: Processes in leather making

Tanning, the process of transforming the animal hides to leather, involves the conversion of skin, a natural renewable resource, which is putrescible and inflexible when dried, to a chemically and structurally modified form with long shelf life and high flexibility. The leather production process is made up of several operations and typically includes hide and skin storage and beam house operations, tanning operations, post-tanning operations and finishing operations **(Fig. 1.3).**

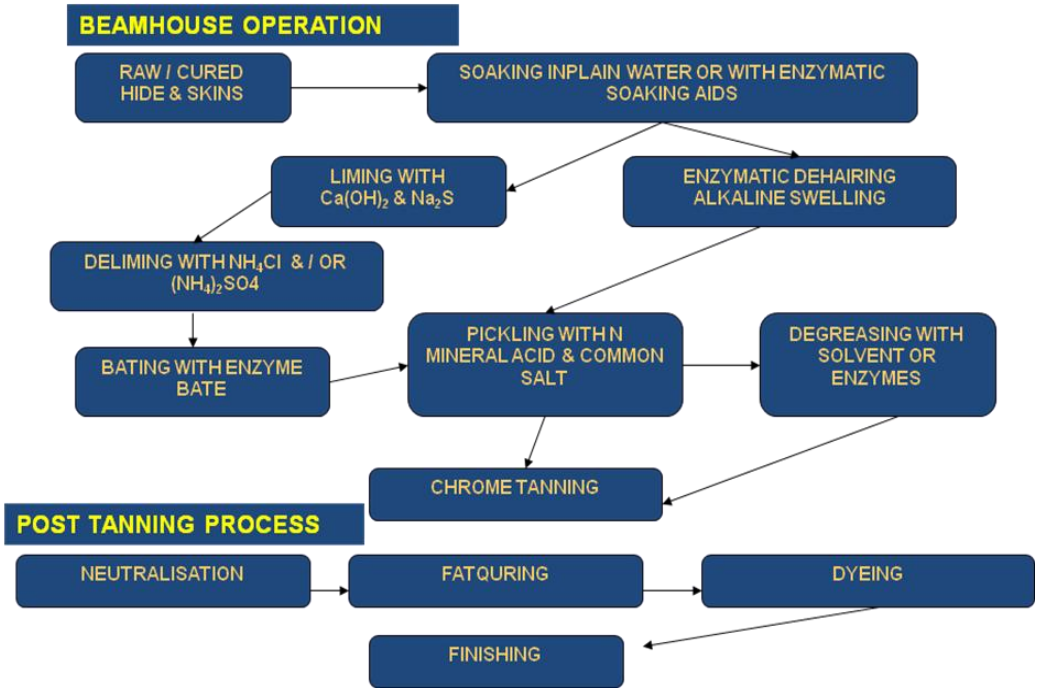


Fig. 1.3: Flow chart showing the tannery processes (Puvanakrishnan and Dhar, 1999**)**

Leather-making occurs in three broad steps, viz, removal of all materials that are not a part of the final product, rendering the remaining hide not easily biodegradable i.e. tanning and finishing the tanned material to desired characteristics of the final material (Ramasami et al., 1999; Thanikaivelan et al., 2005). Pre-tanning processes also called beam house operations collectively represent all the processes of conversion of hide to pelt. At this stage, hides/skins are fleshed, dehaired and shaved to the required thickness (**Fig. 1.4**).

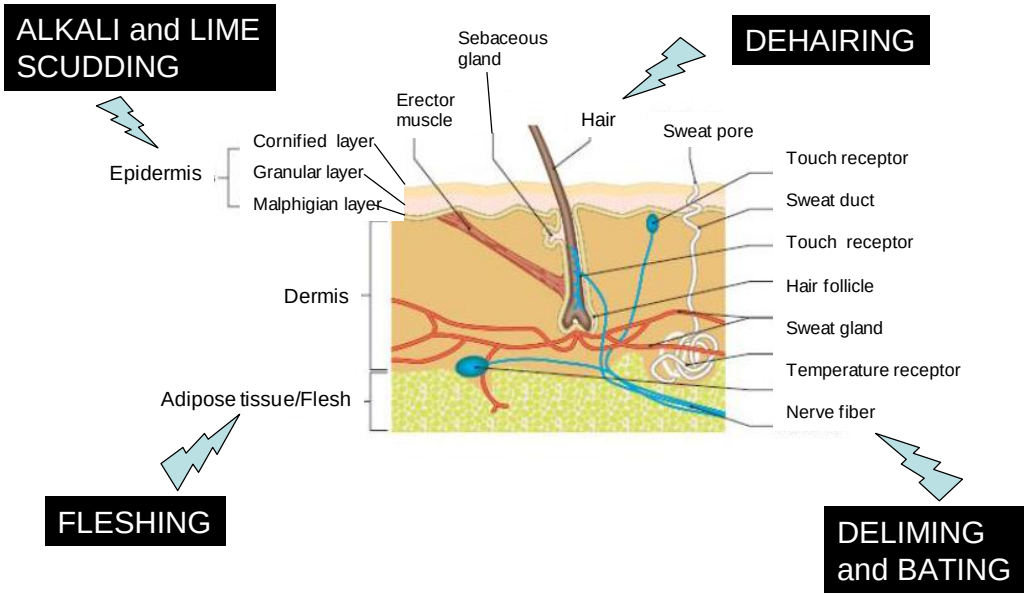


Fig. 1.4: Structure of skin and its components showing the pre-tanning operations that remove the specific unwanted components during leather making

The major component of hide and the biochemical modifications they undergo during leather making is depicted in **Fig. 1.5**. From the point of view of leather making, hides consist of the following major proteins: collagen, elastin, albumin, and keratin. Among the fibrous proteins of skin, collagen constitutes about 98%, elastin 1% and keratin 1%. About 3.5% of total skin proteins are globular proteins which have to be removed in the finished product. Keratins of epidermis and hair are also not usually part of the finished leather. The cementing substances, including nonfibrillar collagen molecules are also to be eliminated from the final product, since without removal of the interfibrillar proteins, leather has a hard and horny structure due to the cementing all of the corium fibers together. In pretanning procedures usually carried out by the application of lime and sulfides, inter-fibrillary proteins as well as the keratinous dermis is removed to make the leather soft and smooth. The soft, jellylike cells and ECM have little resistance and are readily disintegrated by alkalies, especially sodium sulfide or hydrosulfide. The collagen fibres being most resistant part of the skin are not lost upon this treatment, and the tightly woven surface of the dermis is exposed as a smooth layer during pre-tanning processes, on which the grainy appearance is revealed. Toward the centre of the dermis the collagen fibres are coarser and stronger, and their interlacing determines the properties of the resultant leather: if upright and tightly woven, the leather will be firm and hard, with

little stretch, while if they are more horizontal and loosely woven, the leather will be softer and stretchable.

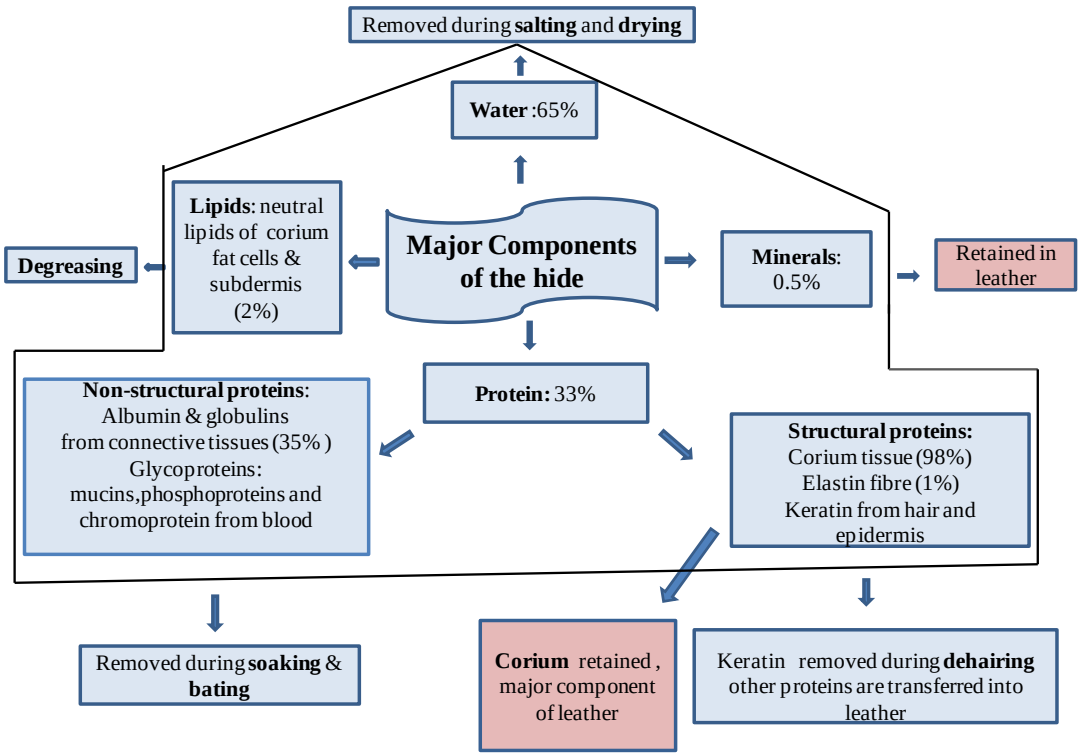


Fig. 1.5: Over view of major components of hide and their fate during leather making (compiled from Uhligh, 2006)

1.3.1: Pre-tanning process

Pre-tanning process is called as **beam house** process, because traditionally fleshing, dehairing and shaving operations were carried in between wooden beams. Now the term **beamhouse** is collectively representing all the process of conversion of hide to pelt, finally leading to the tanning stage (Covington, 2009).

1.3.1.1: Soaking

Soaking is the first operation in the tannery, where the fresh or preserved hides and skins are treated with water for making them clean and soft. Hides/skins received in the tannery are of four categories, viz, fresh immediately after flaying, wet salted, dry salted and dried. Prime purpose of soaking are the removal of extraneous matter, which otherwise are good nutrients for bacterial growth, and restoration of moisture lost during curing and preservation. Furthermore large amounts of salt have to be removed some of which adheres to the outside of the hide and the rest that is well penetrated in the entire cross section of the hide.

Soaking process rehydrates the dried interfibrillary proteins and loosens the cementing substance of the fibres. The collagen fibers and keratin cells of the hair and epidermis also takes up water and become more flaccid and flexible. Green/fresh hides are soft and can be processed without soaking, whereas the salt cured hides, the moisture content is increased from 45 to greater than 55% by soaking. Soaking aids used to improve the efficiency of soaking are antimicrobial compounds and surfactants.

1.3.1.2: Dehairing

Hair on animal skins varies depending upon the animal species, its age and health (**Fig. 1.6**). Unless the hair is desired for the final product it is removed by chemical and/or physical means. Dehairing is an essential step of the pre-tanning processes of leather manufacture where in the hair is removed along with epidermis and during the process non-collagenous proteins and other cementing substances are removed from the skin. Upon dehairing and removal of the keratinous epidermis, the distribution pattern of hair on the surface of the skin is exposed in the form of grainy texture and this forms the characteristic grain pattern of the leather (**Fig. 1.6**). The process of unhairing is achieved by either hair-destruction methods, where the hair is reduced to a pulp, or by hair-saving methods, where the epidermal tissue surrounding the hair bulb is destroyed or modified, so that the hair is loosened and can be removed intact. Hair and wool can be used as valuable by products of leather industry by hair saving dehairing methods.

In modern leather production, sulfide as Na_2S or NaSH is applied at about 2-4% based on the weight of the hides in the presence of lime in quantities sufficient to make the pH to about 12. The chemical process consists of painting the flesh side of the washed and soaked skins with a thin paste composed of lime (20-30%)-sulphide (6%) mixture. After painting, skins are piled up and incubated until the hair becomes loose and can be removed either by a blunt knife or by mechanical means (Puvanakrishnan and Dhar, 1985).

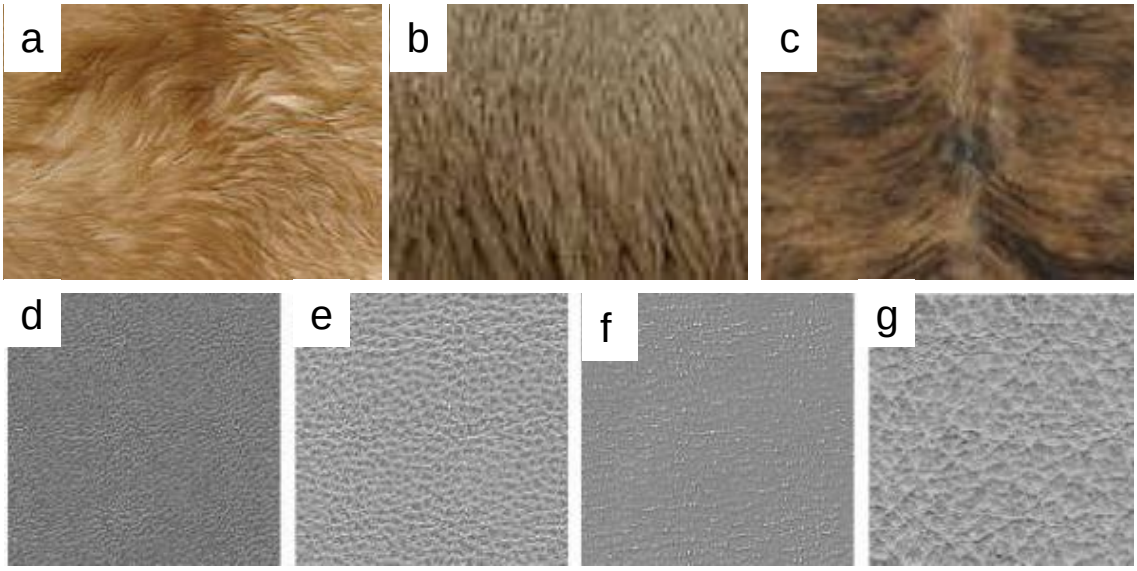


Fig. 1.6: Textures of untreated animal hides and finished leather. a: Goat hide; b: Sheep hide; c: Bovine hide; d: Bovine leather; e Ovine leather; f: Goat leather; g; Pig leather

1.3.1.3: Bating

To make leather pliable, the hides and skins require an enzymatic treatment before tanning known as bating. During bating, scuds (short hairs) are loosened and other unwanted proteins are removed and degree of stretch increases. Bating de-swells swollen pelts and prepares leather for tanning. It makes the grain surface of the finished leather clean, smooth and fine. The main objective of bating is to remove non-leather forming proteinaceous materials like albumins, globulins and muciods from hides and skins and to allow the splitting of collagen fibres so as to help the penetration of tanning materials and other processing chemicals thereby giving the finished leather the desired feel, softness, pliability and other characteristics properties. The most suitable temperature for bating is 37 °C. At high temperature, pelt is damaged and large amount of hide substance is dissolved. Appropriate bating time is few hours to overnight bating depending on the type of leather, preferred pH is 8.0-9.0 and for deliming, good proportion of ammonium salts are used.

Traditional methods for bating employed use of animal manure which is unpleasant, unreliable and slow. Bio-technical developments have now completely replaced this method with use of industrial enzymes. In bating, pancreatic enzymes are used in combination with neutral and alkaline bacterial or fungal proteases. Bating

with enzymes is an indispensable operation of leather processing to obtain best quality of leather and cannot be substituted with a chemical process.

1.3.2: Tanning and Post-tanning processes

Tanning is the stabilising reaction of collagen by modifying peptide links at different reaction sites, while hide is getting converted to leather. Traditionally done with plant tannins, now-a-days chrome tanning is routinely applied. Of the total leather production in India, more than 80% is based on chrome tanning and the rest is based on vegetable tanning. Basic chromium sulphate [$Cr_2(SO4)_3$] (7-10 %) containing 25% Cr_2O_3 and sodium sulphate (25-30%) is used in chrome tanning. The pH is raised to 3.8-4.0 (from 2.5) at the end of chrome tanning process which is called basification. The semi-finished leather after chrome tanning is called wet blue. Plant extracts are used for the purpose of vegetable tanning in which the pH falls down from 4- 4.5 to 3-3.5. Though this process is free of any heavy metal use, the leather developed from this process has comparatively weaker capacity of heat resistance and dye-holding.

Post-tanning operations comprise of re-chroming of semi-finished wet blue leather, neutralization, dyeing, fat liquoring and finishing. In case of post-tanning of vegetable tanned semi-finished leather, the operations involved are semi-chrome tanning, neutralization, dyeing, fat liquoring and finishing. However the operations vary depending on the final product. Phenolics, melamine, acrylics, polymers, naphthalene, etc., are used for finishing to impart fullness to the leather (http:/.tanneryindia.com/)

1.4: Environmental concerns of leather-making

The leather industry exists in cottage, small and medium scale sector and being labour intensive, provides direct and indirect jobs to lakhs of men and women. In recent times tannery is the third largest foreign exchange earner for India (Nagesha and Balachandra, 2006) and the Indian leather industry is considered a successful industry in terms of exports and employment opportunities (Muchie, 2000). Unfortunately, one tonne of the hide gets converted only to 200 kg of leather, rest all are wastes, byproducts and pollutants. The effluent arising from chemical dehairing process are high in alkaline and suspended solids. Use of sulphides liberates toxic hydrogen sulphide which is a serious hazard for tannery workers. Also, hydrogen

sulphide is liable to be oxidised by bacterial action to sulphuric acid, which can cause damage to iron and concrete work. Dissolved sulphide and pulped hair contribute to high biological oxidation demand (BOD) and chemical oxidation demand (COD) in the effluent. In addition to the noxious gases such as ammonia and hydrogen sulfide are released in to the atmosphere. The tannery effluent is high in total dissolved solids (TDS), sulfides, chlorides, sulfates, chromium, etc (**Table 1.1, Fig. 1.7**). Apart from this, solid waste in form of lime sludge and chrome sludge are generated. Conventional liming-reliming effluents contribute 50-70% of the total BOD and COD load and 15-20% of (TDS) load.

A main concern from tannery effluent is the chromium pollution due to presence of its compounds in the discharges from this industry. Chromium and other chemicals are recovered in India, but technically and economically it is not possible to recover all the chromium or other heavy metals present in the effluent. In such cases some amount of chromium remains and becomes responsible for adverse impacts on the environment. Besides this, tanneries are criticized for their consumption of water. Generally about 30–40 m^3 of waste water is generated per tonne of raw material processed.

Table 1.1: Composition of the tannery tffluents and their permissible levels (UNIDO report, 2000 www.unido.org**)**

Parameter	**Composite Tannery Effluent (mg/l except pH)**	**Permissible levels**
pH	8.7	7-9
BOD (Total) 20°C, 5 days	2500	30
COD Total	6000	250
Total Solids	31,600	2200
Dissolved solids	26,600	2100
Suspended solids	4900	100
Chromium as Cr (III)	200	0.5-2.0

In order to curtail the environmental impact, it is necessary to develop strategies for pollution management in the tanneries in countries where this industry plays an important role in the economic development. Some of the large-scale tanneries are adopting cleaner technologies which minimize the use of toxic chemicals such as lime and sulfides by involving the use of enzymes Since small scale producers are apprehensive about the cost involved, it is essential to develop a cost effective and eco-friendly technology by screening for efficient enzymes from microbial sources and producing them in large quantities by applying recombinant DNA technology.

Fig. 1.7: Polluted water body due to the effluent discharge from tannery (http:// tanneryindia.com/)

1.5: Enzymes in leather processing

In recent years enzymes including proteases, amylases and lipases, find application in the soaking, dehairing, bating and degreasing operation in leather making (**Table 1.2**). Proteases are used mainly in the soaking, dehairing and bating. Application of cellulases and xylanases in soaking are proposed for removal of extraneous matter. Among the different industrial proteases pepsin, trypsin and rennin are the most widely used enzymes in leather manufacturing (Choudhary et al., 2004). Pepsin has been reported being applied during pickling and chrome tanning. Another class of important industrial enzyme is transglutaminase ,which catzlyses acyl transferase reaction between carboxamide group of peptide bound glutamine residues as acyl donors and primary amine as acceptors (Puvanakrishnan et al., 1999). This enzyme

can be useful in improving the quality of the bated pelt by stabilizing the collagen fibre bundles.

The major impediment in the application of enzyme technology is its high cost, requires careful control of conditions such as pH and temperature. Variations in the conditions may produce inconsistent results which may give rise to inefficiency of desired action or unwanted action against leather forming protein affecting the quality of the finished product.

Table 1.2: Role of enzymes in different stages of leather processing (Choudhary et al., 2004**)**

Stages	Enzymes involved	Functions of enzyme
Curing	Enzymes are not directly involved	To preserve skin/hides
Soaking	Acidic, neutral proteases and pancreatic proteases	To remove non-fibrillar proteins
Dehairing	Alkaline and neutral proteases	To remove immature keratin and other interfibrillary cementing material and waste water quality improvement
Degreasing	Lipases and proteases	To remove lipids and adipose layer
Bating	Trypsin, neutral and alkaline proteases	To make pelt soft, supple and pliable
Tanning	Enzymes are not directly involved	To influence the quality of tanning
Waste processing	Trypsin and other proteolytic enzymes	Chrome-tanned waste processing

1.6: Enzymes in the management of waste from tannery

The waste material generated from hide and skin consists mainly of proteins and lipids. Significant research work has been carried out in development of newer

methods of recovery and utilization of trimmings and fleshing wastes. Conventionally, fleshings from the tannery industry are treated by a chemical thermal process, which needs high amounts of energy and is time consuming. An alternative is an enzyme process, which is more favourable in terms of environment protection. Furthermore, protein products obtained can be used as fertilizers or for other purposes. A procedure for the extraction of protein from skin, bone and cartilage wastes by phosphoric acid hydrolysis was developed and analysed (Chakarska et al., 2006). Fleshings were hydrolyzed using pancreatic enzymes with a view to evolve a simple method for solid waste management. Treatment with pancreatic enzyme preparation showed a six fold increase in proteolysis with total liquefaction, against the control at the end of 7 days. (Kumaraguru et al., 1998). The extraction of collagen from raw sheepskin trimmings was studied in a two step process in which the first step involved use of trypsin (Hervas et al., 2007). A heat stable alkaline protease produced by *Paecilomuces lilacinus*, a fungal isolate, was studied for its effectiveness in fleshing hydrolysis (Chakraborty and Sarkar, 1998). Animal fleshing was used as the substrate for the production of alkaline protease by Pseudomonas aeruginosa (Kumar et al., 2008). The production of a mesophilic protease with solvent stability in solid-state fermentation using a proteinaceous solid waste is studied (Kumar et al., 2009). Animal fleshing, the major proteinaceous solid waste discharged from leather manufacturing industries was used as the substrate for the production of alkaline protease by Pseudomonas aeruginosa (Kumar et al., 2008). The microbiological method for utilization of hide trimmings, green and limed fleshings from leather manufacturing industries, for the production of value added products like enzymes, is an unconventional methodology compared to existing chemical and thermal methods for the disposal of solid wastes. The production of a mesophilic protease with solvent stability in solid-state fermentation using a proteinaceous solid waste is studied (Kumar et al., 2009). Wet leather fleshing (LFs) from sheep and goatskins had high alkaline pH (12.1) and ash (18.1%) due to liming of fresh skins for leather production. Treatment of limed fleshings with 0.1% H2O2 (1:10v/ v) followed by 0.2 HCL (1:10w/v) solutions produced very little or no H2S. Delimed fleshings, mixed with 19.5% (v/w) Pediococcus acidolactici culture, 20% (w/w) sugar and 2% (w/w) common salt, on fermentation at 30 ± 2 °C yield silage (protein 7%, fat 8% and ash 3.6%), which can be used in animal feeds. This can also be used in aquaculture and

animal feeds to supplement arginine, phenylalamine and tyrosine (Bhaskar et al., 2007).Enzyme are also employed for by-products utilization and effluent treatment. Enzymes could be used in the treatment of fleshings and effluent from tannery processes. A ombination of hydrolytic enzymes, viz. proteases, carbohydrases, and lipases would be required. The advantages to be realised include a protein by-product suitable for animal feed as well as energy conservation and fat recovery (Ganeshkumar et al., 2007)

1.7: Proteases in Pre-tanning

Biochemically, leather can be considered to be composed of a modified structural protein-collagen and the processing of hides during leather making require specific and limited proteolysis. Proteases contribute a major role in different stages pretanning where alkaline proteases and keratinases are employed.

1.7.1: Proteolytic soaking aids

Proteolytic enzymes increase the rate of soaking of dried fur skins. Acidic to neutral pH has the advantage of loosening the scud and production of leather with less wrinkled grain and a decrease in soaking time. Proteases solubilize the non-leather forming globular proteins and thereby help in the removal of interfibrillary cementing substances from the hide. Grimm (1996) has described a soaking method using proteolytic enzymes and carbohydrases in the pH range of 5.5 to 10.0. Alkaline proteases from *Aspergillus parasiticus*, *A. flavus*, *A. oryzae*, and *Bacillus subtilis* have been used alone or in mixtures with the use of carbohydrase from the mold culture A. *awamori* in soaking (Dutta, 1985). Soaking is usually carried out using a combination of proteolytic enzymes that are optimally active in the neutral or alkaline pH range. For enzymatic soaking, the average soaking period for salted raw stock is about 4 h and for dried raw stock is about 8–10 h (Taylor et al., 1987). A water soak without auxiliary agents takes 24 h for salted hides, and 36–48 h for dried hides. Maps (www.maps enzymes.com) offers a formulation of protease and lipase for soaking which work in different pH conditions. A mixture of protease and lipase for soaking in alkaline pH conditions “palsoak” and a mixture of protease and lipase for soaking in acidic pH conditions “Palkosoak ACP” are some of the popular soaking formulations.

1.7.2: Proteases in unhairing

As early as 1910, enzyme preparations were suggested for unhairing. A systematic analysis of more than 30 different enzyme preparations having proteolytic and/or amylolytic activities from microbial, plant and animal sources. Importantly, their experiments involved pre-treatment of hides with lime, under these conditions they found that microbial preparations were more effective than non-proteolytic preparations as well as than pancreatic preparations. It was concluded that depilatory action was not correlated protease activity as measured by caseinolytic ability. It is now understood that not all proteases are effective at dehairing of hides and specific substrate preferences are probably required for the protease to be effective at dehairing.

Advantages of enzymatic dehairing are: the significant reduction or even complete elimination of the use of sodium sulphide, recovery of hair of good quality and strength with a good saleable value and creation of an ecologically conducive atmosphere for the workers (Arunachalam and Saritha, 2009). Residual hair has a high protein content of 82 percent, with nitrogen content of 13 percent and constitutes an important source of protein uses as biological fertilizers in agriculture or horticulture and animal feed (Serrano et al., 2003). About 5% of dry hair is recovered based on the raw hide weight in case of bovine and ovine hides. Enzymatically dehaired leathers have shown better strength properties and greater surface area and retain undisturbed nature of the original hide structure (Cantera et al., 1996; Valeika et al., 2009). Another significant advantage of the enzymatic dehairing process is the time factor involved, it can be accomplished comparably if not faster than as the lime-sulphide process. Industrial scale enzymatic process has started from 1990. It has been suggested that pelts unhaired by using an enzymatic depilant containing microbial proteases do not require further liming, deliming and bating. The effluent resulting from the enzymatic unhairing methods is found to be easily biodegradable because of its lower pH value. The difficulty arising from the gelatinization of the sludge derived form the hair could be avoided by the use of enzymes. Variations in the conditions may produce inconsistent results which may be inefficiency of desired action or unwanted action against leather forming protein affecting the quality of the finished product. Controlled tannery trials and laboratory experiments show that the quality of the finished leather in terms of its physical attributes such as tensile

strength, elongation, tearing strength, is comparable if not better than conventionally depilated (lime/sulphide method) leather (Choudhary et al., 2004; Sivasubramanian et al. 2008). It has been shown that the use of enzyme for dehairing results in 2% increase in area of the final leather (Thanikaivelan et al. 2004).

1.7.3: Bating enzymes

Rohm (1907) first patented the production of bating agent by using a mixture of pancreatic extracts and ammonium salts. Modern bating processes employ pancreatic enzymes or proteolytic enzymes of bacterial origin. Different commercial formulations are available as bating enzymes. Verma batzyme, an alkaline protease, is a bating agent which contains ammonium salts and buffers and is suited for all kinds of pelts. It improves the grain clarity and smoothness will be improved and all excess proteins will be dissolved and removed from the hides and skins. Verma cidbate, an acid protease to be used in acid bating of the hide and skin and is specially designed for bating of hides and skin already pickled or tanned by either chrome or vegetable or vegetable synthetic method. This enzyme can be used for bating of carbon dioxide delimed pelts or pelts limed in acidic conditions with oxidizing agents (www.enzymes-India/leather-enzymes.html). Alkobate and alkocid are bating formulations from MAPS (www.mapsenzymes.com) which works under alkaline and acidic conditions respectively. Dermilize PGC is a low duset granular alkaline protease derived from a genetically modified strain of *B. subtilis* is a bating agent for hide and skin. Dermalize BA100 is a concentrated granular powder formulation also a commercial bating enzyme

1.8: Application of pre tanning enzymes on hide

Soaking, enzymes are added into the soak respect to the hide weight and the nature of the hide. Most popular method of soaking is the pit soaking (**Fig. 1.8**).

Fig. 1.8: Different types of pre tanning processes: a, pit soaking; b, drum soaking; c, paint method on flesh side; d, hide partially deahaired by dip method; e, drum bating.

Proteolytic enzymes are added at a concentration of 0.5% and 0.3% of the hide weight for green and salted hides respectively (Puvanakrishnan and Dhar, 2000).Three methods of application are commonly used in the enzymatic dehairing process: (i) paint method (Malathi and Chakraborty, 1991; Purushotham et al., 1994; Pal et al., 1996; Raju et al., 1996), (ii) dip method (Macedo et al., 2005; Jaouadi et al., 2009; Rai and Mukherjee, 2010), and (iii) spray method (**Fig. 1.8**). Of late, dehairing by drumming is being practiced, and industrially this should be feasible. Bating is mostly doll-drum process and enzymes are added into the drum along with the bating liquid (**Fig. 1.8**).

1.9: Sources of proteases used in pre-tanning

Pancreatic proteases and trypsin are used mostly for beam-house process. Apart from that other sources of proteolytic enzymes employed in pre-tanning are depicted in **Table 1.3**. Dehairing proteases may be derived from fungi, bacteria, or

actinomycetes. Gram positive bacteria are far more preponderant in the production of dehairing enzymes, among which *Bacillus* sp. are undoubtedly the most well studied and largest studied group of organisms to produce dehairing enzymes (**Table 1.3**). Among different *Bacillus* species, *B. subtilis* strains have been reported maximally to produce enzymes suitable for dehairing of hides and skins.

Table 1.3: Sources of proteases for pre-tanning processes

Process	Microorganism	Reference
Soaking	*Aspergillus parasiticus*	Jareckas et al., 1985
	Aspergillus flavus	Monsheimer and Pfleiderer, 1974
	Bacillus subtilis	George et al.,1995
	Rhizopus rhizopodiformis	Kamini et al.,1999
Dehairing	*Aspergillus flavus*	Malathi and Chakraborty, 2001
	Rhizopus oryzae	Pal et al., 1996
	Lactobacillus sp	Malathi and Chakraborty, 1991
	Condiobolus sps	George et al., 1995
	Streptomyces griseus	Gehring et al., 2002
	Streptomyces fradiae	Chandrasekharan and Dhar, 1983
	Streptomyces moderatus	Chandrasekhar and Dhar, 1983
	Bacillus subtilis	Macedo et al., 2005
	Bacillus subtilis	Mitra and Chakrabartty, 2005
	Bacillus licheniformis	Tiwary and Gupta, 2010a
	Bacillus cereus	Nilegaonkar et al., 2007
	Bacillus sp	Giongo et al., 2007
	Bacillus pumilus	Jaouadi et al., 2009
	Bacillus subtilis	Sivasubramanian et al., 2008
	Bacillus halodurans	Prakash et al., 2009
Bating	*Aspergillus parasiticus*	Muthukumaran and Dhar,1982
	Streptomyces rimosus	Pfleiderer et al.,1984
	Bacillus licheniformis	Jareckas and Proizrod, 1974
	Bacillus subtilis	Kamini et al., 1998
	Penicillium janthinellum	Sztajer and Zboinska, 1988

1.10: Conditions for the action of pre-tanning proteases

Ideal conditions for soaking are alkaline pH of 9.8-10.5, temperature of 20°C for aduration of 4-6h (Puvanakrishnan and Dhar, 2000). Raising the temperature of the water aids in the dispersion of globular proteins, but results in the loosening of the fibre bundle (Thorstensen, 1985).

The optimum conditions for the dehairing of hides using proteases from different sources are compiled in **Table 1.4.**

Table 1.4: Optimum conditions for hide dehairing by microbial enzymes.

Isolate	pH	Temperature (°C)	Time for Dehairing (h)	Reference
Aspergillus tamarri	9-11	30-37	16, 6 with SDS	Dayanandan et al., 2003
Aspergillus flavus	9	32	20	Malathi and Chakraborty, 2001
Rhizopus oryzae	3-11	33-35	11-12	Pal et al., 1996
Acaligenes feacalis	8-11	30	24	Thangam et al., 2001; Thangam and Rajkumar, 2002
Bacillus sp	7.5-9	37	18-24	Raju et al., 1996
B. altitudinis GVC11	9-11	30	24	Vijay kumar et al., 2011
B. amyloliquefaciens P7	7-11	25	18	Giongo et al., 2007
B. cereus MCM B-326	6-12	28	24	Nilegaonkar et al., 2007; Zambare et al., 2007
B. cereus VITSN04	8	30		Sundararajan et al., 2011
B.s circulans	11	35	12	SubbarRao et al., 2009
B. halodurans PPKS-2	11	28	16	Prakash et al., 2009
B. halodurans AH-101	5-12	35	20	Takami et al., 1999
B. licheniformis AP-1	9-12	35	16	Zhang et al., 2003; Tang et al., 2004
B. licheniformis ER-15	11	30	21, 16 with 3% Ca $(OH)_2$.	Tiwary and Gupta, 2010a
B. pumilus	10	35	14	Zhang et al., 1998
B. pumilus CBS	9-11	37	24	Jaouadi et al., 2009
B. subtilis P6	7-11	25	18	Giongo et al., 2007
B. subtilis IIQDB 32	8	30	12	Varela et al., 1997
B. subtilis BA 06	9.6	35	12	Wang et al., 2007
B. subtilis MTCC 6537	7	30	18h	Sivasubramanian et al., 2008
B. subtilis S14	9	24	9	Macedo et al., 2005
B. subtilis DM-04	10	37	6	Rai and Mukherjee, 2010
B. subtilis IH-72	8-9	37	7	Mukhtar.and Ikram-ul-Haq, 2008

B. velesensis P11	7-11	25	18	Giongo et al., 2007
Microbacterium sp. kr 10	6-8	30	22	Riffel et al., 2003; Brandelli et al., 2008
Chryseobacterium sp. kr 6	6-8	30	22	Riffel et al., 2003; Brandelli et al., 2008
Pseudomonass aerogenosa MCM-B327	7	28	21	Zambare et al., 2011
P. aeruginosa PD100	8	50	2-3	Najafi et al., 2005
Vibrio sp. Kr2	5-12	30	20	Riffel et al., 2003; Brandelli et al., 2008
Streptomyces griseus	7.5	37	20, 4 with bicarbonate	Gehring et al., 2002
S. nogalator	7.5-8.5	28	12 (overproducing mutant), 22 (wildtype),	Mitra and Chakrabartty, 2005

It can be seen that dehairing proteases show a broad range of pH optima. Representative enzymes functioning in acidic, neutral as well as alkaline pH range are observed, however, most dehairing proteases can be categorized to be functioning efficiently at near neutral pH or at alkaline pH. Some enzymatic preparations need sodium sulfide to be added (Cantera et al., 1996) while others are more effective with addition of lime (Tiwary and Gupta, 2010) or detergents (Dayanandan et al., 2003). Those with preference for high pH may work efficiently with limed hides, as was observed for *B. licheniformis* ER-15, wherein addition of the alkali reduced the time of dehairing (Tiwary and Gupta, 2010). In case of *A. tamarri*, it was observed that the enzyme caused damage of the leather grain when applied alone but was more efficient and caused less damage in the presence of the anionic detergent SDS, presumably due to the removal of fats as well as some soluble proteins from the fleshy portions of the hide (Dayanandan et al., 2003). In most cases however, the enzymes work without any additives and bring about efficient dehairing within 6-20 h, the notable exception being the enzyme preparation from *Pseudomonas aeruginosa* PD100 which is reported to act within 2-3h (Najafi et al., 2005).

Ideal pH for the action of bating enzymes is in the range of 8.0-9.0 and the most suitable temperature for bating is around 37 °C. Temperature above this can lead to loosening of the hide structure and also the distortion of grain nature (Puvanakrishnan and Dhar, 2000).

1.11: Mechanism of dehairing

The differences in the amino acid chemistry of the hide collagen and the hair keratin are the basis of the lime sulfide unhairing system. Keratin has a sulfur content of 3 to 5 % due to the presence of cysteine residues. This amino acid cross links the polypeptide chains of mature hair keratins. The sulfide breaks the polypeptide S-S linkages and the integrity of the epidermal layer is greatly affected (El Baba et al., 2000). Unhairing without sulfide may take several days or weeks. Sulfide degrades the hair from the terminal ends towards the hair follicles, but frequently leaves behind portions of undegraded hair in the follicles, which have to be removed by scudding or it may be visible in finished leather. The mature keratins of hair can be easily hydrolysed after the breakdown of the hair fiber structure and the hair can be removed by gentle mechanical pressure. The basement membrane, which is fragile and sensitive to chemical and mechanical treatment, is consequently removed along with the epidermis on liming, leaving a surface of the grain layer (Cantera, 2001). The collagen fibers are unaffected by the presence of the sulfides as this molecule lacks cysteine residues and is crosslinked by unique lysine-lysine bridges intra-molecularly and by hydroxypyridinium structures between different collagen molecules in the bundles. Lime contributes to swelling of skins by removing the electrical charge from the basic groups in collagen and changing the dimensions of its structure (Menderes et al., 2000). Lime also opens up the collagen fiber bundles by removing much of the charged glycosaminoglycans (GAGs) from proteoglycans through carbonyl elimination (Alexander et al., 1986).

Mechanism of protease action for dehairing is less understood. In order to reach the hair bulb, the enzyme has to overcome the barrier of mature keratinous layer constituted by the upper part of the epidermal layer which is tightly cross-linked with disulpide linkages, making the enzyme inaccessible to its target sites. Without the breakdown of the disulphide linkages by sulfitolysis or sulphide bond reduction, the mature keratins are refractile to enzymatic hydrolysis (Onifade et al., 1998). Sufitolysis can be brought about by cell-bound redox system, intra- and/or

extracellular disulfide reductases, or the release of sulfite and thiosulfate (Kunert, 1989; Bockle and Müller, 1997; Ramnani et al., 2005). Keratinolytic action of proteases can be increased over 50 folds by the combined action with disulphide reductase (Yamamura et al., 2002). The immature keratins found in hair roots, hair root sheaths, and the lower layer of the epidermis, however, may be more easily degraded by proteases without sulfitolysis.

For the enzyme action on the hair bulbs and follicles, it is expected that the enzyme not only penetrate through the epidermis (which keratinases might well do) but it also diffuses deep in to the corium where the hair bulb is embedded. The cementing substances in dermis crosslink the collagen fibers making them highly compact and impervious to the diffusion of substances including enzymes (Cantera, 2001). If the enzyme preparation has the ability to remove the cementing substances, it is possible that the enzyme could reach the hair bulb to mediate its action. Different enzyme preparations may exhibit differences in their specificities towards these components of cementing substances. The detailed interactions of enzymes on different components of the ECM and the finer differences in the enzyme substrate preferences and how these factors affect on the unhairing efficiency as well as the quality of the pelt are important aspects that have to be more clearly understood.

The mechanism of opening of collagen fiber bundles, caused by the chemical and enzymatic methods is also different. Lime hydrolyzes the amide side chains from the basic groups such as asparagine and glutamine in collagen, resulting in negative charge on the collagen units resulting in the electrostatic repulsion which eventually lead to splitting up of fiber bundles (Ramasami et al., 1999), leading to a shortening and thickening of each fiber and in increase in thickness (swelling) of the whole fiber structure. Lime also catalyzed the β-carbonyl elimination of an alkoxide from an O-substituted serine with the concomitant separation of the charged GAGs from protein core of PG and opened up the collagen fiber bundles (Alexander et al., 1986). In case of the enzyme on the other hand, proteases attack and degrade protein core of glycoproteins, which in turn could release GAG side chains and N-linked oligosaccharides, lead to a drop in the viscosity of cementing substances around the collagen fibers, resulting in the opening up of fiber bundles, favouring further penetration of enzyme. A major advantage of such an enzymatic dehairing process is that bating step in leather processing could be avoided.

1.12 : Specificities of proteases to be used in pre-tanning

Generally usage of proteases in pre-tanning is often on a 'trial and error basis' due to a lack of understanding of specific targets from among the different hide proteins. Examination of protease activities in different enzyme preparations could lead to a directed degradation. On the basis of the substrate specificties, proteases may be categorized as keratinases, collagenases, gelatinases etc. Typically, general, nonspecific protease activity is monitored using casein as substrate as it is most readily digested by many proteases. These are further denoted as keratinases, collagenases etc. based on the ratio of the caseinolytic activity and activity on specific substrate. Potential proteolytic enzymes for unhairing are keratinases, elastases and collagenases (**Table 1.5**). Although proteases in general hydrolytically cleave the peptide bond of proteins, it has long been understood that the amino acids adjacent to the bond to be cleaved are very important in determining the cleavage efficiency on by different enzymes. Synthetic short peptides are routinely used to determine the specificity of proteases regarding this aspect. Some of the commonly studied artificial substrates are Suc-Ala-Ala-Pro-Phe- *p*Na , CBz-Phe- *o*Np, Suc-Leu-Leu-Val-Tyr-AMC, Bz-Phe-Val-Arg- *p*Na, Bz-Ile-Glu-Gly-Arg- *p*Na , L-Leu- AMC, CBz-Phe-*p*Na and -Pro-Gly-Leu-Asn- Ser-Thr (Brandelli et al., 2010). Proteases independent of their catalytic type, cleaves hydrophobic and aromatic aminoacid residues at the P1 position, resembling the specificity of chymotrypsin. The presence of Arg at P1 appears to be preferentially cleaved only by some keratinases from *Bacillus* spp. (Manczinger et al., 2003; Macedo et al., 2008) and *Chryseobacterium* sp. (Silveira et al., 2009), a preference also showed by trypsin. In this sense, the specificity of keratinases towards keratinous materials may arise from the amino acid composition of keratins, which contains about 50–60 % of hydrophobic and aromatic residues (Arai et al., 1983; Gregg et al., 1984; Barone and Schmidt, 2006; Gradisar et al., 2005).

Keratinases display a high capability of degrading insoluble keratin substrates such as found in hair, nails and feathers. Increased attention has been devoted to these enzymes because of their several potential uses associated to the hydrolysis of keratinous substrates and other applications (Gupta and Ramnani, 2006; Brandelli, 2008; Brandelli et al., 2010). Keratinolytic activity may be measured using any of the

natural keratinous materials as substrates and checking for the release of soluble peptides or amino acids. Yet another method is to use azo-dye conjugated insoluble substrates such as hide-azure and estimate the quantity of dye released due to proteolytic action. Similarly, collagenase activity is measured by action on collagen or on the synthetic substrate Azo-coll and likewise, elastase acticity may be measured by using congo red-elastin (Foroughi et al., 2006; Sivasubramanian et al., 2008). As seen in **Table 1.6**, most dehairing enzymes are keratinolytic in their substrate specificity. On the other hand, many dehairing preparations are low in if not totally lack collagenolytic activity. Macedo et al., (2005) reported that keratinolytic proteases lacking collagenase activity more suitable for dehairing process. Collagens, being the leather forming proteins, keratinolytic enzymes not showing collagenolytic activity, are being increasingly realized for their importance the leather industry.

The significance of gelatinase activity in dehairing proteases is poorly understood. Gelatin is derived from collagen as product of its structural and chemical degradation particularly obtained by heating collagen to high temperatures. Gelatin lacks many distinct properties of collagen, such as high molecular weight, basic isoelectric point, triple helix structure, high resistance to protease, and ability of fibril formation (Zhang et al., 2005). Some dehairing preparations have gelatinase activity and gelatine based zymography is useful for visualization of the enzyme band on gels. Whether the gelatinase activity is desirable property of unhairing enzymes or whether it is a harmless activity with no role in leather making remains to be understood. The substrate preferences for some dehairing proteases are that, *Bacillus subtilis* DM-04 showed casein > haemoglobin > gelatine > chicken-feather keratin (β-keratin) > and bovine serum albumin. No activity on human hair (α-keratin) and collagen was observed (Rai and Mukherjee, 2010). *Pseudomonas aeruginosa* MCM B-327 preferred casein > gelatn > bovine serum albumin >> elastin. No activity on keratin azure and collagen (Zambare et al., 2010). Keratinase from *Chryseobacterium* sp. kr6 was reported to hydrolyze keratinous substrates in the following order: stratum corneum from human sole > porcine skin > chicken feathers > chicken nails > wool > hair keratin (Riffel et al., 2003). Enzyme from *Bacillus licheniformis* ER-15 showed a preference of azocasein > haemoglobin > fibrin > BSA = casein > hooves keratin > meat protein > feather keratin > keratin azure. It did not hydrolyze gelatine, elastin and hair (Tiwary and Gupta, 2010a).

Table 1.5: Substrate preference of certain commercial proteases finds application in pre tanning: compiled from Foroughi et al., (2006).

Process	Commercial proteases	Azocoll (A)	Elastin (E)	Keratin (K)	Order of preference
Soaking	Buzyme 148	√	√	√	E>A>K
	Merpizym 4581	√	√	X	A>E>K
	Merpizym 8009	√	√	X	A>E>K
	Pelvit DPH	√	√	X	A>E>K
	Vinkol A	√	√	X	A=E>K
Dehairing	Buzyme 7705	√	√	√	E>A>K
	E-zyme	√	X	X	E>A>K
Bating	Oropon AN2	√	X	X	A>E=K
	Oropon ON2	√	√	X	A>E>K
	Pancreol PBWI	√	√	X	A>E>K

√ indicates presence and X indicates absence of activity.

1.13: Molecular aspects of pre-tanning enzymes

Cloning and heterologous over-expression of pre-tanning enzymes is important for their biotechnological production in a large scale and cost effective manner. One of the earliest report has been on the cloning of the AP gene encoding alkaline serine protease with dehairing function from a *Bacillus pumilus* strain (Pan et al., 2004). The cloned gene was not able to show activity in *E. coli* although protein could be visualised on SDS-PAGE. After the mature protease region was cloned into an expression in *B. subtilis* shuttle vector functional expression was achieved. Recently, a Mn (2+) dependent alkaline serine protease gene of *Bacillus pumilus* TMS55 has been cloned. Sequence analysis showed that this polypeptide is composed of 29 residues N-terminal signal peptide, a propeptide of 79 residues and a mature protein of 275 amino acids. Docking analysis was used to identify residues that interact with Mn (2+) (Ibrahim et al., 2011). Other keratinolytic protease genes have been overexpressed from *Bacillus licheniformis* in *Bacillus megaterium* (3 fold increase) in *Pichia pastoris* (2.9 fold increase) (Radha and Gunasekaran, 2009) and into *E. coli* (Tiwary and Gupta, 2010).

Table 1.6: Biochemical characteristics of dehairing proteases from different sources

Isolate	Keratinase	Collagnase	Elastase	Geltinase	Reference
Bacillus cereus MCM B-326	X	X		X	Nilegaonkar et al., 2007; Zambare et al., 2007
Bacillus halodurans PPKS-2	√				Prakash et al., 2009
Bacillus licheniformis AP-1	√	Low	√		Zhang et al., 2003; Tang et al., 2004
Bacillus licheniformis ER-15	√		X	X	Tiwary and Gupta, 2010a
Bacillus pumilus CBS	√				Jaouadi et al., 2009
Bacillus subtilis MTCC 6537		X	Low		Sivasubramanian et al., 2008
Bacillus subtilis S14	√	X			Macedo et al., 2005
Bacillus subtilis DM-04	√	X		√	Rai and Mukherjee, 2010
Microbacterium sp. kr 10	√				Riffel et al., 2003; Brandelli et al., 2008
Chryseobacterium sp. kr 6	√				Riffel et al., 2003; Brandelli et al., 2008
Pseudomonas aeroginosa MCM-B327	X	X	√ Low	√	Zambare et al., 2011; Pandeeti et al., 2011
Vibrio sp. kr2	√				Riffel et al., 2003, Brandelli et al., 2008

√ indicates presence and X indicates absence of activity. Blank cell indicates non-availability of information

By using the tools of genetic engineering, proteases are selectively modifed to remove non collagenous material depending on the end use of leather (Rao et al., 1998). TfPa (thermostable broad pH range) serine protease (1 kb) cloned from *Thermomonospora fusca* YX in *P.pastoris* finds apllication in dehairing and bating

(Kim and Xen, 2005). Pandeeti et al., (2011) recently reported the cloning of *las*B gene encoding the elastase responsible for dehairing activity of *Pseudomonas aeruginosa* strain. The purified protein was subjected to identification by mass spectroscopy and the corresponding homologue from their organism was isolated by PCR-amplification using primers designed from the *Pseudomonas* genome database. The cloned gene was expressed in a surrogate host which was safer than the potentially pathogenic native bacterium and the over-production of the enzyme was noted but fold icrease is not reported. The deharing activity demonstrated using the enzyme preparation from the heterologous host.

1.14: Rationale behind the current investigation

Specificity of proteases to be employed for pre-tannery process is not well documented. Based on a comparative analysis of the dehairing enzymes studied till date, a potential dehairing protease may have the following characteristics: broad pH range, with maximal activity at neutral to near alkaline range, be active at ambient temperature (30-35 °C); thermostable to be active at room temperatures up to 12-20 h, keratinolytic activity particularly acting on feather keratins, elastase and gelatinase activity may be beneficial in the last stages of pre-tanning, i.e. bating and enzyme should be collagenase free. A thorough understanding of the mechanism of action would require knowledge about the true substrates for dehairing. Substrate preference is the most varied property of the dehairing enzymes and a rationalization of the substrate preferences with their efficacy at dehairing is not as yet possible. The cloning and over-expression of dehairing enzymes and demonstrating the dehairing function in heterologous system is an important area which may provide economically viable enzyme preparations for the biotechnological market. Cost-effectiveness is one of the major apprehensions in the use enzymes in tannery. Bioprocess where any protein waste can be reused for the generation proteases can be an added advantage in terms of waste management and by product recovery. Present investigation aims to identify a bacterial extracellular-protease producer, which meet the above requirements and also to study the biochemical and genetic and application aspects of such a protease.

1.15: Objectives of the present study

1. To isolate and characterize a bacterium producing an extracellular protease suitable for pre-tanning applications.
2. To develop and optimize a medium for the production of protease from the selected isolate through statistical tools
3. To explore and develop a process for the in-house management of chrome shavings, solid -waste from post-tanning using the selected isolate
4. To purify and characterize the protease
5. To clone, over-express the gene encoding the pre-tanning enzyme

Chapter 2

Isolation and characterisation of bacterial isolate producing extracellular protease for pre-tanning applications

When you have eliminated the impossible, whatever remains, however improbable, must be the truth.
- Sir Arthur Conan Doyle

2.1: Introduction

Microbial proteases are the major enzymes utilised in different pre-tanning processes (Puvanakrishnan and Dhar, 2000). Of the 3000 different enzymes described to date the majority have been isolated from mesophilic organisms (Govardhan and Margolin, 1995). These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzymes insufficient. Thus, the search for new microbial sources is a continual exercise, keeping in mind the vast biodiversity. The microorganisms from diverse and exotic environments, extremophiles, are considered an important source of enzymes, and their specific properties are expected to result in novel process applications (Robertson et al, 1996).

Microbial proteases are classified into various groups, dependent on whether they are active under acidic, neutral or alkaline condition and also on the characteristic of the active site group of the enzyme, i.e. metallo- (EC.3.4.24), aspartic - (EC.3.4.23),cysteine or sulphydryl- (EC.3.4.22) or serine type-(EC.3.4.21; Kalisz,1988 ; Rao et al., 1998).Among them alkaline serine proteases are commercially well explored for the dehairing process in pre-tanning (Varela et al., 1997). Alkaline proteases speed up the process of dehairing , because the alkaline conditions accelerate the swelling of the hair root and subsequent attack of proteases on the hair follicle proteins allow the easy removal of the hair (Gupta et al., 2002). Reported literature for the last decade indicates that most of the dehairing enzyme producers are soil, tannery effluent or animal hide isolates belonging to the geneus Bacillus. B. *altitudinis* (Vijay kumar et al.,2011), *Bacillus licheniformis,* (Tiwary and Gupta 2010), *Bacillus halodurans* (Prakash et al., 2009), *Bacillus pumilus* (Jaouadi et al. 2009), *Bacillus subtilis* (Sivasubramanian et al. 2008), *Bacillus pumilus* (Wang et al., 2007), *Bacillus sp* (Giongo et al., 2007), *Bacillus cereus* (Nilegaonkar et al., 2007), *Bacillus subtilis* (Macedo et al., 2005); (Mitra and Chakrabartty, 2005) etc are a few to name. Apart from bacilli, depilatory enzyme producers belonging to Streptomyces.sp (Mitra and Chakrabartty 2005), vibro sp (Riffel et al., 2003), Pseudomands (Zambare et al., 2010) are isolated from different habitat. Distant from dehairing, soaking and bating are the other two major pre-tanning processes.Since dehairing is a highly polluting process, most of the enzymatic studies are

concentrated on the same. Hameed et al (1996) reported *B. subtilis* K2, which produces an alkaline protease, finds application in bating.

As described in previous chapter, the ideal pH and temperature varies from process to process and also depends on the nature of the finished leather (Puvanakrishnan and Dhar,2000).Optimum temperature requirement, thermostability are also critical, as all these operations are carried out under ambient conditions. Seasonal fluctuations can hamper the action of biological catalyst, unless they are thermo-stable with broad temperature and pH optimum. To be competent for the purpose, specificity towards non-leather forming protein, keratin, is also necessary.

The present chapter deals with the isolation of a protease producing bacterial strain having broad range of substrate specificity, pH and temperature optimum. Potential isolate selected on the basis of above said requirements were identified, by different biochemical tests according to Bergy's manual of Systematic Bacteriology and genetically by 16SrDNA analysis. Crude enzyme preparations were characterized biochemically and evaluated at tannery level for soaking, dehairing and bating.

2.2: Materials and Methods

2.2.1: Isolation and screening of protease producing bacteria

Screening for potential isolates was carried out from different habitats by serial dilution and spread plating on Luria-Bertani (LB) agar with 1% skimmed milk (LBSMA). Potential protease producing bacterial isolates from different habitats, viz, soil samples from hotspring, cattle sheds and fish market were initially selected based on the ratio of clearance zone diameter and colony size (CZ/CS ratio), further screened for protease activity in a broad pH (5.0-12.0) and temperature(10-50◦C) range.

Protease production in liquid media was monitored using basal medium (BM), ie LB SMA and also using protease production medium reported for *Bacillus*.sp by Puri et al.(2002), containing (w/v) K_2HPO_4, 0.7%; KH_2PO_4, 0.3%; $MgSO_4$, 0.01%; yeast extract, 0.5% and soyabean meal, 1% . Supernatant collected by centrifugation at 12,000 *g* for 10 min during different stages of growth was used as the enzyme preparation.

2.2.2: Characterization of the bacterial isolate

Identification of the bacterial isolate was carried out based on 16S rRNA gene sequence and biochemical characterization according to Bergey's manual (Holt et al. 1994). Approximately 1500 bp of the 16S rRNA gene was amplified using the universal eubacterial primers 27F and 1541R (Table2.1). For template DNA preparation, bacteria were grown on LB agar plates and a freshly grown colony was suspended in 50µl of nuclease free water, lysed in sterile microfuge tubes by placing in a boiling water bath for 20 min. The suspension was then centrifuged and the supernatant was then used as template for PCR amplification. The PCR reaction mixture was incubated in a thermal cycler (Applied biosystems model 2720) under the following cycling regime.

Lid temp – 105 ºC

Step -1 (Initial denaturation)		: 94 °C - 3 min
Cycle: 1	Step - 2 (Denaturation)	: 94 °C - 30 sec
	Step -3 (Annealing)	: 58 °C - 45 sec
	Step – 4 (Extension)	: 72 °C - 1.5 min
Steps 2, 3, 4	repeated for **30 cycles**	
Step 5	Final extension	: 72 °C - 10 min.
Hold		: 4 °C - 20 min

PCR system

Ingredient	**Quantity**
10X Taq DNA polymerase buffer	5 µl
10mM dNTP mix	1 µl
1mM forward primer	1 µl
1mM reverse primer (1541r, 1107r, 534r)	1 µl
Template DNA	10 or 15 µl (bacterial colony lysate)
Taq DNA polymerase (3U/µl)	0.5 µl
Total volume made by using sterile Distilled water	Up to 50 µl

The PCR product was sequenced using primers 27F, 1541R and also with following set of primers (**Table2.1**),viz, 340F 1053F 534R and 1107R (Shivaji et al.,

2000). Approximately 1500 bp sequence information of the amplified fragment was obtained through the sequencing services provided by LabIndia Instruments Pvt. Ltd., Gurgaon, India. The DNA sequence data were matched using the tools provided at NCBI database and the Ribosomal Database project II *(http://rdp.cme.msu.edu/)*.

Table 2.1: Details of the primers used

Primer code	Sequence	Used for
27F	5'-GAGAGTTTGATCCTGGCTCAG-3'	initial amplification
340F	5'-CCTACGGGAGGCAGCAGA-3'	sequencing ofs internal regions
1053F	5'- CATGGCTGTCGTCAGCTCGT-3'	
534R	5'- ATTACCGCGGCTGCTGG-3'	
1107R	5'-GCTCGTTGTGGGACTTAACC-3'	
1541R	5'-AAGGAGGTGATCCAGCCGC-3'	initial amplification

2.2.3: Determination of caseinolytic activity

Hydrolysis of casein by enzymatic action was used as the routine assay method for protease activity measurements. Caseinolytic activity was assayed by modified method of Anson, as described by Joo et al. 2002, using 1% casein dissolved in 100mM phosphate buffer pH 7.0. The reaction mixture was incubated at 50 °C for 10 min and reaction was stopped using 5% trichloroacetic acid (TCA). After 15 min, tyrosine released was estimated from the supernatant collected using Folin-Ciocalteau's reagent and measuring absorbance at 660nm. One unit of protease was considered as the amount required for the release of 1 µmol of tyrosine per min.

2.2.4: Determination of keratinase activity

Keratinase activity was measured by using modified method of Dozie et al. (1994). Keratin solution (1%) used as the substrate was prepared in Tris Cl pH 7.0 as described by Gradíšar et al (2000). Insoluble residues were removed by filtration through Whatman No. 1 paper. Assay mixture containing 1 ml of substrate and 1 ml of appropriately diluted aliquot of enzyme was incubated at 50 ºC for 10 min and the

reaction was stopped using 5% TCA. Determination of tyrosine released and unit definition are asgiven in section 2.2.3..

2.2.5: Azocol and azocasein hydrolysis assays

Collagenase as well as caseinolytic activities were estimated using azo-dye linked substrates, azocol and azocasein (Sigma-Aldrich Chemicals Pvt. Ltd, .USA), respectively, both substrates prepared in 50mM Tris-Cl buffer pH 8.0. Reaction mixtures comprising of 0.4 ml of 1% substrate and 0.2 ml of appropriately diluted enzyme were incubated at 50 ºC for 10 min, stopped using 15% chilled TCA and kept on ice for 15 min. Absorbance of the supernatant collected after centrifugation at 12,000 g for 10 min was measured at 450 nm for azocasein (Thys et al., 2004) and 520 nm for azocol assay (Janssen et al., 1994). One unit is defined as the amount of enzyme bringing about an increase in 0.1 absorbance /min at 50 ºC.

2.2.6: Determination of Total Protein Content

The total protein contents of the samples were determined according to the method described by Lowry (Lowry et al., 1951); the protein standard used was Bovine Serum Albumin (Sigma). Absorbances of the samples were measured spectrophotometrically at 660 nm using Schimadzu UV-Visible spectrophotometer.

2.2.7: Influence of pH and temperature on protease activity

For determining the optimal pH, the protease assay with different substrates like casein, keratin, azocasein and azocol was carried out using 100mM of the following buffers in the assay systems: citrate buffer (pH 5.0 and 6.0), Tris-Cl (pH 7.0 and 8.0), borate buffer (9.0), glycine- NaOH buffer (pH 10) and sodium- tetraborate buffer (pH 11.0). The temperature optimum of the enzyme was determined using casein as the substrate by incubating the reaction mixtures at different temperature

2.2.8: Effect of protease inhibitors, detergents and reducing agents on protease activity

Enzyme preparation in 100mM phosphate buffer was incubated at 50ºC for 10 min with the following inhibitors, each separately at concentrations mentioned: 0.1mM phenyl methyl sulfonyl fluoride (PMSF), 5mM EDTA, SDS (0.1% and 0,5%) and Triton X100 (5%) and reducing agent, β-mercapatoethanol (βME) (0.1% and 0.5%) (Bressollier et al.,1999). Enzyme samples were assayed along with untreated control using Anson's method (Joo et al., 2002) as described above.

2.2.9: Goat hide dehairing assays

Freshly salted goat hide was cut into pieces of approximately 5 cm x 5 cm, washed with distilled water repeatedly to remove salt and extraneous matter. After brief air drying, the hide was weighed to maintain between 2-3 g) and transferred to 40 ml capacity glass tubes containing 10 ml sterile distilled water. The pH was adjusted to 8.0 with 0.1% lime (Macedo et al., 2005) and subjected to various combinations of treatments in which enzyme preparation and different additives such as βME (0.1%) and PMSF (0.1mM) were added as mentioned in the Figure legends. Incubations were carried out for 18h after which hair was scraped off gently from the hides. Traditional tannery dehairing was also performed using 1.5% lime and 1.5% sodium sulfide (Thanikaivelan et al., 2004). Results were observed using a stereomicroscope (Leica –M2 –16A) at 70X magnification.

2.2.10: Feather degradation studies

The potential of the bacterial strain at feather degradation/disintegration was studied by inoculating LB containing sterile chicken feather with 2% of 0.6 OD bacterial culture. Efficiency of hydrolysis was observed visually, by light microscopy after monochrome staining and also by scanning electron microscopy during different stages from 12h to 24h. Feather fibril detachment and hydrolysis was also studied using 70% ethanol washed chicken feathers using enzyme treatment (50°C, 30 min; Böckle et al., 1995) along with additives such as βME and PMSF in different combinations. Patterns of detachment were recorded by periodic observation of the treated feathers using compound light microscope at 10x magnification.

2.2.11: Keratin hydrolysis studies

Quantitative estimations of hydrolysis of natural alpha and beta keratins were carried out at 37ºC using ethanol washed ground chicken feathers (beta keratin) and finely trimmed human hair (alpha keratin) as the substrates (0.1 g/L suspension in 100mM Tris-Cl buffer pH 7.0). Filter sterilized enzyme preparation along with 0.1% βME were added, aliquots withdrawn after every 4 h were centrifuged at 12,000 g and hydrolysis products in the supernatant were monitored on the basis of peptides released as assessed by absorbance at 280 nm (Böckle et al., 1995). Residual dry weights were also monitored at every 4 h interval after washing the pellet thrice with distilled water.

2.2.12: SDS-PAGE and protease zymography

SDS- PAGE was carried out at 4°C using 12% polyacrylamide gel, based on the recipe described in **Appendix I**. Protein samples were diluted in 50mM Tris-Cl pH 6.8 containing 2% (w/v) SDS, 0.1% bromophenol blue and 10% (w/v) glycerol (Sambrook and Russell, 2001) and applied on the gel without heat treatment for protease zymogram analysis, and after heat treatment for protein staining by Coommassie brilliant blue R250 (CBBR- 250) or silver staining. Protease activity staining procedure for zymography was followed from Bressollier et al. (1999). Breifly, the gel was washed with 2.5% triton X- 100 for 30 min, then with Tris-Cl (pH 7.2) for 30 min to remove the SDS and renature the enzyme. Gelatin (2% w/v) prepared in 50mM Tris.Cl (pH 7.2) was poured on the acrylamide gel slab. After 1 h of incubation at 40 ºC, the gel was washed with distilled water and stained using CBBR-250 and then destained.

2.2.13: Tannery Application studies

In order to understand the feasibility of protease preparation for all pre-tanning processes, trials same were carried out on buffalo hide at Adam Tannery, Chrompet, Chennai, India.Pre tanning conditions and the processes are mentioned in **Table 2.2**.

All the results are represented as mean of three independent experiments and vertical bars represent the standard deviation

Table 2.2: Pre-tanning conditions of tannery trials carried on buffalo hide

Process	Conditions
Soaking	Water-500%, enzyme: 1.5%of hide weight at 30°C, pH 7.0 Duration: 18 h, alternate exposure of hair side and flesh side
Dehairing	1.5% of extra enzyme added and proceeded in the same soaking pit for another 8h.Temperature and pH maintained same as soaking
Bating	Drum bating, water:50%, $(NH_4)_2SO_4$: 0.5%, pH 8.5, Temperature: 35°C

2.3: Results

2.3.1: Characterizations of bacterial isolate P13

Among the 20 isolates, P13 was selected based on CZ/CS ratio at different temperatures and also based on the protease activity at broad pH values (**Appendix II**). The isolate P13 (**Fig. 2.1a**) produced colonies on skimmed milk agar plates with a CZ/CS ratio of 1.28 at 15 ºC, 1.34 at 37 ºC and 1.42 at 50 ºC and **Fig.2.1b** depicts the scanning electron micrograph indicating rod shaped cells. Biochemical tests performed according to Bergey's manual (Holt et al., 1994) indicated that this isolate, (**Fig.2.1b**) belonged to the genus *Bacillus*. Approximately 1500 nucleotide sequence of 16S rRNA gene (Genbank accession number DQ 681073.3, **Appedix III**). Phylogenetic analysis using 16S rRNA gene sequence showed maximum similarity to *Bacillus* sp.B144, an isolate from a marine sponge (*Halichondria rugosa*) from South China Sea (**Fig2.2**), and belongs to *Bacillus subtils* group (**Appendix VI**). Based on the morphological, biochemical and molecular characteristics, the organism is therefore henceforth designated as *Bacillus subtilis* P13.

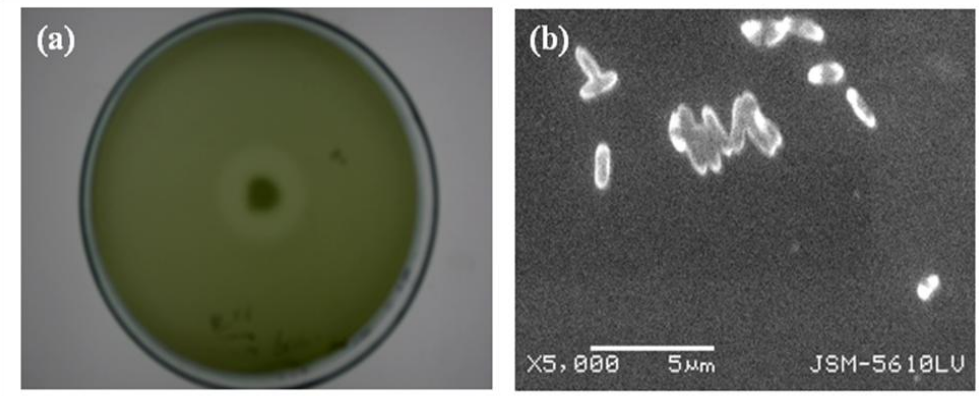


Fig.2.1: *Bacillus subtilis* P13
a: Colony phenotype on LBSMA; b: Scanning electron micrograph

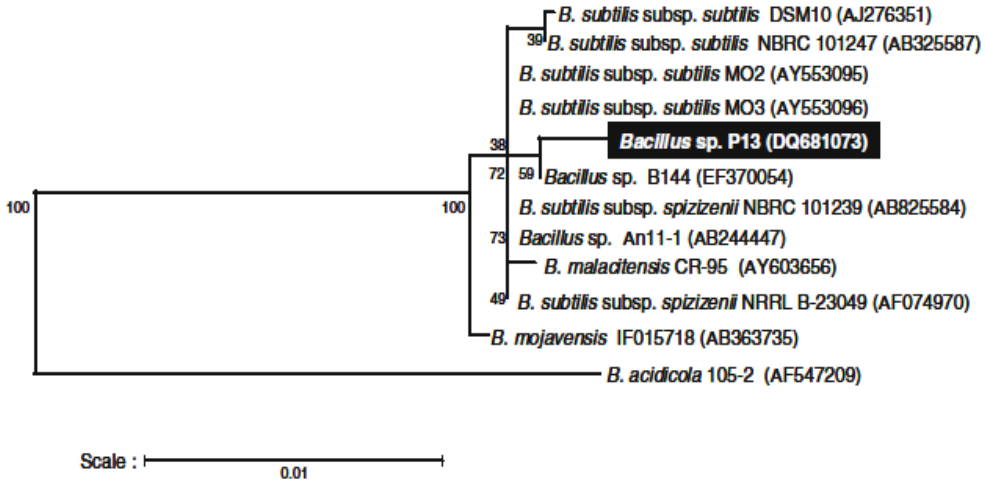


Fig. 2.2: 16S rRNA gene sequence based pylogenetic relationship of isolate P13 within the genus Bacillus. The branching pattern was generated by **neighbour weighted neighbour-joining tree building software.** The gene bank accession number of the 16s rRNA nucleotide sequences are indicated in brackets. The number at each branch indicates the bootstrap values out of 100. The bar indicates the Jukes–Cantor distance of 0.01. *Bacillus acidicola* is used as out-group.Isolate P13 clustered along with *Bacillus subtilis* strains.

2.3.2: Protease production by *B. subtilis* P13

Growth associated protease production by *B. subtilis* P13 was carried out in basal medium (BM) which is LBSM and production medium (PM). Optimum growth and production temperature is depicted in **Fig. 2.3**. Though the optimum growth temperature of this isolate is 40°C, due to the interest of having cost-effective process, all the enzyme production studies were restricted to 30°C.

A maximum activity of 1.5U/ml was observed in the PM after 48h and activity declined to 0.84 U/ml after 72h **(Fig. 2.4)**. Since the extracellular production of proteases is inducible in most strains, protein source is an important factor that regulates their production, which may explain the difference between BM and PM. The decrease in activity after 72h might be due to autocatalytic effect or due to end product inhibition.

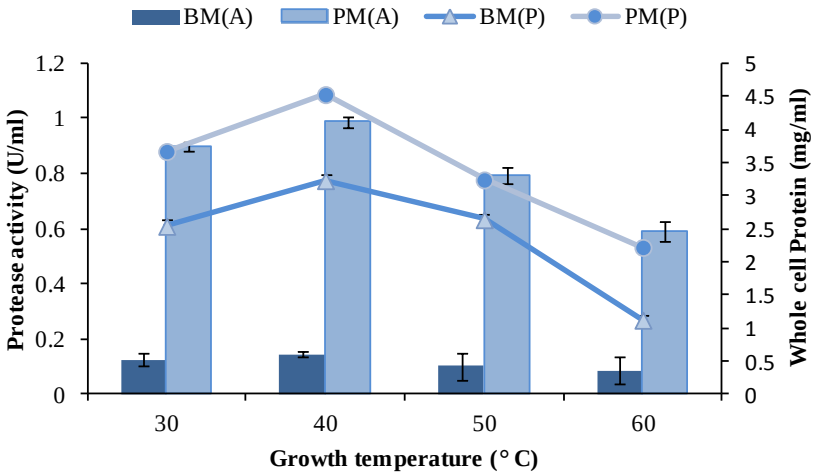


Fig. 2.3: Optimum growth and protease production temperature of *B. subtilis* P13. After 48h of growth to identify the ideal conditions for protease production

BM (A) &PM (A) : Protease activity in basal and production media

BM (P) & PM (P) : Whole cell protein from basal and production media

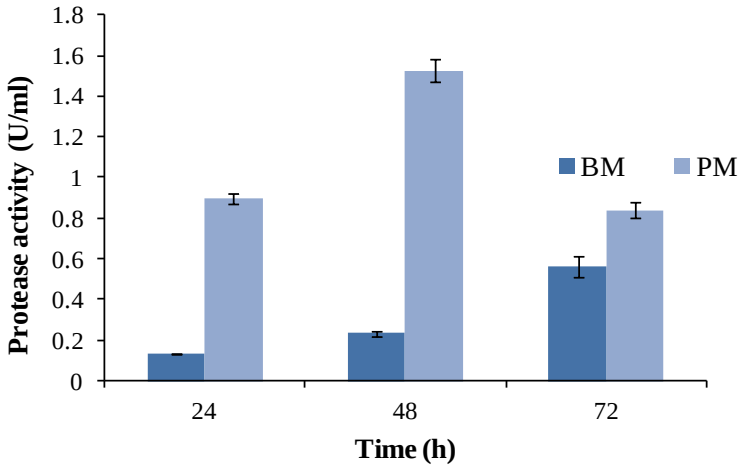


Fig. 2.4: Comparison of protease production by *B. subtilis* P13 in basal medium (BM) and production medium (PM) at 40° C

2.3.3: Influence of pH, temperature and substrate nature on protease activity

The optimum pH for caseinolytic activity of the enzyme preparation was found to be 7.0, allowing its classification as a neutral protease (**Fig. 2.5**). Approximately, 40% of the activity at pH 7.0 could be obtained at lower (pH 5.0) as well as higher (pH 11.0) pH values. With keratin, azocasein and azocol, the pH optimum was

nearly similar to that with casein. At its optimum, about 95% of the total protease activity as determined by caseinolytic method was found to be accounted for as keratinase activity (**Fig. 2.5**). The ratio of keratin hydrolysis activity and casein hydrolysis activity of greater than 0.5 confirms the specificity for keratin (Gradíšar et al., 2005). It is generally accepted that proteases to be applicable as depilatory enzymes should have minimum action on collagen, the leather forming protein (Saravanabhavan et al., 2004), so as to break the collagen bundles without action on collagen fibers. The ratio of azocol and azocasein activities at optimum pH was found to 0.1, suggesting lower preference of collagen.

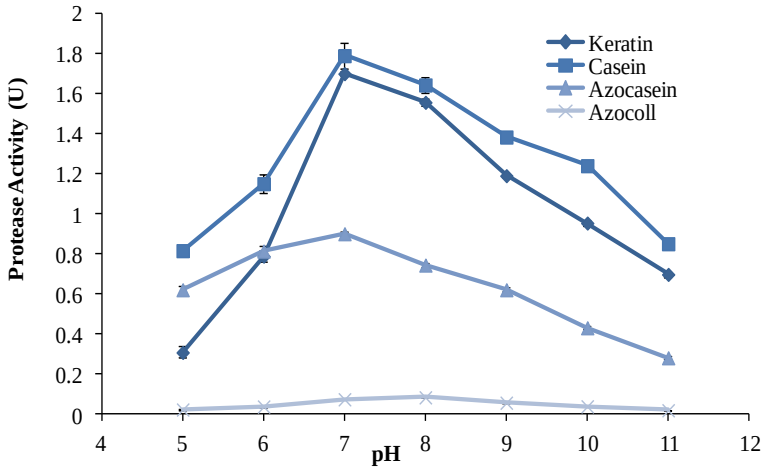


Fig. 2.5: Effect of pH on protease activity of *B. subtilis* P13 using different substrates.

As shown in **Fig. 2.6** the temperature optimum for the protease preparation is 65^{o}C, indicating the moderately thermophilic nature of the enzyme in accordance with the isolate being obtained from hotspring.

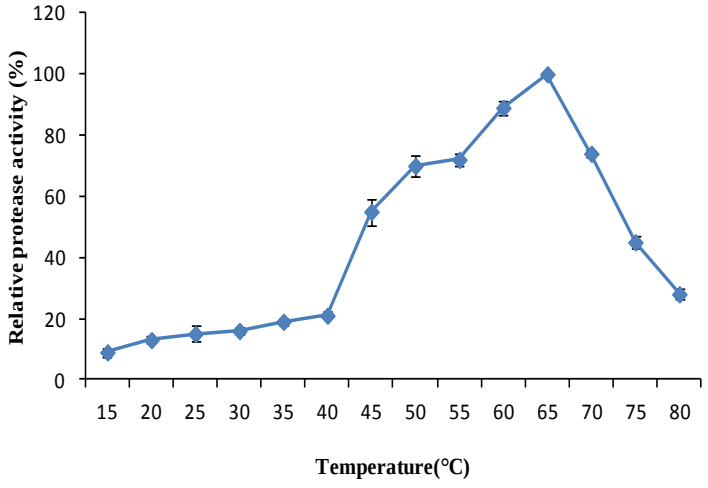


Fig. 2.6: Temperature optima of protease from *B. subtilis* P13

2.3.4: Effect of inhibitors, detergents and reducing agents on protease activity of *B. subtilis* P13

Protease activity determined using casein as substrate was almost completely inhibited by serine protease inhibitor, PMSF (0.1 mM) (**Table 2.3**) indicating presence of the serine group in the active sites. On treatment with EDTA (5mM), 82% of the caseinolytic activity was retained. Nonionic detergent Triton X100 enhanced the activity by 10% while presence of 0.5% SDS had a negative effect, by loss in 83% of the total activity. In the presence of 0.5% βME, 94% of the total activity was found to be retained, confirming that this reducing agent has minimal effect on enzyme activity.

Table 2.3: Effect of inhibitors/additives on the protease activity *of B. subtilis*. P13

Additive	Concentration	Residual caseinolytic activity (U)	% Residual activity
None	-	2.29 ± 0.26	100
PMSF	0.1mM	0.016 ± 0.005	0.02
EDTA	5mM	1.88 ± 0.21	82
SDS	0.1% 0.5%	0.530 ± 0.06 0.39 ± 0.044	28 17
β-Mercapto ethanol	0.5%	2.15 ± 0.24	94
Triton. X-100	0.5%	2.5 ± 0.28	110

2.3.5: Proficiency of protease enzyme of *B. subtilis* P13 at goat hide dehairing

Complete dehairing of goat hides was achieved with 2 U of enzyme/g of hide at 18 h (**Figs. 2.7b** and **c**). No dehairing was observed of the specimen when uninoculated bacterial growth medium and lime (0.1%) were used (**Fig. 2.7a**).

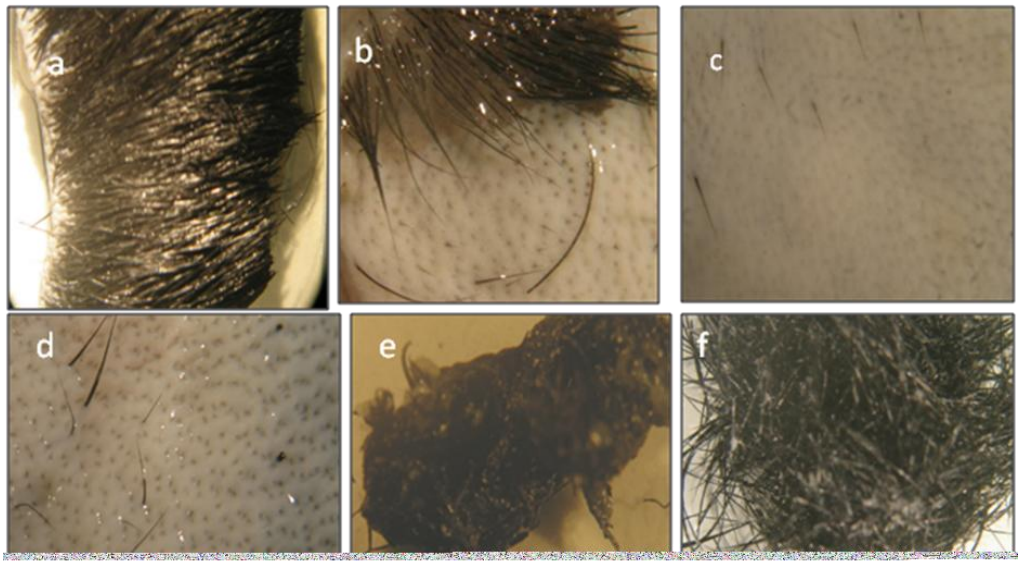


Fig. 2.7: Dehairing studies on goat hide using crude protease preparation from *B. subtilis* P13. Protease preparation used: 2U/g of hide; a) 0.1% lime + uninoculated growth medium; b) enzyme + 0.1% lime 12 h, c) 18 h d) 1% lime + 1.5% Na_2S, e) hair pulp from treatment -d f) intact hair from treatment –b.

Traditional chemical process of hide dehairing uses lime and sulfide, hence in the hide depilation experiments were also done with these agents and complete dehairing was achieved in 12 h (**Fig. 2.7d**), but showed the stubbles of the hair, indicating the requirement of prolonged treatement to decement hair from the hair roots. When protease was used along with 0.1% PMSF, no depilation occurred (data not shown), thus confirming serine protease is responsible for dehairing. Hair collected from the lime-sulfide process (traditional unhairing) was found to be gelatinized and converted into a pulp (**Fig. 2.7e**) where as that from enzymatic dehairing process was intact (**Fig. 2.7f**).

2.3.6: Feather disintegration studies with *B. subtilis* P13 protease

Progressive debarbulation (disintegration of barbs) was observed at 12, 16, 20and 24 h visually when *B. subtilis* P13 was allowed grow on sterilized whole chicken feather in LB in the absence of reducing agents (**Fig. 2.8**).

Fig. 2.8: Chicken feather degradation by *B. subtilis* P13
a) Uninoculated control; b, c, d and e are stages after 12, 16, 20 and 24 h of growth.

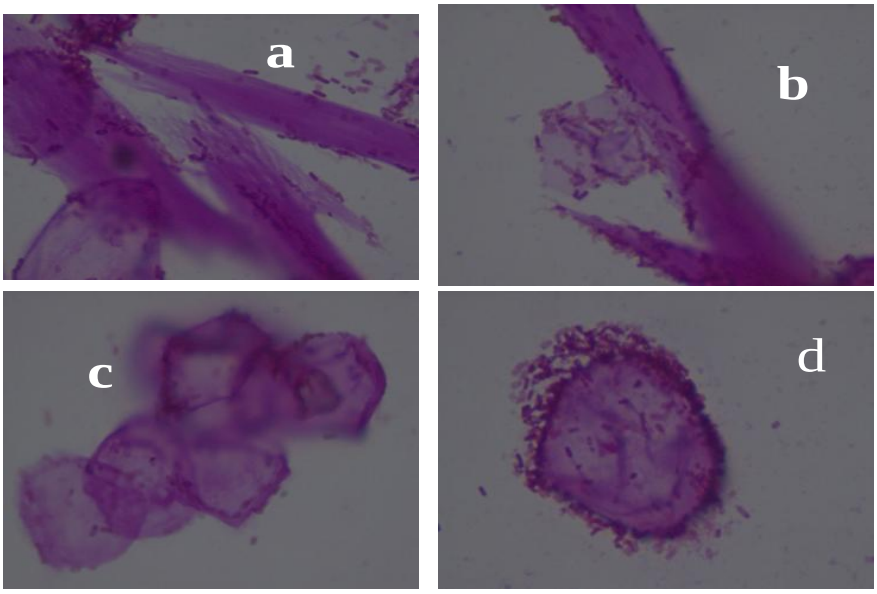


Fig. 2.9: Growth of *B. subtilis* P13 on chicken feather–monochrome stained light micrograph under 100X magnificaton

Feather pieces and residues when stained with crystal violet showed the presence of rod shaped bacterium on the surface of various parts of the feather (**Fig. 2.9a**), indicating that debarbulation resulted due to attached bacterial growth (**Fig. 2.9b**), followed by the detachment of the polygonic ridges which holds the barbs on the rachis (**Fig. 2.9c**). Since the ridges are highly keratinased compared to the barbs, the growth of bacteria appears to progresses from barbules to barbs, followed by barbs to polygonic ridges and then on the hollow mid rachis. **Fig. 2.9d** depicts an individual polygonic unit with bacterial growth. Scanning electrom microscopy studies were carried for the same and the micrographs are shown in **Fig. 2.10**. Bockle and Muller, 1997, reported cell-bound redox system for disulfide bond

reduction in keratin. The disintegration potential displayed by *B. subtilis* P13 in the absence ßME (**Fig. 2.8**) can be attributed to cell bound redox system. Gupta et al., (2005) reported similar potential of *Bacillus licheniformis* RG 1in feather degradation.

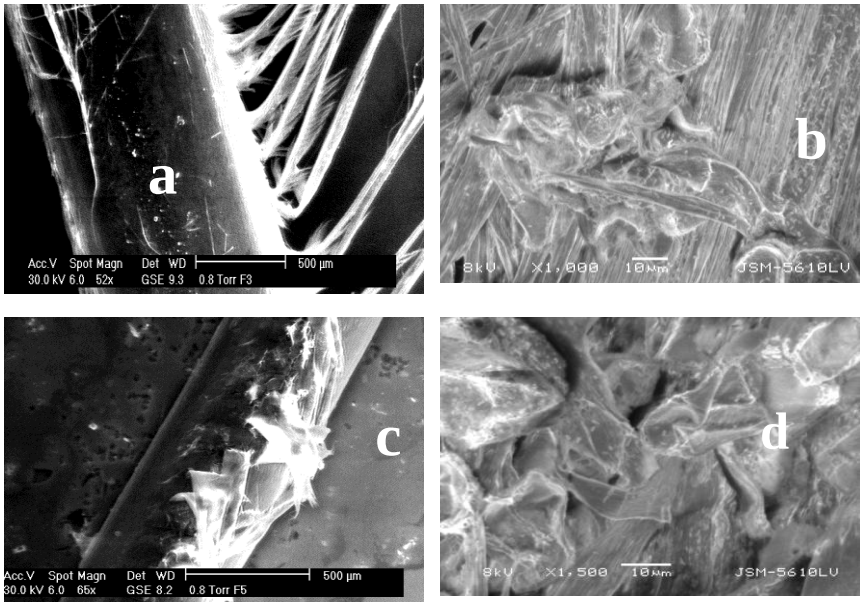


Fig. 2.10: Scanning electron micrograph of chicken feather disintegration by *B. subtilis* P13 after 12h (fig.2.10.a**),16h(**fig.2.10b**),20h(**fig.2.10c**) and 24h(**fig.2.10.d**)**

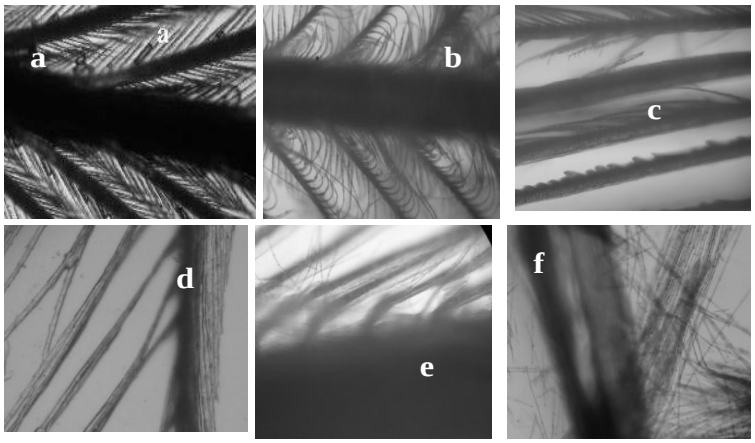
To understand the role of protease in disintegration, crude protease preparation was used to treat the feathers. Gradual changes in the feather upon enzymatic treatment with 0.1% βME could be visibly observed and clean shaft was obtained after 18 h (**Fig. 2.11**).

a : Control;
b, c, d :Treatedwith4U of protease+0.1%βME after 8,12h and 18h respectively

Fig. 2.11: Chicken feather disintegration by *B. subtilis* P13 crude protease preparation–Visual observation

The hollow supporting shaft (rachis) of feathers and its side branches (barbs) to which are attached a set of fine barbules could be clearly observed in microscopic images of control untreated feathers (**Fig. 2.12a**). Disintegration of barbules

apparently started after 4 h of enzymatic treatment with βME (**Fig. 2.12b**) and after 8 h complete debarbulation was seen (**Fig. 2.12c**). After 12 h, barbs started disintegrating (**Fig. 2.12d**) and within 18 h, degradation of the barbs, leaving behind the thick basal portion of the rachis was seen (**Fig. 2.12e**). Efficiency of degradation in the absence of reductant was lower and took nearly 48 h to show clean shaft (data not shown). The base of the shaft remained undigested even after prolonged incubation for 7 days in the presence of enzyme.



a: Treated with0.1% βME+Uninoculated media,
b, c, d, e, f: Treated with 4Uenzyme + 0.1 % βME
after 8, 12, 14 and 18 h respectively

Fig. 2.12: Chicken feather disintegration by *B. subtilis* P13 crude protease preparation (10X magnification)

In order to compare the hydrolytic efficiency of the enzyme with keratins of feather and hair, quantitative experiments with these substrates were performed (**Fig. 2.13b**). Protease preparation could degrade 50% of the feather meal in 4 h and 72 % after 24 h of incubation. Keratin of human hair origin was found to be less prone to attack since only 5 % was degraded within 4h and 24 % after 24 h incubation. Relative peptide release (as ratio of absorbance at 280 nm) was 4 times higher with feather as compared to hair at 24 h (**Fig. 2.13a**).

(a) (b)

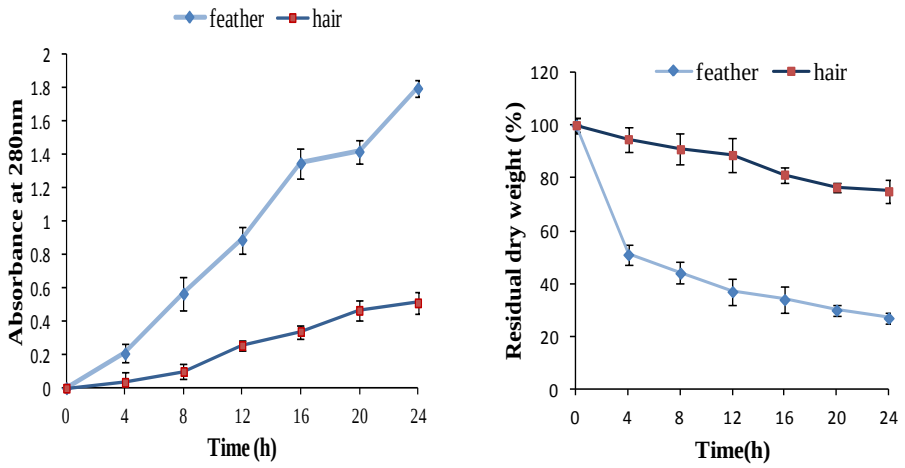


Fig. 2.13: Hydrolysis of feather and hair keratins by protease preparation from *B. subtilis* P13 (a) Dissolved peptides released as assayed by absorbance at 280 nm; (b) Residual dry weight.

2.3.7: Protease zymography analysis

When 30µg of crude protease preparation was electrophoresed on SDS-PAGE, many bands were observed upon Commassie blue staining (**Fig. 2.14a**). However, a single major protease band appeared as a clear zone in blue background on zymography to visualise protease bands (**Fig. 2.14b**). The apparent molecular mass of the major band was calculated as a 31kDa on the basis of relative mobility on the SDS-PAGE, which correlates with most of the reported serine proteases whose masses ranges from 18-35 kDa (Kato et al. 1992; Yamagata et al.1995). Lack of appearance of major band when zymogram was developed in the presence of PMSF confirmed the active site serine of this protease. In EDTA treated sample, the 31 kDa band was unaffected.

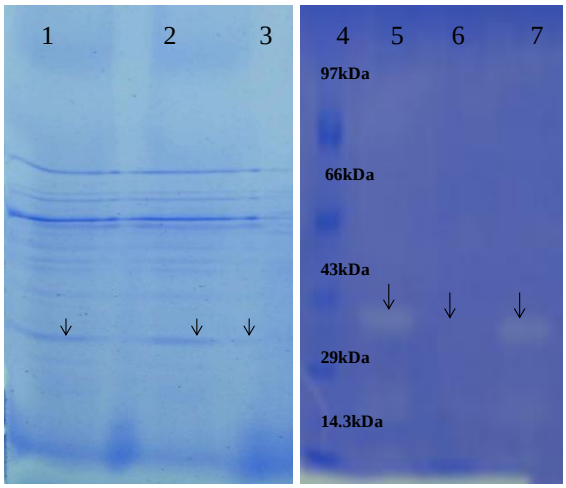


Fig. 2.14: (a) Total protein profile and (B) gelatin based protease zymogram of crude protease preparation from *B. subtilis* P13 on 12 % SDS-PAGE.

Lane 1: Control; lane 2: PMSF treated, lane 3: EDTA treated; lane 4: MW marker; lane 5: control; lane 6: PMSF treated; lane 7: EDTA treated.

2.3.8: Preliminary tannery trials with protease preparation from *B. subtilis* P13

Tannery trials were carried out at Adam tannery, Chennai, India to understand the potential of the crude enzyme preparation in pre-tanning processes to understand whether the isolate P13 could be used to develop an eco-friendly process for tannery applications (**Fig. 2.15**). Based on the water absorbance and swelling rate, soaking was stopped after 14 h. A swelling rate of 25% was achieved during the period. Ideal swelling rate in a proper soak condition varies from 25-30% and is an indication of the degradation of non-leather forming fibrous proteins, opening up of collagen fibers. Lack of bulging indicated the intactness of collagen fibers, the major leather forming units of the hide. Blood stain removal was 100% compared to the commercial soaking enzyme control, BASF, where only 80% could achieved. This is an added advantage, as the traces of blood stains can leave patches on finished leather.

About 30% dehairing was achieved in the soak liquor and dehairing continued in the same pit with additional supply of 1.5% of crude preparation from *B. subtilis* P13 and could achieve complete dehairing in another 4 h. Buffalo hide is a tougher system to work due the presence of thick scuds (short hair), which needs extra attention after bating. In the case of this preparation, after dehairing about 95% of the scuds were removed during dehairing step itself.

Fig. 2.15: Buffalo pelt after soaking, dehairing and bating using crude protease preparation from *B. subtilis* P13

In short, soaking and dehairing completed in 18h, under operational conditions (pH 7.5 and 30 °C) in the same pit. Bating proficiency of the enzyme was confirmed based on clean pores, white pelt, thick, smooth and slippery nature with proper grains. Shrinkage during the conversion of hide to pelt was only 10%. Usually for commercial bating enzymes like TFL, Dorpan WB, SPIC alkali bate etc. shrinkage ranges from 20-30%.

2.4: Discussion

Microbes producing proteases suitable for pre-tanning/beam-house applications have been reported from diverse sources indicating their ubiquity in variety of environments. The proteases find application in tannery for dehairing, which is well studied due to the ecological impact of the process, and other two applications, viz, soaking and bating. Among microbial proteases, microbial keratinases have been described for various biotechnological applications in feed as well as leather industries, yet the growing demand for these enzymes necessitates the screening for novel keratinolytic microorganisms with potential applications. The properties and applications of a keratinase preparation from *Bacillus subtilis* P13, a novel isolate from hot springs at Vajreshwari, Mumbai, India is described in this study.

The lack of inhibition of protease from *B. subtilis* P13 by EDTA and near complete inhibition with PMSF confirms the active site serine nature and is in agreement with the lower preference to collagen, since majority of collagenases reported are metalloproteases (Rao et al., 1998). The protease activity is maximal at

neutral pH with casein as well as keratin as substrate and shows a temperature optimum of 65^{o}C. The neutral keratinase exhibits a dual property of dehairing and feather detachment. Dehairing was comparatively a faster process reaching completion in the absence of any dehairing aids like sodium sulfide and the hair released was intact. This indicates the ability of this neutral protease to selectively breakdown of keratin tissues in the hair follicle, thereby pulling out intact hair (Macedo et al., 2005). Since most of the proteases available as dehairing aids belong to alkaline proteases group (Varela et al., 1997), requiring a pH of 10-12, attaining hide depilation at a pH of 7.5 – 8.0 is advantageous, because it avoids high pH effluent like those that occur in sulfide dehairing process as well as because deliming process can also be reduced. Intact hair recovered after unhairing using *B. subtilis* P13 protease suggests the inability to hydrolyze mature α-keratin and provides a hair-saving dehairing process, avoiding semi-gelatinous organic matter in tannery waste water (Macedo et al., 2005).

Many keratinase preparations exhibit activity against collagen (Lin et al., 1992; Böckle et al., 1995; Nam et al., 2002; Gousterova et al., 2005), which like keratin, is a fibrous protein but of different organization, consisting of a triple helix conformation, stabilized by covalent intermolecular cross-linkages involving lysine and lysine derivatives (Voet and Voet 2004). The poor collagenase activity of the proteolytic preparation of *B. subtilis* P13 is of advantage in the leather industry since collagen, the major leather-forming protein (Macedo et al., 2005), would not be not significantly degraded.

Microscopic examination of feather disintegration studies using filter-sterilized enzyme in presence of the reducing agent, revealed that fibril bundles of barbs are loosened and separated out, which can be attributed to the combined action of βME and keratinolytic activity of the enzyme preparation. In the absence of βME, the process with filter-sterilized enzyme preparation was very slow. However, *B. subtilis* P13 unfiltered enzyme preparation could bring about feather disintegration in the absence of βME within 24h, indicating that cell-bound redox system for disulfide bond reduction (Böckle and Müller, 1997) was perhaps responsible, as it is likely that some cells may be inadvertently introduced through the unfiltered enzyme preparation.

B. subtilis P13, when allowed to grow on feather, showed a sequential process of disintegration in the order of barbules, barb, barb ridge separation and slow degradation of mid rachis. The preferential action of the protease of the isolate on feathers over hair indicated that the *B. subtilis* P13 enzyme preparation is relatively less effective in hydrolysis of native α-keratins even in the presence of reducing agents while under similar conditions the feather β-keratins were nearly completely degraded *in vitro*.

The detection of a single major PMSF-sensitive protease band in zymogram analysis, coupled with the observation that addition of PMSF prevents hide depilation as well as feather degradation, suggests that a single enzyme probably functions in both applications. Giongo et al., (2007) have reported that feather degrading enzyme preparations from *Bacillus* spp. are suitable as depilating agents. However, their preparation was a mixture of several proteases and hence it cannot be concluded that a single enzyme is responsible for both activities. In this study, a keratinolytic protease producer, *B. subtilis* P13 were characterised, hide depilatory property and feather disintegration capability of the crude enzyme was studied.The properties of the hide dehairing proteases are summarised in **Table 2.4**.

Table 2.4: Biochemical nature of protease produced by *B. subtilis* P13

Properties	
Optimum temperature for production	40° C
Optimum temperature for activity	65°C (active at a range 15-70°C)
Optmum pH	7.0 (active at a pH range of 5-12)
Prefered substrates	Casein, keratin, gelatine
MW	31kDa
Catalytic type	Serine protease

2.5: Conclusion

The isolate *B. subtilis* P13 from Vrajeswari hotspring is a moderately thermophilic organism, producing thermophilic keratinolytic serine protease having broad pH range (5.0-12.0).The molecular weight of the enzyme was found to be 31kDa based on gelatine zymography**.** The potential of the crude protease preparation from this isolate for all the three pre-tanning processes are confirmed at

the tannery level. The substrate preference exhibited by *B. subtilis* P13 are as follows: casein>keratin>>azocoll. It showed good activity on feather keratin and gelatine but not on human hair keratin. Studies carried out on feather and hide confirm that *B. subtilis* P13 produces keratinolytic protease which acts efficiently on immature keratin, where the number of di-sulfide linkages are less. Inorder to act on mature keratin, where large amount of di-sulfide linkages are present , protease preparation from this isolate requires the assistance of additional reducing system, either cell bound or external supply of reducing agents. Based on the co-relation studies on feather detachment, disintegration and hide deharing, it can be confirmed that any protease which can perform feather detachment can be a potential depilatory enzyme.

Chapter 3

Statistical optimization of production and tannery applications of a keratinolytic protease from Bacillus subtilis P13

There are no such things as applied sciences, only applications of science.
- Louis Pasteur

3.1: Introduction

The ability of *Bacillus* species to secrete large quantities of various extracellular enzymes has made them the most important producers of industrially significant enzymes (Schallmey et al., 2004). Production of extracellular enzymes by micro-organisms depends strongly on various aspects of growth media such as C/N ratio, availability of easily metabolizable sugars such as glucose (Beg et al., 2002), easily assimilable nitrogen sources such as amino acids and presence of metal ions (Varela et al., 2002). Conventional experimental approach used for media optimization employing 'Change-one-factor-a-time' is extremely time consuming and expensive and laborious for screening a large number of variables.

Several statistical methods are available for optimizing the parameters (Felse and Panda, 1999; Montgomery, 2002), which reduce the time and expense of the experiment. The primary selection of components for statistical designs is either by borrowing or random selection. The method of borrowing involves survey of literature to select components used by other workers for the same desired product from similar organisms. The limitation of this method is that there are fewer options if the organism under study has not been previously investigated for the production of desired product. Alternatively, it becomes essential to screen large number of possible components. Optimizing the medium constituent by component replacement is the widely used method for screening of carbon, nitrogen and phosphorous sources. The effect of other components such as surfactants, metal ions, antibiotics, etc. can be studied by one-factor-at a time approach. However, this approach becomes extremely time-consuming, expensive and unmanageable when large numbers of variables have to be studied and does not depict the combined effect of all the factors involved.

Optimizing all the affecting parameters by statistical experimental designs can eliminate these limitations of a single factor optimization process collectively (Montogomery, 2000). Different statistical designs have gained lot of attention in media optimization and also in understanding the interaction of various physico-chemical parameters using minimum number of experiments (Oh et al., 1995). Statistical methodologies are preferred because of various advantages in their use such as rapid and reliable short-listing of nutrients, understanding the effect of the nutrients at varying concentrations and significant reduction in total number of

experiments resulting in saving time, glassware, chemicals and manpower (Srinivas et al., 1994; Carvalho et al., 1997). Several statistical designs are available such as full factorial, fractional factorial or Plackett–Burman design, Taguchi's robust design and response surface methodology (Montogomery, 2000). Of these, Taguchi approach does not explicitly include model building and optimization (Ross 1996, Simpson, 2001). Taguchi methods have been used extensively in engineering design, however, reports of Taguchi method in biological science are scarce (Han et al., 1998). In full factorial designs the number of factors increase exponentially leading to an unmanageable number of experiments (Hunter, 1985). Hence, fractional factorial design like Plackett-Burman becomes a method of choice for initial screening of medium components. Plackett and Burman's statistical method involves a two level fractional factorial saturated design that uses only k+1 treatment combinations to estimate the main effects of k factors independently (assuming that all interactions are negligible) (Plackett and Burman, 1944). Saturated designs are used in the early stages of experimentation to screen out unimportant factors from amongst a large number of possible factors.

Further optimization and interaction effects between the components can be studied by response surface methodology (RSM). The response is a quantitative continuous variable (e.g. yield, purity or cost) and the mean response is a smooth but unknown function of levels of k factors. The mean response when plotted as a function of the treatment combinations is a surface in k+1 dimensions, called response surface. The commonly used response surface designs include Box-Behnken and central composite design (CCD) (Dean and Voss, 2006), involving three levels and five levels, respectively, for each factor needed for quadratic terms to be estimable in the second-order model. There has been major research and development in the recent years on the use of statistical approach methods involving various statistical software packages for the optimization studies with the aim of obtaining high yields of amylase, protease, biosurfactants, neomycin etc (Hajii et al., 2008).

In spite of the numerous advantages, applications of proteases for proteinaceous waste management as well as in tannery applications has limitations such as the high cost of production, additional operational costs for concentrating the enzyme and for maintaining controlled conditions of pH and temperature for their optimum action

(Thanikavelan et al., 2004). Isolation and characterization of novel microbes producing robust enzymes with a broad range of pH and temperature requirements as well as the optimization of enzyme production by such organisms to minimize the production cost are important factors that will increase the use of green chemistry in tanneries.

Preceding chapter described the studies on crude extracellular-protease preparation from a novel hot spring isolate, *Bacillus subtilis* P13 and the proficiency of the enzyme in efficient disintegration of intact feathers and also at depilation of hide in the absence of any additives. Current chapter deals with the statistical optimization of the bacteriological medium for maximizing the production of the enzyme and the application potential of the preparation for pre-tanning processes at tannery level studies carried out at the Central leather research institute, Chennai India. It is found that *B. subtilis* P13 produces several proteases in a growth phase dependent manner; out of these, one isoform isimplicated in having the abilities to hydrolyse feathers and depilate hide.

3.2: Materials and Methods

3.2.1: Micro-organism used and culture conditions

B. subtilis P13, a thermophilic keratinolytic protease-producing bacterium isolated from Vajreshwari hot springs, India (mentioned in Chapter 2) was routinely grown and maintained on Luria-Bertani (LB) agar with 1% skimmed milk which was used as the basal medium for the protease production. Inoculum of 1 % (1×10^9 CFU/ml) was used from 12 h old culture and growth was carried out at 30^oC on a rotary shaker.

3.2.2: Identification of significant media components

Initial screening of significant media components was carried out by one-variable-at-a-time approach. Four different protein sources, viz, soyabean meal (SBM), green gram powder (GG), bengal gram powder (BG) and milled feather (MF) each to a final concentration of 1%, 0.5% of glucose or sucrose as carbon sources, 0.25% of either ammonium sulphate, ammonium phosphate, ammonium chloride, sodium nitrate and potassium nitrate as inorganic nitrogen sources was studied individually in LB media. The effect of following salts, $BaCl_2$, $CaCl_2$, $MnCl_2$, $ZnSO_4$, $FeSO_4$, $CoCl_2$ and $CaCO_3$ (0.01%) and amino acids like tyrosine and tryptophan (0.01%)

were also studied. The media (50ml) were prepared in a 250ml Erlenmeyer flasks and autoclaved at 121^{o}C for 20 min. *B. subtilis* P13 was inoculated and incubated on a rotary shaker (170 rpm) for 72 h. An aliquot of 2 ml was collected in every 24 h interval, centrifuged at 12,000×*g* for 15 min to remove bacterial cells and the supernatant was used to check the protease activity.

3.2.3: Plackett –Burman Design

To find out the important medium components, Plackett-Burman design (Plackett & Burman 1946) was followed. Total number of trials carried out was according to Plackett-Burman design as k+1 where k is number of variables (medium components). Each variable was represented at two levels, high and low which are denoted by (+) and (-) respectively. The number of positive signs and negative signs per trial were (k+1) / 2 and (k-1) / 2 respectively. Each column contained equal number of positive and negative signs. Ten variables (assigned) and five dummy variables (unassigned) (**Table 3.1**) were screened in sixteen trials, each variable being a medium constituent.

Table 3.1: Concentration of selected media components for Plackett – Burman studies

	Variables	Levels (g/l)	
		+	-
X1	Soyabean meal	10.0	1.0
X2	Glucose	5.0	0.5
X3	Yeast extract	5.0	0.5
X4	NH_4Cl	2.5	0.25
X5	K_2HPO_4	7.0	0.7
X6	KH_2PO_4	3.0	0.3
X7	$MgSO_4$	0.1	0.01
X8	$FeSO_4$	0.1	0.01
X9	$CaCl_2$	0.1	0.01
X10	$BaCl_2$	0.1	0.01
D1-D5	Dummy/unassigned variables		

Table 3.2 depicts the variables used and the design of experiment where each row represents a trial and each column represents an independent or dummy variable. The effect of each variable was determined by the equation $E(Xi) = 2(\Sigma Mi+ - Mi-) / N$, where E(Xi) is the concentration effect of the tested variable. Mi+ and Mi- are the

protease activities from the trials where the variable (Xi) measured was present at high and low concentration respectively and N is the number of trials (twelve). Experimental error was estimated by calculating the variance among the dummy variables as $V_{eff} = \Sigma(Ed)^2/ n$, where V_{eff} is the variance of the concentration effect, Ed is the concentration effect for the dummy variable and n is the number of dummy variables. The standard error (SE) of the concentration effect is the square root of the variance of an effect and the significance level (P value) of each concentration effect was determined using Student's t test t(xi) = E(Xi) / SE, where E(Xi) is the effect of variable Xi.

X_1-X_6 represent different assigned variables and D_1-D_5 are the unassigned/dummy variables; the sign '+' is for high concentration of variables and '-' is for low concentration of variables and actual concentration is as given in **Table 3.2**. The pH of the medium was adjusted to 7.0 and the medium was sterilized by autoclaving at 10 psi for 15min.

Table 3.2: Plackett-Burman design matrix for optimization of protease production by *B. subtilis* P13

Trial NO.	Variables / Levels														
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	D_1	D_2	D_3	D_4	D_5
1	+	+	+	+	-	+	-	+	+	-	-	+	-	-	-
2	-	+	+	+	+	-	+	-	+	+	-	-	+	-	-
3	-	-	+	+	+	+	-	+	-	+	+	-	-	+	-
4	-	-	-	+	+	+	+	-	+	-	+	+	-	-	+
5	+	-	-	-	+	+	+	+	-	+	-	+	+	-	-
6	-	+	-	-	-	+	+	+	+	-	+	-	+	+	-
7	-	-	+	-	-	-	+	+	+	+	-	+	-	+	+
8	+	-	-	+	-	-	-	+	+	+	+	-	+	-	-
9	+	+	-	-	+	-	-	-	+	+	+	+	-	+	-
10	-	+	+	-	-	+	-	-	-	+	+	+	+	-	+
11	+	-	+	+	-	-	+	-	-	-	+	+	+	+	-
12	-	+	-	+	+	-	-	+	-	-	-	+	+	+	+
13	+	-	+	-	+	+	-	-	+	-	-	-	+	+	+
14	+	+	-	+	-	+	+	-	-	+	-	-	-	+	+
15	+	+	+	-	+	-	+	+	-	-	+	-	-	-	+
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.2.4: Optimization of screened components by Box-Behnken design

To select the optimum concentration screened variables (SBM, K_2HPO_4, $FeSO_4$ and $BaCl_2$) and study their interactions, RSM using Box- Behnken (BB) (Box1952)

was applied with the levels of the four variables. A 2^3 factorial design, with five replicates at the centre point, with a total of 29 trials was employed. The experimental model was evaluated using response surface regression procedure, using the following second order polynomial equation $Y= \beta 0 +\Sigma\beta iXi+\Sigma\beta ijXiXj+\Sigma\beta iiXi^2$, where Y is the predicted response; β0 is the offset term; βi is the linear offset; βii is the squared offset and βij is interaction effect .Xi is the dimensionless coded value of Xi. The statistical software package Design-Expert (version 7.1.3, Stat-Ease, Minneapolis, U.S.A.) was used to design and analyze the experiment.

The statistical model derived based on the regression equation was validated by performing shake flask experiment using 10 different concentrations which were not included in the original design. The prediction capacity of the model was evaluated by comparing the experimental protease, keratinase, gelatinase, azocasein and azocoll activities with the one predicted from the model.

3.2.5: Scaleup studies

The optimized media was scaled up to 1L in 2.5L and to 2L in 5L Erlyenmeyer flasks. Fermenter level scale up was carried in a 5L fermenter (**Fig. 3.1**) (Bioflo110, New Brunswick Scientific.Co.Inc.USA) and whole system was sterilized in an industrial scale autoclave. Two percentage inocululm of 0.6 O.D was maintained throughout the study and sterile portals were used for the inoculation and addition of filter sterilized $FeSO_4$.

3.2.6: Protease zymography

Gelatin based zymogram of proteas preparation was done according Bressollier et al., 1999. The intensity of the protease band (from nine media BB-design) was obtained based on densitometry reading done using Alfa-Ease FC software (accompanying AlfaInnotech Gel documentation system).

Fig. 3.1: Fermentor set up for protease production using *B. subtilis* P13

3.2.7: Gel purification of protease

Approximately 31 kDa PMSF sensitive band was excised (without staining) from one half of the SDS-PAGE gel (Sambrook and Russell, 2001) by comparing it with the other half of which a part was stained by CBBR-250 and part was subjected to gelatine zymogram (as described earlier). The band was electroeluted by inserting it into a micropipette tip which was sealed with 0.8% agarose prepared in SDS–PAGE running buffer. The narrow region of the tip was connected to a dialysis bag, and subjected to electrophoresis at 50V for 4h to elute the protein. Purity of the eluted protein was confirmed by SDS-PAGE followed by silver staining (Sambrook and Russell, 2001).

Casienolytic activity (Joo et al., 2002) and keratinase activity (Dozie et al., 1994) were measured as described in section 2.2.5. Peptide release from milled feather by the purified enzyme was done as mentioned in section 2.2.11.

3.2.8: Determination of the N-terminal amino acid sequence of the keratinolytic protease

The electroeluted enzyme, from gelatine zymogram was applied on SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After brief staining with CBBR-250, the band corresponding to the 31 kDa protease was excised and the N-terminal amino acid sequence was determined by the automated Edman protein degradation method on an ABI Procise 492/610A

protein sequencer (Applied Biosystems) at the National sequencing facility at Indian Institute of Technology Bombay, Mumbai, India.

3.2.9: Sulfitolysis - disulfide bond-reducing activity

Sulfitolysis activity was determined spectrophotometrically by measuring the absorbance of yellow coloured 2-nitro-5 mercaptobenzoic acid at 412 nm, formed by the conversion of colourless 5,5´-dithio-bis-(2-nitro benzoic acid) (DTNB) in presence of thiol groups released by keratinolysis of feather by keratinolytic protease. The estimation was carried out at 30 and 50°C using 1% solutions of milled chicken feathers and commercially available keratin powder (Hi-Medi Limited, India) prepared in potassium phosphate buffer pH 7.0 as described by Jaouadi et al., (2010). One unit of sulfitolysis is defined as the amount of enzyme that catalyses the formation of 1µmole of sufide/min.

3.2.10: Enzyme stability studies

Upon growth of the culture in optimized medium for 24 h, the culture supernatant was collected by centrifugation at 12,000 g for 10 min and ammonium sulphate precipitation to 70% saturation was carried out. Precipitate was air dried and ten different formulations were made using the following stabilizers: silica gel (3%), borax (3%), lactose (5%), $CaCl_2$ (0.1%), sucrose (10%), starch (10%), carboxymethyl cellulose (10%), NaCl (5%), $BaCl_2$ (0.1%), Na_2SO_4 (0.1%) (w/v) with 1% ammonium sulphate precipitated enzyme. Ammonium sulfate precipitated enzyme without any additive was considered as the control for the efficiency evaluation. Caseinolytic activity was monitored monthly for one year at 4^oC and at room temperature. Dehairing efficiency of the formulations at regular time intervals was carried out on goat hide.

3.2.11: Tannery application studies

Tannery application studies were carried out on salted and dried goat hide (right half as control and left as test) at the tannery facility of Central Leather Research Institute (CLRI, Chennai, India). The efficiency of ammonium sulfate precipitated enzyme preparation from *B. subtilis* P13 was compared with the commercial formulations for soaking, dehairing and bating processes. Soaking competence was determined with respect to swelling rate and proteolysis, which was quantified indirectly by nitrogen released in the soak water as estimated by Kjeldal's method. Dehairing efficiency was evaluated visually with respect to the area unhaired, removal of the hair along with the root and also the quality of the hair recovered

(Puvanakrishnan et al., 1988). Quality evaluation in bating process included visual observation to check the removal of scuds (short thick hair), mechanical testing including increased stretching, air permeability and microscopic studies.

3.3: Results

3.3.1: Single parametric screening of significant components

B. subtilis P13 produced 0.5±0.007 U/ml of protease (as determined by caseinolytic assay) after 48h of incubation in LB medium, which was used as the routine growth medium. Easily assimilable carbon source like glucose when added in LB medium resulted in the increase in protease production to 1 U/ml in 24 h after which medium turned acidic and the activity declined to 0.4 U/ml after 48 h. In the presence of sucrose, activity was 0.8 U/ml after 48 h. Inorganic nitrogen sources like KNO_3, $(NH_4)_2SO_4$, $(NH_4)_2PO_4$, NH_4Cl were individually screened at a concentration of 0.25% in basal medium LB. Addition of NH_4Cl brought about significant increase in activity to 1.5 U/ml and similarly among the metal salts, increased protease production to1.6 and 1.7 U/ml in 24h was observed with $BaCl_2$ and $FeSO_4$, respectively, whereas incorporation of $COCl_2$ resulted in 1.5 U/ml after 48h. None of the other metal salts were found to be inhibitory to protease production.

In order to study the effect of different natural proteinaceous sources on enzyme production, they were supplemented at 1% (w/v) to LB medium, and protease activities of 0.6, 0.45, 0.32, 0.9 and 1.2 U/ml were observed after 48h in case of skimmed milk (SM), green gram powder (GG), bengal gram powder (BG), milled chicken feathers (MF), soyabean meal (SBM), respectively, as compared to an activity of 0.5 U/ml in unsupplemented LB, thus indicating SBM to be the most optimum proteinace

ous component. **Fig. 3.2** shows the zymogram analysis of the extracellular proteases produced in media supplemented with different natural proteinaceous substances. In 24 h samples, the bands were very faint, while good intensity is observed at 48 h correlating with high activity at the latter time point.

The protease zymogram was similar in presence of all additives and showed about 4-5 isoforms at 48 h. hus, addition of media components glucose, yeast extract, NH_4Cl, K_2HPO_4, KH_2PO_4, $MgSO_4$, $FeSO_4$, $CaCl_2$, $BaCl_2$ etc. brought about a

positive effect in protease activity during similar growth duration. These media components along with SBM, were selected for the Plackett-Burman (PB) design to identify the significant ones among them.

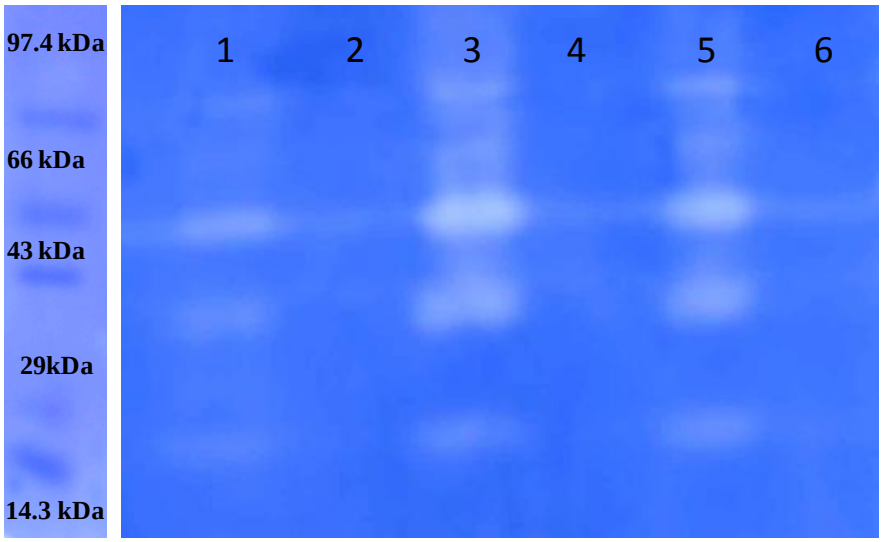


Fig. 3.2: Zymogram analysis of proteases produced by *B. subtilis* P13 during growth on different protein sources. Lane: 1, 3, 5 are 48 h and lanes 2, 4 and 6 are 24 h enzyme samples from media containing skimmed milk, soya bean meal and milled feathers, respectively

3.3.2: Screening of significant media components by Plackett-Burman design

Based on the single parametric study, ten components were selected for the Plackett-Burman design, at low and high concentrations as specified in (**Table 3.3**).The combinations having higher concentration of SBM (1%) showed higher protease activity. The confidence level of the variables X_1 (SBM), X_6 (KH_2PO_4), X_8 ($FeSO_4$), X_9 ($CaCl_2$) and X_{10} ($BaCl_2$) were calculated based on the p-value were 99.87, 92.85, -91.79, 91.36 and 99.83 respectively and confidence level above 90% are considered to be significant. The positive effect of SBM, K_2HPO4, $CaCl_2$ and $BaCl_2$ bringing about increase in protease activity and negative effect of $FeSO_4$ decreasing the activity were showed as paretograph (**Fig. 3. 3**). When the value of concentration effect (Exi) of the tested variable was positive, the influence of the variable was greater at the high concentrations tested and when negative, greater influence of the variable at low concentration.

Table 3.3: Response of Placket-Burman studies for protease by *B. subtilis* P13 after 24h of growth

S. No.	Variable media components (g /l)										Protease activity (U/ml)
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	
1	10	5	5.0	2.5	0.7	3	0.01	0.1	0.1	0.01	1.19 ±0.045
2	1	5	5.0	2.5	7	0.3	0.1	0.01	0.1	0.1	0.95 ±0.052
3	1	0.5	5.0	2.5	7	3	0.01	0.1	0.01	0.1	0.31 ±0.043
4	1	0.5	0.5	2.5	7	3	0.1	0.01	0.1	0.01	0.38 ±0.032
5	10	0.5	0.5	0.25	7	3	0.1	0.1	0.01	0.1	1.20 ±0.025
6	1	5	0.5	0.25	0.7	3	0.1	0.1	0.1	0.01	0.85 ±0.52
7	1	0.5	5.0	0.25	0.7	0.3	0.1	0.1	0.1	0.1	0.76 ±0.054
8	10	0.5	0.5	2.5	0.7	0.3	0.01	0.1	0.1	0.1	1.10 ±0.027
9	10	5	0.5	0.25	7.0	0.3	0.01	0.01	0.1	0.1	1.65 ±0.033
10	1	5	5.0	0.25	0.7	3	0.01	0.01	0.01	0.1	0.70 ±0.022
11	10	0.5	5.0	2.5	0.7	0.3	0.1	0.01	0.01	0.01	1.46 ±0.019
12	1	5	0.5	2.5	7.0	0.3	0.01	0.1	0.01	0.01	0.28 ±0.015
13	10	0.5	5.0	0.25	7.0	3	0.01	0.01	0.1	0.01	2.10 ±0.048
14	10	5	0.5	2.5	0.7	3	0.1	0.01	0.01	0.1	1.60 ±0.029
15	10	5	5.0	0.25	7	0.3	0.1	0.1	0.01	0.01	1.28 ±0.016
16	1	0.5	0.5	0.25	0.7	0.3	0.01	0.01	0.01	0.01	0.15 ±0.057

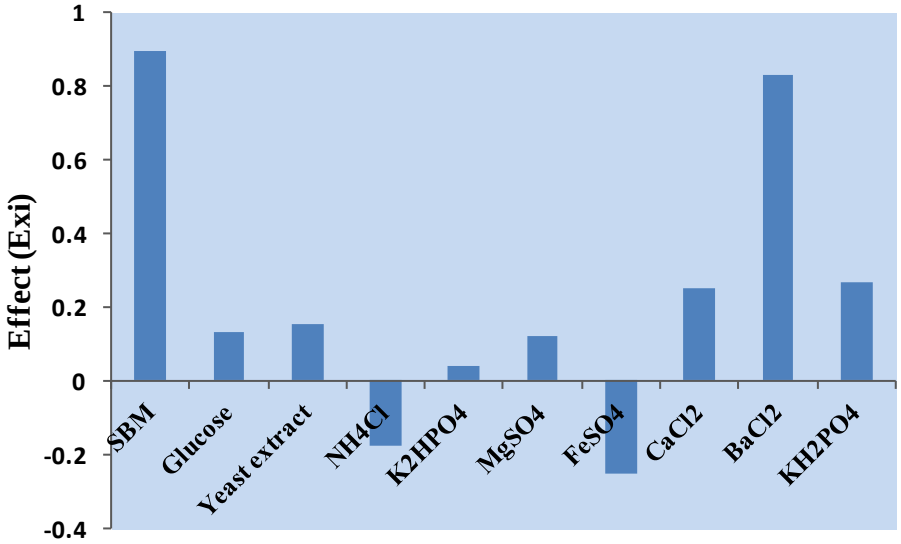


Fig. 3.3: Pareto graph showing the effect of media components used in Plackett-Burman design on protease production by *B. subtilis* P13.The values on Y-axis the effect (Exi) obtained from the PB-design

3.3.3: Optimization of protease production by response surface methodology

The concentrations of the significant parameters identified by Plackett-Burman (SBM, K_2HPO_4, $BaCl_2$ and $FeSO_4$) were further optimized by response surface methodology (RSM) using Box-Behenken (BB) design and the interaction of the selected parameters were studied. The protease activities obtained was analyzed using Design-Expert software were in good agreement with the predicted values (**Table 3.4**). Maximum protease activity was observed after 24h . Activity was stably maintained for 48 h in the medium and declined after 72 h (by approximately 30%, data not shown). Analysis of variance (ANOVA) appropriate for the design was used to evaluate the data after 24 h from the experimental analysis (**Table 3.4**). The regression equation obtained was $Y = 0.91 + 0.90A + 5.00B – 0.068C + 0.016D + 0.11AB + 1.00 AC + 0.097 AD - 0.046BC - 0.033 BD - 0.36CD + 0.11A^2 -1.57 B^2 - 0.049C^2 - 3.075 D^2$, which showed the variation of protease activity (Y) as a function of variables such as SBM (A), K_2HPO_4 (B), $FeSO_4$ (C) and $BaCl_2$ (D).

Table 3.4: Experimental design and results of response surface methodology studies using Box- Behnken design for the production of protease by *B. subtilis* P13 after 24h of growth.

Media #	Variable media components (g/l)				Protease activity (U/ml)	
	SBM	KH_2PO_4	$FeSO_4$	$BaCl_2$	Experimental	Predicted
1	5.5	1.65	0.0055	0.055	0.867	0.857
2	5.5	1.65	0.010	0.010	0.832	0.810
3	5.5	1.65	0.001	0.010	0.95	0.91
4	1	1.65	0.0055	0.010	0.167	0.21
5	5.5	1.65	0.0055	0.055	0.916	0.912
6	10	1.65	0.0055	0.100	2.11	2.03
7	1	1.65	0.0055	0.100	0.048	0.045
8	5.5	1.65	0.010	0.100	0.693	0.742
9	1	1.65	0.001	0.055	0.166	0.17
10	10	1.65	0.001	0.055	1.854	1.93
11	5.5	3	0.010	0.055	0.757	0.783
12	10	0.3	0.0055	0.055	1.65	1.643
13	5.5	0.3	0.0055	0.010	0.88	0.865
14	5.5	1.65	0.0055	0.055	0.91	0.912
15	5.5	1.65	0.001	0.100	0.96	0.951
16	10	1.65	0.0055	0.010	1.813	1.844
17	1	1.65	0.010	0.055	0.012	0.023
18	5.5	3	0.001	0.055	0.98	0.97
19	1	3	0.0055	0.055	0.050	0.0413
20	5.5	1.65	0.0055	0.055	0.859	0.912
21	1	0.3	0.0055	0.055	0.28	0.255
22	5.5	1.65	0.0055	0.055	0.913	0.911
23	5.5	0.3	0.010	0.055	0.864	0.857
24	5.5	0.3	0.0055	0.055	1.043	0.952
25	10	3	0.0055	0.055	2.11	2.09
26	5.5	3	0.0055	0.010	0.835	0.93

27	5.5	0.3	0.001	0.055	0.875	0.88
28	10	1.65	0.010	0.055	1.787	1.83
29	5.5	3	0.0055	0.055	0.855	0.850

Ten different combinations of medium components, that were not included in the Box-Behnken design, were selected to validate the predictability of the model (point prediction) derived by ANOVA at the shake flask level. The concentration range of the components in coded value along with all the above response, both experimental (actual) and also predicted are given in **Table 3.5.** The minimal difference between actual and the predicted values indicated the good predictability of the model.

Table 3.5: Validation of the model through point prediction.

Media	**SBM (gm/l)**	**KH_2PO_4 (gm/l)**	**$FeSO_4$ (mg/l)**	**$BaCl_2$ (mg/l)**	**Caseinolytic**	
					Actual	**predicted**
1	5.5	1.65	5.5	55	0.947±0.99	0.898
2	10	3	1	100	2.2 ±1.24	2.28
3	8.05	2.42	1	81.76	1.67±1.56	1.70
4	10	1.03	1	100	1.89±0.87	1.76
5	10	30	5.26	100	1.92±0.94	1.90
6	5.62	2.27	1.62	56.22	1.05±1.92	1.0
7	10	1.21	1.61	11.22	1.69±0.76	1.49
8	3.55	2.27	1.16	79.32	0.49±0.99	0.50
9	10	1.39	7.57	41.62	1.79±0.92	1.81
10	11	3	10	98.78	0.69±0.83	0.55

The significant model terms for protease production are A, C, A^2, AB and AD. A positive interaction between AB and negative interaction between AC were shown as isoresponse 3D plots in **Fig. 3.4** and **3.5** respectively. The optimal value obtained from isoresponse 3D plot is almost in agreement with the response from regression equation. A relatively lower value for co-efficient of variation (% CV= 7.89) indicated precision and relativity of the experiments performed.

Apart from point prediction, another criteria that can be used to evaluate the quality of the model is ANOVA (**Table 3.6**). For protease production, the co-efficient of determination (R^2) is 0.9924 and it explains 99.24% variability in the model. The R^2 value closer to 1 .0 shows a stronger model with better predictability (Haaland, 1989). The statistical analysis is significant in determining the experimental factors generating large signals in comparison to the noise generated due to manual errors. Adequate precision measures the signal to noise ratio. An adequate precision of 40.10 was recorded for this model. The value for lack of fit F and lack of fit P> F were found to be 35.96 and 0.180 respectively, explaining that the lack of fit is insignificant. The predicted correlation co-efficient (predicted R^2) of 0.9568 suggested a fair agreement between predicted and experimental protease activity. The model F value of 131.14 and P> F of <0.0001 indicated the significance of the model.

Thus to conclude statistical optimization of protease production resulted in improved protease activity of 2.21 (U/ml) from a media comprising 10 g/l SBM, 1.65 g/l KH_2PO_4; 5.5 mg/l $FeSO_4$ and 100 mg/l $BaCl_2$, where the predicted response was 2.09 and is strong agreement with the experimental value. In the optimized medium, a maximum activity is 3.5 fold higher as compared to the un-optimized basal medium (LBSM, 0.6 U/ml). Besides, the increased activity was obtained 24 h earlier than that in the un-optimized medium.

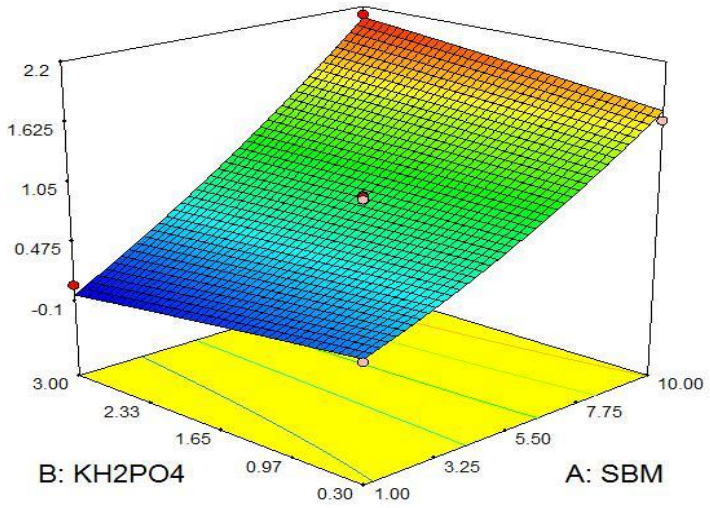


Fig. 3.4: Response plot presenting positive interaction between Soyabean meal (SBM) and KH_2PO_4

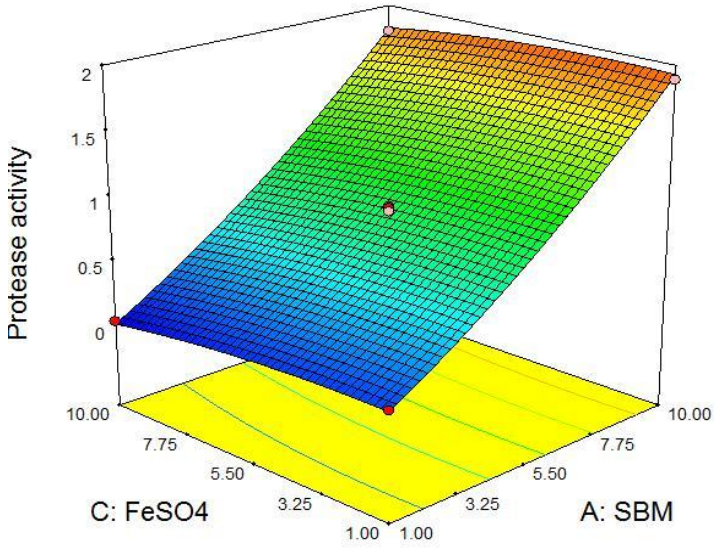


Fig. 3.5: Response plot depicting negative interaction between Soyabean meal (SBM) and $FeSO_4$

Table 3.6: Analysis of variance and regression analysis for Box-Behnken design for protease production by Bacillus subtilis P13. Coefficient of determination (R^2) 0.9924; correlation coefficient (Adj. R^2) 0.9849; Pred. R^2 0.9568; adequate precision 40.100.

Source	Sum of Squares	Degrees of freedom	Mean Squares	F-value	P>F
Model	10.08	14	0.071	131.14	<0.0001
Residual	0.076	14	5.449E-003		
Lack of Fit	0.075	10	7.544E-003	35.96	0.081
Pure error	8.392E-004	4	2.098E-004		
Corrected total	10.08	28			

3.3.4: Growth and enzyme production in optimized medium

To understand whether the high production of protease on optimized medium is because of the better biomass build up provided by SBM and/or induction of the enzyme in its presence, growth associated enzyme production was compared in the optimized medium and in the basal medium (**Fig. 3.6**). In the optimized medium, culture entered in to late log phase around 18-20 h of growth, whereas in the case of un-optimized basal medium, the late log phase was observed after 30 h. A maximum enzyme activity of 2.26 U/ml was observed in the optimized medium around 24 h, whereas in unoptimized medium, maximum activity was observed after 48 h.

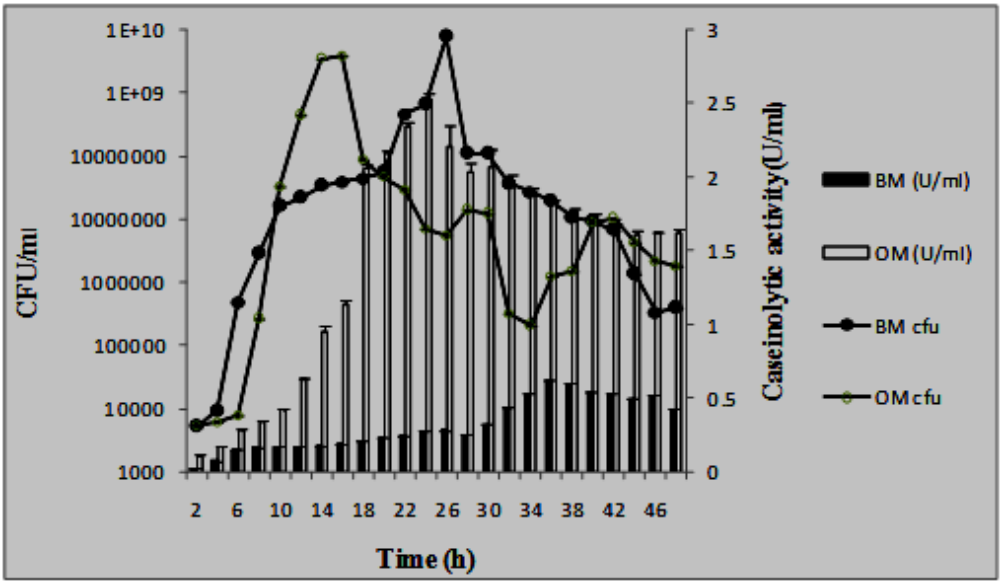


Fig. 3.6: Growth associated protease production by *B. subtilis* P13: Protease activity and growth (cfu/ml) in basal medium (BM) and optimized medium (OM).

Protease zymogram profile at different stages of growth on optimized medium showed no visible protease bands in the initial log phase of growth, but after 22 h (late log phase) the presence of an approximately 31 kDa protease band was observed and its intensity decreased at 32 h (**Fig. 3.7**). Several additional high and low molecular weight protease bands of ~66, 43 and 20 kDa appeared at this point and persisted till 48 h. Zymogram pattern similar to that of optimized medium in the log phase was observed at the end of 48 h,when protease production was carried out using a medium reported by Puri et al., (2002), containing (w/v) K_2HPO_4, 0.7%; KH_2PO_4, 0.3%; $MgSO_4$, 0.01%; yeast extract, 0.5% and soyabean meal, 1%.

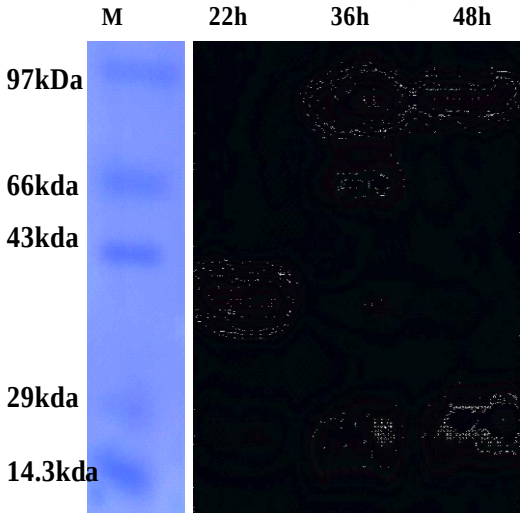


Fig. 3.7: Zymogram of proteases produced in optimized medium at different time intervals. M indicates molecular weight marker

3.3.5: Scale-up studies

Production of enzyme was scaled up to 1L and 2L shake flask and 5L fermenter level consistently. Fermentation parameters were aeration of 6 vvm, dissolved oxygen 100% and agitation of 200 rpm. At the time of inoculation, set pH of the media were maintained at 6.9.batch was harvested after 32 h. The increase in pH from 6.9 to 8.2 was considered as the indicator for the termination of batch termination, based on the observations in 1L and 2L shake flask studies. At 1L and 2L shake flask levels, protease activity of 2.16 U/ml was achieved in 24 h time, whereas at 3L fermenter level, maximum activity of 3.09 U /ml was achieved after 36 h. Compared to the basal level activity of the strain, fivefold increase in activity was achieved at 3L fermenter level. Beg et al., (2002) reported four- fold increase in protease yield from *B .mojavenesis* under fed-batch operations.

3.3.6: Enzyme stability studies

Among the ten stabilizers studied, addition of silca gel, $CaCl_2$, NaCl, $BaCl_2$ and Na_2SO_4 allowed the protease activity to be retained to about 90- 95 % of the original at 4 as well as 37 °C for a period of 6 months, whereas the ammonium sulfate precipitated control sample retained 95- 98 % activity. Activity decreased to 87% in the case of control and 80, 79 and 78% for $BaCl_2$, $CaCl_2$ and Na_2SO_4 respectively at

4°C after 12 months and at 37°C, the decrement in activity was to 80 % for control, 78 % for $BaCl_2$, 75 % in the case of $CaCl_2$ and 73% for Na_2SO_4. Shelf life profiles of protease in presence of best stabilizers viz, $BaCl_2$, $CaCl_2$ and $(NH_4)_2SO_4$ with respect to control at 4 and 37°C for a duration of 12 month are shown in **Fig. 3.8 a and b respectively**

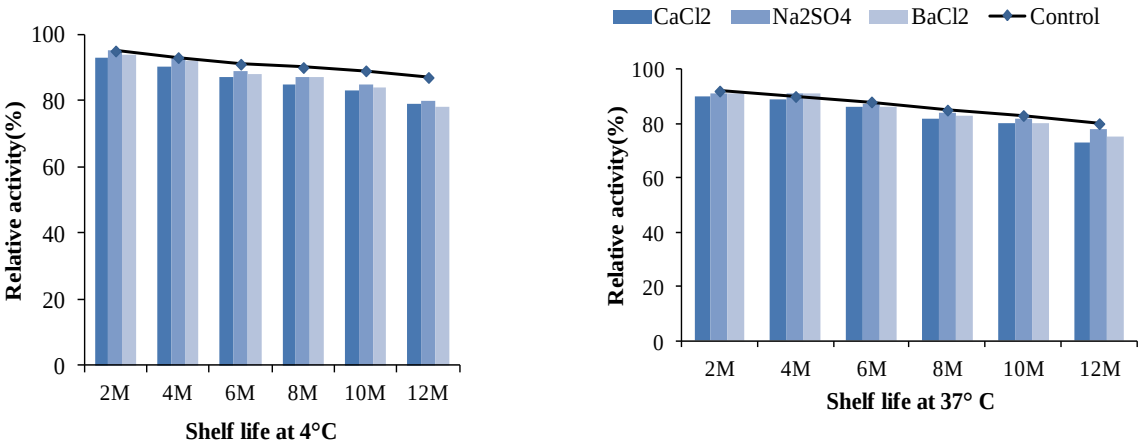


Fig. 3.8: Stability of protease from *B. subtilis* P13 in presence of selected stabilizers M stands for months

3.3.7: Tannery application studies

Soaking is the first process in the tannery in which fresh or dry salted preserved hides are treated for the removal of extraneous matter, rehydration of inter-fibrillary protein and loosening the cementing substance of the fibre. This process is facilitated by the addition of enzymes. When hides were treated with protease preparation *B. subtilis* P13, formulated using $(NH_4)_2SO_4$ (60% saturation), 19.5% swelling was observed after 3 h while 12% swelling was found with commercial soaking enzyme. Amount of N-released was double in the test sample as compared to the control (**Table 3.7**).

Soaking studies carried on goat hide, right half of the dry salted goat hide was used as the control, treated with a commercial soaking enzyme formulation and left half as test, treated with ammonium sulphate precipitated enzyme formulation from *B. subtilis* P13**.**

Table 3.7: Evaluation of soaking efficiency of keratinolytic protease from *B. subtilis* P13.

Sample	Time (h)	%Swelling	N_2 Estimation (mg/ml) (Kjeldhal's method)
Control	1	12	167.65
Test	1	13	251.49
Control	2	12.5	167.66
Test	2	19	287.42
Control	3	12.56	234.72
Test	3	19.5	447.09

After 18 h of treatment with *B. subtilis* P13 protease preparation, intact hair was recovered by simple scraping from the portion of the hide treated with control and test enzyme (**Fig. 3.9**). Good air permeability, retention of the thumb impression and proper grain structure were observed in case of control and test, indicating bating efficiency of the enzyme (**Fig. 3.9**).

Microscopic analysis at 40X magnification, after haematoxylin staining of the enzyme-treated pelts showed digested epidermis, appendages to be absent, and follicles without hair. Degraded hair bulb observed in case of control and test indicated the proper dehairing (**Fig. 3.10a)** and an extensive digestion of the epidermis (**Fig. 3.10b**) reflected better dehairing efficiency. Grain nature of the corium was intact, but appeared flaccid because of the osmotic swelling (**Fig. 3.10c and d**).

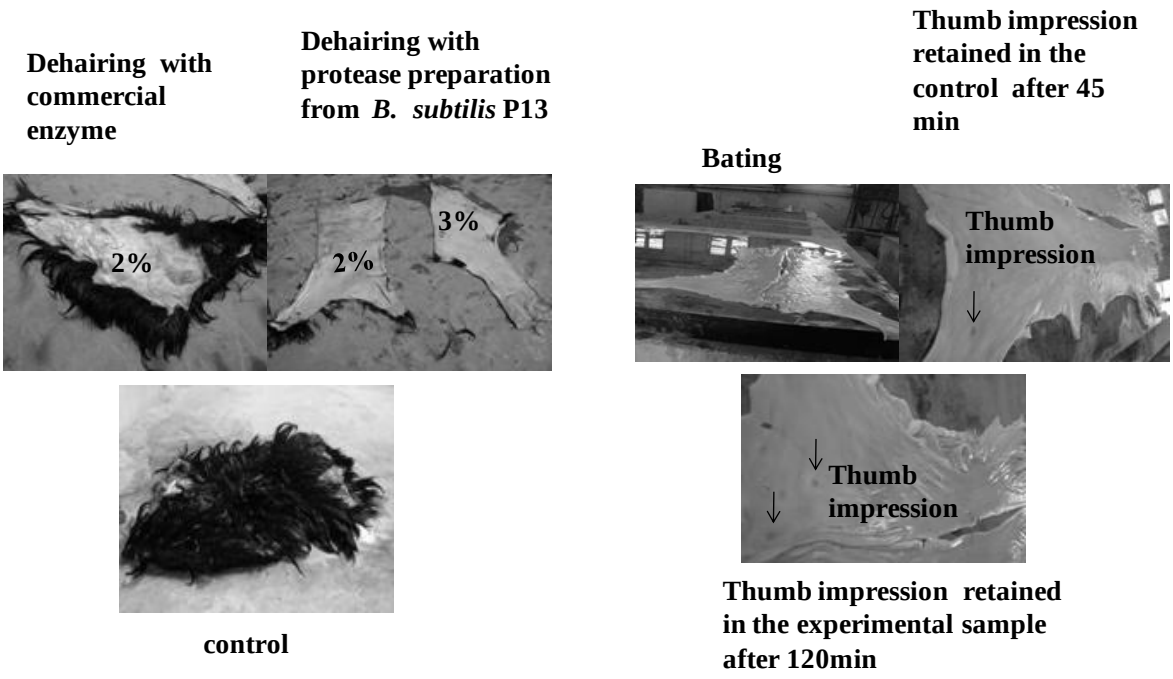


Fig. 3.9: Dehairing and bating studies carried out on goat hide at CLRI Chennai, India. Right half is used as control and left half as test. Standard commercial formulation of dehairing and bating enzyme is used as control and ammonium sulfate precipitated and air dired protease from *B. subtilis* P13 is used as test.

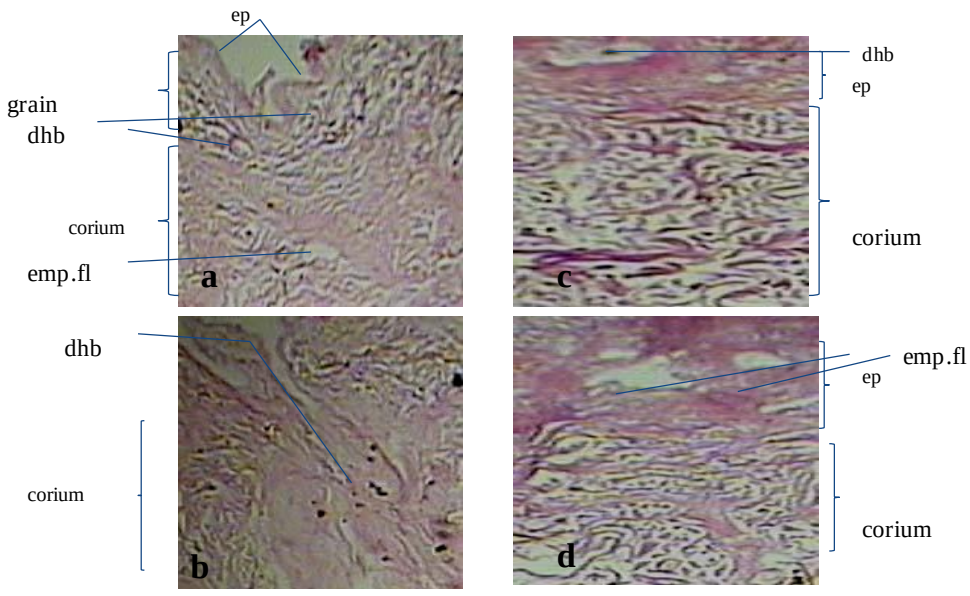


Fig. 3.10: Hematoxylin stain of goat hide subjected to dehairing (a, b) and bating (c, d) using commercial dehairing enzyme (control) (a,c) and protease preparation of *B. subtilis* P13 (test) (b, d). The photographs are taken 40X magnification. [ep: epidermis, Dhb: degraded hair bulb & emp.fl: empty follicle]

3.3.8: Co-relation of feather peptide release and protease activity

Since multiple protease bands were seen during growth in optimized medium, it was important to understand which of these was responsible for feather degradation and hide depilation and whether the same isoform could perform both activities. Feather hydrolysis activity in the culture supernatants from the Box-Behnken design of 29 different media compositions are given in **Table 3.8**. With the feather hydrolysing activity as a response, the software suggested a linear model with a P>F value of 0.0156 and a regression equation of Y = 0.38+ 0.13A-0.086B-2.33C-0.031D. The feather degrading activity correlated poorly with the caseinolytic acitivity in the 29 media combinations (R^2 = 0.214) (**Fig. 3.11**) indicating that feather hydrolysis was probably carried out by only a subset of caseinolytic protease isoforms. The correspondence of peptide release from feather with extracellular caseinolytic activity (24 h media) differed from media to media. Correlation co efficient R^2 = 0.214 indicated that there is poor correlation between the two parameters particularly in certain media combinations such as 4, 21, 22, 23, 25 etc. Interestingly, there were clusters of data points showing good activity as well as peptide release, good activity but poor peptide release, poor activity and good peptide release. Representative media (media combinations 6, 10, 12, 15, 16, 23, 24, 25 and 28) were analyzed in further detail. After providing same units of enzyme for peptide release in each case, combination 6 and 10 were showing similar amount of peptides released, where maximum activity in the media at 24 h was 2.12 U/ml and 1.93 U/ml. In media combination 25, peptide release was of 0.109 AU, which was not correlating with the high activity of 2.11 U/ml. To understand this, enzyme samples from 24h old media from all the above nine combinations were subjected to gelatine based zymogram profiling (**Fig. 3.5 and 3.6**). Area of the activity band corresponds to 31 kDa protein were related with the protease activity and peptide release from crushed feather. Maximum area of 110 was observed in the case of combination 23. R^2 value of 0.5764 confirms that there is a direct relation between peptide release and 31 kDa protease band area but not total caseinolytic activity. Selected samples of the 29 media were subjected to protease zymography and densitometric intensity of the 31 kDa band was relatively better correlated with feather degradation (R^2 = 0.5764) (**Fig. 3.12**). This indicated that the 31 kDa band probably possessed feather degrading activity.

Table 3.8: Feather hydrolysis using protease produced by *B. subtilis* P13using Box-Behken design

Media code	Feather peptide released
1	0.531±0.001
2	0.516±0.0023
3	0.311±0.0041
4	0.186±0.0021
5	0.470±0.0084
6	0.571±0.0011
7	0.115±0.00101
8	0.389±.00256
9	0.180±0.0009
10	0.561±0.00709
11	0.521±0.00176
12	0.537±0.0033
13	0.489±0.00458
14	0.499±0.00335
15	0.518±0.0046
16	0.525±0.0077
17	0.105±0.0004
18	0.471±0.0068
19	0.055±0.00020
20	0.219±0.0026
21	0.449±0.00816
22	0.540±0.00237
23	0.541±0.0009
24	0.216±0.0008
25	0.109±0.0006
26	0.321±0.0049
27	0.495±0.0083
28	0.391±0.0057
29	0.215±0.0005

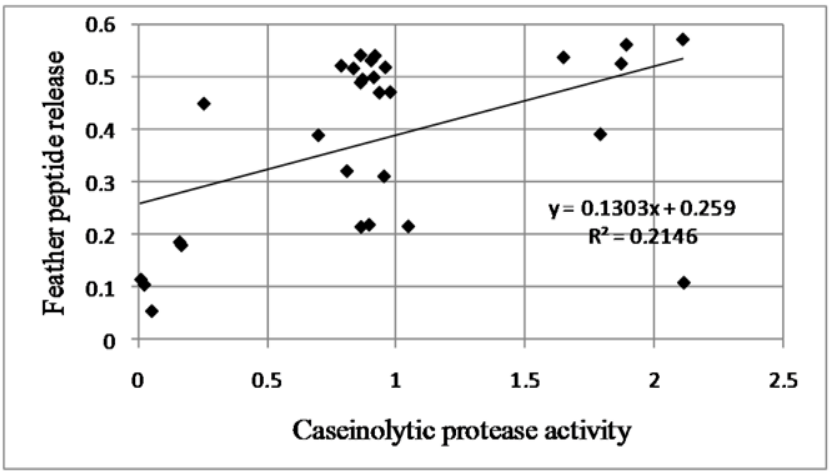


Fig. 3.11. Correlation of feather peptide release with caseinolytic activity from different media combinations used in RSM.

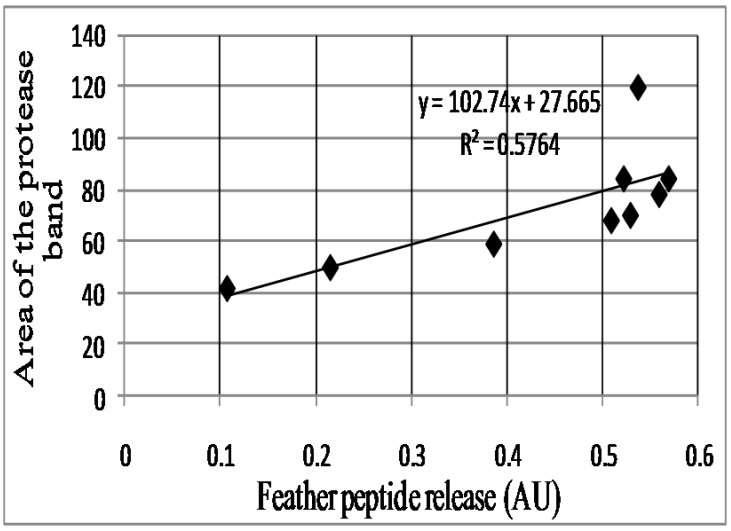


Fig. 3.12: Correlation of intensity of 31 kDa protease band in zymograms with feather peptide release from different media combinations used in RSM

3.3.9: Purification and characterization of 31 kDa protease

To confirm the properties of 31 kDa protease, the band was eluted from the SDS-PAGE gel to obtain a pure preparation (**Fig. 3.13**) and its keratinolytic activity was recorded 0.226 ± 0.0196 U/ml as compared to caseinolytic activity of 0.275 ± 0.0253 U/ml. A ratio of keratinolytic to caseinolytic activity of 0.8 confirms it to be a keratinase. With the gel purified enzyme, peptide release from ground feathers was observed of 0.565 AU after 8 h and it increased to 0.654 AU after 12 h. That the eluted enzyme is a serine protease was confirmed by 94% inhibition of its caseinolytic activity by PMSF.

The eluted 31 kDa protease showed complete dehairing of goat hide after 12 h, using 2% (based on the hide weight) of the enzyme without any additives (data not shown). This confirms our earlier speculation in Chapter 2 that a single keratinolytic serine protease is responsible for feather degradation and hide-depilation. Scanning electron microscopic studies for comparing the goat hide depilated by chemical and enzymatic process (using 31 kDa purified protease), showed that hair remained in the follicles up to 10 h of enzymatic treatment (**Fig. 3.14b)** and interestingly the outer root sheath was released, resulting in loose hair in opened up structure. Complete dehairing with clean pore was observed in enzyme treated sample after 18h (**Fig. 3.14c**), whereas, some portion of the hair were left in the chemically dehaired sample (**Fig. 3.14a**). Collagen was not damaged, flaccidity observed in the case of enzyme treated hide (**Fig. 3.14d**), but in the case of chemical process, the surface of the hide appeared more firm.

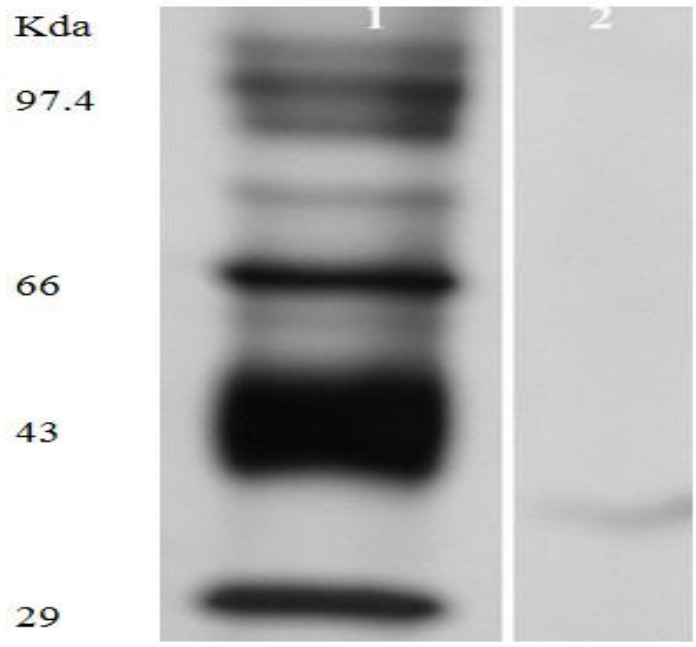


Fig. 3.13: Gel eluted ~31 kDa protease on SDS PAGE showing purity. Lane 1: Molecular weight marker and Lane 2: eluted enzyme

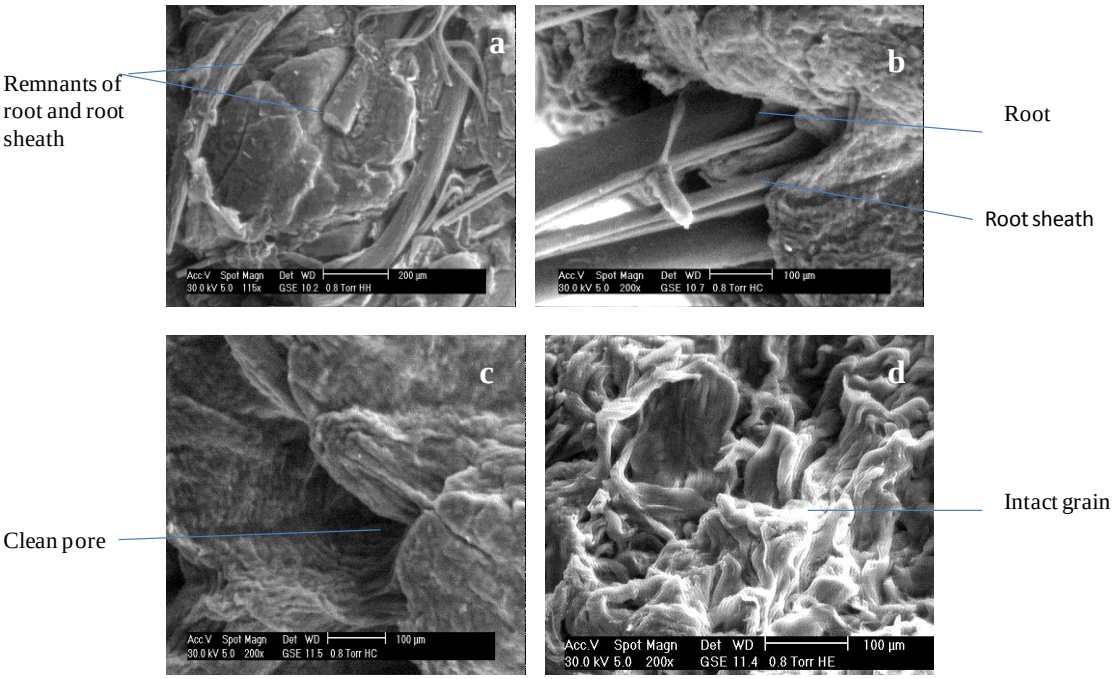


Fig. 3.14: Scanning electron micrographs showing the dehairing of the pelts with protease from *B. subtilis* P13. a: conventional lime–sulfide dehairing; **b** and **c** enzymatic dehairing for 10 h and 18 h, respectively; Complete dehairing observed after 18h is supported byclean pore, indicating complete removal of the hair and root. Stubbles retained in the lime – sulfide treatment reflected as remanants of root and root sheath; **d:** hide with proper grains having flaccidity due to enzymatic dehairing

3.3.10: Sulfitolysis studies with *B. subtilis* protease

Keratin degradation involves two major steps sulfitolysis and proteolysis. The disulfide bond reducing activity (sulfitolysis) of the protease preparation of *B. subtilis* P13 at 30 and 50 °C on milled feathers and commercially available keratin powder is depicted in **Fig. 3.15**. Both the crude enzyme and the purified preparation by gel elution showed better sulfitolysis at 50 °C than 30 °C. Better efficiency of sulfitolysis of culture supernatant compared to the purified enzyme may be due to the presence of other cellular redox systems.

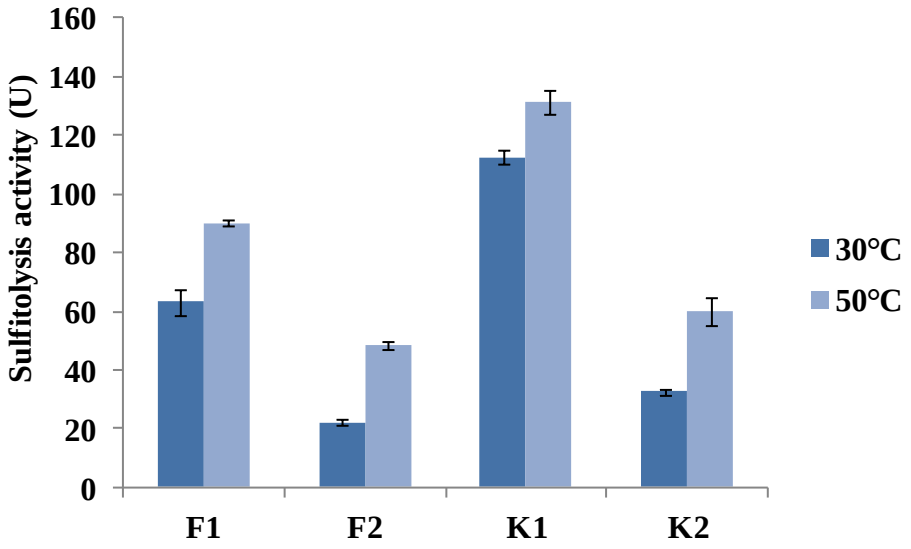


Fig. 3.15: Sulfitolysis (reduction of disulfide bonds) of milled feathers (F) and keratin (K) by proteolytic enzyme preparation of *B. subtilis* P13. 0.5 U of keratinolytic protease. F1, K1: crude culture supernatant and F2, K2: purified 31 kDa protease

3.3.11: N-terminal sequence of the 31 kDa protease from *B. subtilis* P13

The molecular weight of most of the keratinolytic proteases ranges from 27-45 kDa (Peng et al., 2005) and this keratinolytic protease of molecular weight 31kDa also fall in the same category.The first 10 residues from the N-terminal of the purified protease from *B. subtilis* P13 showed an amino acid sequence of AQSVPYGISQ, which is identical to that of subtilisin proteases produced by several other *B. subtilis* strains (**Table 3.9**). The sequence differs to those of other enzymes from *B. subtilis* by two amino acids; Ser3 was replaced by Thr 3 and Ser9 by Pro9.

Table 3.9: Comparision of N-terminal amino acid sequence of keratinolytic serine protease from *B. subtilis* P13 with that of proteases from other *Bacillus* strains

Proteases	N-terminal amino acid sequence (% identity with *B. subtilis* P13 sequence)	Strain	Molecular Mass (kDa)
[a]Keratinolytic serine protease	AQSVPYGISQ	*B. subtilis* P13	31
[b]Subtilisin BSF1	AQSVPYGISQI (100%)	*B. subtilis*	26
[a]Subtilisin E	AQSVPYGISQIKAPA (100%)	*B. subtilis* 168	27.5
[a]KerS14	AQSVPYGISQIKAPA (100%)	*B. subtilis* S14	27
[b]Subtilisin NAT	AQSVPYGISQIKAPALHSYT (100%)	*B. subtilis natto*	27.7
[b]Subtilisin DFE	AQSVPYGVSQIKAPALHSFT (90%)	*B.amyloliquefaciens DC4*	28
[b]Subtilisin FS33	AQSVPYGIPQIKAPA (90%)	*B. subtilis DC33*	30
[b]Subtilisin CK	AQTVPYGIPLIKADD (80%)	*Bacillus sp.* CK 11-*4*	28.2
[c]Keratinase PWD-1	AQTVPYGIPLIKADK (80%)	*B.licheniformis* PWD1	33
[a]Carlsberg subtilisin	AQTVPYGIPLIKADK (80%)	*B.licheniformis*	27.6
[c]Keratinase	XQTVPXGIPYIYSDD (70%)	*B.pseudofirmus*	27.5

[c] Brandelli, 2008; [b] Agrebi et al., 2009; [a]present study

3.4: Discussion

Production of protease is an inherent property of all organisms, mostly constitutive and sometimes inducible (Beg et al., 2002, Kalisz, 1988). Proteases are known to be associated with the onset of stationary phase, which is marked by the transition from vegetative to sporulation stage in the case of spore formers such as *Bacillus* sp. Some reports suggest that sporulation and protease production are just a co-occurrence and are not related. (Fleming et al., 1995). The same group also carried out protease production studies with respect to nucleotide pool analysis (GTP and ATP) on a spore deficient strain of *B. licheniformis*, suggest that it is related to the Gppp ratio in the cell. Bierbaum et al., (1991) concluded that extracellular protease production is a manifestation of nutrient limitation at the onset of stationary phase, particularly due to changes in the transcription apparatus (Malikova et al., 2006). However final yields of protease during this phase are also determined by the biomass build up during the exponential phase (Gupta et al., 2002). Therefore medium manipulation is needed to maximize the growth for better yield.

Bacillus sp use a wide variety of substrates for growth and enzyme production. There are previous reports where *Bacillus sp*. Js-3, RGR-14, ATCC-21415 (Gupta et al., 2002; Puri et al., 2002), preferred SBM over other complex nitrogen sources for protease production. In the present study, *B. subtilis* P13 produced protease preferably using SBM over other 4 different complex nitrogen sources studied. The preference of SBM by this keratinolytic protease producer over MF could be because of the better biomass build up provided by SBM. The efficiency of the culture to degrade MF can be explored in terms of waste management. In single parametric screening glucose appeared as a better carbon source. But in PB studies, glucose was showing negative effect this could be because medium pH reduced to 5.0 after 24h. In the case of positive effect, pH increased from 7.0 to 8.5. The effect of metal ions on keratinase has suggested that divalent metal ions like Ca^{2+}, Mg^{2+}, Mn^{2+} stimulated the enzyme activation. (Mukopadhay and Chandra, 1990). Dozie.et al., (1994) reported that Ba^{2+}, Co^{2+}, and Fe^{2+} lead to the augmentation of keratinolytic activity in *Chrysosporium keratinophilum*. In the present study also, Ba^{2+} and Ca^{2+} showed positive effect in single parametric studies and PB. Interestingly Fe^{2+} showed positive effect in single parametric studies but showed negative effects in both PB and BB designs. Based on single parametric study, Plackett Burman and Box-

Behnken, SBM appears to have strong positive effect on protease activity, as a single component and also in terms of interaction with $BaCl_2$ and, K_2HPO_4

Adıguzel et al., (2009) observed that the secretion of keratinase in two *B. cereus* strains in different media was induced upon entry into stationary phase and they surmise that high keratinolytic activities in cultures grown on native proteinaceous substrates may be a result of nitrogen limitation caused due to poor degradability of the substrate rather than induction. In our experiments, similar interpretation is possible as skimmed milk seems to be more suitable for sustained growth supporting an extended log phase as compared to SBM, however, the former did not support good enzyme production.

The sequential secretion of multiple proteases during different stages of growth is in accordance with other reports on growth associated zymography from *Bacillus* strains (Balaji et al., 2008 and Zuidweg et al., 2005). *B. subtilis* 168 has at least six proteases in the extracellular secretome of which four are serine proteases (Tjalsma et al., 2004). In our studies, *B subtilis* P13 produced about 4 major protease isoforms in the optimized medium distributed differently at various stages of growth.

Studies on the production of a subtilisin-like protease in *B. intermedius* showed that maximum accumulation of protease activity corresponded to 24-26 h and 44 - 46 h of growth (Malikova et al., 2007). In *B. pumilus*, maximum proteolytic activity was reported after 32 h, at the onset of the stationary phase. In the case of other bacilli also, high enzyme synthesis occurred when the growth decreased and when the cells entered into the stationary phase (Malikova et al., 2007). *B. subtilis*.P13 produced maximum protease activity of 0.6U/ ml in the basal medium, LBSM, after 48h. But after BB optimization, maximum activity was observed after 24h. Protease activity band on the gelatine zymogram from 24 h old enzyme sample corresponds to the PMSF sensitive protease band reported in our previous studies. In the zymogram profile, many other protease bands were observed after 48h. Since there was no increase in protease activity after 24h and peptide release was at its best with the enzyme from 24h media, the assumption is that the other proteases found in 48h and 72 h media may be associated with massive cell lysis and endospore release.

Use of statistical design in biotechnology to optimize culture conditions and medium components are widely accepted (Hajji et al 2008). However no reports are available, where there were correlation of media optimization, zymogram and a specific property of the enzyme to confirm the suitability for the application for

which it has to be used. When the protease activity, peptide release and intensity of the activity band on the zymogram were correlated, a good correspondence between peptide release and area of the 31kDa band was observed, but the total protease activity is mostly based on the SBM concentration. In media combination 23, protease activity was only 0.86U/ml, but peptide release was 0.541 and the area of the protease band was maximum (110). High concentration of $FeSO_4$ could be a reason for lower activity (interaction is negative for $FeSO_4$), but at the same time reasonably better peptide release and highest recorded area of protease band is in agreement with the observation that Fe^{2+} augment the keratinolytic activity for *C. keratinophylum* (Dozie et al.1994). In medium 24, K_2HPO_4 at its highest level and $FeSO_4$ at medium concentration, interaction between these components are highly negative and that might be the reason for the lowest peptide release and lesser area of the activity band. The higher activity of 2.11 and 1.82 U /ml in media 25 and 28 could have been because of the maximum SBM concentration.

By employing statistical design, RSM, there was maximum production of protease of 2.2U/ml after 24h. The 3.5 fold increases in activity and reduction in fermentation time from 48h to 24h which could be advantageous to reduce the cost of production. Rao and Satyanarayana, (2003) and Singh and Satyanarayana, (2006) reported similar kind of achievement by RSM for α-amylase and phytase production respectively. Correlation studies offered that a media having 10g/l SBM, 1.65g/l,K_2HPO_4: 10 mg/l $FeSO_4$ and 100mg/l $BaCl_2$, is the most suitable for protease yield improvement, but protease from, a medium with 5.5g/l SBM., 0.3g/l K_2HPO_4:, 5.5 mg/l $FeSO_4$ and 55mg/ l $BaCl_2$ might be more appropriate depilation and other feather applications.

Enzyme stabilizers such as dry powders added directly to enzyme preparations, constitute low molecular weight substances like co-factors, buffers, chelators, metal ions, carbohydrates and antioxidants which are the preferred stabilizers over proteins, glycol, polyethylene glycol and dithiothretol (Durham et al., 1987). An alcohol based downstream processing and formulation of fungal protease showed a stability of 90, 80 and 75% at 4, 25 and 37 °C respectively for 12 months. Here in the case of protease from *B. subtilis* P13, $(NH_4)_2SO_4$ was the best stabilizer and the same might be due to the shielding effect provided by the salt helps in conformational stability.

Media optimization followed by fermenter level scale up studies was reported for different bacterial and fungal strains (Rani et al., (2002). Bhosale et al., (1995) presented a fermentation condition of pH 7-7.5, temperature 28 °C, agitation of 220 rpm over a period of 48h for *Conidobolus coronatus*. Lee et al., (1996) reported fermentation studies at 50 °C, at a pH of 7.2, agitation of 250 rpm for short fermentation duration of 16h for *Thermoactinomyces* sp.E79. Fermentation conditions varied from a pH range of 7.0-10.0, temperature of 30-50°C, agitation of 150-500 rpm and fermentation duration of 16-96 h for different species of *Bacillus* (Kaur et al., 2001;Puri et al., 2002;Oberoi et al., 2001;Beg et al 2002).Apart from the most preferred nitrogen source, SBM, Bacillus sp preferred ammonium nitrate (Bhosale et al., 1995) corn steep liquor (Kumar,2002), casein ans casaminoacids (Beg et al.,2002) KNO_3 (Jhonvesly and Naik,2001).Some strains of Bacillus even required carbon sources like glucose, sucrose, lactose , glycerol etc (Gupta et al. , 2002). In the case of *B. subtilis* P13 also, SBM appeared to have strong positive effect on protease production, as a single component and also in terms of its interactions. In conclusion, media optimization resulted in an improved protease (caseinolytic) activity of 2.26 U/ml, keratinase activity of 2.07U/ml and gelatinase activity of 0.98U/ml (equivalent to 434, 376 and 178 U/ml when expressed in terms of µg of tyrosine released per min) in the optimized medium comprising of 10 g/l SBM, 1.65 g/l KH_2PO_4; 5.5 mg/l $FeSO_4$ and 100 mg/l $BaCl_2$, which was 3.5 fold higher as compared to the un-optimized basal medium. Also, the increased activity was obtained at 24 h, which is earlier than that obtained in the un-optimized medium, which recorded highest activity at 48 h. A subtilisin like serine protease (alzwiprase) production was optimized in the similar manner from *B. subtilis* DM-04 reported maximum units of 465µg/ml/min after 72 h of fermentation (Rai and Mukherji, 2010). As of now not a single report of such a formulation which was worked out for all the three pre-tanning processes. Dual property of the single enzyme was confirmed by gel elution of the respective zymogram band and dehairing studies using the eluted protein.

Specific terms for hair removal based on the mechanism include depilation i.e. removal of hair from the surface, or epilation, which is used to indicate removal of hair along with the root with consequent breaking of follicular system (**Fig. 3.16**). Lime and sulphide are chemical depilatory agents. On the other hand, enzymatic dehairing can be accurately considered as epilation, although the term 'depilation' in

the context of enzymatic dehairing is commonly used in literature (Everette et al. 1962; Yates, 1972; Puvankrishnan and Dhar 1986; Malathi and Chakraborty 1991; Pal et al. 1996; Riffel et al. 2003; Choudhary et al. 2004; Macedo et al. 2005; Mitra and Chakrabartty 2005; Pandeeti et al. 2011), perhaps originating from the comparison and usage of this term for chemical unhairing method and due to a lack of thorough understanding of the enzymatic unhairing mechanism. With histological and electron microscopic studies (Zambare et al., 2007; Sivasubramaniam et al. 2008;, it is becoming increasingly clear that the so called "depilatory enzymes" in fact leave empty hair follicles on the hide and remove the hair intact from its root, and may be termed 'epilation'. Micrographs of the dehaired hides from tannery trials using protease preparation from *B. subtilis* P13 also revealed empty follicles, confirming the epilatory nature of the enzyme. Although the exact substrates of the dehairing enzymes are not understood, it is apparent from the enzyme specificities that they might not directly act upon hair; instead, they might cause epilation by degrading the components of basement membrane at the dermal–epidermal junction. Disruption of this region would lead to the simultaneous detachment of both basement membrane and epidermis from the dermal layer. Hair could be dislodged from the surrounding follicles, as the sac of the hair bulb is less in fibrous keratin (El Baba et al. 2000). It may be reasonable to hypothesize that enzymes selectively destroy the surrounding tissue of the hair follicles, without destroying the mature keratins of the hair and perhaps also the epidermis thus loosening the hair from its shaft as well as detaching the epidermal layer from the basement membrane. The immature keratins found in hair roots, hair root sheaths, and the lower layer of the epidermis, however, may be more easily degraded by proteases without sulfitolysis.

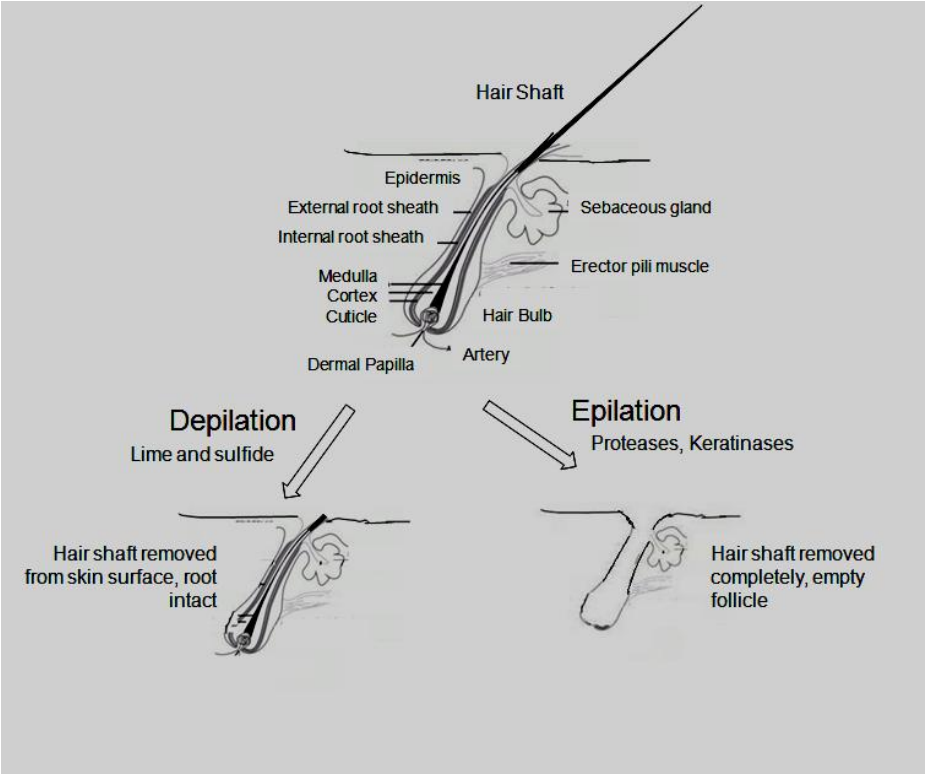


Fig. 3.16: Longitudinal section of a hair follicle showing different components and the effect of two types of dehairing methods on the hair follicle.

Most investigators have contended that one of the possible mechanisms of keratin breakdown is the reduction of disulphide bonds. However, confirmatory studies in this respect are scanty. Profound insight into sulphitolysis during keratin degradation was provided by the investigations of Kunert, (1989, 1992). Contributing evidences were also obtained from the studies by Ruffin et al., (1976) and Malviya et al., (1993). There is a consensus that a complete hydrolysis of keratin can be achieved only after its denaturation by the cleavage of the disulphide bridges representing the main source of the extraordinary stability and resistance to proteolytic digestion. The sulphite reacts at neutral to alkaline pH with cystine cleaving it to cystein and S-sulphocystein, according to the equation below (Kunert, 1992):

Cys - S - S - Cys + HS03 ~Cys - SH + Cys - SS03

According to Kunert (1992), this reaction takes place also with cystine combined in proteins, including keratin, hence keratin is denatured prior to the attack by keratinases by excretion of sulphite, which causes sulphitolysis of the disulphide bonds. Substantiating evidence of the parallel decomposition of keratin by sulphitolysis of the disulphide bonds has been proffered (Kunert, 1989; Malviya et

al., 1992). In accordance to previous observations, crude and purified (gel elution) keratinolytic protease from *B. subtilis* p13 exhibited sulfitolysis at 30 and 50°C. It can be presumed that here also feather disintegration and degradation were initiated as sufitolysis and followed as proteolysis. The N-terminal sequence was having homology with other subtilisin type proteases reported from *B. subtilis* strains and other Bacillus species. Among the ten sequences, there were 100% similarity with some of the reported keratinases (Agrebi et al., 2009) and maximum dissimilarity of 30%. Characterizations of the purified enzyme in terms of substrate specificity and kinetics are important for the better understanding of the system.

3.5: Conclusion

Response surface methodology based statistical medium optimization was carried out for the protease production from *B. subtilis* P13. SBM, $BaCl_2$ and K_2HPO_4 were identified as key factors involved in yield improvement. By this process of optimization fermentation duration was reduced by 24 h with a 3.5 fold yield improvement. The optimized medium was scaled up to 3L fermenter level. Down-streamed and formulated enzyme was evaluated on a commercial scale and its feasibility for all the pre-tanning process, viz, soaking, dehairing and bating were confirmed. Purification based on gel elution, followed by the quality appraisal confirmed that serine protease of 31 kDa is a keratinolytic protease may be having more specificity towards ß keratin. The dual property of hide depilation and feather disintegration exhibited are attributed to the merit of di-sulfide reduction and proteolytic attribute of *B. subtilis* P13. N-terminal sequence of first ten amino acids are matching exactly to the other reported subtilisins, concluding the protease produced by *B. subtilis* P13 as a subtilisin like keratinolytic serine protease which can perform feather hydrolysis and hide depilation.

CHAPTER 4

Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using *Bacillus subtilis* P13 and exploring its chromium resistance mechanisms

For a successful technology, reality must take precedence over public relations, for Nature cannot be fooled.
- Richard Feynman

4.1: Introduction

Leather processing, generates enormous solid and liquid wastes, obnoxious smell due to the degradation of protein from waste hide and the release of gases like H_2S, NH_3 and CO_2. Solid wastes generated are of two categories, chemical waste and high value protein-based waste from untanned and tanned sources (Kangaraj et al., 2006). Untanned wastes are mainly the fleshings, trimmings and are mainly used in glue/gelatine manufacture. The main composition of tanned waste is chromium and protein. Chrome shavings (CRS) are small, thin pieces of leather formed during shaving operation and constitute about 10% of the total weight of raw materials processed. It is estimated that 0.8 million tonnes of chrome shavings generated per year globally (Kanagaraj et al., 2001). Traditional practice of disposal is land filling, however the stringent restrictions are imposed on the disposal of chromium bearing waste in many parts of the world and also the presence of valuable protein necessitates search for other alternatives (Kangaraj et al et al., 2006).

Green chemistry as a concept represents the chemical processes where environmental pollutants can be replaced by ecofriendly alternatives. Eco-friendly options are reuse, by-product recovery, bioremediation etc (Rao et al., 2002). Chrome shavings can be used directly for the manufacture of bonded leather, leather boards, fibrous sheets grafted with acrylates, insulators and building material (Brown et al., 1998) and indirect use of tanned waste is basically the separation of chromium and protein. As a green route, chrome shavings are also used as a reductant for the preparation of basic chromium sulphate (BCS), commonly used tanning material, which otherwise is prepared by the reduction of chromium (VI) (sodium dichromate) in acid medium using molasses or sulphur dioxide as reductant. Combinations of alkali and enzymatic treatment are used to recover chromium from protein from CRS (Raoet al., 2002). Alkaline proteases like alcalase and combination of trypsin (0.05%) and esterase (0.05%) are also used in the preparation of soluble collagen hydrolysate called chrome cake (Marner etal., 1999). Trypsin attacks the peptide bond only where one amino acid is argnine or lysisne, but esterase cleaves the bond between glutamine-histidine, serine-histidine, leucine-valine, leucine-tyrosine, and tyrosine and threonine. Gelatin was recovered from chrome shavings by treating with pepsin A and trypsin at pH 8.0 for a period of 6- 24h at 70°C, where pepsin had

a mild effect on hydrolysis whereas trypsin was more effective .An alkaline protease is reported to completely digest the wet blue to gelable protein and the same formulated with casein and used for leather finishing (Cantera et al., 2000).

Katsifas et al., (2004) reported an *Aspergillus carbonarius* which could perform biodegradation of chrome shavings in solid-state fermentation experiments, where 97% liquefaction of the tannery waste was achieved and the liquid obtained from long-term experiments were used to recover chromium and proteinaceous liquid fertilizer and was used as animal feed additive. Proteinaceous products and recycled chromium were obtained from tannery waste using several chemical methods (Brown et al., 1996). Efforts to obtain chromium after the incineration of shavings did not result in a less toxic waste (Ferreira et al., 1999). Other approaches focused on the extraction of chromium from shavings or chromium oxidation with moisturized air (Tancous et al., 1981)

Microbial enzyme usage for CRS degradation demands a pretreatment, either alkali based denaturation or lime (5-6 %) treatment at 60-65 °C to denature the collagen (Kanagaraj et al., 2006). The use of microorganisms able to grow in highly concentrated chromium environments and transform the waste into an easily recycled byproduct offers a promising perspective for successful chromium recovery. The present chapter focuses on the development of a microbiological procedure using the hotspring isolate *B. subtilis* P13 for the biodegradation of chrome shavings, a solid waste generated during tanning process and the recovery of protease, a byproduct which has application in pre-tanning process .

4.2: Materials and Methods

4.2.1: Microorganism and growth conditions

Bacillus subtilis P13, an isolate from the sediment sample of hot spring at Vajreshwari, Mumbai, India was maintained on Luria-Bertanni (LB) agar, containing 1% skimmed milk. Inoculum was prepared by growing a colony of *B. subtilis* in LB broth up to 0.6 OD_{600nm} at 30° C. An aliquot amounting to 1-2% v/v of the above culture was added to the growth media. CRS containing media were autoclaved at 121 °C, 101 kPa for 15 min. Growth was monitored at regular intervals by spread plating on LB agar.

4.2.2: Microbial hydrolysis of chrome shavings

To understand their degradation pattern, 1% (w/v) CRS were added to LB and inoculated with *B. subtilis* (0.6 OD culture). Degradation efficiency was checked based on weight reduction and microscopic observations. Smears were made from CRS collected during the study duration and observed under oil immersion light microscope (Olympus CX41). UV sterilized chrome shavings (1%) were added to another set of LB flask and experiment were performed in the similar manner to study whether initial thermal processing is required for the degradation of these proteins. Suitability of the organism to degrade chrome shavings (CRS) was studied by using heat and UV sterilized CRS in LB and production medium (OM) containing 1, 5 and 10% of chromeshavings. Protease production was assessed based caseinolytic activity and zymogram profiling, as described in section 2.213.

4.2.3: Microbial hydrolysis of chrome shavings from a solid substratum column

A glass column of 30 cm height was filled with CRS (3 g) and small silicon tubing was attached the bottom and closed with a stopper. Top portion of the column were plugged with cotton and the whole setup was autoclaved at 15 PSI/15 min. The column was saturated with sterile OM and inoculated with 2% wt/vol of 0.6 OD (600nm) culture. Initial height of the packed material in the column was noted and it was regularly monitored when incubated at 30°C for liquefaction of CRS, protease activity and reduction in the height of the column

4.2.4: Determination of Minimum Inhibitory Concentration of chromium

Freshly grown culture of 0.6 OD was inoculated into 10 ml media containing different concentrations of chromium. K_2CrO_4 (10ppm – 50ppm) and $CrCl_3.6H_2O$ (100-500ppm) concentrations were used to study the tolerance of the culture to Cr (VI) and Cr (III) respectively. Optical density at 600nm was recorded after 24h growth at 30°C, 180 rpm, uninoculated LB having similar Cr concentration was used as blank. The minimal inhibitory concentration (MIC) of chromium was defined as the concentration at which no turbidity due to growth was observed in the broth (Luli et al., 1983).

4.2.5: Chromium estimation

Concentration of Cr (VI) from different experimental samples was estimated by the Diphenyl carbizide (DPC) method of (APHA, 1992, Camargo et al., 2003). Cr (III) estimation was carried out by the method of Sankalika et al., (2004). Chromium chloride hexahydrate equivalent to 1.0 mg of Cr (III) was subjected to heating in a muffle furnace in a silica crucible at 550°C for 6h and the ash was dissolved in 0.2 M H_2SO_4 (pH 1 ±0.5), to obtain a stock of 5 µg/ml of Cr (VI). From this stock solution, suitable aliquots were taken and 0.2 ml of DPC dye was added to each, allowed to stand for 5 min for full color development, volume was made up to 10 ml with distilled water to obtain a final concentration of the range 0.1- 0.8 µg/ml and the absorbance was measured at 544 nm. Cr (III) from culture pellet was estimated in similar manner after pre-weighing the pellet, where as 1ml of the culture supernatant was concentrated by heating in muffle furnace proceeded in similar manner.

4.2.6: Recovery of chromium from culture supernatant

Precipitation of chromium from 100ml of the culture supernatant was carried out by converting it to insoluble $Cr(OH)_3$ by raising the pH to 10.5 with the addition of 50% (w/v) NaOH, followed by the addition 1.16 g of MgO and simultaneous heating at 55°C for 2h with stirring, after which the sludge was left to settle at room temperature for 10 h and was then separated by filtration (Katsifas et al., 2004). To determine the chromium that can be recovered from culture pellet, pre-weighed cell pellet was resuspended in 10ml of sterile distilled water and subjected to above-mentioned treatment and estimated by the method of Sankalika et al. (2004).

4.2.7: Chemical Oxygen Demand (COD) by Open Reflux Method and Biological Oxygen Demand (BOD)

The chemical oxygen demand (COD) and biochemical oxygen demand (BOD) during 5 d were determined based on APHA methods (1992) from chromate-containing liquid phase. COD was defined as the concentration of the specified oxidant, which reacted with a sample under controlled conditions as mg/ l of O_2, the quantity of oxidant consumed is expressed in terms of its oxygen equivalence. BOD was estimated and is defined as the measure of the amount of O_2 necessary for the decomposition or stabilization of a given amount of waste and is expressed quantitatively as parts of O_2 per million parts of the material under study.

4.2.8: Estimation of Biosorption efficiency of Chromium by microbial biomass

B. subtilis P13 was inoculated into 100 ml LB in 500 ml conical flasks and incubated on a shaker at 150 rpm for 24 h at 30°C. The cells were grown to late exponential phase, harvested by centrifugation at 12, 000 xg for 15 min at 4 °C and washed three times with deionized water. Cell suspension for the biosorption assay potential of live bacteria was prepared by resuspending the cell pellet in deionized water. Biomass concentrations in cell suspensions were determined by drying an aliquot in a pre-weighed aluminum foil container to constant weight at 80 °C (Puranik and Paknikar, 1999).

Cr (VI) (30mg/l) and Cr (III) (250mg/l) solutions were adjusted to pH 4.0 with 0.1 M sodium hydroxide and 0.1M nitric acid. About 50 mg dry weight of the living cells was added in triplicates to 25 ml chromium solution in 150 ml conical flasks. The flasks were shaken at 150 rpm at 30°C for 1 h. Metal-free and biosorbent-free solutions were prepared as controls (Srinath et al., 2002). The cells were harvested by centrifugation at 12, 000 xg for 10 min at 4 °C and washed twice with sterile distilled water. The biomass was then dried at 80 °C in an oven and weighed and digested with the acid mixture as mentioned in section 4.2.10. The amount of chromium biosorbed was calculated as mg Cr/g dry weight.

4.2.9: Bioaccumulation of Chromium by *B. subtilis* P13

B. subtilis P13 grown to 0.6 OD and 1% of culture suspension was inoculated into 100ml LB containing 30 ppm Cr (VI) and 250 ppm of Cr (III) and incubated at 30°C for 24 h shaking at 150 rpm. After 24 h, bioaccumulated chromium was estimated by harvesting and processing the cells by digesting the samples with a mixture of concentrated nitric (six parts) and perchloric (onepart) (Srinath et al., 2002). The amount of chromium accumulated was calculated as mg Cr/g dry weight. Uuinoculated LB containing the appropriate concentration of Cr (VI) was used as a blank. All the tests were performed in triplicates. To understand the amount Cr(III) accumulated in the system, dry weight of the pellet was noted and subjected to thermal oxidation by heating in a muffle furnace in a silica crucible at 550°C for 6h (Bose et al.,2005) and proceeded as mentioned in section 4.2.10.

4.2.10: Chromate reductase assay

The bacterial culture was grown in 50 mL LB broth for 24 h at 30^0C with and without Cr (VI) (10ppm). Chromate reductase activity was checked from three fractions viz, extracellular, cell bound and intracellular. Extracellular activity was checked from the culture supernatant of culture spun at 12,000xg for 5 min. Pellets recovered were washed twice with 10mM potassium phosphate buffer, pH 7.0 and cell bound activity was checked after resuspending the same in 1mL of 50mM phosphate buffer. To study the intracellular activity, cells were harvested by centrifugation at 12,000 x g for 15 min. Pellets were washed twice with 10 mM potassium phosphate buffer (pH 7.0) and were suspended in 3 mL of 50mM potassium phosphate buffer. Cells were disrupted by sonication for 5 min in cold condition. The resultant homogenate was centrifuged at 12,000 x gfor 30 min at 4^0C; the supernatant was used as a crude extract (Rehman et al. 2008).

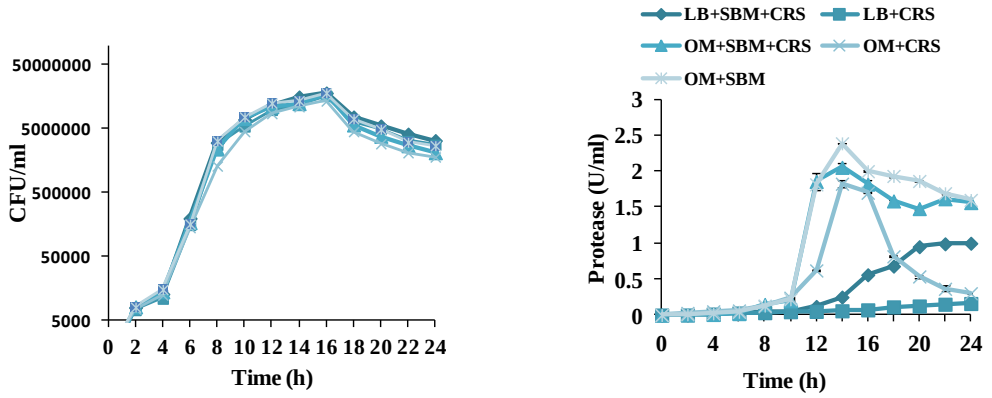
Chromate reductase activity of all the three fractions viz, cell free extract (CFE), cell bound and extracellular fractions were assayed following the procedure of Camargo et al., (2003). The reaction mixture (1.0 ml) contained 0.6 ml of 1mM K_2CrO_4 and 0.3ml of 100mM Phosphate buffer pH 7 .A n aliquot of 0.1 ml of CFE was added as the enzyme to initiate the reaction. Reduction of Cr (VI) was measured by estimating the decrease in Cr (VI) in the reaction mixture after 30 min of incubation at 30^0C. Chromate reductase activity was defined as the amount of enzyme that reduced 1.0µM Cr (VI) per min at 30^0C and the unit is expressed interms of mg protein. Cr (VI) was quantified clorimetrically using 1, 5-diphenylcarbazide (DPC) as the complexing reagent as mentioned in section 4.2.5.

4.3: Results

4.3.1: Growth and protease production by *B. subtilis* P13 in presence of CRS

Growth profile and protease activity of *B. subtilis* P13 were compared in two different media LB and PM using CRS as a protein source and also in combination with SBM (1:1). Considering optimized medium (**OM**) as the control, growth profile appeared to be similar in all the combinations studied (**Fig. 4.1a**). Bacterial count was slightly less in the media containing only CRS compared to other combinations of SBM and CRS. CRS might not be a preferred protein source due to the fact that it is a collagen rich leather waste having chromium Cr (III), compared to SBM which

is a rich source of easily assimilable protein. Although growth was similar in all media, there was a significant difference in the protease activity in the media studied. Maximium activity of 2.4U/ml was observed from production medium with SBM after 24h. Optimized medium (OM) containing SBM and CRS (1:1) and also PM containing 1% CRS showed activity of 2.05 and 1.8 U/ml were obtained after 24h (**Fig. 4.1b**) and was 2.14 and 10 times higher than the respective basal media. Another significant difference observed was that in PM irrespective of the protein sources maximum activity was observed after 18 h. LB being a richer medium might be the reason for prolonged log phase.



a **b**

Fig. 4.1: Growth and protease production by *B. subtilis* P13 in medium containing SBM and CRS as protein source.

a: Growth profile in OM and LB; b: Protease activity profile in OM and LB;

4.3.2: Hydrolysis of chrome shavings by *B. subtilis* P13

About 90% weight reduction was achieved in 24h, whereas degradation efficiency was found to be about 56-60% with 5% CRS in 24h and increased to 90% after 48h. In the case of 10% CRS containing media, degradation was even slower and weight reduction of 18-20% and 30-40% were attained at 24 and 48h respectively (**Fig. 4.2**). The different stges of degradation in **O**M containing 1% CRS is depicted in **Fig. 4.3.a.** Wherever 90% weight reduction was achieved, there was complete liquefaction of the waste and whole medium had taken up bluish tinge of chromium (**Fig. 4.3b**). When same experiment was repeated using UV-sterilized **and non-sterilized CRS, no degradation** was observed. Comparable observations were reported from the studies carried out using *A.carbonicus*, where they reported that double sterilization at 15PSI at 15 min. was essential for the degradation. Oil

immersion micrograph (**Fig. 4.4**) also clearly indicates the disintegration finally resulting in finer particles of the CRS.

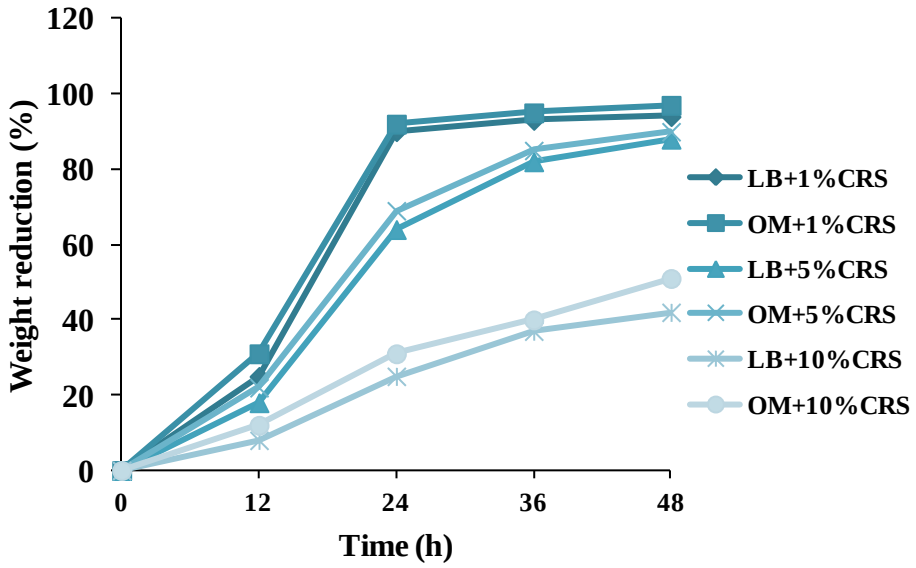
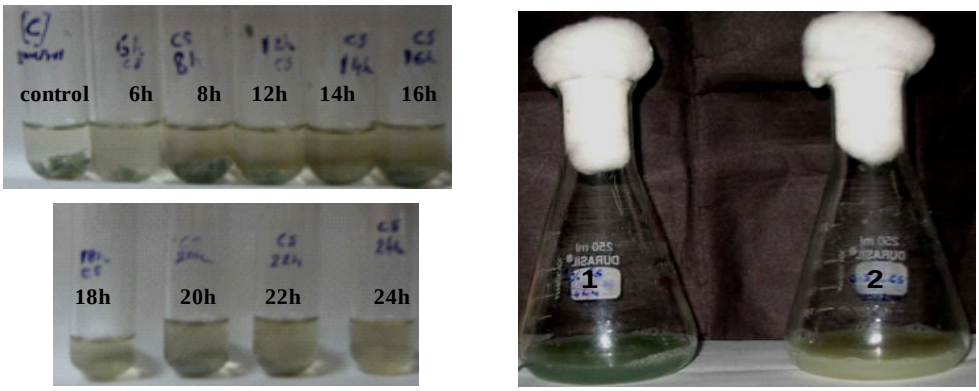


Fig. 4.2: Hydrolysis of chromeshavings by *B. subtilis* P13 with respect to weight reduction



a **b**

Fig. 4.3: Liquefaction of CRS by *B. subtilis* P13.

a. Different sequences in the hydrolysis of CRS by *B. subtilis* P13

b. Complete hydrolysis of 1% CRS leaving a bluish tinge to the supernatant after 24h-Flask1; Flask2- control containing OM +1%CRS, at 0^{th} h.

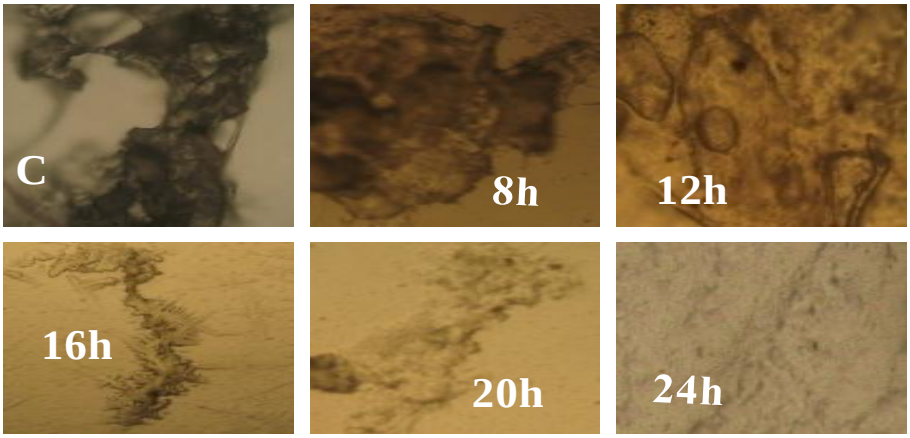
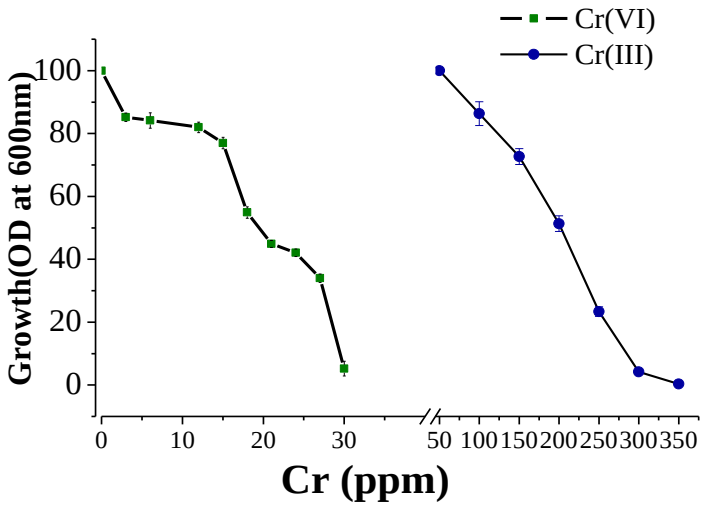


Fig. 4.4: Oil immersion micrograph of chromeshavings during different stages of hydrolysis

4.3.3: Chromium tolerance of *B. subtilis* P13

Since *B. subtilis* P13 could degrade CRS, it was of interest to study its tolerance to Cr. The bacterium could tolerate 35 ppm of Cr (VI) and 350ppm of Cr III (**Fig. 4.5**). No visual morphological changes was observed on the plate after growing in presence of Cr. Growth had reduced drastically after a concentration of 25ppm of Cr (VI) and become negligible at 35ppm concentration. But in the case of Cr (III), the decrement in growth rate was more or less linear and could grow up to 250ppm, decreased to 4% at 300, becomes negligible at 350ppm (**Fig. 4.5**).



4. 5 : Effet of Chromium [Cr(III) and Cr(VI)]on growth (24h) of *B. subtilis* P13

4.3.4: Biosorption of chromium by *B. subtilis* P13

Biosorption efficiency of12 and 24h old live biomass (50mg dry weight) when studied separately for Cr (III) and Cr(VI)showed that the biosorption efficiency was

better with 24 h old culture (**Fig. 4.6 a & b**). Maximum biosorption of 20µg/g was observed in the case of Cr (VI) and 350µg/g for Cr (III) when provided with 30ppm and 250ppm of Cr (VI) and Cr (III) respectively. Srinath et al., 2002 reported two *Bacillus* strains showing biosorption of 10mg/ml, when 100ppm of chromium was provided. Better biosorption efficiency shown by 24h old culture can be attributed to the extrapolymeric substances produced by *B. subtilis* P13

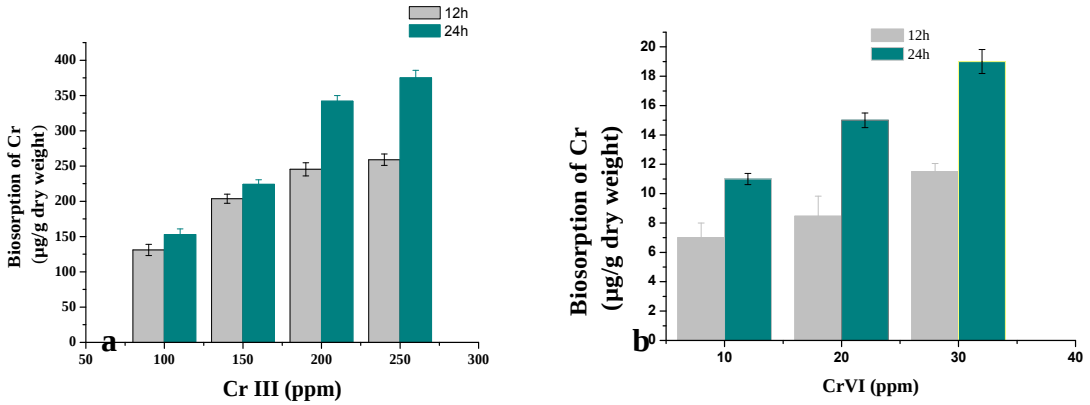


Fig. 4.6: Biosorption of Cr by *B. subtilis* P13

a: Biosorption of Cr(III) and b: Biosorption of Cr(VI)

4. 3. 5: Bioaccumulation of Chromium by *B. subtilis* P13

Bioaccumulation efficiency of *B. subtilis* P13 was evaluated separately using 12 and 24h old cultures and both the cases an increment of 6-10% in bioaccumulation shown by *B. subtilis* P13 after 24h. Residual Cr (VI) concentration in the supernatant was 5±1.09, 8± 2.143 and 19.8±0.962µg/ml in the case of 10, 20 and 30ppm respectively. At higher concentration, residual Cr were more in supernatant and similar observations are already reported (Srinath et al., 2002) In the case of Cr (III) also maximum bioaccumulation were shown after 24h, leaving a residual Cr(III) of 40±0.894; 52±2.18; 76± 1.60 and 99± 0.921 µg/ml respectively when the concentration of 100,150,200 and 250ppm of Cr(III) were studied (**Fig. 4.7a&b)**. Better bioaccumulation efficiency shown with respect to Cr (III) might be the attributed to the less toxicity of Cr (III) compared to Cr (VI). Better accumulation at lower concentration might be due to better growth rate.

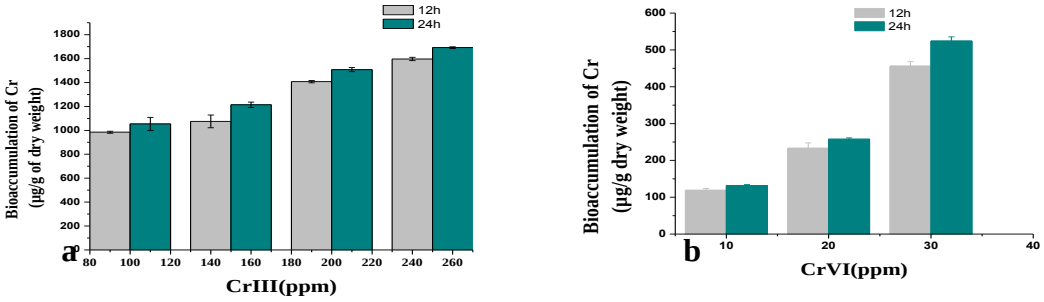


Fig. 4.7: Chromium accumulated by 12 and 24h old cultures of *B. subtilis* P13

4.3.6: Chromium reductase activity of *B. subtilis* P13

Cr (VI) reduction experiments were conducted using cell free extract (CFE), cellbound (CB)and extracellular enzymes of *B. subtilis* P13, to understand the component responsible for biotransformation of Cr(VI).The specific Cr (VI) reduction activity obtained for CFE at 30 °C and a pH of 7.0 was 11.6/mg protein/ min, whereas cell bound and extracellular reductase activity were about 4U and 0.38/ mg of protein respectively (**Fig. 4.8**). The supernatant obtained after harvesting the cells (extracellular enzymes) showed less chromium reductase activity (Cr(VI) reduction/mg protein/min) compared to CFE. This clearly showed that the Cr (VI) reduction was associated with the soluble fraction of the cells and not with extracellular enzymes.

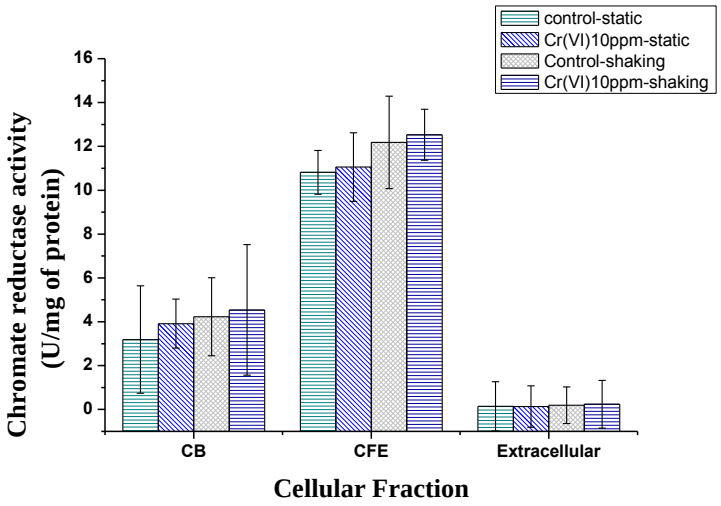


Fig. 4.8: Reduction of Cr (VI) by different cellular fractions of *B. subtilis* P13 under static and shaking conditions in presence of Cr(VI) 10ppm.

CB - cell bound; CFE -cell free extract, control is 24 h old culture and treated is the culture grown in presence of 10ppm of Cr (VI).

Chromium reducing capability of the bacterial isolate was checked by adding Cr (VI) at 10ppm concentration in the culture medium and estimating the amount of residual chromium in the CFE (**Fig. 4.9**). *Bacillus subtilis* P13 could reduce about 35% in 12h, 65% in 24 h, 82% in 36 h and 90% of chromium from the medium after 96 h. Rehman et al., (2008), reported a *Bacillus* sp.ev3 capable to reduce Cr(VI) (100 mg/L) at 91% in 96 h.

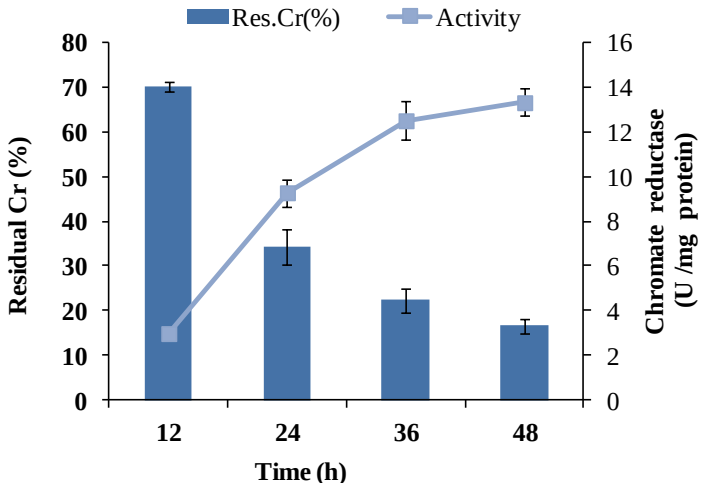


Fig. 4.9: Time course of Cr (VI) reduction in cell free extract of *B. subtilis* P13

4 .3.7: Biochemical characterization of chromate reductase from *B. subtilis* P13

4.3.7.1: Temperature optima of chromate reductase

Maximum chromium reductase activity was observed at 60°C for a pH of7.5 (**Fig. 4.10**). Activity at 50°C was about 50% of that at 60 °C and at 40 and 30 °C the activity was only 15 -20% of the optimum activity. 70°C, enzyme showed only 25% of its optimal activity.

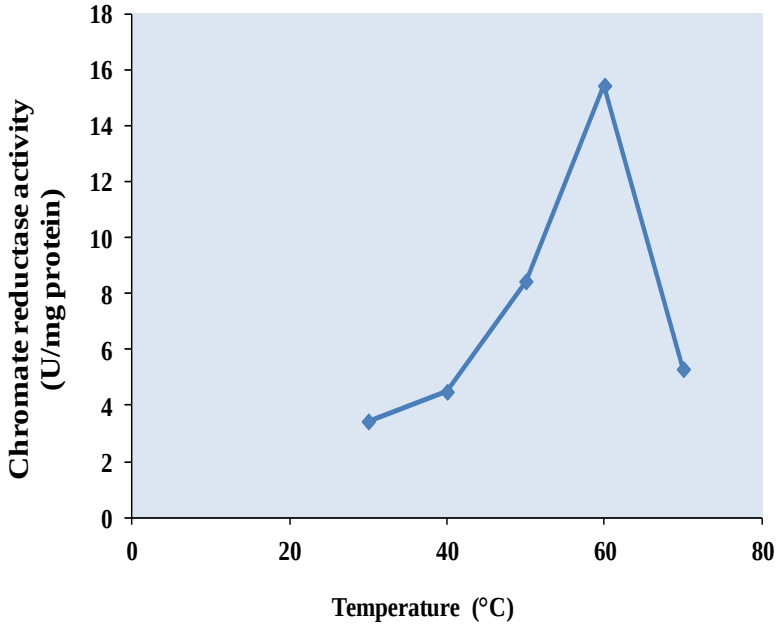


Fig. 4.10: Optimum temperature of chromate reductase from *B. subtilis*.P13

4.3.7 2: Thermostability of chromate reductase

$t_{1/2}$ of chromate reductase was reached after 12h and 24h at 50-55°C and 60-65°C respectively (**Fig. 4.11**). But at temperatures lower than 50°C, enzyme retained more than 50% activity even after 24h. At 70°C, thermal inactivation was quiet fast. From the above observations, it can be concluded that the enzyme under present study is probably a thermostable enzyme. Similar studies with purified enzyme will give more insight to the thermal denaturations kinetics of chromate reductase from *B. subtilis* P13

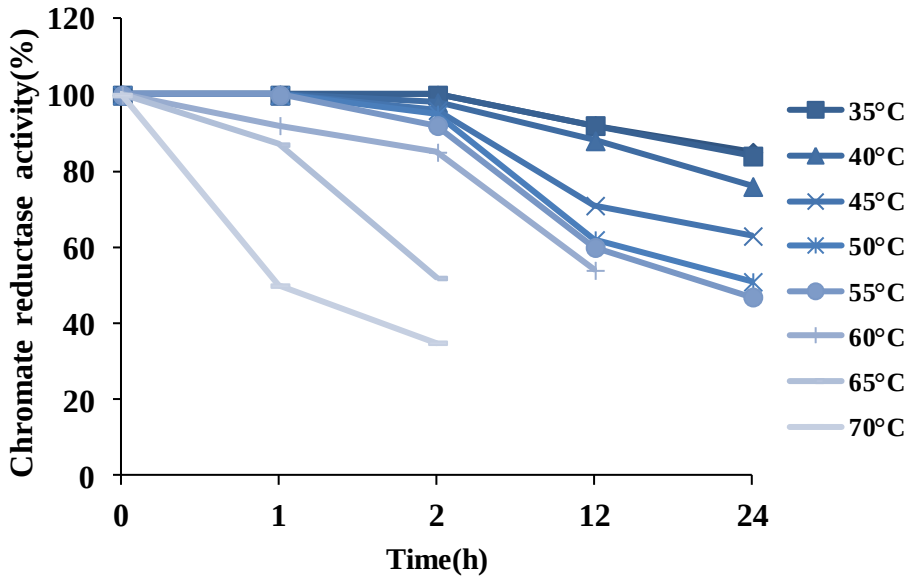


Fig. 4.11: Thermostability of chromate reductase from *B. subtilis* P13

4.3.7.3: pH optimum of chromate reductase

Increase in chromate reductase activity observed from acidic range,decreased drastically at alkaline pH having an optimum at pH 7.5 and about 10% decrease in activity at pH8.0 (**Fig. 4.12**).

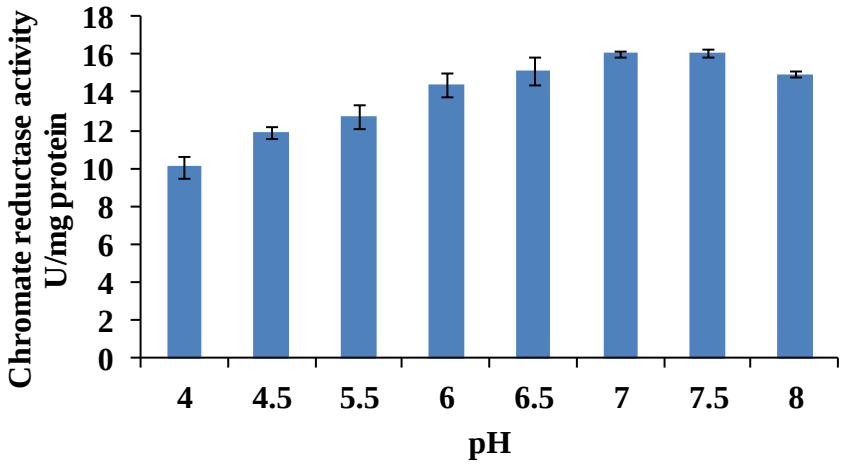


Fig. 4.12: Optimum pH of chromate reductase from *B. subtilis* P13

4.3.8: Biological degradation of CRS

The biological degradation was assessed on the basis of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) and bioaccumulation by precipitating as Cr $(OH)_3$ from the pellet (**Table 4.1**) and supernatant (**Table 4.2**) of the media containing 1, 5 and 10% CRS. The efficiency of hydrolysis varied with respect to the amount CRS in both LB and OM. With 1% CRS complete hydrolysis (reduction in weight) achieved in 24h,5% took 48h and 10% was poorly hydrolysed even after 72h. Reported amount of Cr (III) in chromeshavings varies from 200-300ppm and estimated as 242± 2.1 ppm in the present study. Amount of chromium recovered from 1, 5 and 10 % varied in the range 100 - 1400 and 65 – 350 ppm respectively in the case of supernatant and pellet. Both the parameters increased with increased concentration of CRS. All the parameters studied above were beyond the permissible limits for effluent.

Table4.1: Effluent parameters, protease activity and Cr (III) recovered from the culture pellet of *B. subtilis* P13

Combinations	BOD (ppm)	COD (ppm)	Protease (U/ml)	Cr(III) (µg/gof pellet)
LB+1%CRS	325±2.87	610±3.031	0.12 ±0.0056	65±1.028
PM+1%CRS	356±3.56	520±4.11	0.83±0.0062	80±2.221
LB+5%CRS	419±2.76	1000±5.63	0.19±0.0073	136 ±1.32
PM+5%CRS	429±1.94	890±4.94	0.55±0.0099	158±1.905
LB+10%CRS	478±1.12	1640±3.11	0.22±0.0027	370±2.280
PM+10%CRS	491±2.34	1230±2.807	0.33±0.0079	361±4.10

Table4.2: Chromium recovered, effluent parameters and protease activity from the culture supernatant of *B. subtilis* P13

Combinations	BOD (ppm)	COD (ppm)	Protease (U/ml)	Cr(III) (µg/g of pellet)
LB+1%CRS	130±3.27	450±2.997	0.6 ±0.0054	95±3.028
PM+1%CRS	112±2.56	370±1.904	1.2±0.0062	108±2.561
LB+5%CRS	175±3.06	750±3.70	0.54±0.0043	449 ±1642
PM+5%CRS	150±1.84	520±4.14	1.55±0.0029	503±2.034
LB+10%CRS	221±4.01	950±1.765	0.11±0.0017	940±2.42
PM+10%CRS	256±3.46	825±2.37	0.19±0.0067	965±1.990

4.3.9: Solid substratum fermentation of CRS by *B. subtilis* P13

To develop a process for the microbial hydrolysis of CRS with minimum generation of waste, a solid substratum column with minimal addition of nutrients was set up. **Fig. 4.13** depicts the visual observation of column during different stages of the study. There was a 54% reduction in column height after 7 days (**Fig. 4.13**). The remaining 46% of the column height after 7d was occupied by solubilized viscous remnants of CRS containing bacterial biomass. When this was reused as inoculum for a second refill of the column reactor similar performance was achieved confirming reusability of the biomass. The molecular weight of the protein and zymogram nature (**Fig. 4.15**) confirms that the major protein present in the effluent corresponds to 31 kDa protein which is a dehairing keratinolytic protease. The effluent collected during the entire incubation period was about 10 ml and had protease activity of 4 U/ml (**Fig. 4.14**) and was not only higher than batch cultures on CRS but also 1.5 folds higher than the protease activity from the optimized medium with soya bean meal. A concentration 0.2U of this preparation could perform dehairing on 5x5 cm of hide (**Fig. 4.16**) indicating that one batch of the

small reactor with 3g of CRS produced enough enzyme to carry out dehairing of ten pieces of 100x100 cm of hide.

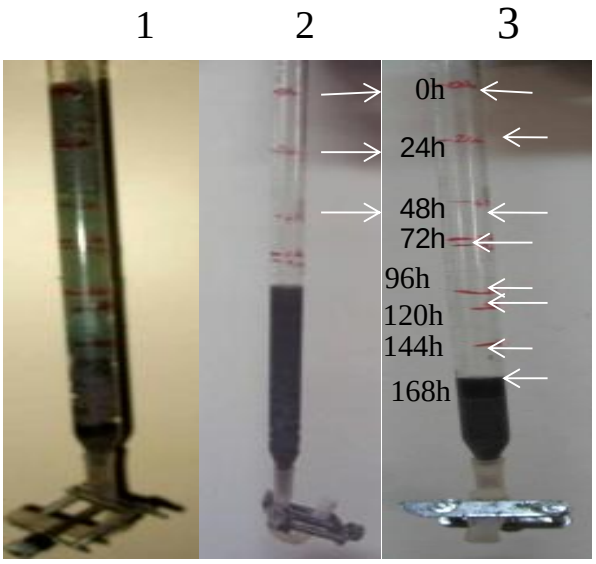


Fig. 4.13: Hydrolysis of chromeshavings by *B. subtilis* P13 from CRS substratum glass column

1,2 and 3 are control at the begining of the experiment and after 122h and 168h respectively**.**

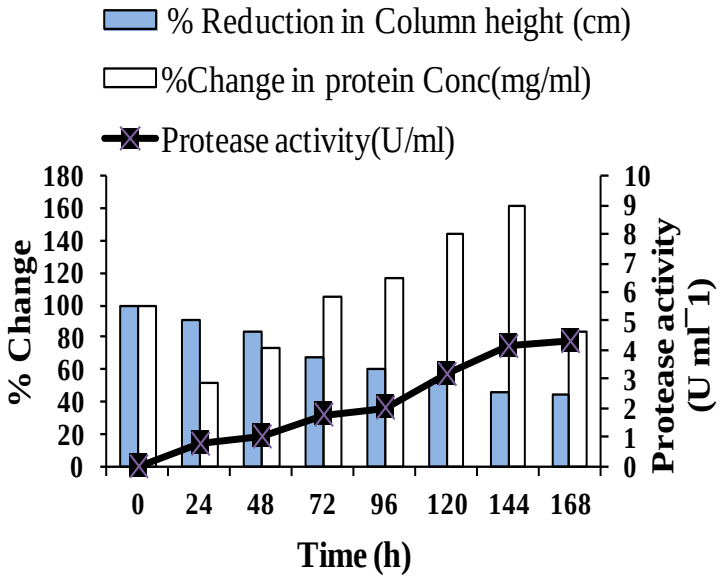


Fig. 4.14: Correlation of CRS degradation meassured as reduction in column height and protease activity from substratum column hydrolysed by *B. subtilis* P13

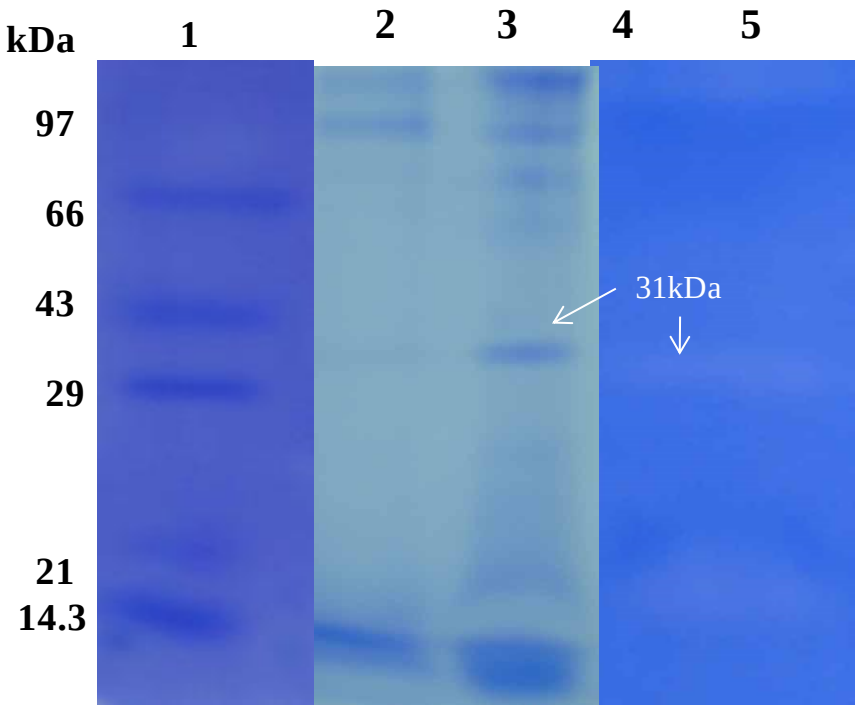


Fig. 4.15: SDS-PAGE and zymogram of the CRS hydrolysate collected from the column

Lane 1:Mw.marker; 2,3: protein profile and 4,5 are gelatin zymogram of effluent collected after 122 and 168h

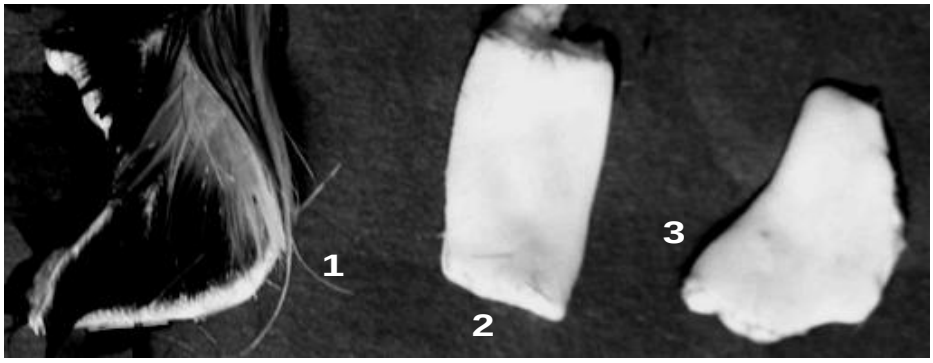


Fig. 4.16: Dehairing of goat hide using the hydrolysate from CRS column.

Effluent from the column was diluted and applied at a protease activity of 0.2U/g hide

4.4:Discussion

Current study revealed that *Bacillus subtilis* P13, a keratinolytic serine protease producer could hydrolyse CRS by virtue of its tolerance to chromium and capability to utilize thermally processed collagen present in the CRS as a protein source. CRS are small particles, in a variety of shapes, mainly consisting of collagen cross-linked with complexes. Upon chrome tanning the collagen matrix of hides becomes highly stabilized due to the formation of metal ion-mediated coordinated cross links in the protein, involving side-chain carboxyl ions of aspartic and glutamic acids of the collagen (Usha and Ramasami 2000). This has been shown to impart collagen with both hydrothermal stability and stability against enzymatic degradation (Gayatri et al., 2001).The lack of growth of indigenous microorganisms in the waste can be attributed to the recalcitrant nature of the waste. Chrome tanned leather as well as its shavings are therefore not prone to bacterial or enzymatic attack. CRS is recalcitrant to proteolytic action without chemical pretreatment to denature the collage fibers (Taylor et al., 1998). In this light, the degradation of CRS by *B. subtilis* P13 is remarkable. Autoclaving of CRS was important in rendering it amenable to attack by the bacillus, since no degradation was observed when UV-sterilized CRS used. Thermal denaturation due to autoclaving might result in the conversion of collagen to gelatin, which is known to be efficiently degraded by *B. subtilis* P13. Comparable observations were reported from the studies carried out using *Aspergillus carbonicus*, where it is reported that double sterilization of CRS at 121 °C, 101 kPa for 15 min was essential for the degradation (Katsifas et al., 2004). Barring studies carried out by the latter group no other reports exist on the use of microorganisms directly for the biodegradation of CRS. *B. subtilis* P13 is much more efficient at CRS degradation than *A. carbonicus*, which is reported to liquefy 1% CRS after 12 d whereas *B. subtilis* P13 achieved similar effect in 24h.

Mechanism of metal tolerance or bioremediation is by biosorption, bioaccumulation (Teitzel and Mathew, 2003; Meriah and Tebo, 2002; Katiyar and Katiyar, 1997) or by transformation of heavy metals, entrapment in extracellular capsules, protein DNA adduct formation, induction of stress, transformation of components by oxidation, reduction, methylation and demethylations and by binding cytosolic molecules (Lovely and Coates, 1997; Gadd, 1990; Ksheminska et al.,

2003). *B. subtilis* P13 exhibited both biosorption and bioaccumulation as mechanisms of Cr tolerance. Srinath et al., 2002 reported two *Bacillus* strains showing biosorption of 10mg/g, when 100 ppm of Cr (VI) was provided. Rehman et al., (2008) reported a *Bacillus* sp.ev.3isolated from metal contaminated waste water which could tolerate 4800 ppm of Cr (VI). Four different species of Bacillus were isolated from Brazil were able to tolerate chromium concentration in the range of 500-2500 mg/l. Apart from *Bacillus.sp*, *Acinetobacter* and *Ochrobactrum* (Francisco et al., 2002), *Arthrobacter* (Megharaj et al., 2003), *Pseudomonas* sp (Rajkumar et al., 2005), *Serratia marcescens* (Campos et al., 2005), *Ochrobactrum* sp (Thacker and Madamwar, 2005),were also reported for its tolerance to chromium.

In the case of *B. subtilis* P13 mechanisms like, bioaccumulation, biosorption and the presence of reductase system have been attributed as the mechanism of chromium tolerance. Studies from different cellular fractions confirmed that chromate reductase in *B. subtilis* P13 present in the intracellular fractions of *B. subtilis* P13. The temperature optimum of 60°C confirms the thermophilic nature of chromate reductase from the under study. Wang et al., (1990) reported that no chromate reduction was observed at 4 and 60 °C by *E. coli.* The optimum pH and temperature for chromate reductase extracted from Bacillus sp. ES 29, *Bacillus sphaericus* 303, *E.coli* ATCC 33456, Actinomycete, and *Arthrobacter crystallopoietes* ES 32 were reported to be between pH 5 to 9 and 30°C, respectively (Camargo et al., 2003; Bae etal., 2005). Chromium recovery studies carried out using *B. subtilis* P13 indicated the better recovery from the supernatant compared to the pellet and similar observation was reported by Katsifas et al 2004; using *A. carbonarius* and could recover 25-28µg/g of chromium. COD was higher in the supernatant and BOD in the pellet in our study and also in the previous report (Katsifas et al.,2004).

Reports on hydrolytic degradation of CRS are focused on the use of enzymes or the combination of alkaline treatment and enzymatic hydrolysis (Kanagaraj et al. 2006). *A. carbonarius* strain reported by Katsifas et al. (2004) to degrade CRS has been envisaged as useful tool in the tanning industries resulting in the degradation of CRS and recovery of valuable Cr. Similarly *B. subtilis* P13 can also be considered useful for solid waste treatment and Cr recovery. The advantages of this organism

are that it is more efficient and during management of CRS it also exhibits protease production which is of use in pre-tanning processes. Thus the CRS management process described here offers a valuable by product which is useful for the upstream tannery processes. This additional potential of the *B. subtilis* P13 pronounce it as a proficient candidate for in-house waste management and saving the cost for the purchase of a valuable consumable commodity needed for the pretanning processes.

The proposed eco-friendly process could be scaled up to tannery application by designing the projected continuous reactor (**Fig. 4.17**) where chrome shavings (thermally denatured at 100°C) could be added as feed at 10% in PM in a vessel, followed inoculation with 2% (w/v) of 0.6 OD *B. subtilis* P13. Liquid oozing out (effluent) will have highly concentrated keratinolytic protease enzyme, which could be applied in pre-tanning processes such as soaking, dehairing and bating. Bimass generated can be reused in a continuous manner as the seed for hydrolysis and this process could be a better way of in-house waste management and byproduct recovery.

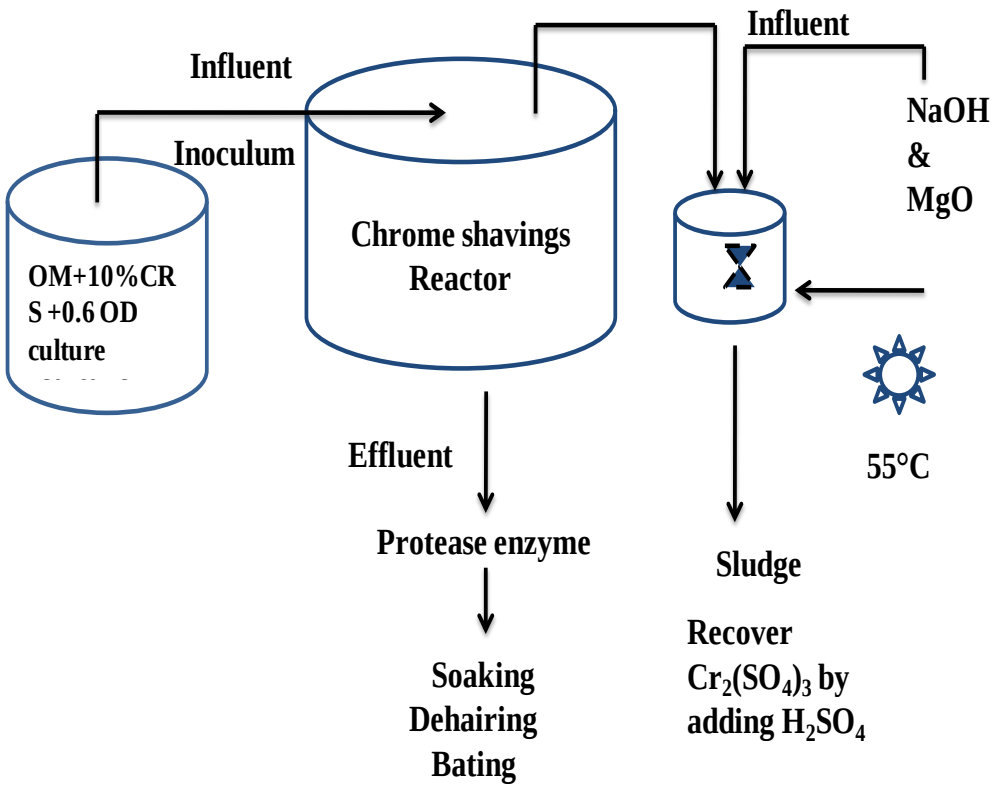


Fig. 4.17: Schematic representation of process for the byproduct recovery and inhouse waste management

4.6: Conclusions

B. subtilis P13, a hot spring isolate, was able to effectively degrade and grow using chrome shavings as the protein source and the spent medium showed high production of keratinolytic serine protease which is efficient in dehairing of hides. The bacterium was moderately chromium resistant tolerating up to 35 ppm and 350 ppm of Cr (VI) and Cr (III), respectively and showed bioaccumulation and bio-sorption of Cr (III) and Cr (VI). Growth profile and enzyme production were comparable in basal and production media containing chrome shavings. An efficient waste management process was developed using solid substratum column reactor leading to the liquefaction of the proteinaceous waste, recovery of the dehairing protease as concentrated product. A continuous reactor scheme is proposed, where the biomass can be reused as the seed for hydrolysis for in-house waste management and byproduct recovery for tannery industry. Apart from the capacity to produce a protease which finds application in pre-tanning processes, this additional potential of the *B. subtilis* P13 pronounces it as a potential candidate for in-house waste management and the recovery of a valuable by-product, protease. Findings from the present study can be developed as a sustainable, eco-friendly and cost-effective process in tanneries.

Chapter 5

Purification, kinetic characterization and thermostability studies of keratinolytic protease from *Bacillus subtilis* P13

The beginning of knowledge is the discovery of something we do not understand.
- Frank Herbert

5.1: Introduction

Tannery is one of the industries which find multiple applications of proteases in pre-tanning processes depending mainly on the substrate specificity, thermostability, pH stability of the enzyme. In tanning industry, alkaline proteases have been shown to be useful in application to various aspects of beam house processes due to the environmental problems (Thanilaivelan et al., 2004) Therefore, proteases from different sources have been characterized and evaluated for their potential application (Nilegaonkar et al., 2007; Macedo et.al., 2005). Several alkaline proteases have been purified and characterized from different strains of *B. pumilus*, *B. subtilis* and other *Bacillus* species and show variation in their catalytic properties (Takahashi et al., 2003; Yasuda et al., 1999) indicating potential application in various industries (Aoyama et al., 2000; Miyaji et al., 2006), although their amino acid sequences share great similarity (Jaouadi et al., 2008). One of considerations for leather tanning is the restricted substrate specificity of the protease (Macedo et al., 2005).

Thermostable enzymes offer robust catalyst alternatives, able to withstand the often relatively harsh conditions of industrial processing. An enzyme or protein is called thermostable when it possesses a high defined unfolding (transition) temperature (Tm), or a long half-life at a selected high temperature which should be a temperature above the thermophile boundary for growth (>55°C). Most, but not all proteins from thermophiles are thermostable. Extracellular enzymes generally show high innate thermostability, as they are not stabilised by cell-specific factors like compatible solutes (Santos and da Costa, 2002). In addition, a few thermostable enzymes have also been identified from organisms growing at lower temperatures, for example *B. licheniformis* amylase. Fundamental reasons to choose thermostable enzymes in bioprocessing is possibility for prolonged storage at room temperature, increased tolerance to organic solvents which reduced risk of contamination, as well as low activity losses during processing when stored below the Tm of the enzyme and even at the elevated temperatures, often used in raw material pre-treatments (Kristjansson, 1989). Discovery and use of thermostable enzymes in combination with recombinant production and development using site-directed and enzyme

evolution technologies, have erased the limited access and substrate specificity for use in industrial biocatalysis.

Most thermally adapted proteins share common structural features that increase thermal stability. These include a decrease in loop length and a concomitant increase in secondary structure, a decrease in labile residues such as cysteines, asparagines and glutamines, an increase in aromatic stacking, increased hydrophobic interactions, increased metal-binding capacity, and increased oligomerization (Yano and Poulos, 2003). It is often speculated that thermozymes should have higher maximal catalytic rates (Zeikus et al., 1996) since classic reaction rate theory states that the rate of a chemical reaction increases with temperature. These higher maximal catalytic rates, however, are not found experimentally. Despite their activity at high temperatures, thermophilic enzymes catalyze reactions at these temperatures with Km and Vmax values similar to those of their mesophilic counterparts at their respective optimal temperature (Cowan et al., 1987). As

expected, thermozyme activity is driven by variation in temperature-dependent substrate kinetic energy alone. If the enzyme structure were to change significantly with different temperatures, one would expect to find nonlinear Arrhenius plots, which is not the case,but depends on the dissociation and conformational changes of the enzyme (More et al., 1995). However, thermozymes do have a broader temperature range in which they are active (Peek et al., 1992).

An important disadvantage of enzymatic processes at higher temperatures is the loss of selectivity and formation of by-products. Furthermore, cofactors required, substrate (Wilkinson et al., 1998), or products might be unstable, and side reactions, such as Maillard reactions in sugar-enzyme mixtures, may occur. Some processes will have higher costs owing to additional heating, and other processes are less likely to need cooling (Peek et al., 1992). It depends on the overall process and its implementation whether operation at higher temperatures is beneficial or not. Often applications with thermozymes are found when the enzymatic process is compatible with existing (high-temperature) process conditions.

In the previous Chapters it was shown that the keratinolytic serine protease from *Bacillus subtilis* P13, a hotspring isolate, is a thermostable subtilisin-like protease which finds application in pre-tanning processes such as soaking and dehairing of hides and also in the management of chrome shavings, a solid waste generated

during chrome tanning. Understanding the enzymatic properties such as substrate specificity, kinetic parameters and thermostability of the protease from this organism necessitates its purification. Knowledge about these properties would be helpful in defining optimum conditions for its industrial application. The current Chapter deals with the purification of the protease by affinity chromatography and characterization of the homogenous preparation with respect to catalytic properties, substrate specificity and thermal denaturation kinetics of the enzyme.

5.2: Materials and Methods

5.2.1: Protease purification

Protease production was carried out in the optimized medium as described in Chapter 3. The culture supernatant collected (120000x*g*, 4°C, 20 min) after 24h of growth was considered as crude enzyme. The crude enzyme sample was first fractionated by a two step ammonium sulphate precipitation at 0-40% and 40-70% saturation. The 40-70% pellet was dissolved in 50 mM potassium phosphate buffer, pH 7.2, dialyzed against the same and applied to a column with 4 ml of casein agarose (ICN, Meckenheim, Germany) equilibrated with potassium phosphate buffer (50mM), pH 7.2. After allowing the binding of the enzyme to the column matrix, it was washed by passing 20 column volumes of 5 mM potassium phosphate buffer, pH 7.2. Elution of the bound protein was performed with NaCl gradient from 0.1 to 0.5 M (100 ml) at a flow rate of 0.25 ml/min.All the purification processes were carried out at 10°C in a cold room.Protein concentrations were measured spectrophotometrically at 280 nm as well as with Bradford dye reagent using bovine serum albumin as standard (Bockle et al., 1995; Bradford, 1976).

The protease activity assay was performed with casein as described in Section 2.2.4 and keratinase activity was measured as mentioned in Section 2.2.5. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μ mol of tyrosine per min under assay conditions. Molecular mass of the purified enzyme, SDS-PAGE was performed on 12% polyacrylamide gel according to the method of Laemmli, along with molecular weight markers (Banglore Genie TM, India; PMWH range: 14.9-98 kDa) and the gel was stained using silver salts (Sambrook and Russel, 2001). Gelatin based zymogram of purified protease was carried out as mentioned in Section.2.2.13.

5.2.2: Enzyme assay with synthetic substrates

Estimation of esterase activity was carried out spectrophotometrically at 405nm by *p*-nitrophenyl acetate (*p*-NPA) hydrolysis method (Oceguera-Cervantes et al., 2007). The reaction was carried out at 50°C for 15 min in 1ml final volume containing 250µl of reaction buffer (100mm of phosphate, pH 7.5), 100µl of enzyme extract, 400µl of distilled water and 250µl of 20mM solution of p-NPA in acetonitrile. Substrate and enzyme blanks were provided to measure the chemical hydrolysis and the reading from the blanks was subtracted from the experimental samples with enzyme preparations. A standard curve was constructed by incubating different concentration of the p-NP under conditions similar to those used for enzymatic assay by using 100µl of 1M NaOH.

Four synthetic para nitroanilide (*p*NA) linked peptide substrates from Sigma-Aldrich (St. Louis, MO, USA) were used here to probe the substrate specificity. They are N-Succinyl-Ala-Ala-Pro-Phe-*p*NA (AAPF-pN), N-Succinyl-L-Phe-*p*NA (LF-*p*N), N-Succinyl-Ala-Ala-Val-Ala-*p*NA (AAVA-pN), and N-Succinyl-Ala-Ala-Ala-*p*NA (AAA-*p*N).These peptides were dissolved in dimethylsulfoxide as a stock of 0.1 M and stored at −20 °C. Assay was performed as described by Izotova et al. (1983). The standard assay mixture contained 1.25 ml of 50 mM Tris-Cl buffer (pH 7.2), 12.5µl. of the substrate from the above mentioned stock solution 50ul of appropriately diluted enzyme solution. After 15 min of incubation at 50°C, the reaction was stopped with 0.25ml of 2 M sodium citrate buffer (pH 5.0), and the released p-nitroaniline was assayed spectrophotometrically (UV1430 -Shimadzu, Japan) at 410 nm. Standard graph was prepared for pNA. One unit of enzyme activity was defined as the amount of enzyme liberating 1 µ mol of *p*-nitroaniline per minute.

5.2.3: Effect of inhibitors, metal ions and detergents on protease activity

Effect of inhibitors and detergents was quantified as mentioned in Chaper 2, Section 2.2.9 using the caseinolytic activity at 50°C using Tris-Cl pH 7.2. The relative activity was calculated with respect to the control without treatment. The effect of metal ions (Ca^{2+} , Ba^{2+}, Mg^{2+} , Zn^{2+} and Hg^{2+}) on purified protease activity was investigated using 5mM working concentration of different metal ions solution. The enzyme solution was mixed with different metal solutions and pre-incubated for 30 min at RT, after which caseinolytic assay was performed.

5.2.4: Determination of optimum temperature, activation energy, temperature quotient (Q_{10}) and thermal inactivation of purified protease

Optimum temperature and activation energy (*E*a) were determined by incubating appropriate amount of the enzyme with 1% casein at various temperatures ranging from 20° to 70°C in 50mM potassium phosphate .buffer (pH 7.0). *E*a was calculated from the slope of the Arrhenius plot (Siddiqhi et al., 1996) of 1000/*T* versus ln [protease activity] (*Ea*= -slope×R), where R (Gas constant) = 8.314 J/K/mol. The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (*Q*10), which is the factor by which the rate increases due to a rise in the temperature by 10°C. *Q*10 was calculated by rearranging the equation given by Dixon and Webb (1979): Q_{10}=antilog$_{\varepsilon}$ (Ex10/RT^2**),** where, *E* = *E*a = activation energy.

Thermal inactivation kinetics of the purified protease was determined by first order expression:

$$dE/_d t = -K_d E \quad (1)$$

So that

$$\ln [E_t/E_0] = -K_d t \quad (2)$$

The K_d (deactivation rate constant or first order rate constant) values were calculated from slopes obtained by a plot of ln[E_t/E_0] or ln[residual activity] versus *t* (time) at a particular temperature and apparent half-lives were estimated using equation (3). The half-life is defined as the time where the residual activity reaches 50%.

$$t_{1/2} = \ln 2/K_d \quad (3)$$

In order to obtain energies of protease deactivation, absolute rates of reaction theory were used (Eyring and Stern, 1939) where the rate of any reaction at a given temperature depends only on the concentration of an energy rich activated complex.

Energy of deactivation was calculated from the slope of a linear plot of ln[*K*d] versus 1/*T* using the Arrhenius equation (5) (Laidler and Peterman, 1979)

$$K_d = Ae^{(-E/RT)} \quad (4)$$

So that

$$[K_d] = -E/RT + \ln A \quad (5)$$

Thermodynamics of irreversible inactivation of the protease was determined by rearranging the Eyring's absolute rate equation (6) derived from the transition state theory.

$$K_d = (k_b T/h) \times e^{(-\Delta H^*/RT)} \times e^{(\Delta S^*/R)} \quad (6)$$

where,

k_b Boltzmann's constant (R/N)=1.38×10^{-23} J K^{-1}

T Absolute temperature (K)

h Planck's constant=6.626×10^{-34} Js

N Avogadro's number=6.02×10^{23} mol^{-1}

R Gas constant=8.314 J K^{-1} mol^{-1}

ΔH^* Change in enthalpy

ΔS^* Change in entropy

Eyring's equation was rearranged to give

$$\ln[K_d/T] = -(\Delta H^*/R)(1/T) + (\ln(k_b/h) + \Delta S^*/R) \quad (7)$$

ΔH^* and ΔS^* values were calculated from the slope and intercept of a ln[Kd/T] versus 1/T plot, respectively.

So that,

$$\Delta H^* = -(\text{slope})\, R \quad (8)$$

$$\Delta S^* = R\,[\text{intercept} - (k_b/h)] \quad (9)$$

Free energy change (ΔG^*) for inactivation of protease were calculated by applying the following equations

$$\Delta G^* \text{ (Gibb's free energy change)} = -RT \ln(K_d h/k_b \times T) \quad (10)$$

5.2.5: Determination of kinetic constants and thermodynamic parameters of substrate hydrolysis

Kinetic constants (*V*max, *K*m, *k*cat and *k*cat/*K*m) were determined using Lineweaver–Burk double reciprocal plot (1/[S] versus 1/v, (Whitaker, 1994) by assaying protease activity at a fixed enzyme concentration with varying concentrations of substrates: casein (0.015- 0.2 mg/ml), keratin (0.04-0.4mg/ml) and synthetic substrates like *p*-NPA (0.5-5mM), AAPF-pN (0.03-0.13mM) and AAVA-pN (0.04-0.15mM), in 50mM potassium phosphate buffer pH 7.0 at 50°C for 10 min. Catalytic efficiency i.e. the ratio Vmax/Km, and kinetic constants (kcat, kcat/Km)

were determined. For determination of kcat and kcat/Km the value of Vmax was expressed in terms of μmole/ml/min. To calculate kcat, Vmax (μ mole/ml/min) of protease to specific substrate was divided by the μmoles/ml concentration of the enzyme used and kcat was expressed in seconds.

Thermodynamic parameters for substrate hydrolysis are the Gibb's free energy of activation, free energy for substrate binding. These parameters depend on the velocity of reaction at different temperature.

Gibb's free energy of activation (ΔG^*) was calculated from the following equation:

$$\Delta G^* = -RT \ln (k_{cat}h/k_b \times T) \quad (11)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (12)$$

The free energy of substrate binding and transition state formation was calculated using the following derivations:

$$\Delta G^*_{E\text{-}S} \text{ (free energy of substrate binding)} = -RT \ln K_a \quad (13)$$

Where $K_a = 1/K_m$

$$\Delta G^*_{E\text{-}T} \text{ (free energy for transition state formation)} = -RT \ln (k_{cat}/K_m) \quad (14)$$

$$\Delta H^* = E_a - RT \quad (15)$$

Where activation energy *Ea* of enzyme for substrate hydrolysis (at 50°C) determined using

Arrhenius model is described in Section 2.5.

$$Q_{10} = \left[\text{antilog}_e (E \times 10/ RT^2)\right]$$

Where, E=Ea= activation energy

5.2.6: Optimum pH of protease

The effect of pH on protease activity was determined by measuring activity at 50°C for 30 min, using following different buffers: 50mM Na-acetate buffer (pH 4.0 and 5.0), 50mM phosphate buffer (pH 6.0 and 7.0) and 50mM glycine-NaOH buffer (pH 8.0, 9.0 and 10.0). Casein prepared in respective buffers was used for the study

5.2.7: Thermostability studies of protease

Thermal inactivation of protease was determined by incubating purified protease at 30, 40, 50 and 60 °C. Aliquots were withdrawn at 15 min intervals, cooled on ice for 10 min and assayed for caseinolytic activity. Thermostability studies of protease in presence of $(NH_4)_2SO_4$, $CaCl_2$, $BaCl_2$ and NaCl were carried out with each salt individually at a working concentration of 5mM. The enzyme was incubated each salt with at 4°C for 10 min, unbound salts were removed by dialysis for 1 h followed by thermostability studies at 30, 40, 50 and 60° C. The relative activities at different temperatures were plotted with respect to the maximum activity at a particular temperature considered as 100%. To study the effect of pH on the stability of the enzyme at different temperatures, thermostability studies were performed at pH range from 4.0 to 9.0, after adjusting the and the pH of the enzyme solutions using acetic acid or NaOH (1M concentration)

5.2.8: Determination of pI

Isoelectric focussing (IEF) was carried out to understand the isoelectric point (pI) of the purified protease using 7 cm, broad range (3-10) IPG strips (Bio-Rad laboratories, Inc.USA). IPG strip was rehydrated by resuspending it in 125µl of rehydration buffer provided by the supplier containing 8M urea, 2% CHAPS and 50mM dithiothreitol (DTT) and pure lyophilisied protein sample to a final concentration of 6µg of protein. Electro focussing was carried on Bio-Rad Protean, IEF cell. Focussing conditions used were as mentioned in **Table 5.1.**

Table 5.1: Conditions used for Isoelectric focussing

Step	Start voltage (v)	End voltage (v)	Set Time	Ramp	Temperatue (°C)
1	0V	250V	15min	rapid	20
2	250V	4,000V	1hr	slow	20
3	4,000	4,000	10-20,000V-hr (4hr)	rapid	20

Two-dimensional (2-D) electrophoresis was performed after electro-focussing the purified protein mixed with 2.5 µl of 2-D protein marker on 12% poly acrylamide gel. The pI of the protein was calculated based on the mobility of the protein, with respect to the marker bands.

5.2.9: LC–MS analysis

The enzyme was subjected to LC–MS peptide sequence analysis at The Centre for Genomic Applications, New Delhi (TCGA). In-gel tryptic digestion was performed and the digested mixture of peptides was subjected to peptide sequencing. The data was analyzed using MASCOT search engine.

Goat hide depilation, hydrolysis of milled chicken feather and hydrolysis of thermally denatured CS were carried out using 50nM of purified enzyme as described in Sections.2.2.10, 2.2.11 and 4.2.3 respectively.

5.3: Results

5.3.1: Purification of protease from *B. subtilis* P13

Keratinolytic serine protease produced by *B. subtilis* P13 was purified to apparent homogenity by two step protocol, ammonium sulphate precipitation followed by casein affinity chromatography. Ammonium sulphate precipitation was carried out in two steps, 0-40% and 40-70% (**Table 5.2**) saturation from 50ml of crude supernatant having a total activity and protein concentration of 115 U and 21 mg respectively. In casein affinity chromatography maximum specific activity observed in the fractions eluted with the NaCl concentration of 350mM (**Fig. 5.1**) The overall purification was achieved up to 71.7fold with 22.17% recovery (**Table 5.3**). Purification profile analysed on SDS PAGE is depicted in **Fig. 5.2.**

Table 5.2: Partial purification and concentration of protease from *B. subtilis* P13 by salting out using $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ Saturations	Total activity (Supernatant)	Total activity (Pellet)	Total protein (Supernatant)	Total protein (Pellet)	% Recovery
0 - 40%	125	2.5	80.5	8.5	2.17
40- 70%	0.581	84	53	2.348	73

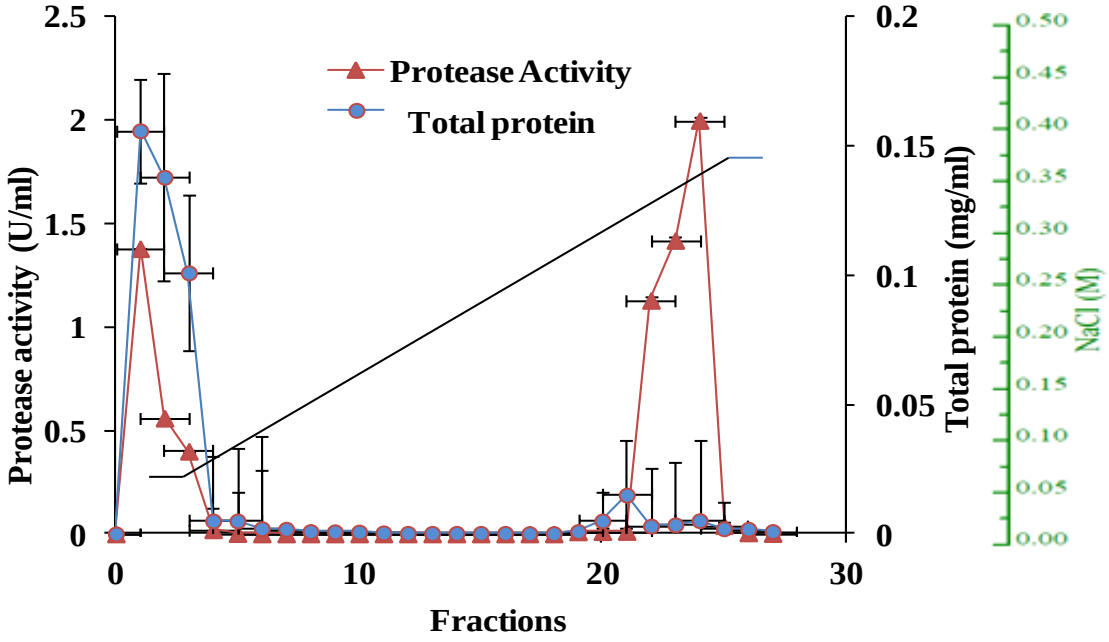


Fig. 5.1: Casein affinity chromatography of protease from *B. subtilis* P13.

Table 5.3: Summary of purification of extracellular protease from *B. subtilis* P13. Values are expressed as Mean ± Standard Deviation from six independent batches of purification

Treatment	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Fold Purification	% Recovery
Crude	115±5.12	21± 1.13	5.47±1.91	-	100
$(NH_4)_2SO_4$ precipitation (40-70% saturation)	84± 2.567	2.348±0.54	35.78±1.20	6.5±1.02	73
Affinity chromatography	25.5±1.86	0.065±0.0005	392±2.21	71.7±0.98	22.17

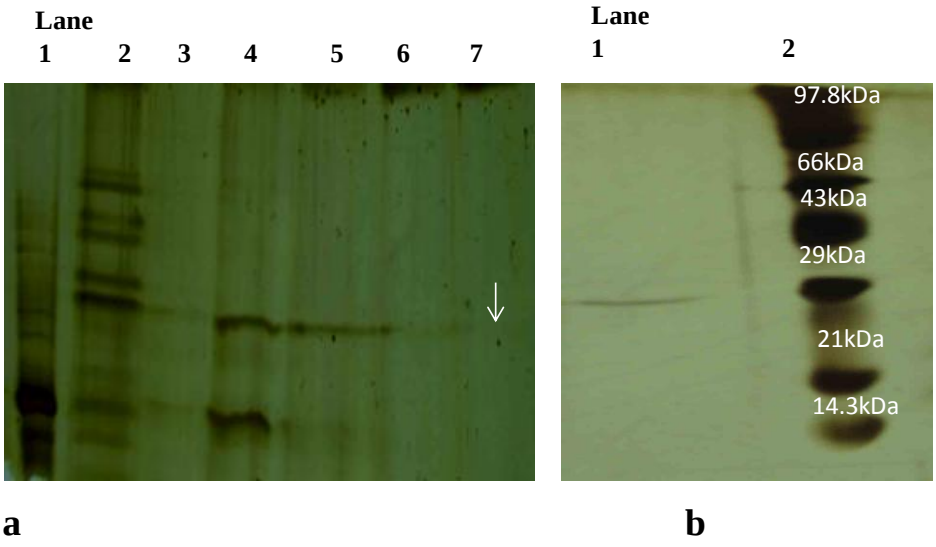


Fig. 5.2: Purification profile of protease from *B. subtilis* P13 on 12% SDS-PAGE and silver staining.

a: Different fractions from casein affinity column. Lane.1: crude enzyme; lane2: $(NH_4)_2SO_4$ ppt (40- 70%) saturation; Lane 3, 4, 5, 6 &7 are F1, F2, F23, F24 &F25 respectively. b: Lane.2: Molecular Weight marker (high range) and lane.1: Pooled, dialysed fractions (F24 and 25)

Protease activity of the purified protease was confirmed by gelatine based zymography (**Fig. 5.3**). After the final purification step, the purified enzyme had a specific activity of 392 units /mg protein, indicated that enzyme was purified by 71.7 fold with approximately 22.17% of recovery

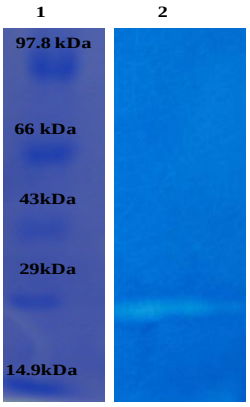


Fig. 5.3: Gelatin zymogram of pooled and dialysed fractions: F24 and 25

The molecular weight of the SDS PAGE band of purified protein was calculated as 27.7kDa (**Fig. 5.4**).

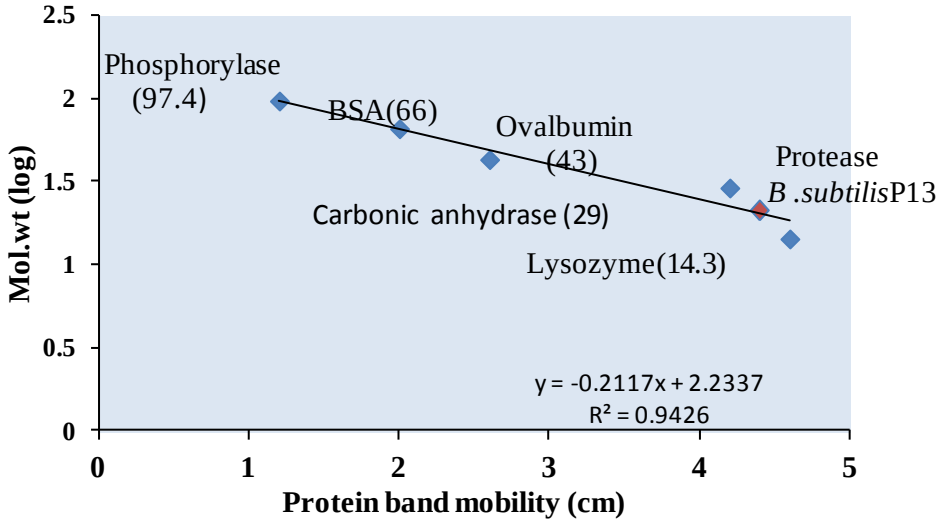


Fig. 5.4: Mobility of the purified protease band from *B. subtilis* P13 on 12% SDS-PAGE .Numbers in parenthesis are Mw in kDa.

5.3.2: Effect of pH and temperature on purified protease from *B. subtilis* P13

The purified protease exhibited highest activity at pH 7.0, with above 80% activity in the range of 6 to 9 indicating that the neutral protease from *B. subtilis* P13 was active over a broad pH range. Even at acidic and alkaline pH of 5 and 12 respectively, 37.7± 5.6 and 38± 2.08% activity was retained (**Fig. 5.5**).

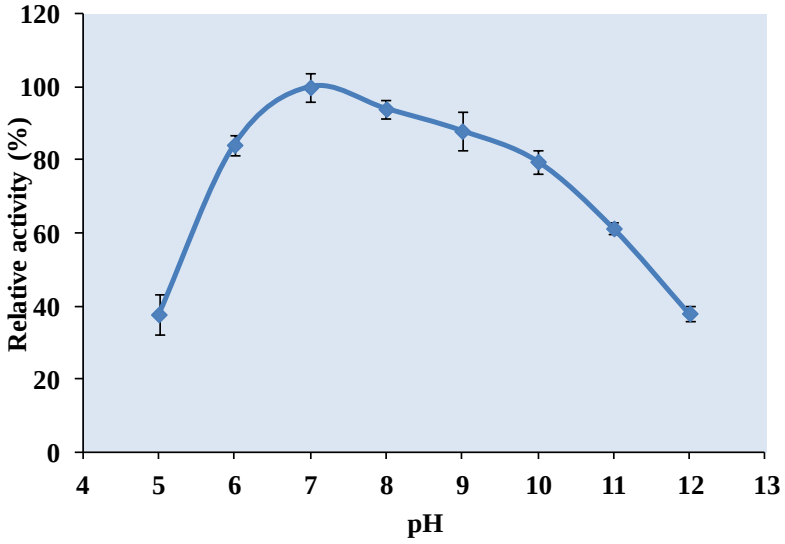


Fig. 5.5: Effect pH on purified protease activity

Optimum temperature for purified enzyme activity was recorded as 60°C (100 ± 0.67%) with relative activities of 74.60 ± 2.32% and 53.3± 2.06% at 50° and 70°C,

respectively (**Fig. 5.6**). The fall in activity was steep at higher temperatures than lower, suggestive of a broad range of temperature for activity in the range of 40 – 65°C of the purified enzyme. It should be noted that in previous Chapters, routine enzyme analysis was carried at a sub-optimum temperature of 50°C and application studies were carried out at 30- 37°C, where the enzyme was active at its 40% efficiency.

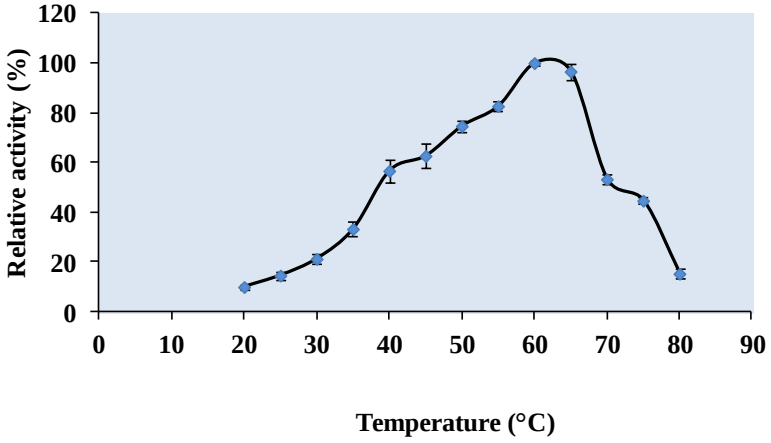


Fig. 5.6: Effect of temperature on purified protease activity

The activation energy (E_a) for the hydrolysis of casein by purified protease was 14.06 KJ/mol as calculated from Arrhenius plot (**Fig. 5.7).** The effect of temperature on rate of reaction was measured in terms of temperature quotient (Q_{10}), which for casein hydrolysis was 1.006 -1.008 between temperatures 30-60°C.

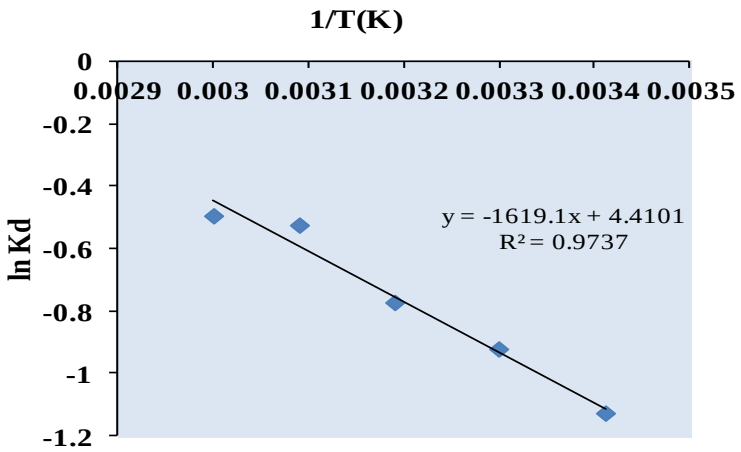


Fig. 5.7: Arrhenius plot to calculate activation energy 'Ea (d)' for irreversible thermal deactivation/denaturation of keratinolytic protease from *B. subtilis* P13.

Relationship used for the calculation: $[K_d] = -$ Ea/RT, where R (gas constant) =8.314kJ/mol

5.3.3: Effect of inhibitors, detergents and reducing agents on purified protease

Protease activity determined using casein as substrate was almost completely inhibited by serine protease inhibitor, PMSF (0.1 mM) indicating presence of the serine group in the active sites. However, 96.17% of the caseinolytic activity was retained on treatment with EDTA (5mM). Nonionic detergent Triton X100 enhanced the activity by 10% while presence of 0.5% SDS had a negative effect, by loss in 82% of the total activity. Of the total activity 94% was found to be retained in the presence of 0.5% βME, confirming that this reducing agent has minimal effect on enzyme activity (**Table 5.4**). Similar observations were reported for DHAP, a dehairing alkaline protease from *B. pumilus* (Qing et al., 2003).

Table 5.4: Effect of inhibitors and detergents on the activity of purified protease from *B. subtilis* p13

Additive	Concentration	Caseinolytic activity (U/ml)	% Residual activity
None	-	5.49 ± 0.26	100
PMSF	0.1mM	0.001 ± 0.0005	0.02
EDTA	5mM	5.28 ± 0.21	96.17
SDS	0.1%	1.090 ± 0.06	19.9
	0.5%	0.99 ± 0.044	18.1
β-Mercaptoethanol	0.5%	5.15 ± 0.24	94
Triton X-100	0.5%	5.95 ± 0.28	108

5.3.4: Effect of metal ions on protease activity

The effect of different divalent metal ions on the protease when checked using 5mM of Ca^{2+}, Ba^{2+}, Mg^{2+}, 1mM of Zn^{2+} and 0.5mM of Hg^{2+}(all chloride salts), showed only Ca^{2+} to bring about approximately 10% increase in protease activity as compared to control. Ba^{2+}, Mg^{2+} and Hg^{2+} ions showed 50-80% activity, whereas Zn^{2+} ions showed significant inhibitory effect on the protease activity, retaining approximately only 10% activity (**Fig. 5.8**). Qing et al (2003) reported 19 and 15% enhancement in activity DHAP activity in presence of Ca and Mg salts, respectively and 20% decrement in presence of zinc salts.

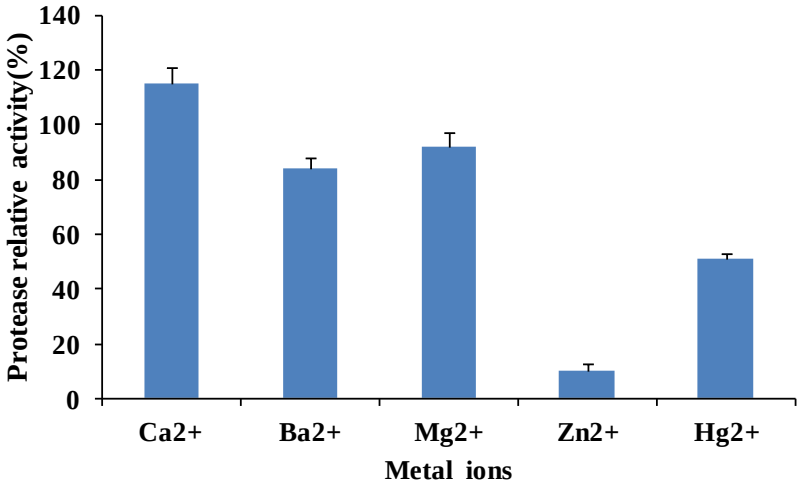
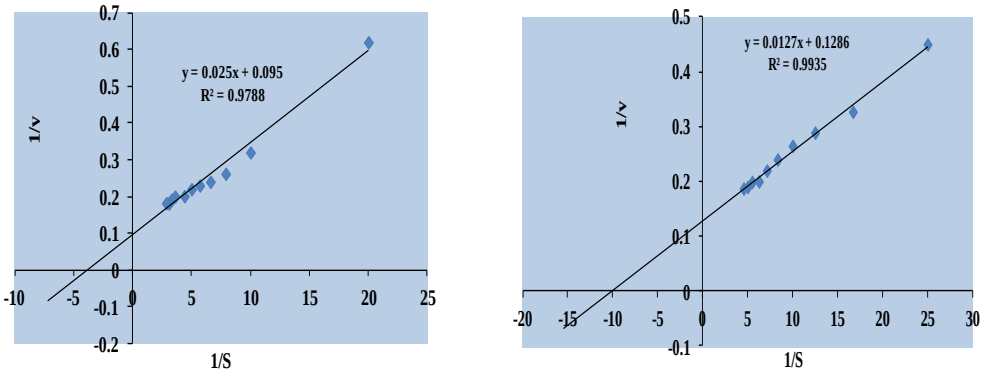


Fig. 5.8: Effect of metal ions on purified protease from *B. subtilis*. P13

$Ca^{2+,}$ Ba^{2+} and Mg^{2+} are used at 5mM concentration; Zn^{2+} 1mM and Hg $^{2+}$ 0.5mM

5.3.5: Kinetics of hydrolysis of natural proteins

Purified protease showed typical Michaelis-Menten kinetics with Km and Vmax values of protease determined through Lineweaver–Burk plot. For the hydrolysis of casein at 50°C Km of 0.283 mg/ml and Vmax of 10.5 µm/ml/min were calculated (**Fig. 5.9**). Km value for keratin hydrolysis was 0.089 mg/ml and Vmax of 7.39 µm/ml/min. Comparatively lower Km value in the case of keratin might indicate more affinity towards keratin than casein. The lower Vmax for keratin can be attributed to the complexity of the substrate



a. Casein **b. Keratin**

Fig. 5.9: Lineweaver-Burk plots for the hydrolysis of natural protein by purified protease from *B. subtilis* P13

Km and V (max) values of proteases from different sources against different substrates are mentioned in **Table 5.5.**

B. subtilis P13 exhibited the kcat value of 15/sec and 10/sec for casein and keratin, respectively. Based on the temperature dependent kinetic study, Beg et al (2001) reported kcat values of 16.46/min, 22.02/min, 25.84/min and 26.78/min at 30, 40, 50 and 60°C respectively for *B. mojavenis* using casein as a substrate. Most of the reports on kinetic parameters for proteases are either based on synthetic peptides or chromogenic substrate like azocasein. Lee (2010) reported the kcat (turnover number) and kcat/Km (catalytic efficiency) values of AOLK-101 protease were 163.5/mg/sec and 3.9×10^{6}/M/sec, respectively for azocasein. The kcat value of this enzyme was higher than 61/sec of *B. megaterium* KLP-98 protease toward azocasein purified from anchovy sauce. Kinetic parameters such as Km, Vmax, kcat, and kcat/Km values are different depending on pH and substrate (Agboola et al., 2003). Catalytic efficiency (kcat/Km) of *B. subtilis* P13 towards casein and keratin were found to be 52 and 112 /mg/sec respectively.

Table 5.5: Comparision of kinetic parameters of protease from *B. subtilis* P13 with proteases from different sources

Enzyme source	Substrate	Km (mg/ml)	V(max)	Reference
B.mojavenis	Casein	0.025	117 µg/ml/min	Beg et al., 2001
Sclerotinia sclerotiorum	Casein	0.8	NA*	Chung,1984
***Saccharomyces* sp. B101**	Casein	1.02	NA*	Hwang et al., 2008
A.oryzae-ALOK-101	Casein	1.04	124 moles/l/min	Lee et al., 2010
***B. megaterium* KLP-98 acid protease**	Casein	2	285µmoles/l/min	Fu et al., 2008.
Alcaligenes faecalis	Casein	1.66	526µmoles/mg/h	Lin et al., 2009
P. aeruginosa	Casein	2.7	3 mmol/ min/min	Lin et al., 2009

PseA				
***B. subtilis* P13**	Casein	0.283	11.3µmoles/ml/min	Present study
***B. subtilis* P13**	Keratin	0.08944	7.33µmoles/ml/min	Present study

***** not available

5.3.6: Kinetics of hydrolysis of synthetic peptides

Of the four synthetic peptides used to evaluate the substrate specificity of this protease, the enzyme has remarkable hydrolytic activity towards AAPF-pN, and AAVA-pN (**Tabl 5.6**). Measurement of the kinetic constants (**Fig. 5.10a and b**) of the hydrolytic reactions revealed the Km values for AAPF-pN and AAVA-pN are in the same magnitude (**Table 5.5**). Wan et al (2009) reported Km values of $3.52x10^{-4}$ and $2.82x10^{-5}$ M, respectively for DHAP, a dehairing alkaline protease from *B.pumilus.* The reported Vmax for these substrates were 4.307 and 0.1246 mmol/mg/min respectively for AAPF-pN and AAVA-pN. The Vmax value of protease from *B. subtilis* P13, 0.256 ± 0.022 mmol/mg/min was higher than that of DHAP for AAVA-pN. Catalytic efficiency of DHAP per second for AAPF-pN (kcat 2230/s) was 2.2 times more than that of the protease from *B. subtilis* P13, but it showed catalytic efficiency 5 times surplus of DHAP with respect to AAVA-pN. The values for kcat/Km were more or less comparable for AAVA-pN, three fold higher for DHAP in the case of AAPF-pN. The kcat and Km values of 0.55mM and 33/S (Bockle et al.1995) by keratinolytic protease reported from *S. pactum* for AAPF-pN was much lower than that observed for *B. subtilis* P13 (**Table 5.7**). The enzyme from *S.pactum* showed nil activity towards AAVA-pN (Bockle et al., 1995). However, protease reported from *B. subtilis* P13exhibited no detectable activity with LF-*p*N and AAA-*p*N. The peptide N-Succinyl-Ala-Ala-Pro-Phe-*p*NA (AAPF-pN) was a better substrate than N-Succinyl-Ala-Ala-Val-Ala-*p*NA(AAVA-pN.

The ability to hydrolyse non-specific p-nitrophenol esters by α-chymotrypsin was demonstrated by Paul and Edward, (1977). They reported anomaly for the binding and accylation and not for deacylation stage. Similarly keratinolytic protease from *B. subtilis* P13 also showed non-specific esterase activity of 5 U/mg of protein. Kinetic

parameters were also studied for the esterification (**Fig. 5.10c**) and catalytic parameters are mentioned in **Table 5.6**.

Table 5.6: Hydrolysis of synthetic peptides by purified protease from *B. subtilis* P13

Substrate	Specific Activity (U/mg)
N-Suc-Ala-Ala-Pro-Phe-*p*NA (AAPF-pN)	135.29 ±4.23
N-Suc-Ala-Ala-Val-Ala-*p*NA (AAVA-pN)	15.1 ± 5.29
N-Suc-Ala-Ala-Ala-*p*NA (AAA-*p*N)	Not detectable
N-Suc-L-Phenylalanine-pNA (LF-*p*N)	Not detectable
p-Nitrophenyl acetate (pNPA)	5± 0.907

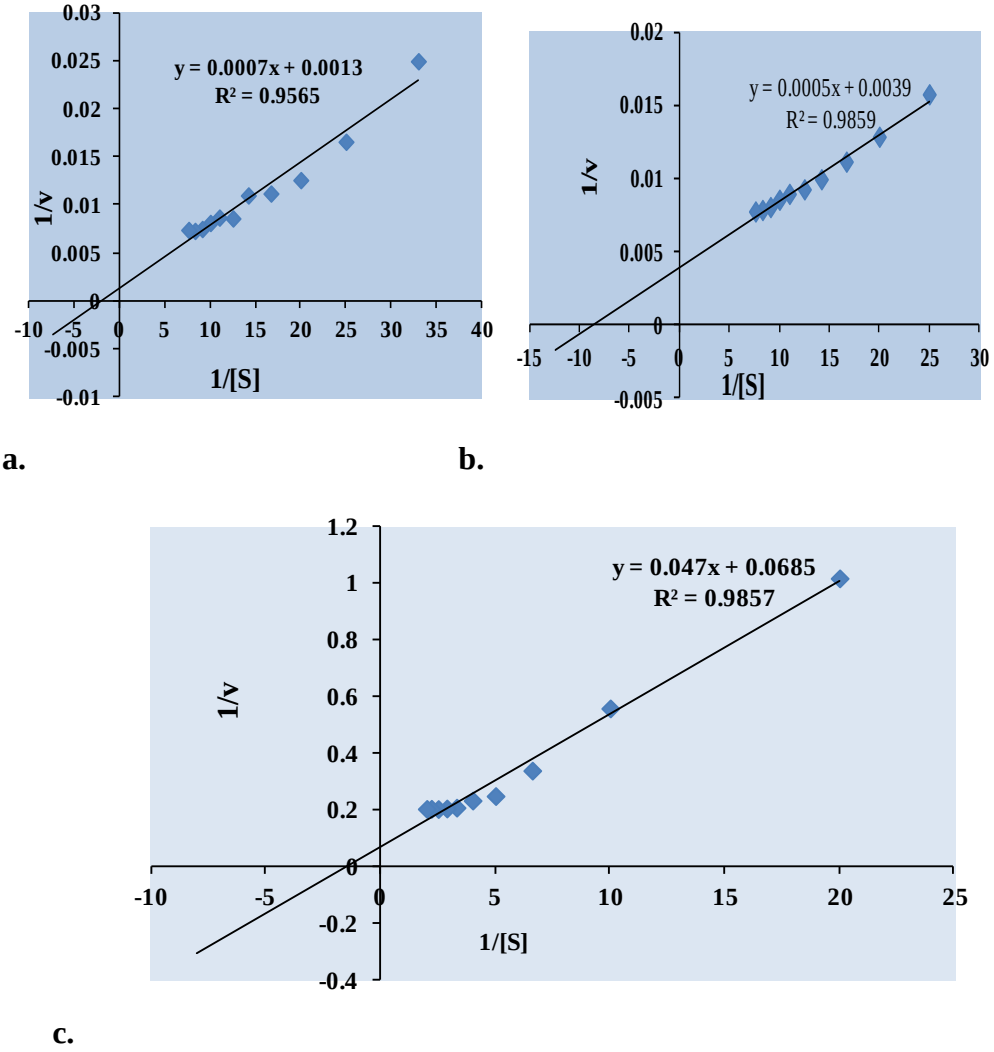


Fig. 5.10: Lineweaver-Burk plots for hydrolysis of synthetic peptides by purified protease from *B. subtilis* P13

a: N-Succinyl-Ala-Ala-Pro-Phe-*p*NA (AAVA-pN); b. N-Succinyl-Ala-Ala-Val-Ala- *p*NA (AAVA-pN) & c. p-NP acetate

Table 5.7: Kinetic parameters for synthetic substrate hydrolysis by purified protease of *B. subtilis* P13

Substrate	Km (M)	Vmax (mmol/mg/ min)	kcat (s^{-1})	kcat/Km (/M/ S)
AAPF-pN	5.38 ±1.145x10^{-4}	0.769 ± 0.0215	1003±1.89	1.864 ± 0.096x10^{6}
AAVA-pN	1.28 ±0.312x10^{-4}	0.256 ± 0.022	333±0.986	2.602±0.189x10^{6}
pNP-A	6.86 ±1.45x10^{-4}	0.0145 ± 0.0011	19±2.91	2.76 ±0.946x10^{4}

5.3.7: Thermodynamic parameters of substrate hydrolysis by purified protease of *B. subtilis* P13

Activation energy required for different substrate hydrolysis by protease was calculated from the Arrhenius plots for each substrate (**Fig. 5.11**). The enthalpy of activation (ΔH*),

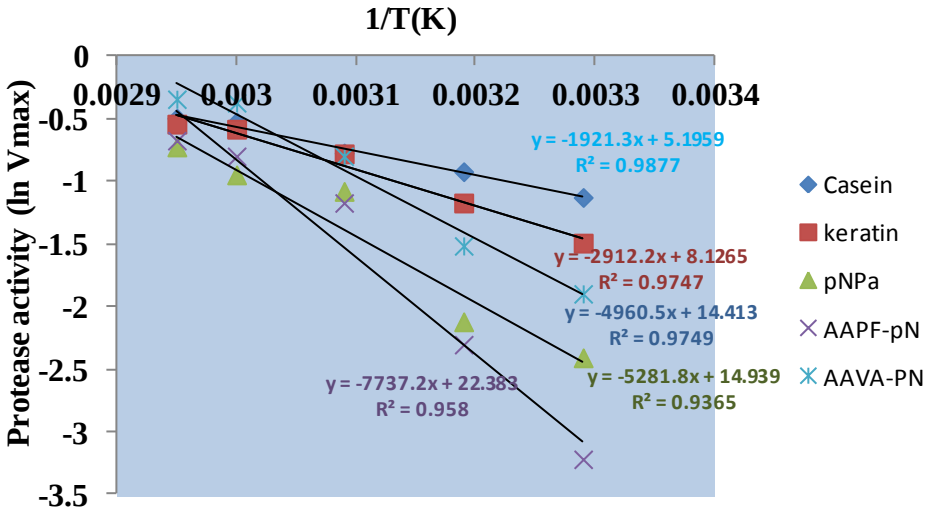


Fig. 5.11: Arrhenius plots for the determination of activation energy (Ea) for the hydrolysis of different substrates, Ea= - slope X R (gas constant)

Gibbs free energy (ΔG*) and entropy of activation (ΔS*) for different soluble substrates by *B. subtilis* P13 are mentioned in **Table 5.8**. The lower enthalpy value indicates the formation of transition state or activated complex between the enzyme-substrate was very efficient. Moreover, lower ΔG*(E-T) value suggested the conversion of its transition complex into product was more spontaneous.

Table 5.8: Kinetic and thermodynamic parameters for substrate hydrolysis by purified protease of *B. subtilis* P13

Substrates	Ea (kJ/mol)	ΔS (J/mol/K)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔG*(E-S) (kJ/mol)	ΔG* (E-T) (kJ/mol)
Casein	13.46	-189.78	10.773	72.1	-3.39	-10.615
Keratin	20.28	-171.98	17.599	73.17	-6.458	-12.677
pNPA	36.92	-55.036	34.23	71.447	-1.011	-8.922
AAPF-pN	51.132	-48.95	53.799	66.951	-1.664	-2.0232
AAVA-PN	31.874	-119.78	34.561	70.58	-5.523	-2.1134

5.3.8: Thermal stability and deactivation rate constants for protease purified protease of *B. subtilis* P13

Kinetics of thermal inactivation of protease with respect to caseinolytic activity from *B. subtilis P13* was measured **(Fig. 5.12).**

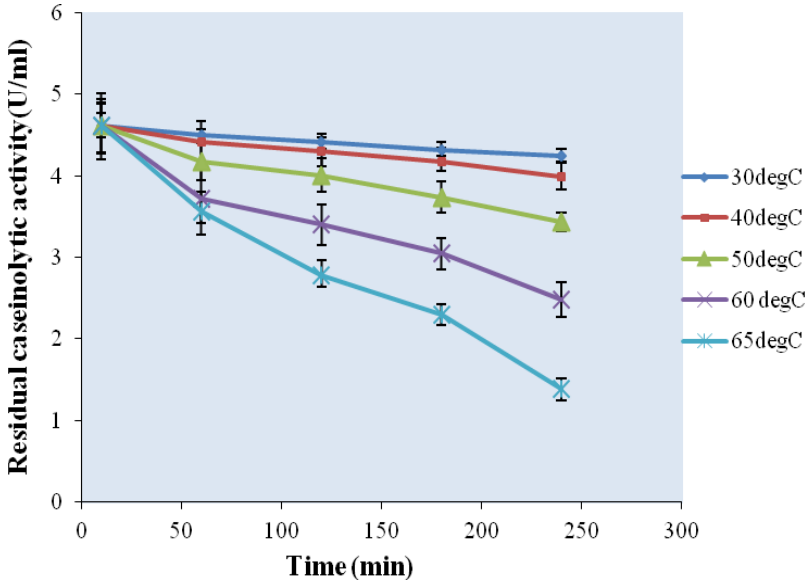


Fig. 5.12: Pseudo first order plots for irreversible thermal denaturation indicating the thermal stability of purified protease from *B. subtilis* P13.

The enzyme was found to be stable at ambient temperatures, where all pre-tanning process is carried out (30-40°C) with $t_{1/2}$ values 8.2 h and 5.02 h at 30 and 40°C respectively. Further increase in temperature increased the inactivation rate. Even at 65°C the enzyme was somewhat stable at with $t_{1/2}$ of an hour.

5.3.9:Thermal deactivation of protease in presence of salts

The deactivation rate for protease was studied at different temperatures ranging from 30-60°C in presence of $CaCl_2$, $BaCl_2$, NaCl and $(NH_4)_2SO_4$ (0.1%), which were relatively good stabilizers as determined in section. 3.3.7. All the selected salts increased the thermal stability of protease (**Fig. 5.12**). Maximum stability of protease was observed in presence of $(NH4)_2SO_4$ with $t_{1/2}$ values of 22h and 4h at temperatures of 30 and 60°C, which was 72.2 and 50% more than that of control at similar conditions (**Table 5.9**). Thermostabilty increases in the presence of salts in the order $(NH_4)_2SO_4$ >$BaCl_2$ > $CaCl_2$ > NaCl (**Fig. 5.13**). Stability improvement is due to the reduction of electrostatic repulsion, which reduces the electrostatic free energy. Increased number of salt bridges /additional hydrogen bond reduces binding factors and smaller accessible surface areas- reduces flexibility, increases the compactness and attribute stiff protein structure (Gouda et al.,2002).

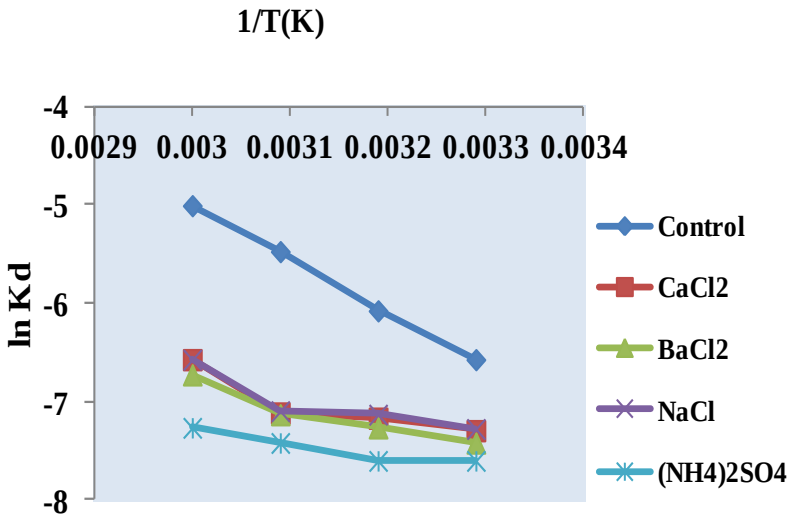


Fig. 5.13: Thermal deactivation of purified protease from *B. subtilis* P13 with different stabilizers. The activation energy for irreversible thermal inactivation ($E_{a(d)}$) was calculated from the relation, Kd=Ea/RT,where R (gas constant)=8.314kJ/mol

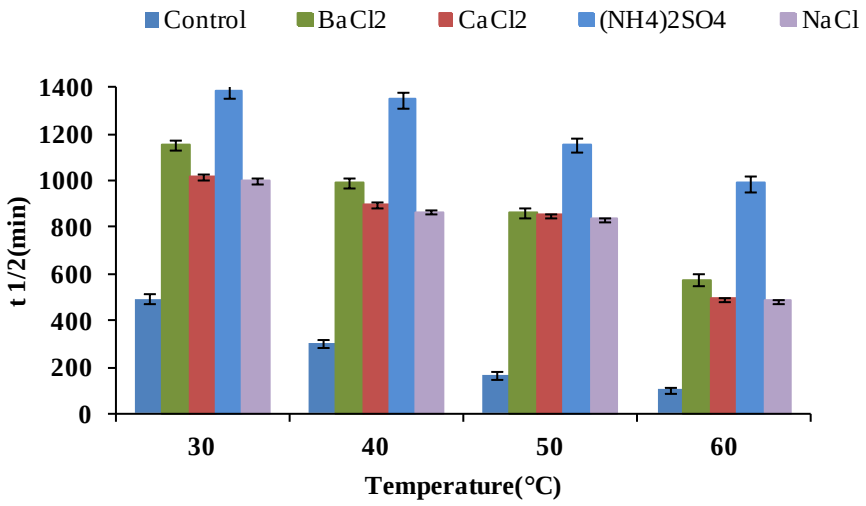


Fig. 5.14: Thermostability of protease from *B. subtilis* P13 in presence of different salts

$t_{1/2}$ values are calculated from **Fig. 5.13** using the relation : $t_{1/2} = \ln 2/K_d$

Table 5.9: Deactivation rate constants (*K*d) of protease at different temperatures in presence of NaCl, $CaCl_2$, $BaCl_2$ and $(NH_4)_2SO_4$

Temperature	**Control**	**NaCl**	**$CaCl_2$**	**$BaCl_2$**	**$(NH_4)_2SO_4$**
(°C)	Kd ($x10^{-3}$)	Kd ($x10^{-3}$)	Kd ($x10^{-3}$)	Kd ($x10^{-3}$)	Kd ($x10^{-3}$)
30	1.4	0.692	0.68	0.6	0.5
40	2.3	0.8	0.772	0.7	0.514
50	4.2	0.829	0.814	0.8	0.6
60	6.67	1.45	1.4	1.2	0.7

5.3.10: Thermodynamic parameters for protease deactivation in presence of salts

Investigation of thermodynamic parameters like change in enthalpy (ΔH^*), change in entropy (ΔS^*), change in free energy (ΔG^*) and activation energy ($E_{a(d)}$) of deactivation of enzyme was carried out to understand the behaviour of molecules at different temperatures in presence of salts which are good stabilizers.Values for the thermodynamic parameters,viz ΔH^* and ΔS^* and are given in **Tables 5.10** and **5.11**.The $E_{a(d)}$ for protease deactivation calculated in presence of salts and was in the order of $(NH_4)_2SO_4$< $BaCl_2$< $CaCl_2$ < NaCl < control, indicating thermal stability in reverse order.

Table 5.10: Thermodynamic parameters for protease deactivation in presence of different salts

Treatment	ΔH^* (KJ/mole)	ΔS^* (J/mole)	$E_{a(d)}$ (KJ/mole)
Control	47.35	-160.22	42.102
NaCl	22.698	-201.33	47.647
$CaCl_2$	18.199	-200.93	38.59
$BaCl_2$	14.921	- 212.11	35.47
$(NH_4)_2SO_4$	10.1587	-243.449	34.627

ΔH^*, change in enthalpy; ΔS^*, change in entropy; $E_{a(d)}$, activation energy for deactivation

Table 5.11: Values of ΔG^* (Gibb's free energy) for deactivation of protease at different temperatures in presence of different stabilizers

Temperature (°C)	ΔG^* (KJ/mole) for deactivation of protease				
	Control	NaCl	$BaCl_2$	$CaCl_2$	$(NH_4)_2SO_4$
30	90.846	92.622	92.981	92.66	93.44
40	92.635	95.382	95.731	95.477	96.66
50	94.059	98.419	98.541	98.469	99.286
60	95.689	100.097	100.522	100.098	102.02

Change in enthalpy was similar for all the temperatures both in the case of control and also in the presence of salts, indicating similar reaction rate at different temperature. The enthalpy change of protease falls in thr range of 20 -150 kJ/ mol, which is reported for biomolecule (Amico et al., 2003). Negative entropy reported for biocatalysts is due to compactness of the molecule, formation of charged particles. Increase in ΔS* imply an increase in the number of protein molecules in the transition active state. Protein stabilization is by decreasing the entropy and increasing enthalpy of activation of protein's unfolded stage (Matsumura et al., 1989). ΔG, Gibbs free energy measures the spontanaeity of a reaction and increased with increase in temperature. Increament of ΔG is minimal in thermophilic with respect to mesophilic and psychrophilic enzymes (Hameed et al., 2000).

5.3.11: Deactivation rate of protease at varying pH

Deactivation of purified protease was studied under different pH and temperature combinations (**Fig. 5.15**). Deactivation rates and half life were studied at 50, 40, 50 and 60°C at different pH values (**Fig. 5.16**) The pH is one of the main factor affecting tertiary and quarternary structure protein and enzyme. In many cases the rate of deactivation enzymes depends on the pH of the enzyme solution (Srinivas and Panda 1999).

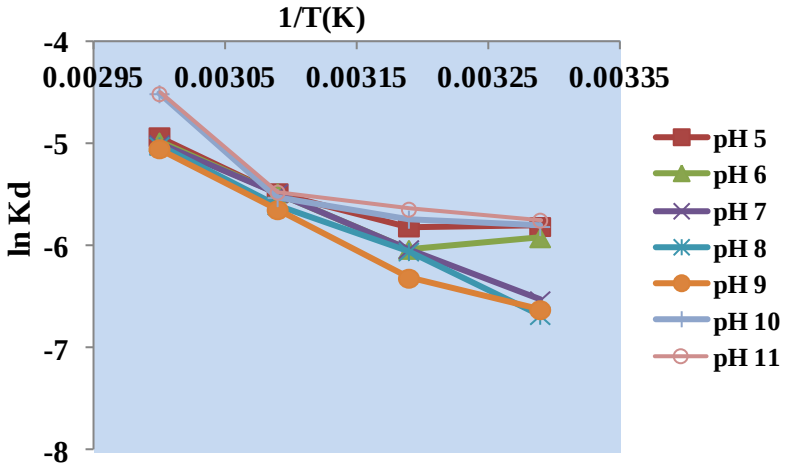


Fig. 5.15: Thermal deactivation of purified protease from *B. subtilis* P13 indifferent pH conditions.$t_{1/2}$ is calculated from the relation : $t_{1/2} = \ln 2/K_d$

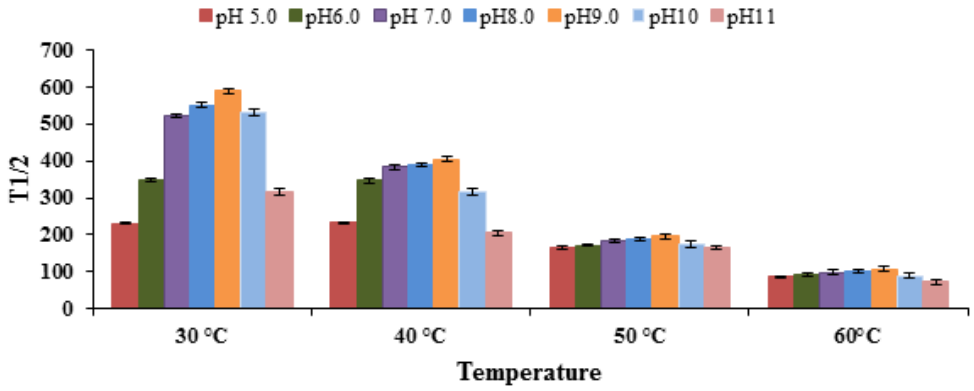


Fig. 5.16: Thermostability of purified protease from *B. subtilis* P13 at different pH conditions

When deactivation parameters are compared, relatively higher ΔG and lower ΔH, ΔS and Ea (d) at pH 9.0 indicated that the thermostability of the protease from *B. subtilis* P13 is best at pH 9.0 (**Table 5.12**).Though the enzyme showed a broad pH range, thermostability is better towards alkaline range than acidic range.

Table 5.12: Kinetic parameters of thermal deactivation of protease from *B. subtilis* P13 at different pH values

pH	ΔG* (KJ/mole)	ΔH* (J/mole)	ΔS* (KJ/mole)	*E*a(d) (KJ/mole)
5.0	92.06	49.13	-158	48.647
6.0	93.15	48.17	-159.57	48.102
7.0	94.059	47.35	-160.22	47.647
8.0	94.891	47.28	-160.59	47.57
9.0	**95.399**	**47.10**	**-160.99**	**47.431**
10	93.012	48. 23	-160.34	48.23
11	92.79	48.19	-159.24	48.50

5.3.12: Determination of pI of purified protease

Iso electric focussing was carried for the purified protein and based on the relative mobility of the IEF marker, pI the protein was found as 6.8 (**Fig. 5.17**).

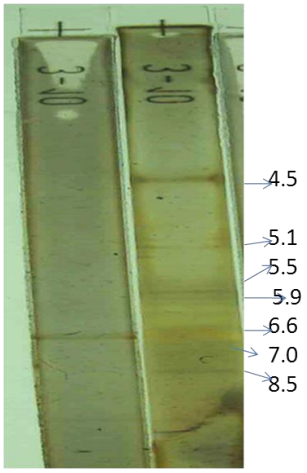


Fig. 5.17: Iso electric focussing pattern of purified proteinfrom *B. subtilis* P13

Two dimensional electrophoresis was performed after mixing the protein with 2-D marker, the molecular weight and pI was confirmed as 28 and 6.8 respectively (**Fig. 5.18**).

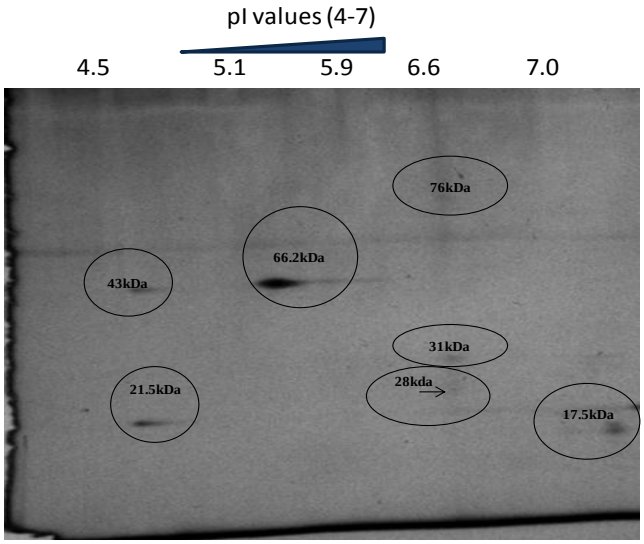


Fig. 5.18: 2-D profile of purified protein in combination with 2D marker

5.3.13: LC-MS analysis

The LC-MS data on MASCOT search showed maximum homology with 25% coverage to Nattokinase (Fragment) from *Bacillus subtilis* subsp. natto, Q5EFD9_BACNA having a nominal mass (M_r): 37231 and a calculated pI value: 7.83 (**Fig. 5.18**). 8. The peptide sequences showed 45% homology with a p value < 0.05 with subtilisins, mostly belong to fibrinolytic group. The calculated molecular mass of the purified protein was 28 KDa and pI of 6.33. Details of the protein hits on MASCOT search is provided in **Table 5.13** and elaborate report is furnished in

Appendix IV . The stretches of peptide sequence obtained after tryptic digestion are as given in **Table 5.14**. The N-terminal sequences obtained were matching exactly with the sequence obtained by Edman protein degradation as mentioned in Section 3.3.

Table 5.13: Summary of protein hits during MASCOT search after LC-MS analysis of purified protease from *B. subtilis* P13

Protein hits -ID	Details
1SCJA	subtilisin e (EC 3.4.21.62) mutant S221C, chain A - *Bacillus subtilis*
Q5EFD9_BACNA	Nattokinase (Fragment).- *Bacillus subtilis* subsp. natto.
SUBSN	subtilisin (EC 3.4.21.62) BPN' precursor - *Bacillus amyloliquefaciens*
1S02	subtilisin (EC 3.4.21.62) BPN' mutant (Q19E, Q271E) - *Bacillus amyloliquefaciens*
Q73PP9_TREDE	Iron compound ABC transporter, periplasmic iron compound-binding protein, putative.-*Treponema denticola*

Table5.14: Peptide sequence from LC-MS analysis

Positions	Peptide sequence
1-45	A**QSVPYGISQIKPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVR**
171 – 186	**YPSTIAVGAVNSSNQR**
238-247	**HPTWTNAQVR()**

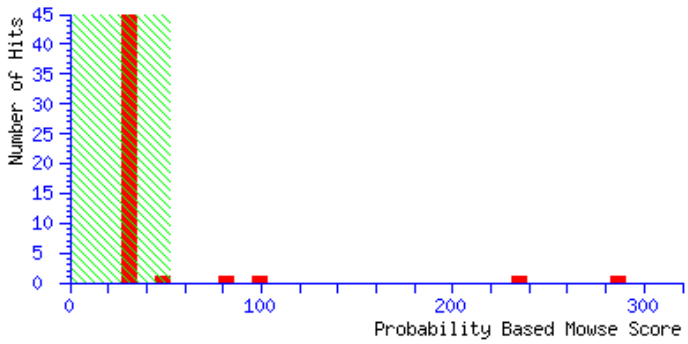


Fig. 5.19: Probability of peptide stretches matched with the reported sequence of subtilisins

5.3.14: Application studies with purified protease

Complete dehairing of goat hide was achieved with 0.28 U/gm of hide wet weight in 4h at 37°C (**Fig. 5.19**). This was considerably more efficient than the crude formulated enzyme, were dehairing was accomplished with 2U/gm of enzyme in16 h.

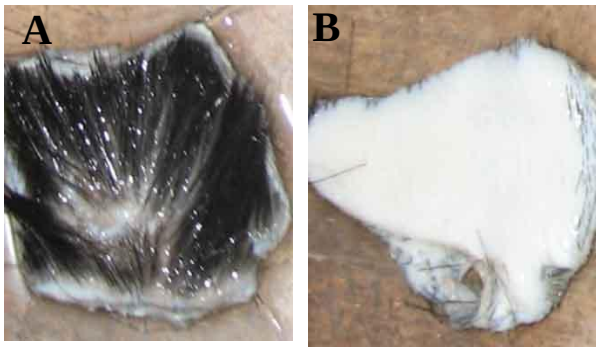


Fig. 5.20: Dehairing studies carried out with purified keratinolytic proteas

Other applications like feather peptide release and hydrolysis of chrome shavings was studied purified enzyme (**Fig.5.20**) to confirm the functional potentials displayed by the crude preparations. In the hydrolysis of chrome shavings, purified enzyme appeared to be less proficient than the crude, reason might be assistance provided by microbial cell to degdrade the denatured collagen.

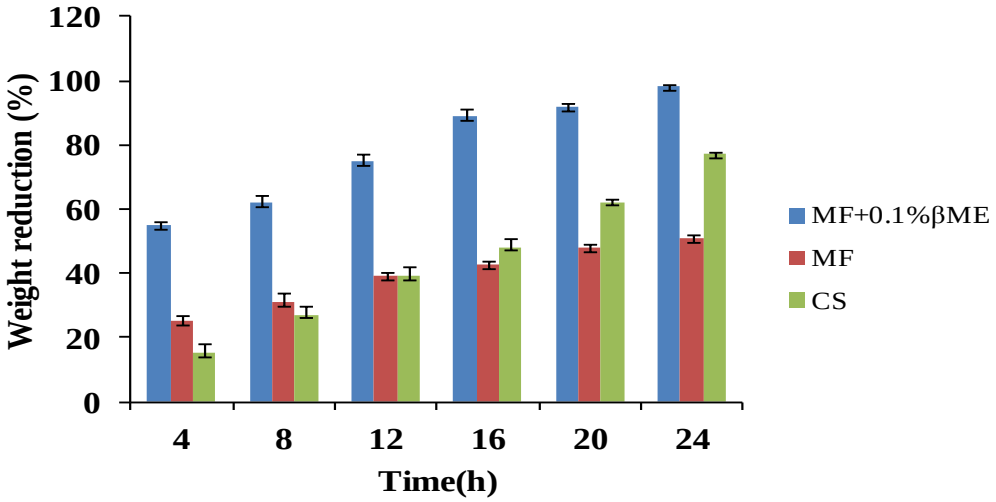


Fig. 5.21: Hydrolysis of milled feather and chrome shavings by the purified keratinolytic protease . MF+0.1% βME : Milled feather and βME; MF: Milled feather alone; CRS: Chromeshavings

5.4: Discussion

Most reports (Thangam and Rajkumar, 2002; Kumar 2002; Bayoudh et al. 2000; Chakrabarti et al. 2000; Poza et al. 2001; Hutadilok-Towatana et al. 1999;Gupta etal.,2002) on protease purifications use ion-exchange followed by size-exclusion chromatography. Compared to affinity chromatography, purification fold and yield for these protocols were in the range of 10-20 fold and 5-40 %, respectively. Protease from *B. subtilis* P13 was purified to 71.7 fold by casein affinity chromatography. Bockle et al., (1995) reported the purification of a keratinolytic protease of 30kDa from *Streptomyces pactum* to 64.8 fold using casein-affinity chromatography. Thus, purification-fold and yield of protease might be different depending on kinds of microorganism, culture medium, culture conditions, and extraction process, etc.

pH and temperature are the most important factors affecting activity and stability of enzymes to be used under harsh industrial conditions and fluctuating environmental conditions. The low value of E_a explains the correct conformation of active site for favourable ES* complex formation, hence requiring less energy. The effect of temperature on rate of reaction was measured in terms of temperature quotient (Q_{10}). Q_{10} values are used to infer whether or not the metabolic reactions being examined are controlled by temperature or by some other factor. Generally enzymatic reactions show Q_{10} values between 1-2 and any deviation from this value is indicative of involvement of some factor other than temperature in controlling the rate of reaction. Q_{10} value of 2 suggests doubling of rate of reaction with every 10°C rise in temperature (Dixon and Webb, 1979). The Q_{10} values were in the range of 1.21-1.29, for casein, keratin, AAPF-pN AAVA-PN and 1.12-1.16 for pNP-A by protease of *B. subtilis* P13 temperatures 30-60°C reflecting that every 10°C raise in temperature increased the rate of reaction by 12-29% respectively.

The purified protease exhibited relatively a moderate affinity for natural proteins, casein and keratin and high affinity for synthetic peptides N-Succinyl-Ala-Ala-Pro-Phe-*p*NA (AAPF-pN) and N-Succinyl-Ala-Ala-Val-Ala-*p*NA (AAVA-pN) and hydrolysed them efficiently as revealed by low k_m and high V_{max} values.Comparision of the specificity towards synthetic peptides with respect to other keratinolytic proteases are depicted in **Table 5.15.**

Table 5.15: Comparision of amino acid specificity of keratinolytic protease from *B. subtilis* P13 with other reported keratinases

Substrate	Enzyme	Reference
Suc-Ala-Ala-Pro-Phe- pNa	Protease (*B. subtilis* P13)	Present Study
Suc-Ala-Ala-Val-Ala- pNa	Protease (*B. subtilis* P13)	Present Study
Suc-Ala-Ala-Pro-Phe- pNa	KerA (*B. licheniformis* PWD-1)	Evans et al., 2000
Suc-Ala-Ala-Pro-Phe- pNa	SAKase (*S. albidoflavus* K1-02)	Bressollier et al., 1999
Suc-Ala-Ala-Pro-Phe- pNa	Sfase-2 (*S. fradias* ATCC 14544)	Kitadokoro et al., 2004
Suc-Ala-Ala-Pro-Phe- pNa	NAPasea (*Nocardiopsis sp.*)	Mitsuiki et al., 2004
Suc-Ala-Ala-Pro-Phe- pNa	Keratinase Pm (*P. marquandii*)	Gradisa et al., 2005
Suc-Ala-Ala-Pro-Phe- pNa	Keratinase Dm (*D. microsporus*)	Gradisa et al., 2005
Suc-Ala-Ala-Pro-Phe- pNa	Keratinase Pa (*P. areudinosa*)	Lin et al., 2009
Suc-Ala-Ala-Pro-Phe- pNa	Keratinase 100409 (*Thermoanaerobacter sp.)*	Kublanov et al., 2009
Suc-Leu-Leu-Val-Tyr- AMC	Keratinase AL20 (*Nesterenkonia sp.)*	Bakhtiar et al., 2005
CBz-Phe- oNp	Keratinase SP (*S. pactum*)	Böckle et al., 1995
CBz-Phe- PNa	Keratinase L (*Lysobacter sp.*)	Allpress et al., 2002
CBz-Phe- pNa	Keratinase Kr10 (*Microbacterium sp. Kr*10)	Thys and Brandelli, 2006
P4 P3 P2 P1		

The specificity of keratinases towards keratinous materials may arise from the amino acid composition of keratins, which contains about 50–60% of hydrophobic and aromatic residues (Arai et al. 1983; Gregg et al. 1984; Barone and Schmidt 2006; Gradisar et al. 2005). Additionally, the nature of amino acids at the vicinity of the cleaved bond was showed to influence the specificity for the P1 position, which might indicate the presence of an extended active site (Kitadokoro et al., 1994; Böckle et al.,1995; Bressollier et al. 1999; Macedo et al. 2008). For instance, substitution of Ala residue of Suc-Ala-Ala-Ala-pNA by Pro at the P2 position increased the kcat/Km value of NAPase from *Nocardiopsis* sp. TOA-1 by 37-fold (Mitsuiki et al., 2004). The preference for longer substrates at both sides of the scissile peptide bond suggests the suitability of keratinases for the conversion of native and complex substrates. The cleavage of peptide bonds in the compact keratin

molecules is difficult due to the restricted enzyme substrate interaction on the surface of keratin particles and accessibility to splitting points. Therefore, the hydrolyzing ability of keratinolytic proteases may be due to its ability and specificity to bind onto compact substrates, and a more exposed active site (Böckle et al., 1995).

Cai et al., (2008) reported a keratinase that was active over a temperature range of 40~70 °C, with an optimum at 55° C produced by *B. subtilis* KD-N2, having affinity towards keratin, BSA and casein and was active at neutral and alkaline conditions, with optimum at pH 8.5, and the most suitable buffer seemed to be Tris-HCl. Most keratinases possess an activity optimum in the range of 30~80 °C, for example, keratinase from *B. pseudofirmus* AL-89 is of 60~70 °C (Gessesse et al., 2003), *Nocardiopsis* sp. TOA-1 is of 60 °C (Mitsuiki et al., 2004), and a few have exceptionally high temperature optimum of 100 °C (Nam et al., 2002). Most keratinases are active in neutral to alkali conditions, from pH 7.0 to pH 9.5. For example, the activity optimum of keratinase from *Microbacterium* kr10 is pH 7.0 (Thys et al., 2004), *B. pumilus* FH9 of pH 8.0 (El-Refai et al., 2005), *Fervidobacterium islandicum* AW-1 of pH 9.0 (Nam et al., 2002), and a few of extreme alkalophilic optima at pH 12~13 (Takami et al., 1999) and pH 12.5 (Mitsuiki et al., 2004).

Thermodynamic parameters for substrate hydrolysis show low values of enthalpy and negative values of entropy suggested formation of a more efficient and ordered transition state complex between enzyme and substrate. Δ*G** is the measure of spontaneity of any reaction. Low Δ*G** values suggest that the conversion of transition state complex (ES*) into product was more spontaneous. The feasibility and extent of chemical reaction is best determined by measuring changes in Gibb's free energy (ΔG*) for substrate hydrolysis, i.e the conversion of E-S complex into product. The lower the free energy change, more feasible is the reaction, i.e the conversion of the reactant to product will be spontaneous. Lower entropy explained that the transition complex had less disorder. Low Δ*G** values suggest that the conversion of transition state complex (ES*) into product was more spontaneous. The feasibility and extent of an enzyme catalysed reaction is best determined by measuring change in Δ*G** for substrate hydrolysis i.e. the conversion of E-S complex into product (Muhammad et al., 2007). Low values of free energy for substrate binding (Δ*G**E-S) and formation of activated complex (Δ*G**E-T)

confirmed that the high affinity of the substrates for protease. Due to the difference in unit definitions, the order of substrate preference by protease preference can be compared between casein – keratin and AAPF-pN – AAVA-pN. Among them, lower values of (ΔG*E-S) and (ΔG*E-T) indicated more preference towards keratin and AAPF-pN

Stability of enzymes is important parameter which determines the economic feasibility for any industrial application. Maximum stability of protease was observed in presence of $(NH_4)_2SO_4$ followed by $BaCl_2$, CaCl and NaCl, as revealed by t1/2 values at 60°C. The high stability of purified protease at temperatures prevailing under ambient conditions (30-40°C) in presence of salts suggested its applicability in tanneries as a formulated product. The stability was high in all the treatments compared to control. Investigation of other thermodynamic parameters like activation energy of deactivation (Ea(d)) and change in enthalpy (ΔH*), entropy (ΔS*) and free energy (ΔG*) of enzyme is necessary to understand the behaviour of molecules in different conditions. These parameters were effective in the order: $(NH_4)_2SO_4$ >$BaCl_2$, > CaCl, >NaCl > control. The low ΔG* value for the heat labile enzyme corresponds to the large ΔH* and ΔS* contributions and conversely the high ΔG* corresponds to the low ΔH* and ΔS* for heat-stable enzyme.

Thermal denaturation of enzymes occurs in two steps (Siddiqui et al., 1999), i.e. N↔U→I, where N is the native enzyme, U is the unfolded inactive enzyme which could be reversibly refolded upon cooling and the inactivated enzyme formed after prolonged exposure to heat and therefore cannot be recovered upon cooling (Eisenberg et al., 1992). The decrease of ΔH* as enzyme stability increases as shown by keratinolytic protease from *B.subtilis* P13 in presence of $(NH_4)_2SO_4$ and $BaCl_2$ mainly reflects the decrease in co-operativity of inactivation and unfolding.The denaturation of enzyme in a shorter temperature range leads to the sharp slope of the Arrhenius plots and subsequently to high activation energy *Ea* and ΔH*. In addition, the heat stable enzyme seems resistant to denaturation before the irreversible loss of activity and conformatio (low ΔS*). Therefore, the heat stable enzyme can be regarded as an intrinsically stable protein that counteracts heat denaturation by a weak cooperativity of unfolding and inactivation (D' Amico et al., 2003). The study of these thermodynamic parameters for protease deactivation in presence of salts at

different temperatures suggested that the thermal stability of enzyme was due to higher value of ΔG* and negative value of Δ*S** which enabled the enzyme to resist against thermal denaturation.

The molecular mass of protease purified (27.7 kDa) by casein affinity chromatography was found to be close to (25-35kDa) other reported serine proteases (Rao et al., 1998).The mascot search following LC-MS analysis of the purified protease revealed its close similarity to nattokinase, a fibrinolytic enzyme from *B. subtilis natto*.

5.5: Conclusion

The properties of the purified enzyme mentioned in **Table 5.15**. The unhairing efficiency of the purified protease was confirmed by goat dehairing studies. Keratinolytic property was confirmed by substrate specificity, catalytic parameters and also by comparing the preference on synthetic peptides with other reported keratinases from different sources. Thermodynamic parameters were studied and ammonium sulphate appeared to be excellent thermal stabilizers. This has an advantage that the concentration by ammonium sulfate precipitation itself improves the shelf life of the enzyme. Best thermal stability was observed at pH9.0.LC-MS analysis of the purified protein showed maximum homology to a fibrinolytic enzyme, nattokinase and the same has to be explored further for confirmation.

Table 5.16: Biochemical properties of purified protease from *B. subtilis* P13

Biochemical properties	
Optimum temperature	65°C (t1/2 approx :2hr)
Optmum pH	7.0 (best thermal stability at pH 9.0)
MW	28 kDa
Catalytic type	serine
Most preferred natural protein	Keratin km : 0.08944mg/ml V(max) : 7.33µmoles/ml/min kcat : 10/sec
Most preferred synthetic peptide	AAPF-pN km :$5.384x10^{-4}$M V(max) :0.769mmol/mg/min kcat : 1003/s
Best thermostabilizer	$(NH_4)_2SO_4$;thermostability increased by 50 % at optimum temperature

CHAPTER 6

Cloning, expression and characterization of keratinolytic protease from *Bacillus subtilis* P13

Prediction is very difficult, especially about the future.
- Niels Bohr

6.1 Introduction

The alkaline proteases from bacteria have been intensively studied. They mainly come from *Bacillus sp.*, such as *B. licheniformis, B. subtilis, B. amyloliquefaciens B.pumilus* and *B. alcalophilus,* and the representative is subtilisin from *B. subtilis.* Genes encoding alkaline proteases have been cloned , sequenced and over-expressed (Jacob et al., 1985; Koide et al., 1986; Jang et al., 1992; Jorgensen et al., 2000; Nakamura et al., 1992). Subtilisins are encoded by aprE gene encoding of 275 amino acid long polypeptide. The expression of bacillar genes is controlled by complex regulatory system, which includes specific regulatory proteins (spoO, AbrB, CepA, DegU, sinR, Hpr and others). Most of the species of Bacilli secretes proteases with two maxima of activity, one in the early (24hr of growth) and the other in late (44hr of growth) stationary phase. Many of the genes of the adaptive response pathway are responsible for the sporulation, but the selection of the pathway, depends on the availability of nutritional sources (**Fig. 6.1**).

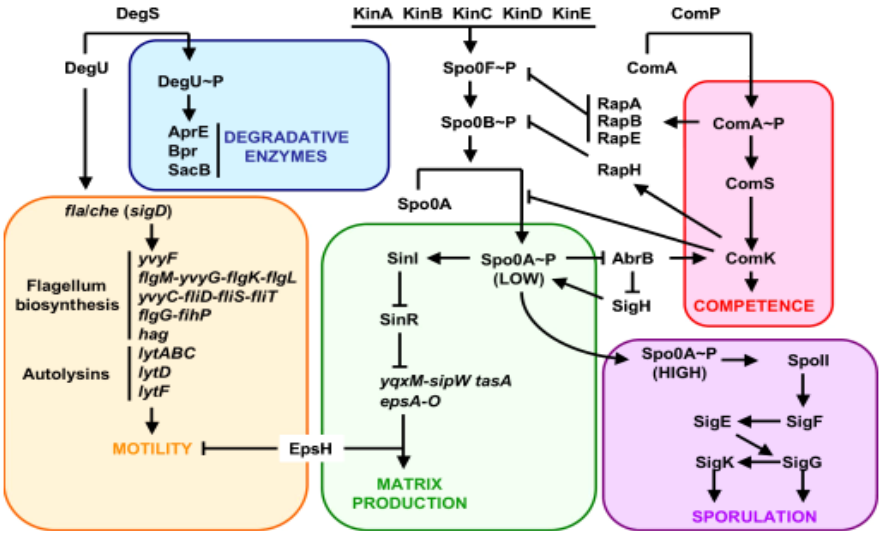


Fig. 6.1: Network of the different genetics pathways related to cell differentiation in *Bacillus subtilis*. Genes related specifically for each differentiation process are located within the specific frame (Lopez et al., 2008)

Many regulatory factors influence *aprE* expression, acting via 5' regions in *cis.* In some cases, direct contact with *aprE* DNA has been demonstrated, and target sites defined, e.g., the transition-state regulator Hpr, affecting subtilisin production negatively interacts. The main reported regulators that control AprE production are DegU/DegS (Henner et al., 1988b; Kunst et al., 1988; Msadek et al., 1990), AbrB

(Strauchet al. 1989), Hpr (also known as ScoC) (Kallio et al.1991), and SinR (Gaur et al., 1991; Mandic-Mulec et al.1992; Bai et al. 1992). Furthermore, direct binding of AbrB, Hpr and SinR to the *aprE* regulatory region has been demonstrated by in vitro gel retardation assays and/or footprinting analysis (Strauch et al., 1989; Gaur et al., 1991; Kallio et al., 1991). SinR binds to the *aprE* promoter at a region between 217 and 263 bp with respect to the transcriptionstart point (Gaur et al., 1991). Furthermore, it is known that high levels of SinR repress production of extracellular proteases and the expression of an *aprE-lacZ* fusion and is generally assumed that SinR is a repressor directly acting on *aprE* (Gaur et al., 1986, 1991).

The LCMS analysis and MASCOT search revealed the similarity of keratinolytic serine protease produced by *B. subtilis* P13 to nattokinase (Section 5.3.11). Nattokinase is an enzyme, (E.C. 3.4.21.62), produced by *B. subtilis* (natto) isolated from natto, a fermented soybean Japanese food. Nattokinase has potent fibrinolytic activity and has attracted worldwide attention because of its health benefits, such as reducing blood cholesterol level, decreasing blood pressure, preventing atherosclerosis and inhibiting osteoporosis (Yokota et al., 1996; Tsukamoto et al., 2000; Milner and Makise, 2002).Three main mechanisms for the breakdown of fibrin by nattokinase have been suggested (Milner and Makise,2002). Action of nattokinase was demonstrated in two stages; consist of direct degradation of fibrin strands, followed by the activiation of urokinase, enhancing the transformation of plasminogen to plasmin which then breaks down fibrin. Third, nattokinase enhances the production of tissue plasminogen activator that promotes the transformation of plasminogen to plasmin, thus fibrin is degraded (Milner and Makise , 2002).

Recently, many fibrinolytic enzymes have been identified from different fermented soybean foods, such as subtilisin DFE (Peng et al., 2003) and subtilisin FS33 (Wang et al; 2006) from douchi in China, subtilisin DJ-4 from doen-jiang (Koide et al., 1986), and subtilisin CK 11-4 from chungkook-jang (Kim et al., 1996) in Korea. It was reported that nattokinase action could be enhanced and prolonged in the plasma when it was taken orally (Sumi et al., 1990; Suzukhi et al., 2003).

Since *E. coli* has a short generation time, an established fermentation procedure, a high production of foreign proteins, it has become the most widely used prokaryotic organisms for recombinant protein production.Cloning and heterologus expression studies carried for many proteases find application in pre-tanning, especially in dehairing. DHAP, dehairing alkaline protease, produced extracellularly by *Bacillus*

pumilus UN-31-C-42 comes from a wildtype strain BA (06), which was isolated from biological waste in Cheng Du, P. R. China was purified, the gene cloned and its expression in *E. coli* and *B. subtilis* WB60 (Pan et al; 2004).

Subtilisins are synthesized as preprosubtilisin precursors consisting of a typical signal peptide of 29 residues followed by the propeptide of 77 residues and then a mature subtilisin segment of 275 residues (Vasantha et al., 1984; Stahl and Ferrari, 1984). When the DNA sequences coding for the subtilisin signal peptide have been fused to the gene encoding the mature protein A from *Staphylococus aureus* (Vasantha and Thompson, 1986) and to the TEM β – lactamase derived from pBR322 (Wong et al., 1995), then the resulting fusion proteins were processed by signal peptidase , and the mature products were secreted into external medium by *B. subtilis.* But when the OmpA (*E.coli* outer membrane protein) signal peptide was directly fused to the mature subtilisin sequence (without the propeptide), protease activity was not detected (Ikemura et al., 1987). So propeptide is essential for the correct folding of the enzyme and not for the release from the plasma membrane (Ikemura and Inouy, 1988). Liang et al., (2008) reported a nattokinase producing bacterium, *B. subtilis*YF38, was isolated from douchi, using the fibrin plate method. The gene encoding this enzyme was cloned by polymerase chain reaction (PCR). Cytoplasmic expression of this enzyme in *E. coli* resulted in inactive inclusion bodies. But with the help of two different signal peptides, the native signal peptide of nattokinase and the signal peptide of PelB, active nattokinase was successfully expressed in *E. coli* with periplasmic secretion, and the nattokinase in culture medium displayed high fibrinolytic activity.Cloning and over expression of a keratinolytic protease having potential tannery applications from *B. subtilis* P13 and the exploration of the proposed fibrinolytic activity of the protein are described in the current chapter.

6.2 Materials and Methods

6.2.1 : Bacteria and strains of plasmid used

Table 6.1 shows the plasmids used in the study along with the bacterial strains and transformants used for the cloning and over-expression of keratinolytic protease from *B. subtilis* P13. All bacteria were routinely grown in Luria-Bertani (LB) medium. Solid medium was prepared by addition of 2.5% agar to LB broth. Ampicillin was used at a concentration of 100µg/ml for the cultivation of *E. coli* DH5α containing pJET1.2 clones, while 50 µg/ml ampicillin was added for the growth of *E. coli* strains bearing pET22b(+).

Table 6.1: Bacterial strains and plasmids used in this study

Strain	Genotype	Reference
Bacillus subtilis P13	Lab isolate from Vrajeswari hotspring, Mumbai,India. Moderately thermophilic with a growth optima of 40˚C	Present work(Pillai and Archana, 2008)
Escherichia coli strains		
DH5α	*supE44* D*lacU(*f*80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi 1 relA1*	Lab. stock (Sambrook and Russell, 2001)
BL-21(DE3)	F-ompT gal(dcm)(lon)hsdSB(rB- mB- an E.coli B strain)with DE3,a λ prophage carrying the T7 polymerase gene	Lab. stock (Sambrook and Russell, 2001)
Plasmids		
pJET1.2 (**Fig. 6.2**)	Blunt ended cloning vector with Ampicillin as the selection marker and a lethal gene eco471 enabling positive selection.	MBI Fermentas
pET22b(+) (**Fig. 6.3**)	N-terminal pelB signal sequence and an optional C-terminal 6X His Tag with Ampicillin as a selection marker .No blue white selection	Novagen

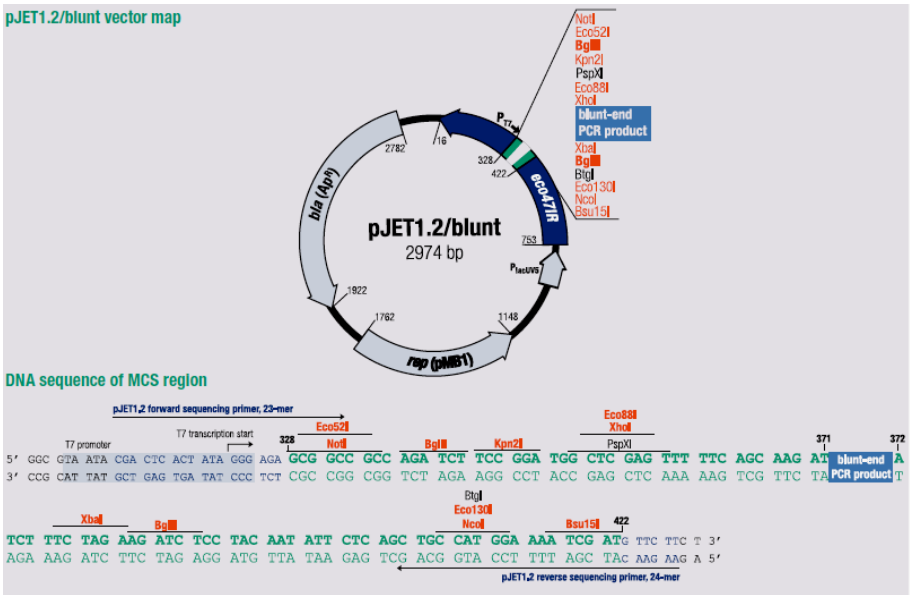


Fig. 6.2: Vector map of pJET1.2

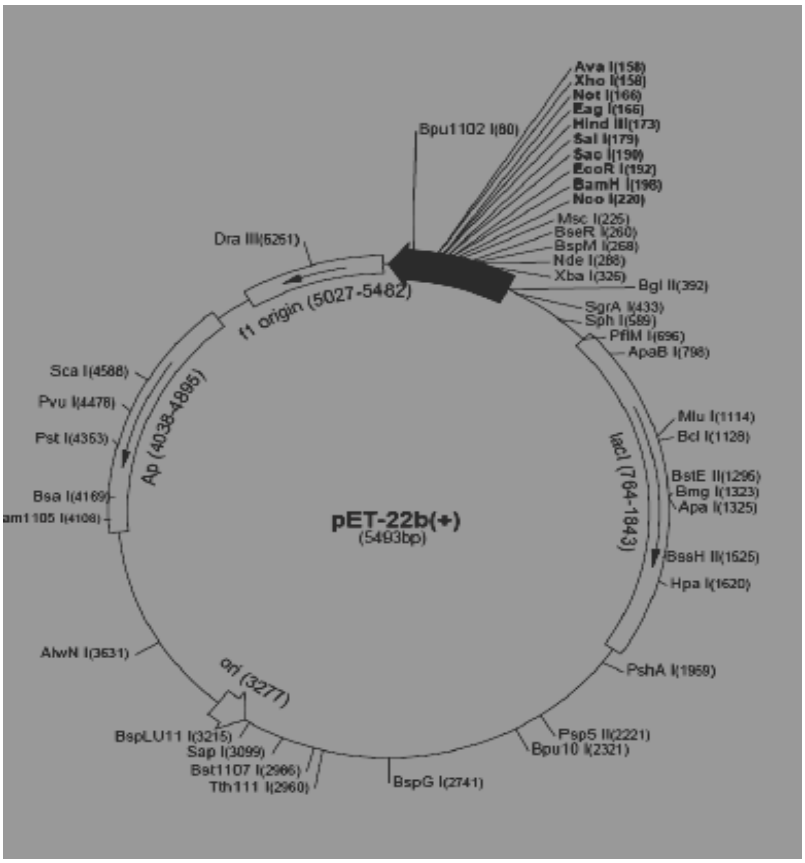


Fig. 6.3: Vector map of pET22b (+)

6.2.2: Molecular biology techniques

Media constituents, agar and agarose were purchased from Hi-Media Pvt. Ltd. India. Inorganic salts, buffers and other chemicals were of analytical grade. Restriction enzymes and PCR reagents like dNTPs, *Pfu and Taq* polymerases were purchased from Bangalore Genei, Bangalore, India. Oligonucleotide primers were procured from Sigma–Aldrich, USA.

6.2.2.1: Genomic and plasmid DNA isolation

Genomic DNA extraction from *B. subtilis* P13 was carried out by CTAB method (Sambrook and Russell, 2001). The culture pellet of 12h old culture was resuspended in 68 µl of Tris.EDTA buffer, pH 8.0 (10:1) and 2 mg lysozyme was added followed by incubation at 50^0C for 1 h. Protinase k, 3 µl from 100µg/ml stock and 10 µl of 10% sodium dodecyl sulfate(SDS) and of was added and incubated at 50°C for another 1 h. After incubation.100 µl of 5M NaCl and 16 µl of 10% hexadecyltrimethyl ammonium bromide(C-TAB) were added and incubated at 65^oC for 10 min. The solutions were then extracted with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) followed by extraction using chloroform: isoamylalcohol (24:1). Double the volume of ice-cold ethanol was added to precipitate nucleic acids. After centrifugation at (12,000 x g for 5 min at RT).the pellets were washed with 70% ethanol, air dried and dissolved in 30µl of autoclaved distilled water at 65^oC for 10 min.

Plasmid Preparation from *E.coli* both miniscale and maxiscale were obtained by alkaline lysis method described by Sambrook and Russel, 2001.

6.2.2.2: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator (Alpha Imager HP).

6.2.2.3: Restriction enzyme digestion analysis

DNA samples (0.5-1.0µg) were used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10µl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

6.2.2.4: Ligation

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5-1.0U and sterile double distilled water to make up the volume. The cohesive end ligation reaction was carried out at 15°C for 12-16h. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

$$pmoles \text{ of DNA} = \frac{\text{Amount of DNA } (\mu g) \times 1{,}515}{\text{Size of DNA fragment (No. of base pairs)}}$$

6.2.2.5: Gel elution and purification

From the sample electrophoresed on 0.8% agarose gels, the DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band The PCR products were purified using the tube in tube protocol described by Sambrook and Russell, (2001). The homemade Sephadex G-10 spin columns consist of a 2 ml and a 0.5 ml micro centrifuge tube without caps and a tiny bit of sterilized glass wool to make such a spin column. A needle or a pin, was heated with a Bunsen burner, the heated needle was lightly stabbed into the bottom of a 0.5 ml micro centrifuge tube to make a small hole, no bigger than 1 mm. The 0.5 ml tube was put into a 2 ml tube to form a homemade spin column. The band(s) of interest were cut out with a clean surgical blade under long wavelength UV illumination. The gel slice was transferred into the small tube and the gel slice was

smashed against the tube. The column was centrifuged for 10 minutes at the maximum speed of the bench-top micro centrifuge at 12,000xg. The DNA solution was transferred to a clean 1.5 ml tube, 1/10 volume of 3 M sodium acetate (pH 5.2) was added and double the volume ice-cold ethanol was used to precipitate the DNA. The DNA pellets were washed twice with ice-cold 70% ethanol and the DNA was dissolved in autoclaved distilled water (D/W). The eluted DNA was analysed on 1% agarose gel.

6.2.2.6: Transformation of *E. coli*

Plasmid transformations of *E.coli* strains were carried out by preparing competent cells using $CaCl_2$ method and heat shock method was used as mentioned by Sambrook and Russell, 2001. Before transforming the main ligated product, the purity of the competent cells was checked. A mock transformation was performed with known concentration of p BlueScript (pBS) and the efficiency of transformation was calculated. The ligated product was transformed only if the efficiency was ~ 10^5 transformants/µg of DNA.

6.2.2.7: SDS-PAGE

SDS-PAGE slab gel electrophoresis was carried out using 12% acrylamide gel by following the procedures described by Sambrook and Russell, (2001). After electrophoresis the gel was stained using the staining solution (**Appendix I**) for about 1h and then de-stained with de-staining solution (**Appendix I**) by incubating overnight under mild shaking conditions. Result was recorded by direct scanning of gel.

6.2.2.8: Polymerase Chain Reaction (PCR)

The chromosomal DNA from *B. subtilis* P 13 was used as a template for the PCR. PCR amplifications were performed in thermocycler, (Applied biosystem, AB: 2720). The amplification reaction was carried out in a total volume of 25µl containing 10X *Pfu* buffer, 0.8mM dNTPs, 1µlof each primer from 20 pmoles stock,approximately 20 ng of genomic DNA and 1.5 U of *Pfu* Polymerase. The PCR conditions were as follows: initial denaturation at 95˚C for 5min; 30 cycles of denaturation at 94˚C for 30s, annealing at 45˚C for 30s followed by an extension at 72˚C for 1:30s; final extension at 72˚C for 10 min.

The first set of primers, NK1 and NK2 (**Table6.2**) were used to validate the similarity of keratinolytic protease from *B. subtilis* P13 obtained by LC-MS analysis as nattokinase.To express nattokinase in *E.coli***,** expression vector was constructed using the PCR amplified product using primers NK3 and NK4 (**Table 6.2**) having unique restriction sites of *BspH1* and *BamH1* on forward and reverse primers respectively. The amplification of coding sequence of nattokinase was carried out by PCR in a total volume of 25µl and *Pfu* polymerase. The PCR conditions were similar, except the annealing condition of 50˚C for 30s.

Table 6.2: Details of primers used

Primer details	RE site (underlined nucleotides)	Reference
NK1: 5'-GCGGTCGACGTATGAAAATAGTTA	EcoRI	Xiaobo et al, 2007
NK2: 5'- GTAGAATTCTCCGGTGCTTGTGAA	SalI	
NK3:5'ATTCATGACGAAAAAGCAGTACAGAA	BspH1	
NK4:5'GTACAAGCAGCTGCACAATCGGATCCGATA	BamHI	

6.2.2.9: Sub-cloning of nattokinase like protease in pET22b+

Since the protease gene to be cloned was having an internal site of *Nco*I, *BspH*I, a restriction enzyme which produces compatible ends to *Nco*I, was incorporated in the forward primer. These primers are reported to amplify the 1100 bp coding region of nattokinase (Xiaobo et al., 2007).The amplified fragment of 1100bp was initially cloned into blunt cloning vector pJET 1.2. Insert of expected size was released from pJET -Kpr using vector specific enzyme BglII and digested with *BspH1* and *BamH1*. Subsequently, the DNA fragment of pET22b was digested with *Nco*I and *BamH*I and subcloned into pEt22b+. The construct pET-AJ-Kpr carries only the propeptide and mature enzyme gene under the control of the T7 promoter and lac operator. The pelB in the vector pET22b+ codes for signal peptide and assist the secretion of the expressed protein to periplasm. The pJET1.2 clones and pET22b (+) clones were confirmed by using the *gene* specific primers. The PCR cycling conditions were kept the same as described in Section 6.2.2.8. The 1.1Kb amplicon was analysed on 1% agarose gel. The sequence analysis of the selected clone was carried out at the

facility available at Bangalore Genei Ltd. The construct was further transformed into protease null strain of E.coli, *Escherichia coli* BL-21 (DE3) for expression analysis.

6.2.2.10: Expression of keratinolytic protease protein

Escherichia coli BL-21 DE3 cells harbouring the expression construct were grown to an OD of approximately 0.4 to 0.5 at 600nm. Gene expression was induced by treatment with 0.7 mM isopropyl-1-thio-L-D-galactopyranoside (IPTG) and cells were further incubated for 18 h at 25^0C.The cells were centrifuged to remove the supernatant and washed with sterile Phosphate Buffered Saline (PBS). PBS 1M solution of pH 7.4 contain 137mM NaCl, 2.7mM KCl, 10mm Na_2HPO_4 and 2mM KH_2PO_4. Cells were stored as frozen pellets at -20^{o}C. The thawed cells were disrupted by sonication in 10mM PBS, centrifuged at 12,000xg for 10 min. The supernatant was collected and mixed with 5X gel loading dye, boiled for 2-3 min and analysed on 12% SDS-PAGE for the expression of recombinant protein with against un-induced cultures. Protein estimation was done by measuring absorbance at 280nm against BSA standard.

6.2.3: Nattokinase/ Fibrinolytic activity

Fibrin plate was used to determine the enzyme activity, which was described by Choi and Kim, (2001), with some modifications. Five millilitres of 6mg/ml(w/v) fibrinogen solution in a 50 mM Tris-Cl buffer (pH 7.5), 5 ml of 2.5% (w/v) agarose solution and 0.1 ml of thrombin solution (1mg/ml) were mixed well in a petri dish and was placed at room temperature for 1 h, to allow the thrombin to convert fibrinogen to fibrin. The culture supernatants were diluted appropriately and 10µl of the diluted supernatant was dropped into a well made in the plate and incubated for 18 h at 37°C. The fibrinolytic activity was observed by measuring the diameter of the clear zone.

Fibrinolytic activity was quantified according to the following protocol (Sigma protocol). Fibrinogen (0.4ml of 6mg/ml) and thrombin 0.1ml of (1mg/ml) were mixed and incubated at 37°C for 10 min under shaking to form the fibrin. A 0.1ml aliquot of appropriately diluted enzyme was added to the above mixture and whole cocktail was incubated at 37°C for 20 minutes. The enzyme reaction was

stopped by adding 0.4ml of 10% trichloro acetic acid (TCA). The enzyme blank was prepared by adding TCA to the mixture prior to the addition of the enzyme. The difference in absorbance at 275nm for test and blank were observed. The standard Urokinase Enzyme Solution was diluted in ice cold 50mM Tris.C.l (Immediately before use, prepare a solution. One unit will activate that amount of porcine plasminogen which will produce an A275nm of 1.0 per ml per minute at pH 7.5 at 37°C, when measuring perchloric acid soluble products from a-casein (1 cm light path). Urokinase from Win medicor, NewDelhi, India was used as the standard.

6.2.4: Purification and characterization of cloned keratinolytic protease

Over expressed enzyme after IPTG induction was recovered by cell lysis as mentioned in Section 6.2.2.10. The cells were separated by centrifugation (12,000*g* for 15 min at 4 °C) and the supernatant was concentrated by ultrafiltration (MW cut off, 50 kDa, Amicon,Millipore Inc.USA) and charged on casein-affinity column and purified in the similar manner as described in section. 5.2.1. The purified enzyme was evaluated for caseinolytic and keratinolytic activity, preference to synthetic peptides, hide depilation and hydrolysis of the milled feather as described in sections 2.2.10 and 2.2.12.

6.2.5: Modelling and phylogenetic analysis of cloned protease

Translated Protein sequence of protease obtained from gene sequencing, was subjected to BLASTP with PDB database to search homologs. Structure of protease was modeled with the best match using MODELLER 9. Modeled structure of cloned keratinolytic protease as validated with Ramachandran plot using PROCHECK and was used to determine structural features of the protein. Protein sequence of protease from *B. subtilis* P13 was subjected to BLASTP to search homologs for multiple sequence alignment. 50 homologs were taken and multiple sequence alignment and phylogenetic analysis was done using **phylogeny.fr** server.

6.3: Results

6.3.1: PCR amplification of keratinolytic protease from *B. subtilis* P13

The protease gene showing homology to nattokinase was amplified by PCR using primers NK1 and NK2 from the total DNA of the isolated bacterium *B. subtilis* P13.

A single band of about 1,500 bp **(Fig. 6.4a)** was obtained. pJET1.2 vector which is an advanced positive selection system for the highest efficiency cloning of PCR products generated with *Pfu* DNA polymerase. This vector pJET1.2 contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site (**Fig. 6.2**), resulting the propagation of cells only with recombinant plasmids. The PCR product was purified by tube in tube method and cloned in pJET1.2 vector and cloned in pJET1.2. Ten colonies were randomly selected and were further analysed by digesting with with Bgl II sites for which flank the insert and 1.5Kb fragment was released (**Fig. 6.4b**). **Fig. 6.5** shows the map of the recombinant plasmid, pJET- AJ-Kpr. A total of 30 colonies were obtained after cloning in pJET1.2 which indicated the putative clones Confirmation of the selected clone (**Fig. 6.4c**) was carried out using Primers NK1 and NK2 (**Fig. 6.5**) and was sequenced at the sequencing facility of Exceleris Ltd. Ahemadabad, India.. RE map of the cloned gene is depicted in **Fig. 6.6** .The sequenced DNA was analyzed via BLAST at NCBI, which revealed that the obtained DNA sequence exhibits 100% homology with the subtilisin NAT (aprN) gene (**Appendix VI & VII**) (Nakamura et al., 1992). The sequence revealed one open reading frame (ORF) of 1,143- bp, with the start codon ATG.

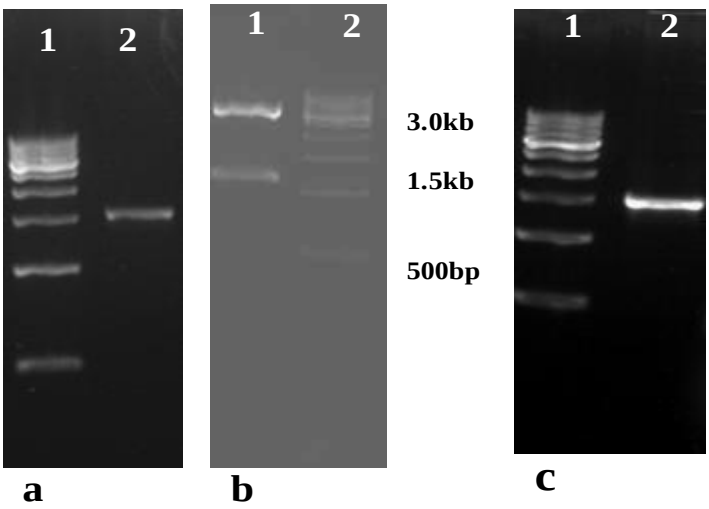


Fig. 6.4 Cloning of keratinolytic protease gene from *B. subtilis* P13.

a. PCR amplification of protease gene using NK 1and NK2; b. RE digestion of pJET-AJ-Kpr clone with vector specific enzyme BglII; c. PCR ocnfirmation of putative clone from blunt ended cloning in pJET vector.Lane1 inFig.6.4a&b : 500bp ladder and lane.2: 1.5 kb amplicon and viceversa in **Fig. 6.4b**

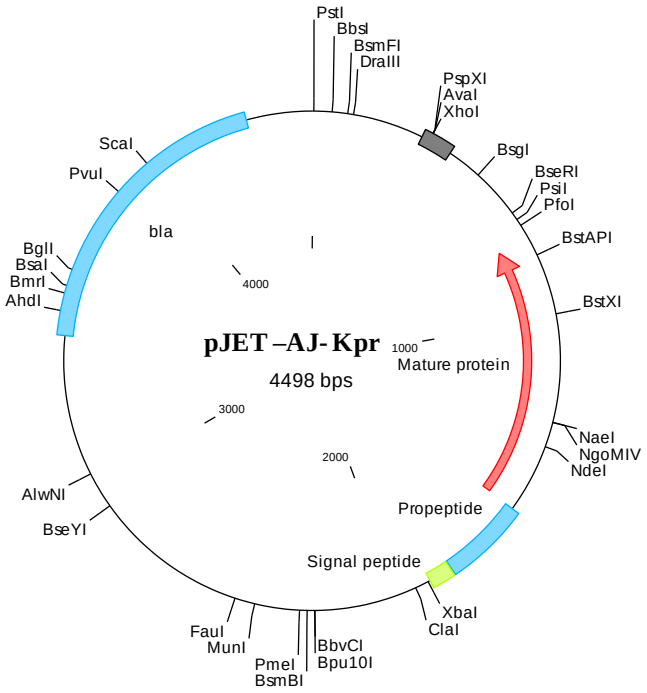


Fig. 6.5: Map of pJET AJ KPr construct harbouring nattokinase like keratinolytic protease gene from *B. subtilis* P13

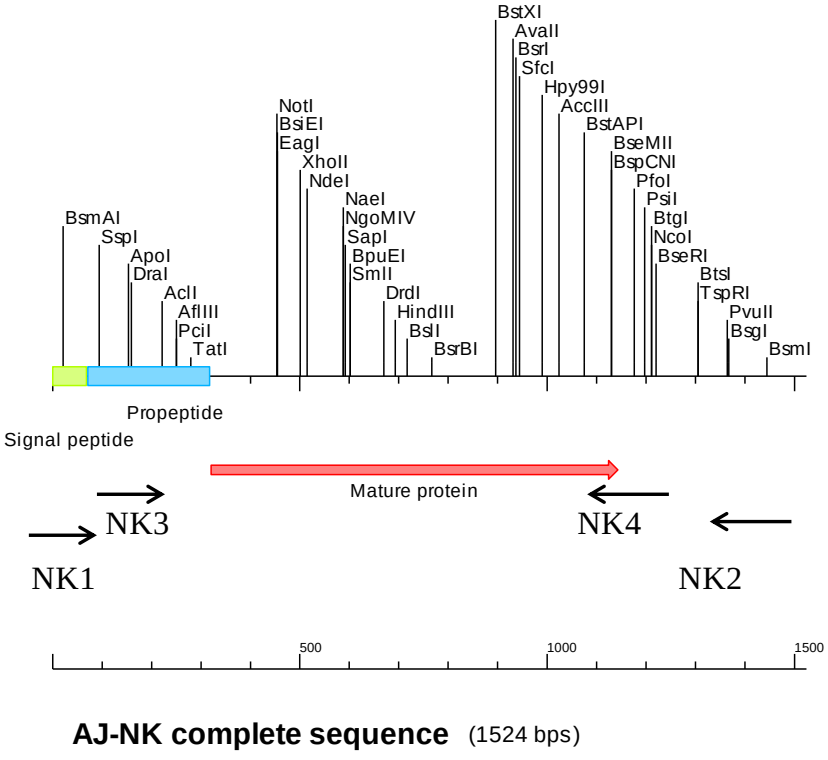
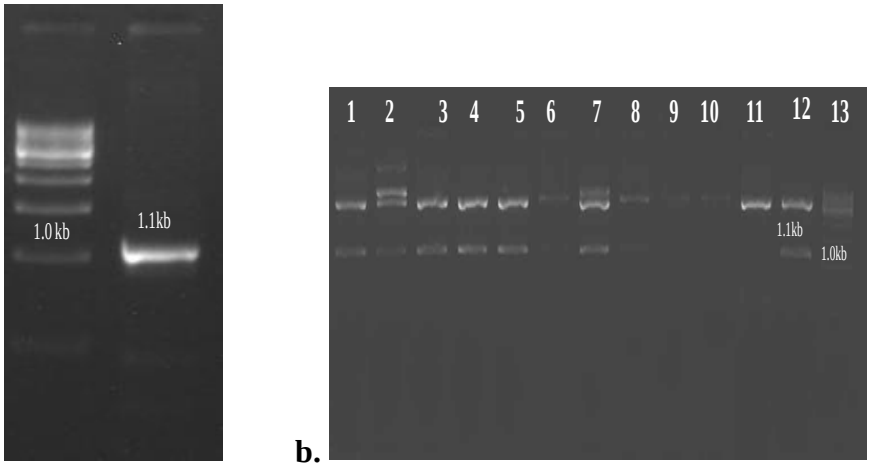


Fig. 6.6: Primer binding sites and RE map of PCR amplified gene for nattokinase like protease from *B. subtilis* P13

PCR amplification of coding region of keratinolytic protease gene having homology to nattokinase (1100bp) was carried out from the genomic DNA of *B.*

subtilis P13 using primers NK3 and NK4 (**Fig. 6.7a**). Amplicon was cloned in blunt end cloning vector pJET 1.2 and putative clones were confirmed by the insert release of 1.1Kb using vector specific RE,*Bgl*II (**Fig. 6.7b**). Clone pJET –AJ-Kpr4 was sequenced and the sequencese deposited at the NCBI gene bank (accession #:JF921199). On BLAST atnalysis , the cloned gene revealed 100% homology to aprN gene,which codes for a fibrinolytic enzyme (**Appendix VIII and IX**). The deduced protein of the ORF contained 381 amino acids. The sequence of the protein-coding region of this fibrinolytic enzyme was found to include a 29-residue signal peptide, a 77-residue propeptide, and a 275-residue mature enzyme.



Lane.1, 500bp ladder
Lane. 2, 1.1 kb PCR amplicon

Lane 1-12: putative clones(pJET-AJ-Kpr)
Lane.13: 500bp marker.
Clone pJET-AJ-Kpr4 from lane 4 selected for subcloning in pET22b (+)

Fig. 6.7: PCR amplification of clones of coding region of nattokinase (a) and confirmation of putative Karatinolytic protease by BgIII digestion (b)

6.3.2 Sub-Cloning of pJET Kpr harbouring the coding region of nattokinase like protease in pET22b (+)

Cloning of gene fragment of *B. subtilis* P13 protease in pET22b(+) was carried out for its expression in *E.coli* under the strong T7 promoter at the *Nco*I and BamHI sites in the MCS. The map of the recombinant construct pET-AJ-Kpr is depicted in **Fig. 6.8**. The recombinant protein is expected to have a 6X His tag at the N-terminus. The vector was digested with with Nco I and BamHI (**Fig. 6.8**). Since the gene has an internal site for NcoI, (**Fig. 6.5**) BspHI an enzyme produces compatible ends with NcoI was used for

cloning. Insert was digested BspHI and BamHI (**Fig. 6.9**) and ligation in to digested pET22b (+) yielded 30 putative clones. Thirty putative clones obtained from the pET22b (+), plasmids were extracted and analysed by using two approaches, digestion with XbaI and Hind III, expecting the the release of of 1.1Kb insert. Except in five cases, two fragments of around 700 and 500bp. were obtained on the gel, (**Fig. 6.10**). The reason for the above said restriction pattern was the presence of HindIII site at the 447th position **(Fig. 6.5)** of the cloned NK gene.Clones were also further confirmed by PCR using gene specific primers NK3 and NK4 **(Fig. 6.11**). An amplicon 1.1Kb confirmed the clones.

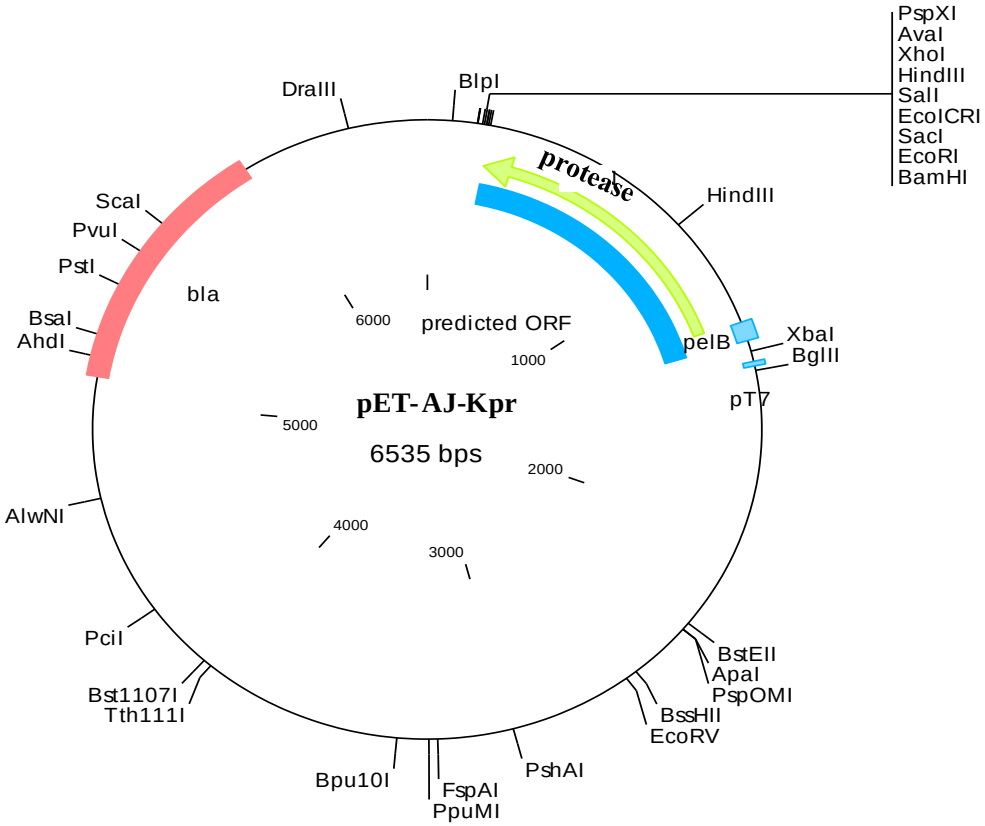
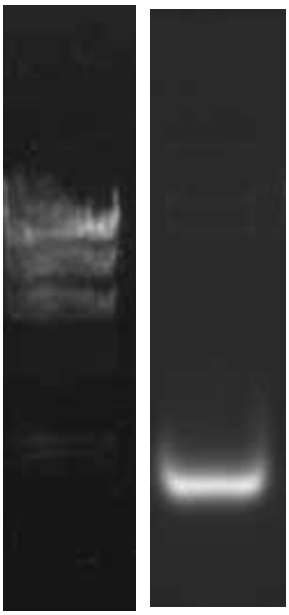


Fig. 6.8: Map of the pET 22+ construct, pET-AJ-Kpr harbouring protease gene



a **b**

Fig. 6.9: pET 22b (+)plasmid preparation(a) and digestion and linearization (b)
Linearized plasmid from lane 2 was used for subcloning

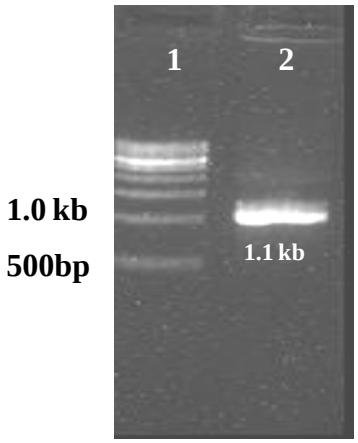


Fig. 6.10. Double digested (BspHI and BamHI) pET-AJ- Kpr.
Insert released after the digestion with BglII was subjected to double digestion.
Lane.1:500bp ladder and lane.2 : insert released

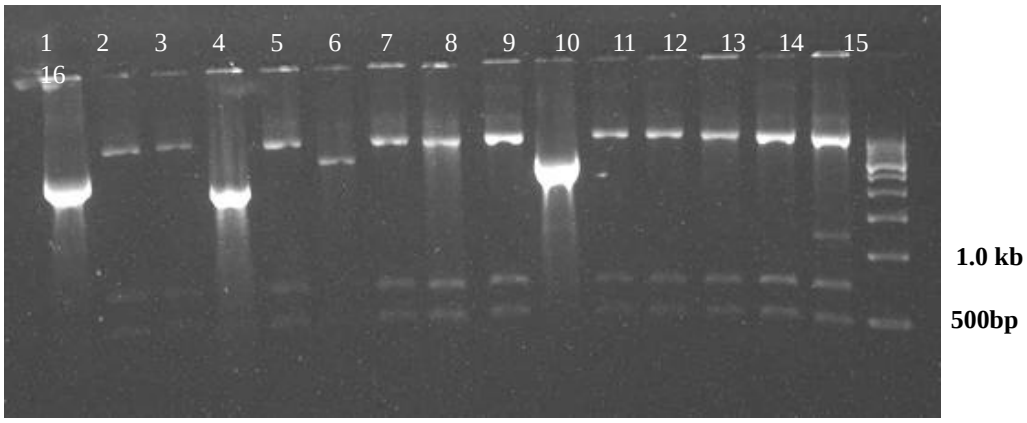


Fig. 6.11: RE digestion of putative clones using XbaI and Hind III, showing the release of two fragments of 460 and 640 bp.

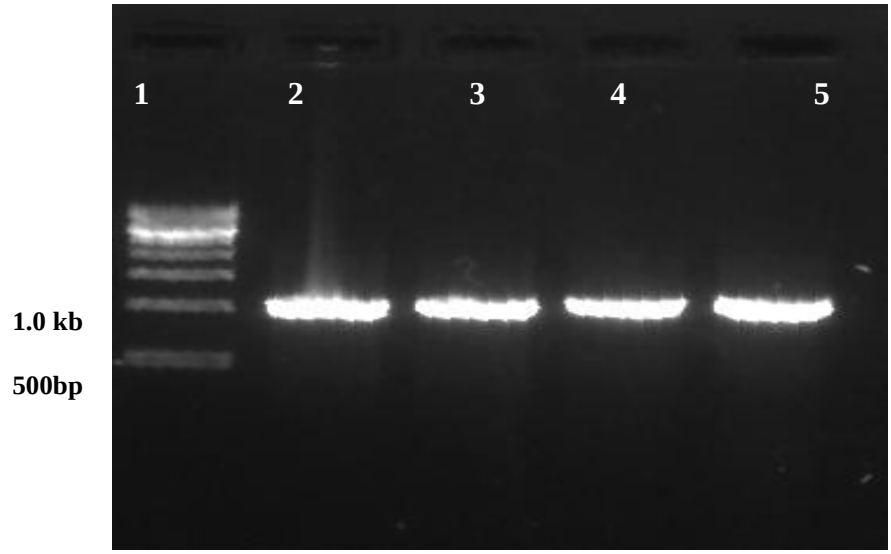


Fig. 6.12: PCR confirmation of selected clones.
Clones from lane # 7,8,12 and 13 of fig.6.11 are considered as pET-AJ-Kpr1,2,3 and 4 respectively.

6.3.3: Expression analysis keratinolytic protease in construct pET-AJ-Kpr

The transformants were called as pET-AJ- Kpr series. The protease expression level of clone pET-AJ-Kpr1,2 and 3 were compared with vector control. All 3 clones showed the presence of a 40 kDa band in induced cultures in contrast to vector control and un-induced culture (**Fig. 6.13**).,thus it indicated that cloned protein was expressed in these clones. pET-AJ-Kpr3 clone in lane 4 was selected for sequencing and further characterization of the protein (**Fig. 6.14**). Functional

analysis based on caseinolytic activity carried with pET-AJ-KPr3.. No protease activity was observed in the extracellular fractions of the clones after induction, activity with respect to different duration of induction was depicted in **Fig. 6.15** .Nil activity was recorded for induced vector control. The molecular weight of the over expressed protein was around 40kDa. The protein band of about 40 kDa appeared in the intracellular lysate, which might be the unprocessed nattokinase containing the signal peptide PelB, the propeptide and the mature peptide of nattokinase according to the deduced molecular weight.

The N-terminal sequence of the secreted protein was confirmed as AQ**S**VPYGI**S**Q by Edman degradation, and LC-MS analysis of the purified protein. The first 10 aminoacid of the of the over expressed protein on translation (from 45^{th}-55^{th} aminoacids) (**Appendix X**.). showed 100% homology with the N-terminal sequences of the processed extracellular keratinolytic protease of *B. subtilis* P13. First 44 Sequences were showing exact homology to the reported propeptide sequence of subtilisin-nattokinase (**AppendixXII**).

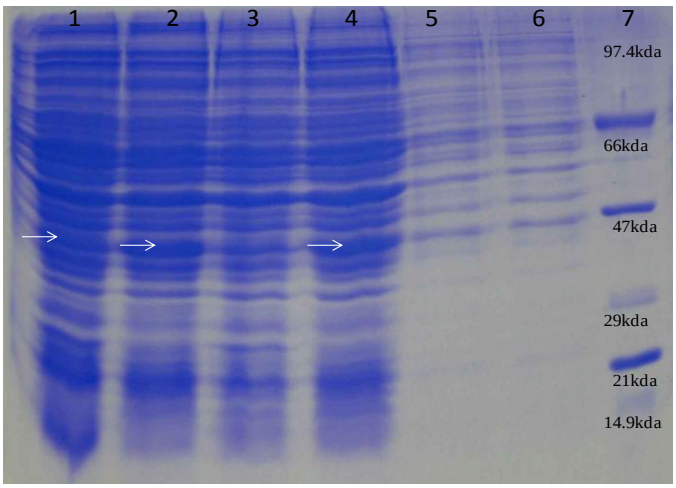


Fig. 6.13: SDS PAGE of NK clones on IPTG induction

Lane.1,2 and 4: IPTG induced pET-AJNK Lane.3. uninduced pET-AJ-NK;Lane.5 : vector control induced;Lane.6: vector control uninduced and Lane7: MW marker (medium range)

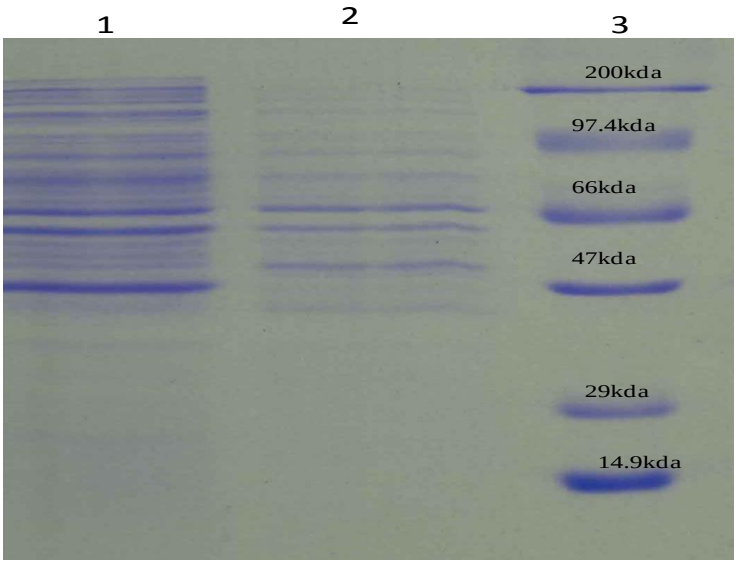


Fig. 6.14: pET-AJ-Kpr 3: clone selected for sequencing

Lane.1 IPTG induced; lane.2: uninduced and lane.3: high Mw marker

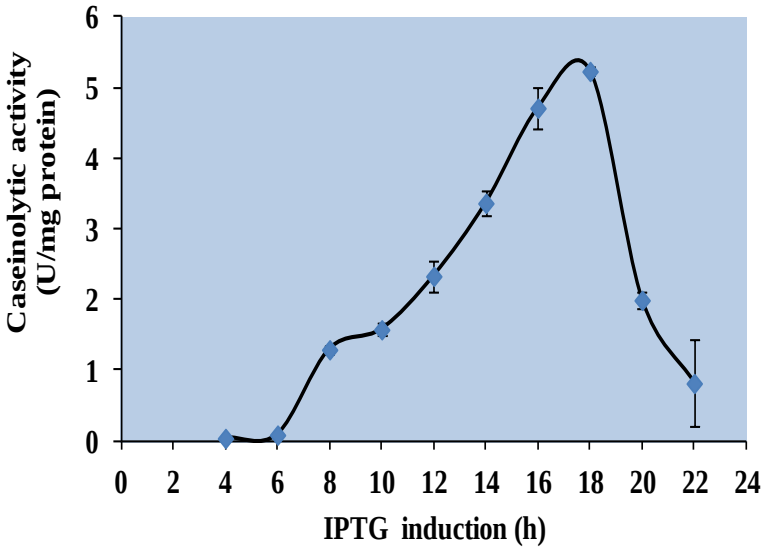


Fig. 6.15: Protease expression levels of pET-AJ-Kpr3 during different durations of IPTG induction

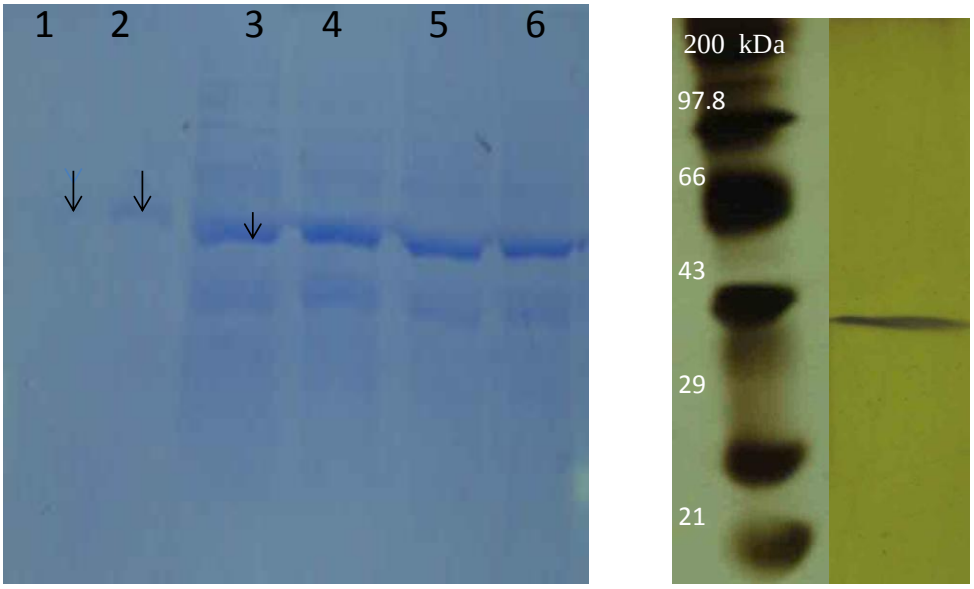
6.3.4: Purification and characterization of keratinolytic protease cloned from *B. subtilis* P13

Cloned keratinolytic protease from *E.coli* BL21DE3 was prepared in the crude form as mentioned in Section 6.2.4 and concentrated by ultrafiltration .The fitereate from 50KDa cut-off was purified to 69.4 fold with a 41.13% recovery by casein affinity chromatography. Purification details are summarised in **Table 6.4.**SDS-PAGE analysis and gelatin zymogram of the purified protease is depicted in **Fig.6.16** and **6.17**

respectively**.** A band of proteolysis corresponding to 40kDa was observed on the zymogarm **(Fig. 6.17)**.

Table 6.4: summary of purification of recombinantkeratinolytic protease

Treatment	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Fold Purification	% Recovery
Crude	26.5	90	0.294	-	100
Ultrafiltration (50kDa filtrate)	22.2	8	2.775	9.4	83.7
Affinity chromatography	10.9	0.434	25.1	85.4	41.13



(A) (B)

Fig. 6.16.Purification profile of the cloned and expressed protein by casein affinity chromatography

(A): Different fractions from column chromatography

(B): Pooled purified fraction showing a protein band of 40kDa

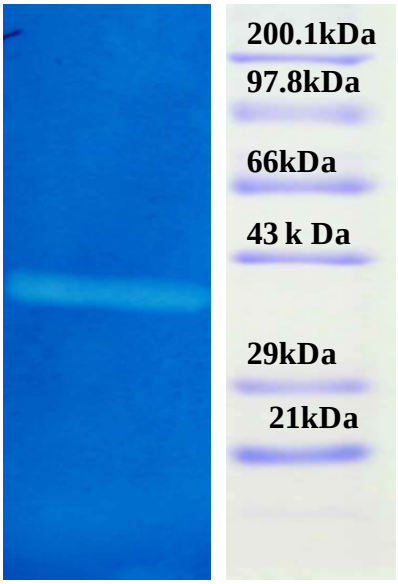


Fig. 6.17: Gelatin zymogram of purified protease from pAJ Kpr

6.3.5: Biochemical properties of cloned keratinolytic protease

Caseinolytic, keratinolytic, collagenase activity and affinity towards synthetic peptides were compared for crude, purified, over expressed and purified enzyme from *B. subtilis* **P13 (Table 6.5).** Other biochemical parameteres like temperature optimum and pH optimum was also performed the biochemical identity of the overexpressed protein sequence.

Table 6.5: Comparision of the activity of crude, purified and cloned keratinolytic and protease from *B. subtilis* P13

Activity (U/mg protein)	Culture supernatant from *B. subtilis* P13	Purified protease from *B. subtilis* P13	Lysate from *E.coli* BL21 (vector control)	Overexpressed protease from *E.coli* BL21
Caseinolytic	0.6± 0.056	4.5±1.06	ND*	5.3±0.987
Keratinase	0.51±0.064	5.1±2.10	ND*	5.19±1.89
Collagenase	0.09±0.012	ND	ND*	ND*
Fibrinolytic	53 ± 2.34	349±5.79	ND*	572±5.054
pNP acetate	0.68±0.12	4.89±1.10	ND*	4.29±3.37
AAA-PFpNA	12.9±3.4	135±6.23	ND*	146±4.91
AAVpNA	0.945±0.897	15± 3.41	ND*	18± 2.53

*Not detected

6.3.6: Evaluation of the cloned enzyme for tannery applications

To confirm functional similarity of the over expressed protein with respect to its native counter part, soaking, dehairing, milled feather degradation and hydrolysis of thermally denatured chromeshavings etc.were compared among crude, purified and overexpressed-purified enzyme (**Table 6.6**). Efficiency to perform above applications were more or less similar with same quantity of purified and overexpressed enzyme, but the whole process was faster with lesser amount of enzyme in comparision to the crude enzyme.

Table 6.6: Comparision of application efficiency of crude, purified and over expressed protease from *B. subtilis* P13

Properties studied	**Culture supernatant From *B. subtilis* P13**			**Purified protease from *B. subtilis* P13**			**Overexpressed protease from pAJKpr**		
	Units	Duration	Efficiency	Units	Duration	Efficiency	Units	Duration	Efficiency
Soaking	2	18h	19% swelling	0.2	4h	22%swelling	0.2	4h	20%swelling
Dehairing	2	16h	100% dehairing	0.2	4h	100% dehairing	0.2	4h	100% dehairing
Feather hydrolysis in presence of 0.1% βME	2	12h	95% weight reduction	0.2	4h	96% Weight reduction	0.2	4h	93.7% Weight reductio
Feather hydrolysis	2	24h	98% Weight reduction	0.2	24h	47% weight reduction	0.2	24h	50% Weight reductio
Hydrolysis of CS	2	24h	98.46% Weight reduction	0.2	24h	54% Weight reduction	0.2	24h	61% weight reduction

6.3.7: Nattokinase activity of cloned keartinolytic protease from *B. subtilis* P13

The fibrinolytic activity of cloned keratinolytic protease to nattokinase was evaluated by urokinase activity (**Fig. 6.18**) and fibrin plate assay (**Fig. 6.19**). The N-terminal sequence of the cloned keratinolytic protease was compared with other reported nattokinases from *Bacillus*.sp.(**Table 6.7**) .The first 10 aminoacids of the nattokinase like keratinolytic protease from *B. subtilis* P13 showed 100% homology with all, except *B. subtilis* CK and *B.amyloliquefaciens*

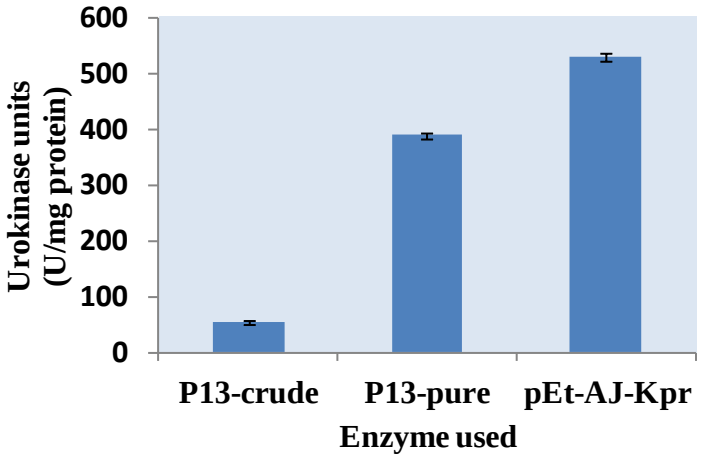


Fig. 6.18: Comparision of fibrinolytic activity of keratinolytic protease

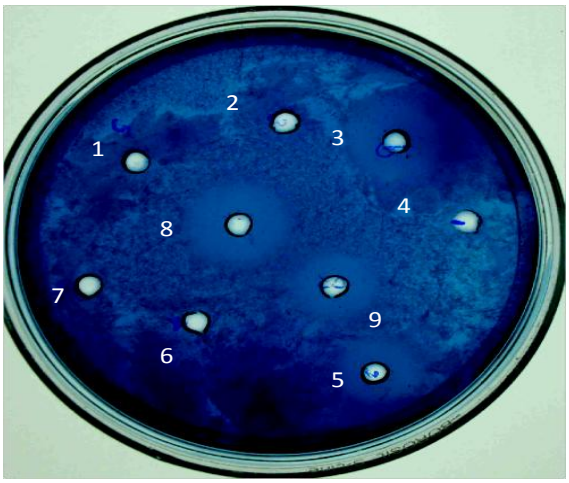


Fig. 6.19: Fibrin plate assay. Clear zone around the wells are due to fibrinolysis

1. Reagent blanks from urokinase assay; 2.Uninduced pET AJKpr: 3.Induced pETAJKpr; 4. Optimized PM of *B. subtilis* P13; 5. Culture supernatant of *B. subtilis* P13 from PM;6. Vector control uninduced;7.Vector control induced: 8. Urokinase standard; 9.Purified protease from *B. subtilis* P13

Table 6.7: Comparision N-terminal sequence of keratinolytic protease from *B. subtilis* P13 with other reported nattokinase from Bacillus.sp

Microorganism	Source	Name of the enzyme	MW.pI. Opt.Temp pH	N-terminal sequence	Reference
B. natto	Natto, Jap	Nattokinase, NK	27.7 kDa, pI 8.6	AQSVPYGISQIKA PALHSQGYTGS	Fujita et al., 1993
B.amyloliquefaciens	Douchi, DC-4 China	Subtilisin DFE	28 kDa, pI 8.0, pH 9.0, 48°C	AQSVPYGVSQIK APALHSQGFTGS	Peng et al., 2003
***Bacillus sp.* CK**	Chungkook-jang, Korea	CK	28.2 kDa, pH 10, 70°C	AQTVPYGIPLIKA D	Kim et al., 1996
***Bacillus sp.* DJ-4**	Doen-jang, Korea	Subtilisin DJ-4	29 kDa, pH 10.0, 40°C	AQSVPYGVSQIK AP	Kim and Choi ,2000
***B. subtilis* QK02**	Fermented soybean	QK-1 andQK-2	28 kDa, pH 8.5, 55°C	AQSVPYGISQIKA PALHSQG	Ko et al.,2004
***B. subtilis* P13**	Hot spring isolate	Keratinolytic serine proteas	28kDa, pH 7.2,60°C pI 6.33	AQSVPYGISQ	Present study

6.3.8: Modelling of cloned keratinolytic protease

All protein structures were modeled by comparative modelling techniques with Discovery studio 3.1. Protein BLAST of pET-AJ-Kpr protein shows high homology to subtilisin proteins having similarity/identity ranging from 98-100%. The homolog which has been chosen for homology modeling of the cloned protein is (PDB ID: 1MEE, 1MEE sequence identity= %) having sequence identity of 99%. The given sequence identity shows the most nearby/best homolog of subtilisin. Homology modeling of the protein was on the basis of 1MEE results in the highly accurate modeled structure with RMSD xyzA0 (**Fig. 6.20**).

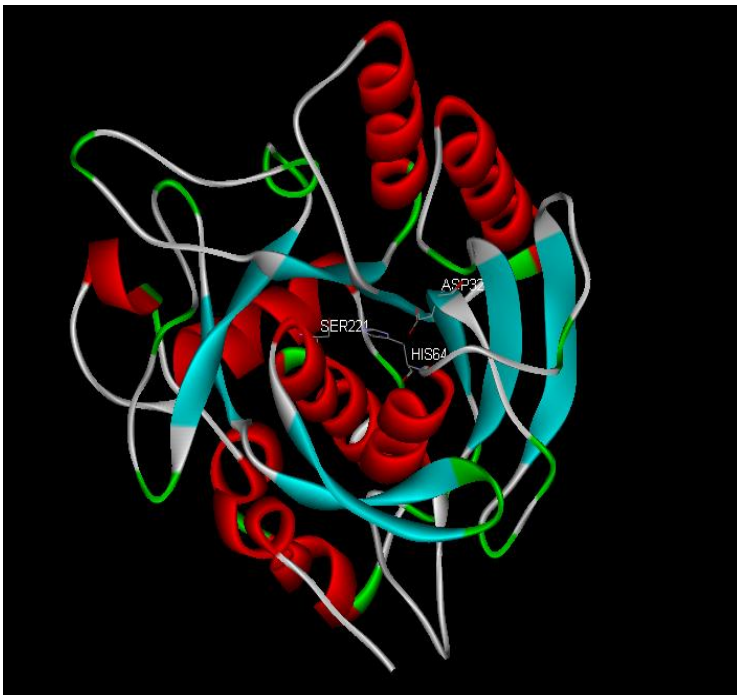


Fig. 6.20: Schematic representation of the secondary structure topology of cloned keratinolytic protease from *B. subtilis* P13

α- helices shown as cylinders and β-sheet strands as *ar*rows. Substrate pocket is in the vicinity of catalytic triad ASP32 – HIS64 – SER221

Model deduced along with 44 sequences of propeptide showed a proper attachment to the subtilisin as separate structure (**Fig. 6.20**).

Energy minimization of modeled subtilisin protein made it energetically stable and orientation of each amino acids became stable and accurate. Ramachandran plot of modeled subtilisin showed that all amino acids of modeled subtilisin fall in allowed regions. These result indicated that the conformation of all amino acids are accurate/standard which provides proper conformation of modeled pAJNK protein. Thus, the results obtained showed that the structure is conformationally accurate.

Structural characteristics/features were determined on the basis of 1MEE. The catalytic triad of ASP32 – HIS64 – SER221 and substrate specificity pocket was identified. These catalytic triad and substrate specificity pocket are very important structural features of the protein as it contributes to its activity (**Fig. 6.21**).

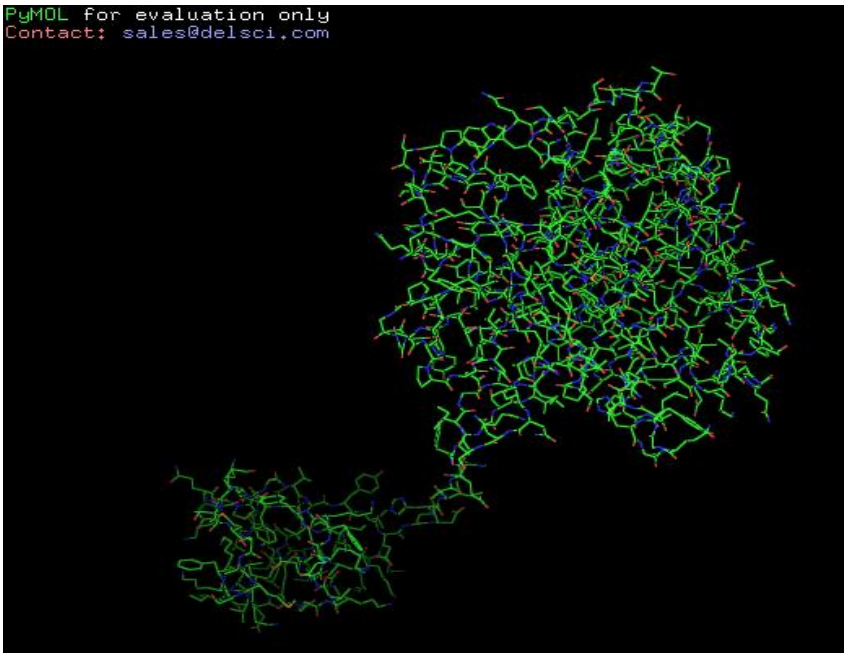


Fig. 6.21: Structure of protease with propeptide attached to it.

Propeptide is having autocatalytic property and gets cleaved once the protease folded into its right conformation

6.3.9: Phylogeny of cloned keratinolytic protease

Phylogenetic tree of nattokinase like keratinolytic protease protein showed, the similarity of a protein with other proteins having the structural and functional relationship. BLASTP of the protein resulted in highly identical homologs. 50 homologs were taken which shows the similarity with subtilisin protein. Phylogenetic tree revels that the one complete group of 8 to 10 proteins having 99% relationship with subtilisin protein which would have similarity in structure and function (**Fig. 6.22**). The analysis shows that the isolated subtilisin protein has maximum evolutionary relationship with subtilisin proteins from *B. subtilis*. Thus, structural and functional similarity analysis of the over expressed protein with other proteins of phylogenetic groups provide the information regarding the function and structural characteristics of the subtilisin protein. The most evolutionary related subtilisin proteins are *B. subtilis* thermostable alkaline serine protease and *B. subtilis* fibrinolytic enzyme AprE8. This observation is reconfirms the similar observation obtained from LCMS analysis and also the accuracy of the structure which has been predicted. The other proteins have approx. 80% similarity with subtilisin protein which indicates the functional evolution of subtilisin.

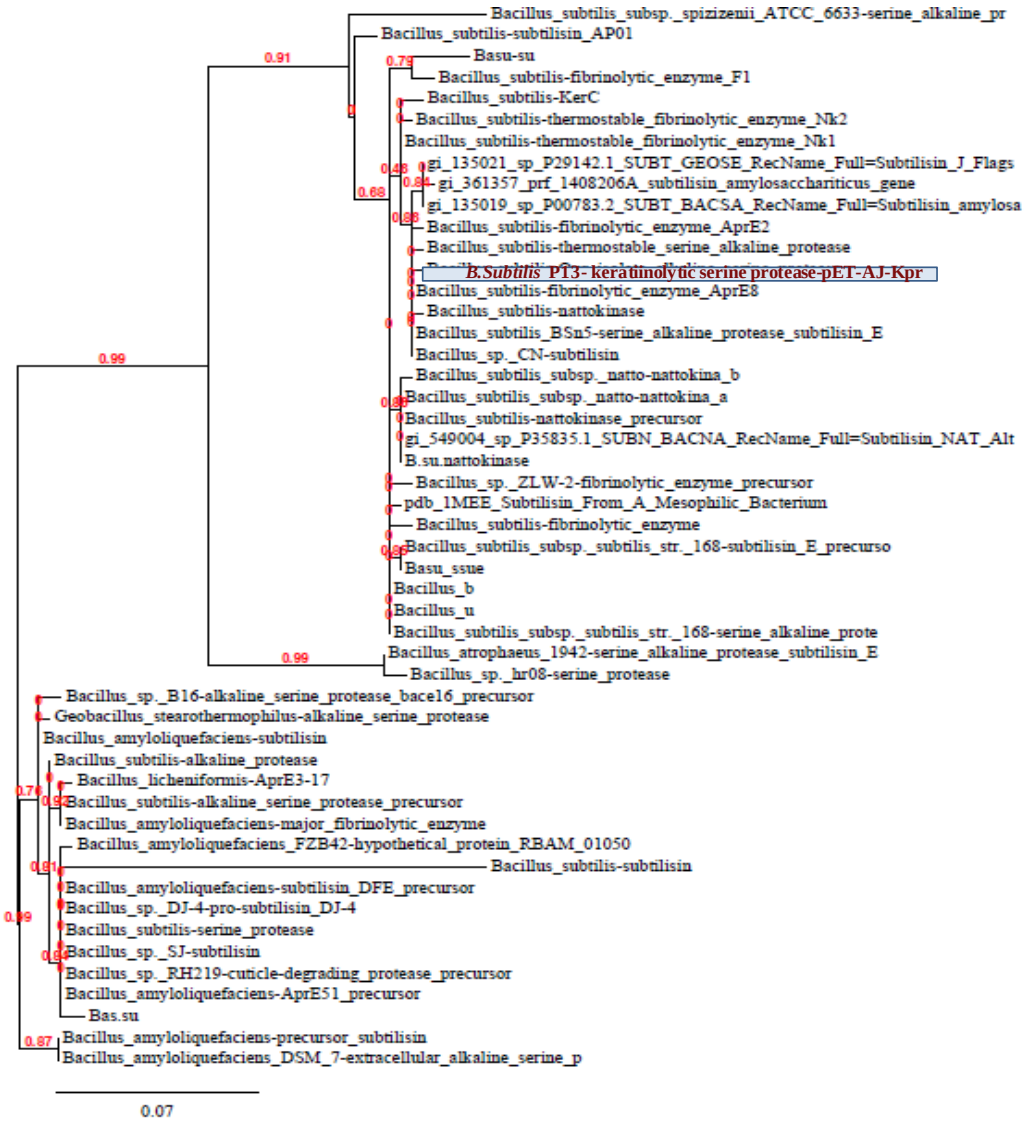


Fig. 6.22: Phylogenetic tree of cloned keratinolytic protease from *B. subtilis* P13where the enzyme is positioned accordind to its functional homology

6.4: Discussion

Keratinolytic protease having fibrinolytic activity was successfully expressed in recombinant *E.coli*-BL21 (pEt-AJ-NK) under T7 promotor. The leader peptide was pelB and maximum activity was observed in the cell lysate. Different induction conditions were tried in presence of IPTG and maximum specific activity of 5.3 caseinolytic units and 500 urokinase units observed after induction at 25°C for 18h. The pelB signal peptide was from Yerwinia and shown to have the capability of directing the synthesized foreign protein experiments through the cytoplasmic membrane of *E. coli* (Lucic et al., 1998). Even though the production of nattokinase

in *B. subtilis* is considerable, the long growth cycle, high endogenous proteases and the excessive byproducts of *B. subtilis* are not favourable for the development of this enzyme into therapeutic medicine for thrombosis. The *E. coli* expression system, by contrast, could surmount such disadvantages, and can be successfully used in the production of heterologous proteins Periplasmic expression of nattokinase in presence of pELB and secretory expression in presence of native promotor was reported by Laing et al., (2007).

The over expressed keratinolytic serine protease from *B. subtilis* P13 showed 100% homology to fibrinolytic nattokinase, subtilisin NAT (Tsukamoto et al. 200). Xiaobo et al, 2007, reported the heterologus expression of fibrinolytic protease, which also showed homology to subtilisin NAT except a serine at 258. The difference between fibrinolytic protease aprE and subtilisin NAT (Tsukamoto et al. 2000) consists only in one amino acid at 258, which is serine in the former instead of asparagine in the latter. The yield of active nattokinase from *B. subtilis* (natto) (Tsukamoto et al. 2000) in the culture of the recombinant *E. coli* is related to the temperature. However, at a relatively low temperature a higher yield of active nattokinase was detected. Pfeifer (Pfeifer etal 2001) proposed that a moderate decrease of culture temperature could improve the expression of recombinant proteins in *E. coli*, and Missiakas and Raina, (1997) suggested that lower temperatures could help propeptide to fold properly. The propeptide of subtilisin (Fu etal 2000) and a-lytic protease (Baker et al 1992) was also reported to be necessary for active expression. Compared to the extracellularly produced protease by the wild strain (0.6U/mg protein for crude lysate from *B. subtilis P13* and 5.3 U/mg protein for the *E.coli* BL21 clone,pET-AJ-NK), protease activity was 8 fold higher.

Other biochemical properties were all compared and confirmed that the serine protease produced by *B. subtilis* P13 is a thermostable keratinolytic enzyme having fibrinolytic activity. There are many reports on recombinant keratinases. In recombinant *P. pastoris*X33 (pPZK3), the *ker* gene was successfully expressed under alcohol oxidase (*AOX1*) methanol inducible promoter and keratinasewas secreted using the *Saccharomyces cerevisiae* a-factor signal sequence. Compared with wild type strain *B. licheniformis* MKU3, recombinant *Pichia* strain has shown 2.9-fold increased keratinase production. Similarly, recombinant *P. Pastoris* strain expressed the *Thermomyceslanuginosus* xylanase (XynA) at high yield (3-fold) after

96 h of induction(Radha and Gunasekaran,2009). However,*P. Pastoris* transformants carrying the keratinase gene from *B. licheniformis* PWD-1 has shown steady increase in keratinase activity (285 U/ml) up to 144 h after the induction (Porres et al.,2002). *Aspergillus fumigates ker* gene in *P. pastoris* produced maximum keratinase (1 U/ml) in 72 h of growth (Noronha et al., 2002). The heterologusly expressed keratinolytic protease from *B. subtilis* P13 was purified to 85.4 fold purity by casein affinity chromatography. Purification of recombinant keratinase from *P. pastoris* (pPZK3) through nickel affinity chromatography with 85.55 fold purity was reported by Radha and Gunasekaran, 2009. The same research group reported a recombinant strain of *B. megaterium* produced keratinase constitutively under a-amylase promoter (3-fold) higher than the wild type strain (Radha and Gunasekaran, 2008).

An alkaline protease gene , named the AP gene) was cloned from *B. pumilus* UN-31-C- 42 and the N_-terminalamino acid sequence of the deduced mature proteaseis identical to that of the purified dehairing protease DHAP (Pan et al.,2004). The AP gene was expressed in *B. subtilis* WB600 successfully and the expressed product had an identical molecular weight to the protease DHAP, which also had alkaline protease activity and dehairing activity (Huang et al.,2003).Aoyama et al,(2000) have reported an extracellularalkaline serine protease, APRP, which can coagulate soybean milk, and the gene aprP from *B. pumilus* TYO-67. However, they have not identified the dehairing effectof this protease.

Cloned keratinolytic protease having fibrinolytic property is a high value protein, par with commercially available fibrinolytic enzyme, urokinase. The microbial fibrinolytic enzymes, especially those from food-grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure cardiovascular diseases. NK has already been developed as drugs in the market, including Nattokinase NSKSD, Jarrow NattoMax JR-154, and Natto-K. (Sherry, 1987).Development of other microbial fibrinolytic enzymes is still ongoing, and much work needs to be done, especially concerning thrombolytic effects in vivo. The new trend for improving thrombolytic agents is to increase the efficacy and fibrin specificity, focusing on developing effective targeted thrombolytic agents. Several reports have illustrated successful construction of the chimeric proteins, in which a thrombus-specific polypeptide or antibody was attached to the plasminogen activator

to enhance the thrombolytic specificity (Ruppert et al., 2003; Tait et al., 1995). These advances direct the way of future research on microbial fibrinolytic enzymes .Keratinolytic protease from *B. subtilis* P13 can also be explored further to develop it further as a food additive or drug, which could be administered with ease. The modelled structure appeared similar to that of reported structure of the subtilisisns (Siezen and Leunissen, 1997***).*** The structure suggests that the triad architecture of ser-His-Asp shares the catalytic site with other serine proteases. Protein engineering can be employed to improve the catalytic property and also to adapt it low temperature.

6.5: Conclusion

Keratinolytic protease from *B. subtilis* p13 was expressed to 9.5 fold higher levels heterologusly in *E.coli* BL21 under T7 promotor. The protein was purified to 85.4% homogeneity by casein affinity chromatography. The expressed protein showed a molecular weight of 40kDa on SDS-PAGE and the same was confirmed on gelatine zymogram. The same keratinolytic protease secreted out by the wild type is of 28kDa. The higher molecular weight of the ecloned protein is attributed to the additional 77aminoacids coding for the propeptide. Propeptide is required for the proper folding of the protein and is autoctalytic and gets cleaved before secretion. The potential of the enzyme for soaking, dehairing, feather hydrolysis and the hydrolysis of chromeshavings were confirmed. The identity of the protein to nattokinase was confirmed by sequencing of the cloned gene and the functionality was confirmed with respect to urokinase activity, and in terms of its phylogenetic position, the enzyme branched along with fibrinolytic enzymes. The 3-D modelled structure was exactly matching with subtilisin having a catalytic traid of ASP32 – HIS64 – SER221.

APPENDICES

A wise man will make more opportunities than he finds.
- Sir Francis Bacon

APPENDIX – I

Composition of SDS-PAGE reagents (Sambrook and Russell, 2001)

(A) Monomer solution (30%) (Store at 4º C in dark)

Acrylamide	14.6 g
Bisacrylamide	0.4 g
D. H20	Till 50 ml

(B) Resolving gel buffer- 1.5M Tris (pH 8.8) Adjust pH with HCl

Tris base	9.1 g
SDS	0.2 g
D. H20	Till 50 ml

(C) Stacking gel buffer 1.0M Tris (pH 6.8) Adjust pH with HCl

Tris base	3.02 g
SDS	0.20 g
D. H20	Till 50ml

(D) Tank Buffer (pH 8.3)

Tris base 6.0 gm
Glycine 28.8 gm
SDS 2.0 gm D.
H20 Till 2 L
Adjust the pH with HCl

(E) Sample Loading buffer (2X)

SDS	4%
Glycerol	20%
Tris-Cl (pH6.8)	0.125M
Bromophenolblue	0.05%w/v
β-mercaptoethanol	10mM
D. H20	Till 10ml

(F) Other reagents

APS (fresh)	10% (w/v)
TEMED	2-3 µl

Water saturated n-butanol

Mixed molecular weight protein marker - 14.9-96 kDa (Bangalore Genei.)

(G) Separating Gel (8%, 10ml)

30% Monomer	2.0 ml
Separating gel buffer (pH 8.8)	1.3 ml
SDS (10%)	50µl
D. H20	1.6ml
10% APS	50 µl
TEMED	2 µl

(H) Stacking Gel (3.9%, 5ml)

30%Monomer	0.5 ml
Stacking gel buffer (pH 6.8)	0.38µl
SDS (10%)	30µl
D. H20	10% 2.1 µl
APS	30µl
TEMED	3 µl

(I) Staining Solution

0.025% Commassie Blue R- 250 in 40% Methanol and 7% Acetic acid

(J) De-staining solution

(10% methanol and 10% Aceticacid)

APPENDIX - II

Characteristics of protease producers from different habitats

Culture #	Cz/Cs 37°C	Basal activity	Cz/Cs 15°C	Cz/Cs 50°C	Relative activity pH 3	Relative activity pH.11	Gram's nature
1	10	0.8	1.25	1.73	17	33	+ ve ,cocci
2	8	0.56	NA	NA	NA	NA	-ve,cocci
3	6	0.9	NA	NA	NA	NA	+ve,rods
4	2.2	1.6	0	0	0	0	+ve,rods
5	1.8	0.6	0	0	0	0	+ve,rods
6	2	1.2	1.25	1.8	0	42	+ve,rods
7	3.3	0.65	NA	NA	NA	NA	+ve,rods
8	1.8	0.9	2.3	2.2	0	6.8	+ve,rods
9	4.6	0.55	NA	NA	NA	NA	-ve,cocci
10	2	0.09	NA	NA	NA	NA	+ve,rods
11	2.5	0.6	NA	NA	NA	NA	+ve,cocci
12	2	1.25	NA	NA	NA	NA	+ve,long rods
13	**1.8**	**0.8**	**1.28**	**1.42**	**20**	**54**	**+ve,short rods (selected)**
14	3	1.2	NA	NA	NA	NA	+ ve, slender rods
15	2	0.8	NA	NA	NA	NA	+ve, coccobacilli
16	2	1.25	1.16	1.7	0	0	+ve,cocci
17	4	0.6	NA	NA	NA	NA	+ve,cocci
18	2.5	1.4	NA	NA	NA	NA	+ve,rods in chain
19	2	1.4	1.13	1.5	0	0	+ve,rods in chain
20	2.5	0.7	NA	NA	NA	NA	+ve, rods

Isolate #13 was selected for the study and identified as *B. subtilis* P13

*NA: No activity.

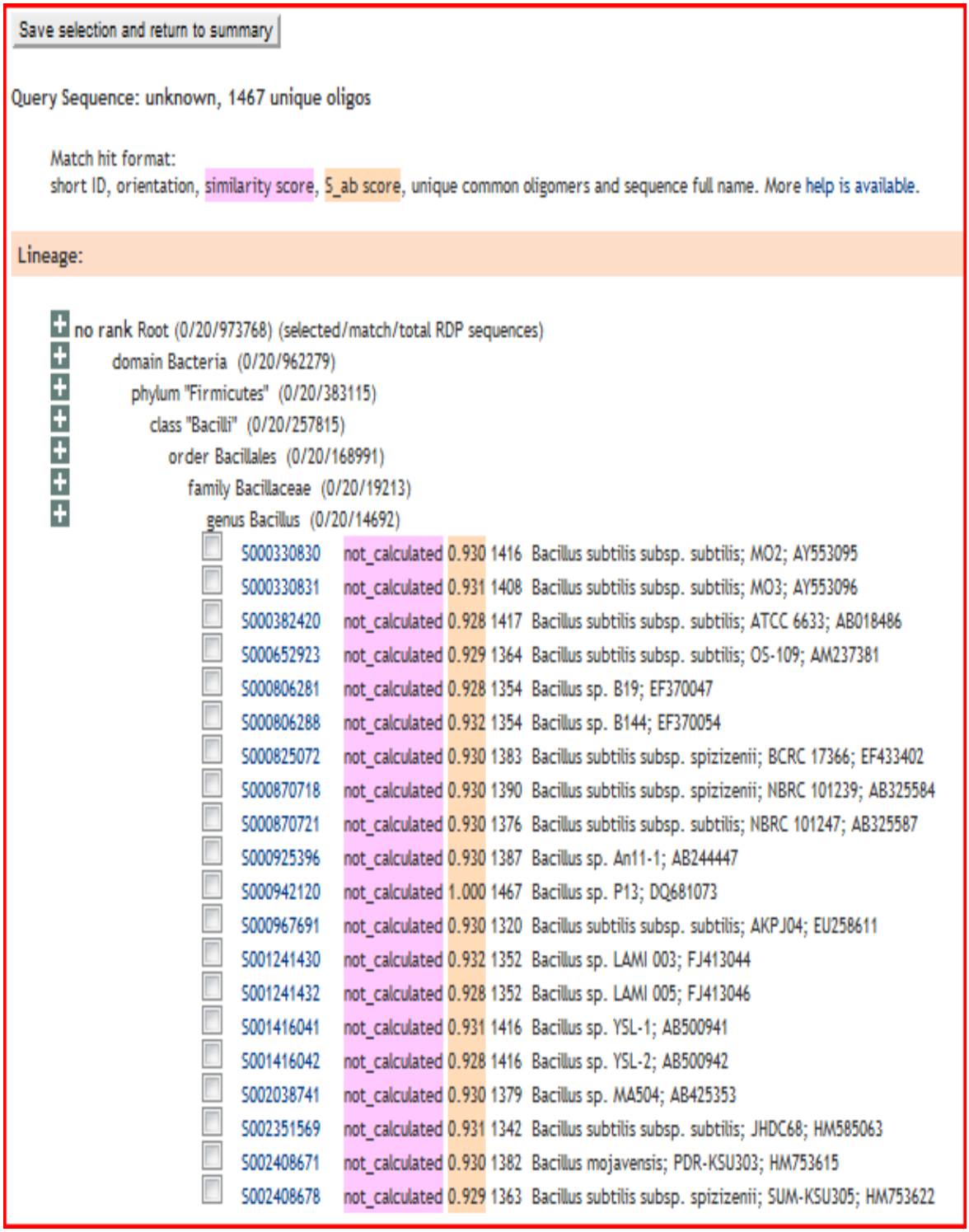
APPENDIX – III

Sequence details of 165rRNAgene from B. subtilis P13

LOCUS DQ681073 1555 bp DNA linear BCT 28-SEP-2007
DEFINITION Bacillus sp. P13 16S ribosomal RNA gene, partial sequence.
ACCESSION DQ681073
VERSION DQ681073.3 GI:157741649
KEYWORDS .
SOURCE Bacillus sp. P13
ORGANISM Bacillus sp. P13
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE 1 (bases 1 to 1555)
AUTHORS Pillai,P.S. and Gayatri,A.
TITLE Thermostable neutral protease of Bacillus sp.: Isolation, partial characteriztion and beam house application studies
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1555)
AUTHORS Pillai,P.S. and Gayatri,A.
TITLE Direct Submission
JOURNAL Submitted (09-JUN-2006) Microbiology, M.S. University of Baroda, Sayajiganj, Vadodara, Gujarat 390 002, India
REFERENCE 3 (bases 1 to 1555)
AUTHORS Pillai,P.S. and Gayatri,A.
TITLE Direct Submission
JOURNAL Submitted (20-SEP-2007) Microbiology, M.S. University of Baroda, Sayajiganj, Vadodara, Gujarat 390 002, India
REMARK Sequence update by submitter
REFERENCE 4 (bases 1 to 1555)
AUTHORS Pillai,P.S. and Gayatri,A.
TITLE Direct Submission
JOURNAL Submitted (28-SEP-2007) Microbiology, M.S. University of Baroda, Sayajiganj, Vadodara, Gujarat 390 002, India
REMARK Sequence update by submitter
COMMENT On Sep 28, 2007 this sequence version replaced gi:157391849.
FEATURES Location/Qualifiers
source 1..1555
/organism="Bacillus sp. P13"
/mol_type="genomic DNA"
/strain="P13"
/db_xref="taxon:393915"
rRNA <1..>1555
/product="16S ribosomal RNA"
ORIGIN
1 tacctggctc aggacgaacg ctggcggcgt gcctaataca tgcaagtcga gcggacagat
61 gggagcttgc tccctgatgt tagcggcgga cgggtgagta acacgtgggt aacctgcctg
121 taagactggg ataactccgg gaaaccgggg ctaataccgg atgcttgttt gaaccgcatg
181 gttcaaacat aaaaggtggc ttcggctacc acttacagat ggacccgcgg cgcattagct
241 agttggtgag gtaatggctc accaaggcga cgatgcgtag ccgacctgag agggtgatcg
301 gccacactgg gactgagaca cggcccagac tcctacggga ggcagcagta gggaatcttc

361 cgcaatggac gaaagtctga cggagcaacg ccgcgtgagt gatgaaggtt ttcggatcgt
421 aaagctcctg ttgttcaggg aagaacaagt accgttcgaa tagggcggta ccttgacggt
481 acctaaccag aaagccacgg ctaactacgt gccagcagcc gcgggtaata cgtaggtggc
541 aagcgttgtc cggaattatt gggcgtaaag ggctcgcagg cgggtttctt aagtctggat
601 gtgaaagccc ccggctccaa ccggggaggg tcattggaaa ctggggaact tgagtgcaga
661 agaggagagt ggaattccac gtgtagcggt gaaatgcgta gagatgtgga ggaacaccag
721 tggcgaaggc gactctctgg tctgtaactg acgctgagga gcgaaagcgt ggggagcgaa
781 caggattaga taccctggta gtccacgccg taaacgatga gtgctaagtg ttaggggtt
841 tccgcccctt agtgctgcag ctaacgcatt aagcactccg cctggggagt acggtcgcaa
901 gactgaaact cagaggaatt gacgggggcc cgcacaagcg gtggagcatg tggtttaatt
961 cgaagcaacg cgaagaacct taccaggtct tgacatcctc tgacaatcct agagatagga
1021 cgtccccttc gggggcagag tgacaggagg tgcatggtgt attcagagcc ttgctcctgg
1081 cactgccccc ccgtgattgg gttaagtccg cacgagcgca cctttgatct tagttggcca
1141 gacattcagt tgggcactct aaggtgactg ccggtgacaa accggaggaa ggtggggatg
1201 acgtcaaatc atcatgcccc ttatgacctg ggctacacac gtgctacaat ggacagaaca
1261 aagggcagcg aaaccgcgag gttaagccaa tcccacaaat ctgttctcag ttcggatcgc
1321 agtctgcaac tcgactgcgt gaagctggaa tcgctagtaa tcgcggatca gcatgccgcg
1381 gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca ccacgagagt ttgtaacacc
1441 cgaagtcggt gaggtaacct tttaggagcc agccgccgaa ggtgggacag atgattgggg
1501 tgaagtcgta acaaggtagc cgtatcggaa ggtgcgctgg tcccccaacc cttaa
//

RDP blast result of 16srRNA sequence of the selected isolate B. subtilis P13



Save selection and return to summary

Query Sequence: unknown, 1467 unique oligos

Match hit format:
short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:

no rank Root (0/20/973768) (selected/match/total RDP sequences)
domain Bacteria (0/20/962279)
phylum "Firmicutes" (0/20/383115)
class "Bacilli" (0/20/257815)
order Bacillales (0/20/168991)
family Bacillaceae (0/20/19213)
genus Bacillus (0/20/14692)

S000330830	not_calculated	0.930	1416	Bacillus subtilis subsp. subtilis; MO2; AY553095
S000330831	not_calculated	0.931	1408	Bacillus subtilis subsp. subtilis; MO3; AY553096
S000382420	not_calculated	0.928	1417	Bacillus subtilis subsp. subtilis; ATCC 6633; AB018486
S000652923	not_calculated	0.929	1364	Bacillus subtilis subsp. subtilis; OS-109; AM237381
S000806281	not_calculated	0.928	1354	Bacillus sp. B19; EF370047
S000806288	not_calculated	0.932	1354	Bacillus sp. B144; EF370054
S000825072	not_calculated	0.930	1383	Bacillus subtilis subsp. spizizenii; BCRC 17366; EF433402
S000870718	not_calculated	0.930	1390	Bacillus subtilis subsp. spizizenii; NBRC 101239; AB325584
S000870721	not_calculated	0.930	1376	Bacillus subtilis subsp. subtilis; NBRC 101247; AB325587
S000925396	not_calculated	0.930	1387	Bacillus sp. An11-1; AB244447
S000942120	not_calculated	1.000	1467	Bacillus sp. P13; DQ681073
S000967691	not_calculated	0.930	1320	Bacillus subtilis subsp. subtilis; AKPJ04; EU258611
S001241430	not_calculated	0.932	1352	Bacillus sp. LAMI 003; FJ413044
S001241432	not_calculated	0.928	1352	Bacillus sp. LAMI 005; FJ413046
S001416041	not_calculated	0.931	1416	Bacillus sp. YSL-1; AB500941
S001416042	not_calculated	0.928	1416	Bacillus sp. YSL-2; AB500942
S002038741	not_calculated	0.930	1379	Bacillus sp. MA504; AB425353
S002351569	not_calculated	0.931	1342	Bacillus subtilis subsp. subtilis; JHDC68; HM585063
S002408671	not_calculated	0.930	1382	Bacillus mojavensis; PDR-KSU303; HM753615
S002408678	not_calculated	0.929	1363	Bacillus subtilis subsp. spizizenii; SUM-KSU305; HM753622

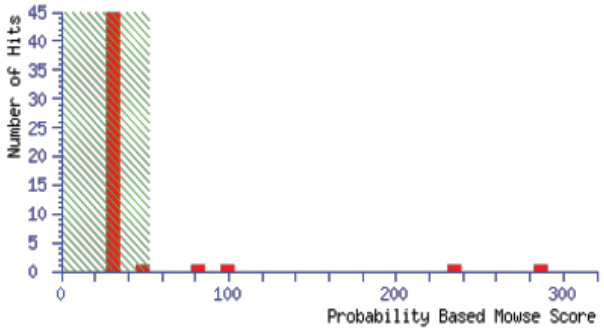
APPENDIX – IV

Peptide summary report of MASCOT search result of LCMS analysis of purified protease from B. subtilis P13

```
Taxonomy        : Bacteria (Eubacteria) (1478095 sequences)
Timestamp       : 25 Aug 2009 at 08:50:42 GMT
Protein hits    : 1SCJA          subtilisin e (EC 3.4.21.62) mutant S221C, chain A - Bacillus subtilis
                  Q5EFD9_BACNA Nattokinase (Fragment).- Bacillus subtilis subsp. natto.
                  SUBSN          subtilisin (EC 3.4.21.62) BPN' precursor - Bacillus amyloliquefaciens
                  1S02           subtilisin (EC 3.4.21.62) BPN' mutant (Q19E, Q271E) - Bacillus amyloliquefaciens
                  Q73PP9_TREDE Iron compound ABC transporter, periplasmic iron compound-binding protein, putative.- Treponema dentico
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 52 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. 1SCJA **Mass:** 27771 **Score:** 286 **Queries matched:** 6 **emPAI:** 0.57
subtilisin e (EC 3.4.21.62) mutant S221C, chain A - Bacillus subtilis
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑ 308	605.2536	1208.4927	1208.6051	-0.1123	0	46	0.19	1	K.HPTWTNAQVR.D
☑ 309	605.6286	1209.2427	1208.6051	0.6377	0	(22)	48	1	K.HPTWTNAQVR.D
347	646.3086	1290.6027	1289.6979	0.9048	0	23	40	2	-.AQSVPYGISQIK.A
☑ 419	765.6086	1529.2027	1528.7634	0.4393	0	61	0.0051	1	K.APALHSQGYTGSNVK.V
☑ 467	832.8086	1663.6027	1662.8325	0.7702	0	95	1.9e-006	1	K.YPSTIAVGAVNSSNQR.A
☑ 473	947.9386	1893.8627	1892.9592	0.9035	0	60	0.0056	1	K.VAVIDSGIDSSHPDLNVR.G

Proteins matching the same set of peptides:
Q3LTM2_BACSU **Mass:** 27816 **Score:** 286 **Queries matched:** 6
Nattokinase (Fragment).- Bacillus subtilis.
Q93L66_BACSU **Mass:** 27596 **Score:** 286 **Queries matched:** 6
Nattokinase (Fragment).- Bacillus subtilis.
E983959 **Mass:** 27639 **Score:** 286 **Queries matched:** 6
POLYPEPTIDE SEQUENCE FOR SUBTILISIN AMYLOSACCHARITICUS.- Bacillus sp.
E983960 **Mass:** 27682 **Score:** 286 **Queries matched:** 6
POLYPEPTIDE SEQUENCE FOR SUBTILISIN 168.- Bacillus sp.

---

2. Q5EFD9_BACNA **Mass:** 37231 **Score:** 242 **Queries matched:** 5 **emPAI:** 0.41
Nattokinase (Fragment).- Bacillus subtilis subsp. natto.
☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
308	605.2536	1208.4927	1208.6051	-0.1123	0	46	0.19	1	K.HPTWTNAQVR.D
309	605.6286	1209.2427	1208.6051	0.6377	0	(22)	48	1	K.HPTWTNAQVR.D
419	765.6086	1529.2027	1528.7634	0.4393	0	61	0.0051	1	K.APALHSQGYTGSNVK.V
467	832.8086	1663.6027	1663.8166	-0.2138	0	74	0.00023	2	K.YPSTIAVGAVNSSDQR.A
473	947.9386	1893.8627	1892.9592	0.9035	0	60	0.0056	1	K.VAVIDSGIDSSHPDLNVR.G

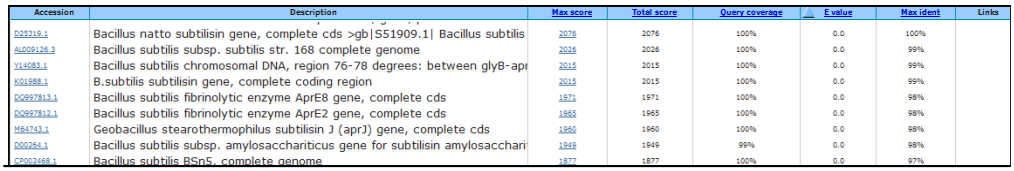
APPENDIX – V

Sequence and NCBI blast of amplified protease gene from B. subtilis P13

Sequence of amplified protease gene from *B. subtilis* P13using primers NK1 andNK2

GTATGAAAATAGTTATTTCGAGTCTCTACGGAAATAGCGAGAGATGATATA
CCTAAATAGAGATAAAATCATCTCAAAAAAATGGGTCTACTAAAATATTATT
CCATCTATTACAATAAATGCACAGAATAGTCTTTTAAGTAAGTCTACTCTGA
ATTTTTTAAAAGGAGAGGGTAAAGAGTGAGAAGCAAAAAATTGTGGATCAG
CTTGTTGTTTGCGTTAACGTTAATCTTTACGATGGCGTTCAGCAACATGTCT
GCGCAGGCTGCCGGAAAAAGCAGTACAGAAAAGAAATACATTGTCGGATTT
AAGCAGACAATGAGTGCCATGAGTTCCGCCAAGAAAAAGGATGTTATTTCT
GAAAAAGGCGGAAAGGTTCAAAAGCAATTTAAGTATGTTAACGCGGCCGCA
GCAACATTGGATGAAAAAGCTGTAAAAGAATTGAAAAAAGATCCGAGCGTT
GCATATGTGGAAGAAGATCATATTGCACATGAATATGCGCAATCTGTTCCT
TATGGCATTTCTCAAATTAAAGCGCCGGCTCTTCACTCTCAAGGCTACACA
GGCTCTAACGTAAAAGTAGCTGTTATCGACAGCGGAATTGACTCTTCTCAT
CCTGACTTAAACGTCAGAGGCGGAGCAAGCTTCGTTCCTTCTGAAACAAAC
CCATACCAGGACGGCAGTTCTCACGGTACGCATGTCGCCGGTACGATTGCC
GCTCTTAATAACTCAATCGGTGTTCTGGGCGTAGCGCCAAGCGCATCATTA
TATGCAGTAAAAGTGCTTGATTCAACAGGAAGCGGCCAATATAGCTGGATT
ATTAACGGCATTGAGTGGGCCATTTCCAACAATATGGATGTTATCAACATG
AGCCTTGGCGGACCTACTGGTTCTACAGCGCTGAAAACAGTAGTTGATAAA
GCGGTTTCCAGCGGTATCGTCGTTGCTGCCGCAGCCGGAAACGAAGGTTCA
TCCGGAAGCACAAGCACAGTCGGCTACCCTGCAAAATATCCTTCTACTATT
GCAGTAGGTGCGGTAAACAGCAGCAACCAAAGAGCTTCATTCTCCAGCGTA
GGTTCTGAGCTTGATGTAATGGCTCCTGGCGTGTCCATCCAAAGCACACTT
CCTGGAGGCACTTACGGCGCTTATAACGGAACGTCCATGGCGACTCCTCAC
GTTGCCGGAGCAGCAGCGCTAATTCTTTCTAAGCACCCGACTTGGACAAAC
GCGCAAGTCCGTGATCGTTTAGAAAGCACTGCAACATATCTTGGAAACTCT
TTCTACTATGGAAAAGGGTTAATCAACGTACAAGCAGCTGCACAATAATAG
TAAAAAGAAGCAGGTTCCTCCATACCTGCTTCTTTTTATTTGTCAGCATCCT
GATGTTCCGGCGCATTCTCTTCTTTCTCCGCATGTTGAATCCGTTCCATGAT
CGAAGGATGGCTGCCTCTGAAAATCTTCACAAGCACC GGA

NCBI-BLAST result of amplified protease gene from *B. subtilis* P13 using primers NK1 and NK2



Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
D25319.1	Bacillus natto subtilisin gene, complete cds >gb|S51909.1| Bacillus subtilis	2076	2076	100%	0.0	100%	
AL009126.3	Bacillus subtilis subsp. subtilis str. 168 complete genome	2026	2026	100%	0.0	99%	
Y14083.1	Bacillus subtilis chromosomal DNA, region 76-78 degrees: between glyB-apr	2015	2015	100%	0.0	99%	
K01988.1	B.subtilis subtilisin gene, complete coding region	2015	2015	100%	0.0	99%	
DQ997813.1	Bacillus subtilis fibrinolytic enzyme AprE8 gene, complete cds	1971	1971	100%	0.0	98%	
DQ997812.1	Bacillus subtilis fibrinolytic enzyme AprE2 gene, complete cds	1965	1965	100%	0.0	98%	
M64743.1	Geobacillus stearothermophilus subtilisin J (aprJ) gene, complete cds	1960	1960	100%	0.0	98%	
D00264.1	Bacillus subtilis subsp. amylosachariticus gene for subtilisin amylosacchari	1949	1949	99%	0.0	98%	
CP002468.1	Bacillus subtilis BSn5, complete genome	1877	1877	100%	0.0	97%	

Sequence details of protease gene cloned from B. subtilis P13 using primers NK3 and NK4

LOCUS JF921199 1124 bp DNA linear BCT 06-NOV-2011
DEFINITION Bacillus subtilis strain P13 nattokinase precursor, gene, partial cds.
ACCESSION JF921199
VERSION JF921199.1 GI:355140961
KEYWORDS .
SOURCE Bacillus subtilis
ORGANISM Bacillus subtilis
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE 1 (bases 1 to 1124)
AUTHORS Pillai,P. and Archana,G.
TITLE Cloning and heterolgous expression of keratinolytic serine protease from Bacillus subtilis P13
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1124)
AUTHORS Pillai,P. and Archana,G.
TITLE Direct Submission
JOURNAL Submitted (03-MAY-2011) Department of Microbiology and Biotechnology Centre, M.S. University of Baroda, Sayajigunj, Vadodara, Gujarat 390 002, India
FEATURES Location/Qualifiers
source 1..1124
/organism="Bacillus subtilis"
/mol_type="genomic DNA"
/strain="P13"
/isolation_source="hot spring"
/db_xref="taxon:1423"
/country="India: Vrajeswari, Mumbai"
CDS <1..977
/note="pAJNK"
/codon_start=3
/transl_table=11
/product="nattokinase precursor"
/protein_id="AER52006.1"
/db_xref="GI:355140962"
/translation=
"VISEKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIA

HEYAQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVR
GGASFVPSETNPYQDGSSHGTHVAGTIAALNNSIGVLGVAPSASLYAV
KVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTALKTVVDK
AVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASF
SSVGSELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILS
KHPTWTNAQVRDRLESTATYLGNSFYYGKGLINVQAAAQ"
mat_peptide 150..974
/product="nattokinase"

ORIGIN

1 atgttatttc tgaaaaaggc ggaaaggttc aaaagcaatt taagtatgtt aacgcggccg
61 cagcaacatt ggatgaaaaa gctgtaaaag aattgaaaaa agatccgagc gttgcatatg
121 tggaagaaga tcatattgca catgaatatg cgcaatctgt tccttatggc atttctcaaa
181 ttaaagcgcc ggctcttcac tctcaaggct acacaggctc taacgtaaaa gtagctgtta
241 tcgacagcgg aattgactct tctcatcctg acttaaacgt cagaggcgga gcaagcttcg
301 ttccttctga aacaaaccca taccaggacg gcagttctca cggtacgcat gtcgccggta
361 cgattgccgc tcttaataac tcaatcggtg ttctgggcgt agcgccaagc gcatcattat
421 atgcagtaaa agtgcttgat tcaacaggaa gcggccaata tagctggatt attaacggca
481 ttgagtgggc catttccaac aatatggatg ttatcaacat gagccttggc ggacctactg
541 gttctacagc gctgaaaaca gtagttgata aagcggtttc cagcggtatc gtcgttgctg
601 ccgcagccgg aaacgaaggt tcatccggaa gcacaagcac agtcggctac cctgcaaaat
661 atccttctac tattgcagta ggtgcggtaa acagcagcaa ccaaagagct tcattctcca
721 gcgtaggttc tgagcttgat gtaatggctc ctggcgtgtc catccaaagc acacttcctg
781 gaggcactta cggcgcttat aacggaacgt ccatggcgac tcctcacgtt gccggagcag
841 cagcgctaat tctttctaag cacccgactt ggacaaacgc gcaagtccgt gatcgtttag
901 aaagcactgc aacatatctt ggaaactctt tctactatgg aaaagggtta atcaacgtac
961 aagcagctgc acaataatag taaaaagaag caggttcctc catacctgct tctttttatt
1021 tgtcagcatc ctgatgttcc ggcgcattct cttctttctc cgcatgttga atccgttcca
1081 tgatcgaagg atggctgcct ctgaaaatct tcacaagcac cgga
//

NCBI blast resultof the protease gene cloned from *B. subtilis* P13 using primers NK3 and NK4 expressed in *E.coli* –BL21

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
JF921199.1	Bacillus subtilis strain P13 nattokinase precursor, gene, partial cds	2076	2076	100%	0.0	100%	
D25319.1	Bacillus natto subtilisin gene, complete cds >gb|S51909.1| Bacillus subtilis	2076	2076	100%	0.0	100%	
AL009126.3	Bacillus subtilis subsp. subtilis str. 168 complete genome	2026	2026	100%	0.0	99%	
Y14083.1	Bacillus subtilis chromosomal DNA, region 76-78 degrees: between glyB-apr	2015	2015	100%	0.0	99%	
K01988.1	B.subtilis subtilisin gene, complete coding region	2015	2015	100%	0.0	99%	
DQ997813.1	Bacillus subtilis fibrinolytic enzyme AprE8 gene, complete cds	1971	1971	100%	0.0	98%	
DQ997812.1	Bacillus subtilis fibrinolytic enzyme AprE2 gene, complete cds	1965	1965	100%	0.0	98%	
M64743.1	Geobacillus stearothermophilus subtilisin J (aprJ) gene, complete cds	1960	1960	100%	0.0	98%	
D00264.1	Bacillus subtilis subsp. amylosachariticus gene for subtilisin amylosacchari	1949	1949	99%	0.0	98%	
CP002468.1	Bacillus subtilis BSn5, complete genome	1877	1877	100%	0.0	97%	
CP002906.1	Bacillus subtilis subsp. subtilis RO-NN-1, complete genome	1871	1871	100%	0.0	97%	
FJ850748.1	Uncultured bacterium clone KSL 79 alkaline serine protease gene, complete	1805	1805	86%	0.0	100%	

NCBI BLAST result of translated protein sequences of the protein expressed in *E.coli* BL21 used for modelling the protein structure

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
P35835.1	RecName: Full=Subtilisin NAT; AltName: Full=Nattokinase; Flags: Precursor >gb|AAC60424.1| subtilisin NAT [Bacillus subtilis subsp. natto] >dbj|BAA04989.1| subtilisin [Bacillus subtilis] >gb|AAK54130.1| serkinase-nattokinase [Bacillus subtilis subsp. natto] >gb|ACJ11220.1| nattokinase [Bacillus subtilis subsp. natto] >gb|ACR46520.1| alkaline serine protease [uncultured bacterium] >dbj|BAI84580.1| alkaline serine kinase [Bacillus subtilis subsp. natto BEST195] >prf||1903156A subtilisin:ISOTYPE=NAT	649	649	100%	0.0	100%	
NP_388911.2	serine alkaline protease (subtilisin E) [Bacillus subtilis subsp. subtilis str. 168] >sp|P04189.3|SUBT_BACSU RecName: Full=Subtilisin E; Flags: Precursor >emb|CAB12870.2| serine alkaline protease (subtilisin E) [Bacillus subtilis subsp. subtilis str. 168] >gb|EHA30202.1| serine alkaline protease (subtilisin E) [Bacillus subtilis subsp. subtilis str. SC-8]	647	647	100%	0.0	99%	G
AAO65246.1	nattokinase precursor [Bacillus subtilis]	647	647	100%	0.0	99%	
AER52006.1	nattokinase precursor [Bacillus subtilis]	644	644	100%	0.0	100%	
ACJ06132.1	nattokinase [Bacillus subtilis subsp. natto]	645	645	100%	0.0	99%	
CAA74536.1	subtilisin E precursor [Bacillus subtilis subsp. subtilis str. 168]	646	646	100%	0.0	99%	
AAX35771.1	thermostable fibrinolytic enzyme Nk1 [Bacillus subtilis] >gb|ABQ02263.1| nattokinase precursor [Bacillus subtilis] >gb|ACU28777.1| serine alkaline protease [Bacillus subtilis]	646	646	100%	0.0	99%	

NCBI blast result of first 44 aminoacids coding for propetide

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AAA22814.1	subtilisin E protease (GTG start codon; putative); putative [Bacillus subtilis]	98.6	98.6	100%	5e-26	100%	
AAA22744.1	pre-pro-subtilisin [Bacillus subtilis]	98.6	98.6	100%	6e-26	100%	
1SCJ_B	Chain B, Crystal Structure Of Subtilisin-Propeptide Complex	94.4	94.4	100%	3e-25	96%	S
AER52006.1	nattokinase precursor [Bacillus subtilis]	99.8	99.8	100%	6e-25	100%	
ADI24411.1	nattokinase [Bacillus subtilis]	100	100	100%	6e-25	100%	
ABO77900.1	fibrinolytic enzyme [Bacillus subtilis]	100	100	100%	8e-25	100%	
ABU93240.1	subtilisin [Bacillus sp. CN]	99.8	99.8	100%	8e-25	100%	
ACJ48969.1	nattokinase [Bacillus subtilis]	99.8	99.8	100%	9e-25	100%	
ACJ06132.1	nattokinase [Bacillus subtilis subsp. natto]	99.8	99.8	100%	9e-25	100%	
ACE63521.1	fibrinolytic enzyme precursor [Bacillus sp. ZLW-2]	100	100	100%	9e-25	100%	

REFERENCES

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.
- Marie Curie

Adıguzel AC, Bitlisli BO, Yasa I, Eriksen NT (2009). Sequential secretion of collagenolytic, elastolytic, and keratinolytic proteases in peptide-limited cultures of two *Bacillus cereus* strains isolated from wool. J. Appl. Microbiol. 107:226–234.

Agboola FK, Thomson A, Afolayan A (2003). Isolation and properties ofcytoplasmic α-glycerol 3-phospahe dehydrogenase from the pectoral muscle of the fruit bat, Eidolon helvum. J. Biochem. Mol. Biol. 36:159-166.

Agrebi R, Haddar A, Hmidet N, Jellouli K, Manni L, Nasri M (2009). BSF1 fibrinolytic enzyme from a marine bacterium *Bacillus subtilis* A26: purification, biochemical and molecular characterization. Process Biochem. 44:1252–12259.Alam G (2002).

Barriers and opportunities for promoting trade in environmentally friendly products— study of India's leather industry (prepared for CUTS, Jaipur and UNEP). Center for Sustainable Development; Jaipur.

Alexander KTW, Haines BM, Walker MP (1986). Influence of proteoglycan removal on opening up in the beamhouse. J. Am. Leather Chem. Assoc. 81:85–102.

Allpress JD, Mountain G, Gowland PC (2002). Production, purification, and characterization of an extracellular keratinase from *Lysobacter* NCIMB 9497. Lett. Appl. Microbiol. 34:337–342.

APHA (1992). Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association. American Water Works Association. Water Environmental Federation, Washington, DC.

Arai KM, Takahashi R, Yokote Y, Akahane K (1983). Amino-acid sequence of feather keratin from fowl. Eur. J. Biochem. 132:501–507.

Arambarri L, Garcia R, Millan E (2001). Application of experimental design in a method for screening sediments for global determination of organic tin by electrothermal atomic absorption spectrometry (ETAAS). Fresen. J. Anal. Chem. 371**:** 955-960.

Arunachalam C, Saritha K (2009). Protease enzyme: an eco-friendly alternative for leather industry. Ind. J. Sci. Technol. 2: No. 12.

Asbeck A, Pfeiffer HF, Plapper T, Schimid R (1980). German Patent No. 2836824.

Bae M, Park PY (1989). Purification and characterization of thermotolerable alkaline protease by alkalophilic *Bacillus.* sp. No. 8- 16. Korean J. Microbiol. Bioeng. 17: 545-551.

Bai U, Mandic-Mulec I, Smith I (1992). SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis*, by protein-protein interaction. Genes Dev. 7:139-148.

Bajza Z, Vrcek V (2001). Thermal and enzymatic recovering of proteins from untanned leather waste. Waste Manage. 21:79–84.

Baker D, Sohl JL, Agard DA (1992). A protein-folding reaction under kinetic control. Nature 356:263–265.

Bakhtiar S, Estiveira RJ, Hatti-Kaul R (2005). Substrate specificity of alkaline protease from alkaliphilic feather-degrading *Nesterenkonia* sp. AL20. Enzyme Microb. Technol. 37:534–540.

Balaji S, Senthil Kumar M, Karthikeyan R, Ramadhar Kumar KS, Sridhar R, Sehgal PK (2008). Purification and characterization of an extracellular keratinase from a hornmeal-degrading *Bacillus subtilis* MTCC (9102). World. J. Microbiol. Biotechnol. 24:2741–2745.

Banerjee R, Bhattacharya BC (1993). Kinetic properties of extracellular alkaline protease of *Rhizopus oryzae*. J. Ferment. Bioeng. 75:380- 382.

Barone JR, Schmidt WF (2006). Effect of formic acid exposure on keratin fiber derived from poultry feather biomass. Bioresour. Technol. 97:233–242.

Beg QK, Saxena RK, Gupta R (2001). Kinetic constants determination for an alkaline protease from Bacillus mojavensis using response surface methodology. Biotechnol. Bioeng. 78:285-291.

Beveridge TJ (1978). The response of cell walls of Bacillus subtilis to metals and to electron-microscopic stains. Can. J. Microbiol. 24 (2):89–104.

Bhosale SH, Rao MB, Deshpande VV, Srinivasan (1995). Thermostability of high-activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20). Enzyme Microb. Technol. 17:136–139.

Bierbaum G, Giesecke UE (1991). Wandrey Ananlysis of nucleotide pools during protease production with *Bacillus licheniformis*. Appl. Microbiol. Biotechnol. 35: 725-730.

Böckle B, Müller R (1997) Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feather. Appl. Environ. Microbiol. 63:790-792.

Box GEP (1952). Multi-factor designs of first order. Biometrika 39:49-57.

Bradford M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brandelli A, Daniel JD, Riffel A (2010). Biochemical features of microbial keratinases and their production and their applications. Appl. Microbiol. Biotechnol. 85:1735-1750.

Brandelli A (2008). Bacterial keratinases: useful enzymes for bioprocessing agro industrial wastes and beyond. Food Bioprocess Technol. 1:105–116.

Brandelli A, Riffel A (2005). Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers. Electron J. Biotechnol. 8:35–42.

Bressollier P, Letourneau F, Urdaci M, Verneuil B (1999). Purification and characterization of a keratinolytic serine protease from *Streptomyces albidoflavus* DSM 40530. Appl. Environ. Microbiol. 65:2570–2576.

Brown EM, Taylor MM, Marner WN (1996). Production and potential uses of co-products from solid tannery waste. J. Am. Leather Chem. Assoc. 91:270-275.

Cabeza LF, Taylor MM, Brown EM, Marner WN (1999). Isolation of protein products from chromium leather waste using two consecutive enzymes and purification of final chromium product: pilot plant studies. J. Soc. Leather. Trades Chem. 83:14-18.

Camargo FAO, Bento FM, Okeke BC, Frankenberger WT (2003). Chromate reduction by chromium-resistant bacteria isolated from soils contaminated with dichromate. J. Environ. Qual. 32:1222-1227.

Campos VL, Moraga R, Yanez, J, Zaror CA, Mondaca MA (2005). Chromate reduction by *Serratia marcescens* isolated fromtannery effluent. Bull. Environ. Contam. Toxicol. 75:400–406.

Cantera C, Goya L, Mingo R (2001). Collagen hydrolysate: soluble skin applied in post tanning process PartI: characterisation. J. Soc. Leather Technol. Chem. 84:29-36.

Cantera CS, Angelinetti AR, Altobelli G, Gaita G (1996). Hair saving enzyme-assisted dehairing. Influence of enzymatic products upon final leather quality. J. Soc. Leather Technol. Chem. 80:83–86.

Carvalho CML, Serralheiro MLM., Cabral JMS, Aire-Barros MR (1997). Application of factorial design to the study of transesterification reactions using cutinase in AOT-reversed micelles. Enzyme Microb. Technol. 21**:** 117-123.

Chakarska I, Goshev I, Idakieva K, Apostolov G (2006) Isolation and characterization of phosphoric acid–soluble collagen from leather wastes of pig breed. Bulg White 90:260–263

Chakraborty R, Sarkar SK (1998) Enzyme hydrolysis of solid tannery wastes: solid-state enzyme production. J. Soc. Leather Technol. Chem. 82:56–63

Chandrasekaran S, Dhar SC (1985) A review on leather processing. Leather Sci. 32: 297–304.

Choudhary RB, Jana AK, Jha MK (2004). Enzyme technology applications in leather processing. Ind. J. Chem. Technol. 11: 659-671.

Chung MJ (1984). Studies on the production of acid protease by *Rhizopus japonicus* and purification of the enzyme. Korrean J. Appl. Microbiol. Bioeng. 12:45-50.

Comfort DA, Chhabra SR, Conners SB, J CC, L EK, Johnson MR, Jones KL, Sehgal AC, Kelly RM (2004). Strategic biocatalysis with hyperthermophilic enzymes. Green Chemistry 6:459-465.

Covington T (2009). Tanning Chemistry: The science of leather. Published by Royal society of Chemistry.www.rsc.org

D'Amico S, Marx JC, Gerday C, Feller G (2003). Activity - stability relationships in extremophilic enzymes. J. Biol. Chem. 278:7891–7896.

Damrongsakkul S, Ratanathammapan K, Komolpis K, Tanthapanichakoon W (2008). Enzymatic hydrolysis of raw hide using papain and neutrase. J. Ind. Eng. Chem. 14:202–206.

Dayanandan A, Kanagaraj J, Sounderraj L, Govindaraju R, Rajkumar GS (2003). Application of an alkaline protease in leather processing: an ecofriendly approach. J. Clean. Prod. 11:533–536.

Dean A, Voss D (2006). Design and Analysis of Experiments. India: Springer Private Limited.

Dixon M, Webb E C (1979). Enzymes. New York: Academic Press

Dozie INS, Okeke CN, Unaeze NC (1994). A thermostable, alkaline active, keratinolytic proteinase from *Chrysosporium keratinophilum*. World. J. Microbiol. Biotechnol. 10:563-567.

Durai G, Rajasimman M (2011). Biological treatment of tannery waste water- A review. J. Env. Sci. Technol. 4:1-17.

Durham DR, Stewart DB, Stellwag EJ (1987). Novel alkaline- and heat-stable serine proteases from alkalophilic *Bacillus* sp. strain GX6638. J. Bacteriol. 169:2762–2768.

Dutta SS (1985). Introduction in principles of leather manufacture, 3rd edn. Indian Leather Technol. Assoc. p:194.

Eisenberg H, Mevarech M, Zaccai G (1992). Biochemical, structural, and molecular genetic aspects of halophilism. Adv. Protein Chem. 43:1–62.

El Baba HAM, Covington AD, Davighi D (2000). The effects of hair saving on unhairing reactions. J. Soc. Leather Technol. Chem. 84:48–53.

Elangovan R, Philip L, Chandraraj K (2010). Hexavalent Chromium reduction by free and immobilized cell-free extract of *Arthrobacter rhombi*-RE. Appl. Biochem. Biotechnol. 160:81–97.

Evans KL, Crowder J, Miller ES (2000). Subtilisins of *Bacillus* spp. hydrolyze keratin and allow growth on feathers. Can. J. Microbiol. 46:1004–1011

Everett AL, Gordon TC, Windus W (1962). Assay of proteolytic depilatory enzymes on keratin: action of metabisulphite. Enzymologia 24:14-24.

Eyring H, Stearn AE (1939). The application of the theory of absolute reaction rates to proteins. Chem. Rev. 24**:** 253-270.

Foroughi F, Keshavarz T, Evans SC (2006). Specificities of proteases for use in leather manufacture. J. Chem. Technol. Biotechnol. 81:257–261.

Fleming AB, Tangney M, Jorgensen PL, Diderichsen B, Priest FG (1995). Extracellular enzyme synthesis in spore-deficient strain of *Bacillus licheniformis*. Appl. Environ Microbiol. 61:3775-3780

Francisco R, Alpoim MC, Morais PV (2002). Diversity of chromium resistant and reducing bacteria in a chromium-contaminated activated sludge. J. Appl. Microbiol .92: 837–843..

Fu XT, Yoo SG, Kim SM (2008). Characterization of a salt-tolerant acid protease produced by *Bacillus megaterium* KLP-98 and its potential as a fermentation starter for the manufacture of fish sauce. J. Food Biochem. 32: 279-298.

Fujita M, Nomura K, Hong K, Ito Y, Asada A, Nishimuro S (1993). Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. Biochem Biophys Res Commun 197(3):1340–1347.

FuX, Inouye M, Shinde U (2000). Folding pathway mediated by an intramolecular chaperone. The inhibitory and chaperone functions of the subtilisin propeptide are not obligatorily linked. J. Biol. Chem. 275: 16871– 16878.

Gadd GM (1990). Heavy accumulation by bacteria and other microorganisms. Experientia 46: 834–840

Ganguli A, Tripathi AK (2002). Bioremediation of toxic chromium from electroplating effluent by chromate-reducing Pseudomonas aeruginosa A2Chrin two bioreactors. Appl Microbiol. Biotechnol. 58:416–420.

Garbisu C, Alkorta I, Llama MJ, Serra JL (1998). Aerobic chromate reduction by *Bacillus subtilis*. Biodegradation 9: 133–141.

Gaur NK, Dubnau E, Smith I (1986). Characterization of a cloned *Bacillus subtilis* gene that inhibits sporulation in multiple copies. J. Bacteriol. 168:860-869.

Gaur NK, Oppenheim J, Smith I (1991). The *Bacillus subtilis sin* gene, a regulator of alternate developmental processes, codes for a DNA-binding protein. J. Bacteriol. 173:678-686.

Gayatri R, Sharma KA, Rajaram R, RamasamiT (2000). Chromium (III)-induced structural changes and self-assembly of collagen. Biochem. Biophys. Res. Commun. 283: 229–235.

Gehring AG, DiMaio GL, Marmer WN, Mazenko C (2002). Unhairing with proteolytic enzymes derived from *Streptomyces griseus*. J. Am. Leather. Chem. Assoc. 97:406–411.

George S, Raju V, Krishnan MRV, Subramanian TV, Kunthala Jayaraman (1995). Production of alkaline protease by *Pseudomonas aeruginosa* using proteinaceous

solid waste generated from leather manufacturing industries Process Biochem. 5: 457–462.

Gessesse A, Hatti-Kaul R, Gashe BA, Mattiasson B (2003) Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enzyme Microb Technol 32:519–524

Giongo LJ, Lucas SF, Casarin F, Heeb P, Brandelli A (2007) Keratinolytic proteases of *Bacillus* species isolated from the Amazon basin showing remarkable de-hairing activity. World. J. Microbiol. Biotechnol. 23: 375-382.

Gonzalez G, Gonzalez C, Merino P (1992). Thermostabilization of Cucurbita ficifolia protease in the presence of additives. Biotechnol. Lett. 14:919–924.

Gousterova A, Brailkova D, Goshev I Christov P, Tishinov K, Tonkova VE, Haertle T, Nedlkov P (2005). Degradation of keratin and collagen containing wastes by newly isolated *Thermoactinomycetes* for alkaline hydrolysis. Lett. Appl. Microbiol. 40: 335- 340.

Gouda MD, Kumar MA, Thakur MS, Karanth NG (2002).Enhancement of operational stability of an enzyme biosensor for glucose and sucrose using protein based stabilizing agents. Biosen.Bioelectron. 17: 503-507.

Govardhan CP, Margolin AL (1995). Extremozymes for industry - from nature and by design. Chem. Ind. 689 – 693.

Gradišar H, Friedrich J, Križaj I, Jerala R (2005) Similarities and specificities of fungal keratinolytic proteases: Comparision of keratinasesof *Pacecilomyces marquandii* and *Doratomyyces microsporus* to some known proteases.Appl.Envir.Microbiol.71: 3420-3426.

Gregg K, Wilton SD, Parry DAD, Rogers GE (1984). A comparison of genomic coding sequences for feather and scale keratins: structural and evolutionary implications. EMBO J. 3:175–178.

Grimm O (1966). German Patent No. 1230169.

Gupta R, Beg QK, Lorenz P (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications, Appl. Microbiol. Biotechnol. 59: 15–32

Gupta R, Beg QK, Khan S, Chauhan B (2002). An overview on downstream processing and properties of microbial alkaline proteases. Appl. Microbiol. Biotechnol. 60: 381-395.

Gupta R, Ramnani P (2006) Microbial keratinases and their prospective applications: An overview. Appl Microbiol Biotechnol 70: 21-33

Haaland PD (1989). Experimental design in biotechnology. New York and Basel: Marcel Dekker, Inc; Statistical Problem Solving. PP 1–18.

Hajji M, Rebai A, Gharsallah N, Nasri M (2008). Optimization of alkaline protease by *Aspergillus clavatus* ES1 in *Mirabilis jalapa* tuber powder using statistical experimental design. Appl . Microbiol. Biotechnol. 79 : 915-923.

Hameed A, Natt MA, Evans CS (1997) . Production of alkaline protease by a new*Bacillus subtilis* isolate for use as a bating enzyme in leather treatment. World J. Microbiol. 20–21.

HanY, Li Z, Miao X, Zhang F (2008). Statistical optimization of medium components to improve the chitinase activity of *Streptomyces* sp. Da 11 associated with the South China Sea sponge *Craniells australiensis*. Process Biochem. 43**:** 1088-1093.

Henner DJ, Yang M, Ferrari E (1988b) .Localization of *B. subtilis sacU* (Hy) mutations to two linked genes with similarities to theconserved prokaryotic family of two signalling systems. J Bacteriol 170:5102-5109

Hervas FF, Celma P, Punti I, Cisa J, Cot J, Marsal A, Manich A (2007) The enzyme activity of Trypsin on sheepskin trimmings in a two-step collagen extraction process. J Am.Leather .Chem Assoc 102:1–9

Holt JG, Krieg NR, Sneath PHA, Staley JT (1994) .Bergey's manual of determinative bacteriology, 9th edition. Williams and Wilkins, Baltimore PP 559-562

Huang Q, Peng Y, Li X, Wang H, Zhang Y (2003). Purification and Characterization of an Extracellular Alkaline Serine Protease with Dehairing Function from *Bacillus pumilus.* Current Microbiol.Vol. 46 : 169–173

Hunter JS (1985). Statistical design applied to product design. J. Qual. Technol. 17**:** 210-221.

Hwang JY,Kim SM, Heo SO, Kim CJ, Lee CH, Lee SK (2008). Purification and characterization of a novel salt-tolerant protease produced by *Saccharomyces cerevisiae* B101 isolated from baker's yeast dough. Food. Sci. Biotechnol. 17: 766-771.

Ikemura H, Inouye H (1988). *In vitro* processing of pro-subtilisin produced in *Escherichia coli*. J. Biol. Chem. 263**:**12959–12963.

Ishibashi Y, Cervantes C, Silver S (1990) Chromium reduction in *Pseudomonas putida*. Appl. Microbiol. Biotechnol. 56: 2268–2270

Izotova LS, Strongin AY, Chekulaeva LN, Sterkin VE, Ostoslavskaya VI, Lyublinskaya LA, Timokhina EA, Stepanov VM (1983). Purification and properties of serine protease from *Halobacterium halobium*. J. Bacteriol. 155**:** 826-830.

Jacobs M, Eliasson M, Uhlen M, Flock JI (1985) .Cloning, sequencing and expression of subtilisin Carlsberg from *Bacilluslicheniformis.* Nucleic Acids Res 13:8913–8926

Jang JS, Kang DO, Chun MJ, Byun SM (1992). Molecular cloning of a subtilisin J gene from *Bacillus stearothermophilus* and its expression in *Bacillus subtilis.* Biochem. Biophys .Res. Commun 184:277–282

Janssen PH, Peek K, Morgan HW (1994) Effect of culture condition on the production of an extracellular proteinase by *Thermus* Sp Rt 41 A. Appl. Microbiol .Biotechnol 41: 400 – 406

Jaouadi B, Abdelmalek B, Fodil D, Ferradji FZ, Rekik H, Zarai N9 (2010). Purification and characterization of a thermostable keratinolytic serine alkaline

proteinase from *Streptomyces* sp. Strain AB1 with high stability in organic solvents. Bioresour. Technol. 101: 8361-8369.

Jareckas, G, Proizrod P (1974). Production of proteolytic enzymes, Milkrobin. Ferment. Prep.1-85

Jiao Pan, Qing Huang, YiZheng Zhang. (2004) .Gene cloning and expression of an alkaline serine protease with dehairing function from bacillus pumilus .Current.microbiol Vol. 49, pp. 165–169.

Johnvesly B, Naik GR (2001) Studies on production of thermostablealkaline protease from thermophilic and alkaliphilicBacillus sp. JB-99 in a chemically defined medium. Process Biochem. 37:139–144.

Joo HS, Kumar CG, Park GC, Kim KT, Paik SR, Chang CS (2002) Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. Process Biochem. 38:155-159

Jorgensen PL, Tangney M, Pedersen PE, Hastrup S, Diderichsen B, Jorgensen ST (2000) Cloning and sequencing of an alkaline protease gene from *Bacillus lentus* and amplification of the gene on the *B. lentus* chromosome by an improved technique. Appl Environ.Microbiol 66:825–827.

Kallio PT, Fagelson JE, Hoch J, Strauch M (1991). The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein.J.Biol. Chem .266: 13411 -13417.

Kamini, NR, Hemachander C,. Geraldine J, Mala S, Puvanakrishnan R (1999). Microbial enzyme technology as an alternative to conventional chemicals in leather industry. CLRI publication.

Kanagaraj J, Velappan KC, Chandra Babu NK, Sadulla (2006). Solid waste generation in the leather industry and its utilization for cleaner environment- A review. J. Sci. Ind. Res.65:541-548.

Katsifas EA, Giannoutsou E, Lambraki M, Barla M, Karagouni AD. Chromium recycling of tannery waste through microbial fermentation. J. Ind. Microbiol. Biotechnol. 2004; 31: 57–62

Kato T, Yamagata Y, Aria T, Ichishima E (1992) .Purification of a new extracellular 90- KDa serine proteinase with isoelectric point 3.9 from *Bacillus subtilis* nattoo and elucidation of its distinct mode of action . Biosci. Biotech . Biochem .56: 1166 - 1168.

Kaur S, Vohra RM, Kapoor M, Beg QK, Hoondal GS (2001).Enhanced production and characterization of a highly thermostablealkaline protease from Bacillus sp. P-2.World. J Microbiol. Biotechnol 17:125–129

Khwaja AR, Singh R, Tandon SN (2001). Monitoring of Ganga water and industry machinery. Curr. Opin. Biotechnol. 10**:**376–381.

Kim SH, Choi NS (2000). Purification and characterization of subtilisin DJ-4 secreted by Bacillus sp. strain DJ-4 screened from Doen-Jang. Bioscience, Biotechnology, and Biochemistry, 64 :1722–1725.

Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y, Oh H, Kwon I, Lee S (1996) .Purification and characterization of a fibrinolytic enzyme produced from Bacillus sp. strain CK 11–4 screened from chungkook-jang. Appl. Environ. Microbiol. 62: 2482–2488.

Kim W, Choi K, Kim Y, Park H, Choi J, LeeY, Oh H, Kwon I, Lee S (1996). Purification and characterization of a fibrinolytic enzyme produced from Bacillus sp. strain CK 11–4 screened from chungkook-jang. Applied and Environmental Microbiology, 62: 2482–2488.

Kim, SH, Choi NS (2000) .Purification and characterization of subtilisin DJ-4 secreted by Bacillus sp. strain DJ-4 screened from Doen-Jang. Bioscience, Biotechnology, and Biochemistry, 64: 1722–1725.

Kitadokoro K, Tsuzuki H, Nakamura E, Sato T, Teraoka H (1994) Purification, characterization, primary structure, crystallization and preliminary crystallographic study of a serine proteinase from Streptomyces fradiae ATCC 14544. Eur J Biochem 220:55–61

Ko JH, Yan JP, Zhu L, Qi YP (2004). Identification of two novel fibrinolytic enzymes fromBacillus subtilisQK02. Comp Biochem Physiol C Toxicol Pharmacol 137:65–74

Koide Y, Nakamura A, Uozumi T, Beppu T (1986). Cloning and sequencing of the major intracellular serine protease gene of *Bacillus subtilis.* J Bacteriol 167:110–116

Kristjansson JK(1989). Thermophilic organisms as sources of thermostable enzymes. *Trends in Biotechnology* 1989, 7:349-353.

Kublanov IV, Tsiroulnikov KB, Kaliberda EM, Rumsh LD, Haertlé T, Bonch-Osmolovskaya EA (2009a). Keratinase of an anaerobic thermophilic bacterium Thermoanaerobacter sp. strain 1004-09 isolated from a hot spring in the Baikal rift zone. Microbiology(Russia) 78:67–75

Kumar CG (2002) . Purification and characterization of a thermostablealkaline protease from alkalophilic Bacillus pumilus. Lett Appl Microbiol 34:13–17.

Kumar GA, Swarnalatha S, Sairam B, Sekaran G (2008). Production of alkaline protease by *pseudomonas aeruginosa* using proteinaceous solid waste generated from leather manufacturing industries. Bioresour Technol 99:1939–1944.

Kumar GA, Venkatesan R, Rao BP, Swarnalatha S, Sekaran G (2009).Utilization of tannery solid waste for protease production by *Synergistes* sp. In solid-state fermentation and partial protease characterization. Eng. Life Sci. 9:66–73.

Kumaraguru S, Sastry TP, Rose C (1998). Hydrolysis of tannery fleshings using pancreatic enzymes: a biotechnological tool for solid waste management. J. Am. Leather. Chem Assoc. 93:32–39..

Kunert J, Krajci, D (1981). An electron microscopy study of keratin degradation by the fungus Microsporium gypseum in vitro. *Mykosen,* 24: 485-496.

Kunert J. (1987). Utilization of various concentrations offree cystine by fungus *Microsporium gypseum.* J. Basic.Microbiol. 27: 207-213.

Kunert, J. (1988). Thiosulfate production from cystine bythe keratinolytic prokaryote *Streptomyces fradiae.* Arch.of.Microbiol. 150: 600-601.

Kunst F, Debarbouille M, Msadek T, Young M, Mauel C, Karamata D, Klier A, Rapoport G, Dedonder R (1988). Deduced polypeptides encoded by the *Bacillus subtilis sacU* locus share homology with two-component sensor-regulator systems. J Bacteriol 170:5093-5101

Kupec J, Dvorackova M, Rudolva S, Rucika J, Kolemaznik K (2002). Deproteination of Chrome waste by washings and enzymatic hydrolysis. J. Am. Leather. Chem. Assoc. 97: 349- 354.

Laidler, KJ, Peterman BF (1979). Temperature effects in enzyme kinetics. *Methods Enzymol*. 63**:** 234-257.

Lee JK, Kim YO, Sunitha K, Oh TK (1998). Expression of thermostable alkaline protease gene from *Thermoactinomycessp*. E79 in *E. coli* and heat activation of the gene product.Biotechnol. Lett. 20:837–840.

Lee SK, Hwang JY, Choi SH, Kim MS,(2010). Purification and Characterization of *Aspergillus oryzae* LK-101 Salt-tolerant Acid Protease Isolated from Soybean Paste. Food Sci. Biotechnol. 19(2): 327-334

Liang X,, Jia S , Sun Y, Chen M, Chen X , Zhong J , Huan L(2007). Secretory Expression of Nattokinase from *Bacillus subtilis* YF38 in *Escherichia coli*. Mol. Biotechnol. 37:187–194

Lin HH, Yin LJ, Jiang ST (2009) Cloning, expression, and purification of Pseudomonas aeruginosa keratinase in Escherichia coli AD494 (DE3)pLysS expression system. J. Agric. Food Chem 57:3506–3511

Lin X, Lee CG, Casale ES, Shih JCH (1992) Purification and characterization of a keratinase from a feather – degrading *Bacillus licheniformis* strain. Appl. Environ. Microbiol. 58: 3271 – 3275.

Lina V, Jenny D (2009). Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*. J. Hazard.Materials. 167: 713–716.

Lopez D, Hera Vlamakis & Roberto Kolter(2009).FEMS Microbiol Rev 33 152–163 Generation of multiple cell types in *Bacillus subtilis*, review article.

Losi ME, Amrhein C, Frankenberger WT(1994). Environmental biochemistry of chromium. Rev Environ Contam Toxicol 36:91–121

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein estimation. J. Biol. Chem.193 :265–275.

Lucic M R, Forbes BE, Grosvenor SE, Carr JM, Wallace J C, Forsberg G (1998). Secretion in *Escherichia coli* and phage-display of recombinant insulin-like growth factor binding protein-2. J. Biotechnol., 61: 95–108.

Luli GW, Talanagi JW, Strohl WR, Pfister RM (1983). Hexavalent chromium resistant bacteria isolated from river sediments. Appl. Environ. Microbiol. 46 (4):846–854.

Macedo AJ, Silva WOB, Gava R, Driemeier D, Henriques JAP, Termignoni C (2005) Novel keratinase from Bacillus subtilis S14 exhibiting remarkable dehairing capabilities. Appl Environ Microbiol 71:594–596.

Mafia GJ, Slomiana M, Davis JF (2002) .Recent advances in collagen based technologies. J. Am. Leather .Chem Assoc. 97:74–82.

Malathi S, Chakraborty R (1991). Production of alkaline protease by a new *Aspergillus flavus* isolate under solid-substrate fermentation conditions for use as a depilation agent. Appl. Environ. Microbiol. 57:712–716.

Malikova LA, Mardanova AM, Sokolova OV, Balaban NP, Rudenskaya GN, Sharipova M (2007). Conditions for the biosynthesis of an extracellular subtilisin-like proteinase by *Bacillus pumilus* KMM62. Microbiologika 76: 273-279.

Manczinger L, Rozs M, Kevei F (2003) Isolation and characterization of a new keratinolytic Bacillus licheniformis strain. World J. Microbiol. Biotechnol. 19:35–39.

Mandic-Mulec I, Gaur N, Bai U, Smith I (1992 Sin, a stage-specific repressor of cellular differentiation. J Bacteriol 174:3561- 3569

Matsumura M, Signor G, Matthews BW (1989). Substantial increase of proteinMcLean J, Beveridge TJ (2001). Chromate reduction by a pseudomonad isolated from a site contaminated with chromate copper arsenate. Appl. Environ. Microbiol. 67: 1076–1084.

Megharaj M, Avudainayagam S, Naidu R. (2003) .Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. Curr. Microbiol. 47:51–54.

Milner M, Makise K (2002). Natto and its active ingredient nattokinase: A potent and safe thrombolytic agent. Alternative and Complementary Therapies. 8 :157–194.

Missiakas D, Raina S (1997). Protein folding in the bacterial periplasm. Journal of Bacteriology, 179: 2465–2471..

Mitsuiki S, Ichikawa M, Oka T, Sakai M, Moriyama Y, Sameshima Y, Goto M, Furukawa K(2004). Molecular characterization of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis* sp. TOA-1. Enzyme. Microb. Technol. 34:482–489.

Miyaji T, Otta Y, Makagawa T, Watanabe T, Niimura Y, Tomizuka N (2006). Letters in Applied Microbiology, 42, 242–247. doi:10.1111/j.1472-765X.2005.01851..

Mohammad BT, Wright PC, Bustard MT (2006). Bioconversion of isopropanol by a solvent tolerant *Sphingobacterium mizutae* strain. J. Ind. Microbiol. Biotechnol. 33: 975-83.

Monsheimer R, Pfleiderer E (1974) .East German Patent No. 2308967.

Montgomery DC (2002). Design and analysis of experiments. Singapore: John Wiley & Sons.

Msadek T, Kunst F, Henner D, Klier A, Rapoport G, Dedonder R(1990). Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in *degS* and *degU*. J Bacteriol 172:824-834

Muchie M (2000). Leather processing in Ethiopia and Kenya: lessons from India. Technol Soc.22:537–55.

Mukhopadhyay RP, Chandra AL(1990). Keratinase of a *streptomycete*. Indian. J .Exp. Biol. 28 :575-557..

Nagesha N, Balachandra P(2006). Barriers to energy efficiency in smallindustry clusters: multi-criteria-based prioritization using the analytic hierarchy process. Energy. 31:1969–83.

Najafi MF, Deobagkar D, Deobagkar D(2005). Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. Elect. J. Biotechnol. 8: 197-203.

Nakamura T, Yamagata Y, Ichishima E (1992). Nucleotide sequence of the subtilisin NAT gene, aprN, of *Bacillus subtilis*(natto). Biosci. Biotechnol. Biochem 56:1869–1871

Nam GW, Lee DW, Lee HS, Lee NJ, Kim BC, Choe EA, Hwang JK, Suhartono MT, Pyun YR (2002) Native-feather degradation by Fervidobacterium islandicum AW-1, a newly isolated keratinaseproducing thermophilic anaerobe. Arch Microbiol 178:538–547

Nilegaonkar SS, Zambare VP,. Kanekar PP, Dhakephalkar PK, Sarnaik SS(2007). Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-32. Biores. Technol. 98, 1238-1245.

Noronha EF, de Lima BD,. de Sa CM,. Felix CR (2002). Heterologous production of *Aspergillus fumigates* keratinase in *Pichia pastoris*, World J. Microbiol. Biotechnol.18: 563–568.

Oberoi R, Beg QK, Puri S, Saxena RK, Gupta R (2001).Characterization and wash performance analysis of an SDSresistantalkaline protease from a Bacillus sp. World J. Microbiol.Biotechnol 17:493–497.

Olmos J, Anda ERDe, Ferrari F, Valle BF(1997). Effects of the sinR and degU32 (Hy) mutations on the regulation of the aprE gene in Bacillus subtilis. Mol. Gen. Genet. 253:562 - 567

Onafide AA, Sane NA, Musallam AA, Zarban S (1998). A Review: Potentials for biotechnological application of keratin-degrading microorganisms and their enzymes for nutritional improvement of feather and other keratins as livestock feed resources. Bioresour.Technol.66:1-11

Paknikar KM, Puranik PR, Pethkar AV(1999). Development of microbial biosorbents - a need for standardizations of experimental protocols. In: Amils, R., Ballester, A. (Eds.), Biohydrometallurgy and the Environment Toward the Mining of the 21st Century. Part B: Molecular Biology,Biosorption, Bioremediation. Elsevier, Amsterdam, pp. 363–373.

Pal S, Banerjee R ,. Bhattacharyya BC, Chakraborty R (1996). Application of a proteolytic enzyme in tanneries as a depilating agent. J. Am. Leather Chem. Assoc. 91:59–63.

Pan J, Huang Q, Zhang YZ. (2004) .Gene cloning and expression of an alkaline serine protease with dehairing function from *Bacillus pumilus* .Current microbial. 49: 165–169

Pandeeti, E.V.P., G.K. Pitchika, J. Jotshi, S.S. Nilegaonkar, P.P. Kanekar and S. Dayananda. 2011. Enzymatic depilation of animal hide: identification of elastase (LasB) from *Pseudomonas aeruginosa* MCM B-327 as a depilating protease. *PLoS ONE* 6(2): e16742. doi:10.1371/journal.pone.0016742

Papadopoulos MC (1986) The effect of enzymatic treatment on amino acid content and nitrogen characteristics of feather meal. Anim Feed Sci Technol 16: 151 -156

Paus R, Cotsarelis G (1999). The biology of hair follicles. In Mechanisms of Disease ed. by Epstein FH. 341: 491- 497.

Peng Y, Huang Q, Zhang RH, Zhang YZ (2003). Purification and characterization of a fibrinolytic enzyme produced by Bacillus amyloliquefaciens DC-4 screened from douchi,a traditional Chinese soybean food. Comparative Biochemistry and Physiology. Biochemistry and Molecular Biology, 134: 45–52.

Peng Y, Yang X, Zhang Y (2005). Microbial fibrinolytic enzymes: an overview ofsource, production, properties, and thrombolytic activity in vivo. Appl. Microbiol.Biotechnol. 69:126–32.

Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C (2001) .Biosynthesis of complex polyketides in a metabolically engineered strain of E. coli. Science, 291: 1790–1810

Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C (2001). Biosynthesis of complex polyketides in a metabolically engineered strain of E. coli. Science, 291: 1790–1792.

Pfleiderer E (1975). *Leder* 26:149–154

Pillai P, Archana G (2008). Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel Bacillus subtilis isolate. Appl Microbiol.Biotechnol; 78:643–50.

Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika. 1944; 33: 305-325.

Porres, J.M M.J. Benito, X.L. Lei (2002). Functional expression of keratinase (*kerA*) gene from *Bacillus licheniformis* in *Pichia pastoris*, Biotechnol. Lett. 24: 631–636.

Prakash, P., S.K. Jayalakshmi and K.Sreeramulu. 2009. Production of keratinase by free and immobilized cells of *Bacillus halodurans* strain PPKS-2: partial characterization and its application in feather degradation and dehairing of the goat skin. *Appl. Biochem. Biotechnol.* doi:10.1007/s12010-009-8702-0

Puranik, P.R., Paknikar, K.M., 1999. Biosorption of lead, cadmium, and zinc by Citrobacter strain MCM B-181: Characterization studies. Biotechnology Progress 15 (2),228–237.

Puri S,Beg QK, Gupta R (2002) Optimization of protease production from *Bacillus* sp. using surface response methodology. Curr Microbiol 44: 286-290

Purushotham, H., S. Malathi, P.V. Rao, C.L. Rai, M.M. Immanuel and K.V. Raghavan. 1994. Dehairing enzyme by solid state fermentation. *J. Soc.Leather Technol. Chem.* **80**:52–56.

Puvanakrishnan R and Dhar CS,1999. Enzyme technology in beam house practice. In house publication,Central leather research institute,Adyar,Chennai-20,India..

Puvanakrishnan R, Bose SM and Dhar SC, (1983). Indian patent N0.151088

Puvanakrishnan, R. and Dhar, S. C., *J. Indian Leather Technol. Assoc*., 1985, 33, 19–22

Puvanakrishnan, R. and S.C. Dhar (1986). Recent advances in the enzymatic depilation of hides and skins. *Leather Sci.* **33**, 177–191

Radha S, Gunasekaran P (2007) . Cloning and expression of keratinase gene in *Bacillus megaterium* and optimization of fermentation conditions for the production of keratinase by recombinant strain, J. Appl. Microbiol.103 1301–1310.

Radha S, Gunasekaran P(2008). Sustained expression of keratinase gene under *PxylA* and *PamyL* promoters in the recombinant *Bacillus megaterium* MS941, Bioresour. Technol.99 5528–5537.

Rai KS, Mukherjee AK. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent –stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. Biochem.Eng. J. 2010; 48: 173-180.

Rajkumar, M., Nagendran, R., Lee, K.J., Lee, W.H., (2005). Characterization of a novel Cr6+ reducing Pseudomonas sp. with plant growthpromoting potential. Curr .Microbiol. 50, 266–271.

Raju AA, Chandrababu NK, Samivelu N, Rose C, Rao NM. 1996. Eco-friendly enzymatic dehairing using extracellular proteases from *Bacillus species* isolate. *J. Am. Leather Chem. Assoc.* 91:115–119.

Ramasami, T., J.R.Rao, N.K.C. Babu, K. Parthasarathy, P.G. Rao, P. Saravavan, R. Gayathri, K.J. Sreeran. 1999. Beam house and tanning operations: process chemistry revisited. *J. Soc. Leather Technol. Chem.* **83**:39–45.

Ramnani P, Singh R, Gupta R (2005) Keratinolytic potential of *Bacillus licheniformis* RG 1: structural and biochemical mechanism of feather degradation. Can J Microbiol 51:191-196Rao MB, Tanksale AM, Ghatge MS Deshpande VV (1998) Molecular and Biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62: 597 -635.

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev.1998; 62: 597-635.

Rao S, Sathish T, Ravichandra P, Prakasham RS (2009). Characterization of thermo-and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. Process Biochem. 44:262–268

Rao, J.L.U.M; Satyanarayana,T; 2003. Statistical optimization of a high- maltose forming ,hyper thermostable and Ca^{2+}- independent alpha- amylase production by a thermophile *Geobacillus thermoleovorans* using response surface methodology. J. Appl. Microbiol. 95, 712-718.

Rao JR, Gayatri R, Rajaram R, Nair BU, RamasamiT (1999) .Chromium(III) hydrolytic oligomers: their relevance to protein binding. Biochim Biophys Acta 1472, 595–602.

RaoJR, Thanikavelan P, Sreeram KJ, Nair BU (2002). Green Route for the utilization of Chrome Shavings (Chromium-Containing Solid Waste) in tanning Industry. Environ. Sci. Technol. 36, 1372-137**.**

Rattray FP, Bockelmann W, Fox PF (1995) Purification and characterization of an extracellular proteinase from Brevibacteriumlinens ATCC 9174. Appl Environ Microbiol 61:3454–3456

Rehman A, Zahoor A, Muneer B, Hasnain S (2008) .Chromium tolerance and reduction potential of a Bacillus sp.ev3 isolated from metal contaminated wastewater. Bull. Environ. Contam. Toxicol. 81, 25–29.

Riffel A, Ortolan S, Brandelli A (2003b) De-hairing activity of extracellular proteases produced by keratinolytic bacteria. J.Chem Technol Biotechnol 78:855–859.

Robertson DE, Mathur EJ, Swanson RV, Marrs BL, Short JM. The discovery of new biocatalysts from microbial diversity. SIM News 1996; 46:3–4.

Rohm O (1910). Dehairing and cleaning of skins.Ger.pat.268.83

Ross PJ (1996). Taguchi Techniques for Quality Engineering. New York, USA: MacGraw-Hill

Ruffin P, Andrieu S, Biserte G (1976). Sulphytolysis in keratinolysis. Biochemical proof *Sabouraudia*, 14: 181-184.

Sambrook J, Russell DW (2001). Molecular cloning a laboratory manual, 3rd Edn, Cold Spring Harbour Laboratory Press, Newyork.

Sankalika MJ, Rajasree CM, Mayur GS, Priti PP, Vijay B (2004). Estimation of trace amounts of Chromium (III) in multi-vitamin with multi-mineral formulation. Ana. Sci 20: 1321-1325.

Santos H, da Costa MS (2002).Compatible solutes of organisms that live in hot saline environments. *Environ. Microbiol.* 4:501-509.

Saravanabhavan S, Aravindhan R, Thanikaivelan P, Rao JR, Nair BU, Ramasami T (2004). A source reduction approach: integrated bio-based tanning methods and role of enzymes in dehairing and fiber opening. Clean. Technol. Environ. Policy 7: 3-14.

Sarkar, KT (1981). Theory and practice of leather manufacture.Pub: Ajoy sorkar,4,Second avenue,Mahatma Gandhi road, chennai-41.P.71.

Schallmey M, Singh A, Ward OP (2004). Developments in the use of *Bacillus* species for industrial production. Can. J. Microbiol. 50:1-17.

Schrooyen PMM, Dijkstra PJ, Oberthur RC, Bantjes A, Feijen J (2001) Partially carboxymethylated feather keratins. Thermal and mechanical properties of films. J. Agric. Food. Chem. 49:221-230.

Serrano AR, Maldonado VM, Kosters K (2003). Characterization of waste materials in tanneries for better ecological uses. J. Am. Leather Chem. Assoc. 98:43–48.

Shen H, Wang Y (1993). Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. Environ. Microbiol. 59:3771–3777.

Sherry, K. 1987. Recombinant tissue plasminogen activator: is it the thrombolytic agent of choice for an evolving acute myocardial infarction? American J. Cardiol. 59:984-989.

Shivaji S, Bhanu VN, Aggarwal RK (2000). Identification of *Yersinia pestis* as a causative organism of plague in India as determined by 16S rDNA sequencing and RAPD –based genomic finger printing. FEMS Microbiol.Lett.189 247-252.

Siddiqhi KS, AzharMJ, Rashid MH, Rjoka ML (1996). Activity and thermostability of carboxymethylcellulose from Aspergillus niger is strongly influenced by non-covalently attached polysaccharides.World J. Microbiol.Biotechnol.12:213-216.

Simpson TW, Peplinski JD, Koch PN, Allen JK (2001). Meta models for computer-based engineering design: survey and recommendations. Eng. Comp. 17: 129-150.

Singh B, Satyanarayana T (2006). A marked enhancement in phytase production by a thermophilic mould *Sporotrichum thermophile* using statistical design in a cost-effective cane molasses medium. J. Appl. Microbiol. 101:344-352.

Sivasubramanian SB, Murali BM, Puvanakrishnan R (2008). Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. Chemosphere 70: 1025–1034.

Srinath T, Verma T, Ramteke PW, Garg SK (2002). Chromium (VI) biosorption and bioaccumulation by chromate resistant bacteria. Chemosphere 48: 427–435.

Srinivas MRS, Chand N, Lonsane BK (1994). Use of Plackett–Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and

enzyme inducers for the production of alphagalactosidase by *Aspergillus niger* MRSS 234 in solid-state fermentation system. Bioprocess Eng. 10**:** 139-144.

Srinivas R, Panda T (1999) .Enhancing the feasibility of many biotechnological processes through enzyme deactivation studies. Bioprocess.Eng.21:363-369.

Stanley JR, Woodley DT, Katz SI, Martin GR (1982). Structure and function of basement membrane. J. Invest. Dermatol. 79: 69–72.

Stirtz T , Schroeder I (1981). Leder, 32: 155.

Strauch MA, Spiegelman G, Perego M, Johnson W, Burbulys D,Hoch J (1989). The transition state transcription regulator *abrB*of *Bacillus subtilis* is a DNA binding protein. EMBO J 8:1615 -1621

Sumi H, Hamada H, Nakanishi K, Hiratani H (1990). Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. Acta. Haematologica. 84:139–143.

Sumi H, Hamada H, Tsushima H, Mihara H, Muraki H (1987). A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese natto; a typical and popular soybean food in the Japanese diet. Experientia 43:1110–1111.

Suzuki Y, Kondo K, MatsumotoY, Zhao BQ, Otsuguro K, MaedaT, TsukamotoY, Urano T, Umemura K (2003). Dietary supplementation of fermented soybean, natto, suppresses intimal thickening and modulates the lysis of mural thrombi after endothelial injury in rat femoral artery. Basic Life Sciences, 73: 1289–1298.

Suzuki Y, Tsujimoto Y, Matsui H, Watanable K (2006). Decomposition of extremely hard –to-degrade animal proteins by thermophilic bacteria. J. Biosci .Bioeng.102: 73-**81.**

Sztajer, H. and Zboinska, E., *Acta Biotechnol*., 1988, 8, 169–175.

Tancous JJ, Bellingham R, Kallenberger W, McDonell A (1981). Conservation of chromium in the tanning industry.J .Am. Leather. Chem Assoc. 76:164.

Tare V, Gupta S, Bose P (2001). Case studies on biological treatment sediments vis-a-vis tannery pollution at Kanpur (India): a case study. Environ Monit Assess.; 68(1):19–35.

Taylor MM, Cabeza LF, Dimaio GL, Brown EM, Marmer WN (1998) Processing of leather waste: pilot scale studies on chrome shavings. Part I. isolation and characterization of protein products and separation of chrome cake. J .Am .Leather .Chem. Assoc 93:61–81.

Taylor MM, DG Bailey and SH Feaiheller. 1987. A review of the use of enzymes in tannery, J. Ame leather Chem Assoc, 82:153-165

Teitzel GM, Mathew RP (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 69(4): 2313-2320

Thacker U, Madamwar D (2005). Reduction of toxic chromium and partial localization of chromium reductase activity in bacterial isolate DM1. World J. Microbiol. Biotechnol. 21: 891–899

Thangam EB, Rajkumar GS (2002). Purification and characterization of alkaline protease from *Alcaligenes faecalis*. Biotechnol. Appl. Biochem. 35: 149–154.

Thanikaivelan P, Rao JR, Nair BU , Ramasami T(2005). Recent trends in leather making: processes, problems, and pathways. Crit. Rev. Environ. Sci. Technol. 35:37–79.

Thanikaivelan P, Rao JR, Nair BU, Ramasami T (2004). Progress and recent trends in biotechnological methods for leather processing. Trend Biotechnol. 22:181- 186.

Thorstensen, TC (1985). Practical leather technology, Third edition.Pub: Robert E.Krieger publishing company,Malabar, Florida,PP: 87-88.

Thys RCS, Brandelli A (2006). Purification and properties of a keratinolytic metalloprotease from *Microbacterium* sp. J Appl Microbiol 101:1259–1268

Thys RCS, Lucas FS, Riffel A, Heeb P, Brandelli A (2004). Characterization of a protease of a feather – degrading *Microbacterium* species. Lett . Appl. Microbiol. 39: 181-186

Tiwary E, Gupta R (2010). Medium optimization for a novel 58 kDa dimeric keratinase from *Bacillus licheniformis* ER-15: Biochemical characterization and application in feather degradation and dehairing of hides Biores. Technol. 101: 6103–6110.

Tjalsma H, Antelmann JDH, Jongbloed PG, Braun E, Darmon DR, Dubois JYF, Westers H, Zanen G, Quax WJ, Kuipers OP, Bron S, Hecker M , van Dijl JM (2004). Proteomics of Protein Secretion by *Bacillus subtilis*: Separating the "Secrets" of the Secretome. Microbiol. Mol. Biol. Rev. 68: 207–233.

Turner P, Mamo G, Karlsson NE (2007). Review: Potential and utilization of thermophiles and thermostableenzymes in biorefining. Microbial Cell Factories, 6:9 doi:10.1186/1475-2859-6-9

Turpeinen R, Kairesalo T, Haggblom M (2002). Microbial activity community structure in arsenic, chromium and copper contaminated soils. J. Environ. Microbiol. 35(6): 998-1002.

Uhligh (2006). Industrial enzymes and their applications: transilated and updated by Elfriede M and Linsmaier B (2005). John wiley and sons Inc.605.Third Avenue-Newyork.NY

Underkofler LA, Hickey RJ (1954). in *Industrial Fermentations*, New York Chemical Publishing Co., NY, , vol. II, p. 109.

Urano T, Ihara H, Umemura K, Suzuki Y, Oike M, Akita S, Tsukamoto Y, Suzuki I, Takada A (2001). The profibrinolytic enzyme subtilisin NAT purified from *Bacillus subtilis* cleaves and inactivates plasminogen activator inhibitor type I. J. Biol. Chem. 27:24690–24696.

Usha R, Ramasami T (2000). Effect of crosslinking agents (basic chromium sulfate and formaldehyde) on the thermal and thermomechanical stability of rat tail tendon collagen fibre. Thermochim. Acta. 356:59–66.

Valeika V, Beleska K, Valeikiene V, Kolodzeiskis V (2009). An approach to cleaner production: from hair burning to hair saving using a lime-free unhairing system. J. Clean. Prod. 17: 214–221.

Varela H, Ferrari MD, Belobrajdic L, Vasquez A , Loprena MD (1997). Skin dehairing proteases of *Bacillus subtilis* :Production and partial characterization. Biotechnol. Lett.19:755-758

Vasantha N, Thompson LD, Rhodes C, Banner C, Nagle J, Filpula D (1984). Genes for alkaline protease and neutral protease from *Bacillus amy/oliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. J. Bacteriol. 159: 811-819.

Vijay kumar E, Srijana M, Kiran Kumar K,. Harikrishna N, Gopal Reddy (2011). A novel serine alkaline protease from *Bacillus altitudinis* GVC11 and its application as a dehairing agent. Bioprocess Biosyst. Eng. 34:403–409.

Voet D, Voet JG (2004). Biochemistry, 3rd Edn. Wiley, New York.

Wang CT, Ji BP, Nout R, Li PL, Ji H, Chen L F (2006). .Purification and characterization of a fibrinolytic enzyme of Bacillus subtilis DC33, isolated from Chinese traditional Douchi. J. Ind. Microbiol. Biotechnol. 33:750–758.

Wang HY, Liu DM, Liu Y, Cheng CF, Ma QY, Huang Q , Zhang YZ (2007). Screening and mutagenesis of a novel *Bacillus pumilus* strain producing alkaline protease for dehairing. Lett. Appl. Microbiol. 44 1–6.

Wang P, Mori T, Komori K, Sasatsu M,. Toda K, Ohtake H (1989). Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. Appl. Environ. Microbiol.55:1665–1669.

Wilkinson D, Reuter S, Zimmermann W (1998). Praznik, W. and Huber, A., eds., WUV Universitaetsverlag, Vienna, Austria, pp. 176–189. in Contributions of the 4th

International Workshop on Carbohydrates as Organic Raw Materials, Vienna, Austria.

Williams CM, Richter CS, Mackenzie JM, Shih JCH (1990). Isolation, identification and characterization of a feather degrading bacterium. Appl. Environ. Microbiol. 56:1509-1515.

Wong SL **(**1995). Advances in the use of *Bacillus subtilis* for the expression and secretion of heterologous proteins. Curr. Opin. Biotechnol. 6**:**517–522.

Yamagata Y, Abe R, Fugita Y, Ichishima E (1995). Molecular cloning and nucleotide sequence of the 90k serine protease gene *hspK* from *Bacillus subtilis* (natto) No.16. Curr. Microbiol. 31: 340-344.

Yamamura S, Morita Y, Hasan Q, Yokoyama K, Tamiya E (2002). Keratin degradation: a co-operative action of two enzymes from *Stenotrophomonas* sp. Biochem. Biophys. Res. Commun. 294: 1138–1143.

Yano JK, Poulos TL, Peek K, Ruttersmith LD, Daniel RM, Morgan HW, Bergquist PL (1992). *BFE* 9: 466–470.

Yasuda M, Aoyama M, Sakaguchi M, Nakachi K, Kobamoto N (1999). Appl. Microbiol. Biotechnol. 51:474–479.

Yates JR (1972). Studies in depilation. Part X. The mechanism of the enzyme depilation process. J. Soc. Leather Technol. Chem. 56:158–175.

Zambare, VP, Nilegaonkar SS, Kanekar PP (2007). Production of an alkaline protease by *Bacillus cereus* MCM B-326 and its application as a dehairing agent. World J. Microbiol. Biotechnol 23: 1569–1574.

Zeikus JG. (1996). *Prog. Biotechnol.* 12, 145–161.

Zhang Z, Lis G (2005). Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. J. Soc. Leather Technol. Chem. 90:23-28.

Zuidweg MHJ, Bos CJK, VanWelzen H (2005). Proteolytic components of alkaline proteases of *Bacillus* strains. Zymograms and electrophoretic isolation. Biotechnol. Bioeng. 14:685–714.

Summary

We judge ourselves by what we feel capable of doing, while others judge us by what we have already done.

- Henry Wadsworth Longfellow

A novel bacterial isolate *Bacillus subtilis* P13, has been characterized for its ability to produce extracellular protease that has application in the pre-tanning processes in leather making. This isolate, obtained from Vajreshwari hotsprings, Mumbai, India, shows characteristics of moderate thermophile, with the protease functioning optimally at around 60^{o}C. Phylogenetic analysis using nearly full length DNA sequence of the 16S rRNA gene of isolate P13 allowed its identification as a *Bacillus subtilis* strain. The crude protease preparation showed keratinolytic activity, acting on the beta-keratins of bird feathers and hide dehairing ability on animal hides. Both these properties were dependent on serine protease activity as seen with the use of different protease inhibitors.

A bacteriological medium for the optimum production of the protease by *Bacillus subtilis* P13 has been designed. Using this, the protease activity was enhanced 3.5 times in shake flask cultures, about 5 fold in 3L fermentor and its production time was reduced by 24 h. The potential of the enzyme produced on large scale for pre-tanning processes, viz, soaking, dehairing as well as bating assessed at two leather making units, the Adam tannery, Chennai, India and Central leather research institute, Chennai, showed that the crude enzyme preparation is suitable for all three operations with its dehairing activity being most remarkable since it did not need liming or any other chemical dehairing aid and intact hair was recovered.

An important observation through microscopic of the enzyme-treated pelts showed digested epidermis, appendages to be absent, and empty follicles without hair and degraded hair bulb. The term “epilation” is thought to be more appropriate for enzymatic dehairing over the term “depilation” which is often used in the literature.

Since mature keratin fibres are highly cross-linked by disulphide bridges, keratin hudrolysis is thought to require sulfitolysis or reduction of disulphide bonds. This activity was provided by the cell-bound redox system when whole cells were allowed to grow on feathers, however needed to be amended with beta-mercaptoethanol when cell-free supernatants were used. However, low amounts of the disulfide bond reducing activity was inherently present in the protease preparation of *B. subtilis* P13 which was better at 50 °C than 30 °C. This level of sulfitolysis may be sufficient for hide unhairing but not for feather disintegration. As most reported dehairing preparations have keratinase activity, it may be interesting to study

whether proteases which can perform feather disintegration are likely to be potential candidates for dehairing.

Besides producing enzyme suitable for pretanning operations, the bacteriun P13 possesses another important characteristic useful for the leather industry, i.e, the abilty to rapidly degrade chrome shavings which are small pieces of leather waste generated during post tanning operations, the main composition being chromium and protein. *B. subtilis* P13 could degrade chrome shavings after thermal hydrolysis possibly due to the thermal denaturation of the collagen fibres. The bacterium has abilty to tolerate Cr(VI) due to bioaccumulation, bio-sorption and presence of chromate reductase enzyme. A solid substratum continuous reactor chrome shavings was developed in which the progression of hydrolysis was accompanied with reduction in column height and oozing out of an effluent hat was rich in dehairing protease activity which was at levels higher than the optimized medium. Chromium could be recovered and reused after bacterial growth on the chrome shavings. Based on the above observations, a continuous bio-reactor is proposed for the tannery to not only manage the solid waste at in-house level, but use it for the production of the valuable by-product, protease, which could be applied in all pre-tanning processes as well as recovery of chromium.

Protease zymography revealed that *B. subtilis* P13 produded several proteases in a growth phase and medium dependent manner. Through correlation studies, a 31 kDa protease band was implicated as the protease that was responsible for the pretanning activities. The purified band eluted from the SDS-PAGE gel ahowed all the properties I,e. keratinolytic activity, PMSF sensitivity, hide unhairing ability and feather protein degradation ability to reside in this protein. The first 10 residues from the N-terminal of the purified 31 kDa protease from *B. subtilis* P13 showed an amino acid sequence identical to that of subtilisin proteases produced by several other *B. subtilis* strains.

Keratinolytic serine protease produced by *B. subtilis* P13 was purified to apparent homogenity by two step protocol, ammonium sulphate precipitation followed by casein affinity chromatography to 71.7 fold with approximately 22.17 % of yield. The molecular mass and PI of the purified protein was found to be 27.7 kDa and 6.8

respectively. The temperature and pH optimum of the purified enzyme was found to be 60 °C and 7.2 respectively. The fall in activity was steep at higher temperatures than lower, suggestive of a lower range of temperature for activity of 40–65 °C. Kinetic parameters for substrate hydrolysis, substaret specificity and for thermal denaturation have been characetrised. Of the four synthetic peptides used to evaluate the substrate specificity of this protease, the enzyme has remarkable hydrolytic activity towards synthetic peptide N-Succinyl-Ala-Ala-Pro-Phe-*p*NA (AAPF-pN). No activity was observed in presence of (Suc-Ala-Ala-Ala-*p*NA), an elastase specific substrate. Thermostabilty increases in the presence of salt in the order $(NH_4)_2SO_4$ > $BaCl_2$ > $CaCl_2$ > NaCl. The low values of enthalpy and entropy and high free energy values in presence of ammonium sulphate signified this salt as a thermostabilizer for this keratinolytic protease. Thermostability improved in alkaline pH and was best at pH 9.0. The molecular mass and pI of the purified protease was determined as 27.7 kDa and 6.8 respectively. The assumption put forth that this keratinolytic protease from *B. subtilis* P13 is having a dual property of hide epilation and feather detachment was confirmed using the purified enzyme preparation.

Mass spectrometrically determined peptide sequence stretches of the purified protease revealed its similarity to nattokinase, a fibrinolytic enzyme reported from *B. subtilis* (natto). Based on this information, primers were designed for PCR amplification of the dehairing protease gene from *B. subtilis* P13, and the gene was successfully cloned and over-expressed in the heterologous host, E. coli under control of T7. Sequence of the cloned showed again 100 % homology to nattokinase gene reported from *B. subtilis* (natto) isolated from fermented soya bean preparation. Expression level of the recombinant enzyme was about 9 fold higher than that of the wild type strain *B. subtilis* P13.The molecular weight of the expressed protein was of around 40 kDa and was due to the presence of propeptide, essential for the proper folding of the protein which is autocatalytic and cleaved during secretion. The cloned protein was purified to 85 % purity by casein affinity chromatography and inherent properties of the enzyme, viz, keratinolytic activity, degradation of chrome shavings, feather hydrolysis and hide unhairing were confirmed. An additional property was attributed to this enzyme i.e. fibrinolytic property, and showed an activity of about 450 urokinase unit. Protein sequence obtained by transalation revealed its identity to nattokinase and first 44 aminoacids

showed 100 % homology to pre-pro subtilisn and nattokinase precursor from the isolate *B. subtilis* (natto) and phylogenetically protein grouped along with fibrinolytic enzymes from *B. subtilis*. Homology modelling was carried out using deduced protein sequence and structure resembled the reported 3D structure of subtilisin with a catalytic triad of ASP32 – HIS64 – SER221.

In conclusion, this might be first report of a keratinolytic protease that finds application in all the three pre-tanning process. The ability of the bacterial isolate to produce the enzyme using chrome shavings as a sole protein source is another significant novelty of this work. This could be further explored to develop an in-house process for solid waste management and by- product utilisation. The fibrinolytic property of the enzyme can be studied more elaborately for developing a product as a food supplement or for intra-venal application.

Presentations

And

Publications

We live on an island surrounded by a sea of ignorance. As our island of knowledge grows, so does the shore of our ignorance.
- John Archibald Wheeler

List of papers published

- **Pillai P**, Archana G (2008). Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. Appl. Microbiol. Biotechnol. 78, 643-650.

- **Pillai P**, Mandge S, Archana G (2011).Statistical optimization of production and tannery applications of a keratinolytic serine protease from *Bacillus subtilis* P13. Process Biochem. 46, 1110-1117

- Archana G, **Pillai P** (2011). Biochemical characteristics and possible mechanism of proteases for dehairing of hides. Chapter X, In: Biotechnology of microbial enzymes. Eds. Vijai Kumar Gupta et al., Nova Science Publishers Inc.

Manuscript under preparation

- **Pillai P** and Archana G. Bioprocess development for management of chrome shavings, a solid waste generated in tanneries, using chromium resistant *Bacillus subtilis* P13

.**List of posters presented**

- **3rd BRSI international Conference** 02-04 Nov 2006 at 3rd BRSI international conference held at School of Biosciences, Vallabh vidyanagar. Gujarat . Entitled: "Novel protease from Bacillus.sp.P13 exhibiting remarkable deharining capability"

- **75th S**ociety of **B**iological **C**hemist conference 08 -11 Dec 2006 at Jawaharlal Nehru University, Delhi Entitled :" **Biochemical characterization of neutral serine protease from Bacillus sp.P13 and its suitability as depilatory enzyme**"

- **A**ssociation of **M**icrobiologist of **I**ndia 18-21 Dec 2007 at IIT Chennai. Entitled: **"Keratinolytic protease of Bacillus subtilis P13 isolate from Vajreswari hotspring- Mumbai**"