STUDIES ON PRODUCTION AND APPLICATIONS OF ALKALINE PROTEASE FROM *BACILLUS PSEUDOFIRMUS*

A THESIS

submitted by

SHAMPA SEN

for the award of the degree

of

DOCTOR OF PHILOSOPHY

CENTRE FOR THE ENVIRONMENT

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

JANUARY 2010

STUDIES ON PRODUCTION AND APPLICATIONS OF ALKALINE PROTEASE FROM *BACILLUS PSEUDOFIRMUS*

A THESIS

submitted by

SHAMPA SEN

for the award of the degree

of

DOCTOR OF PHILOSOPHY

CENTRE FOR THE ENVIRONMENT

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

JANUARY 2010

Dedicated
to
My Parents and Sister

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

CENTRE FOR THE ENVIRONMENT

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Veeranki Venkata Dasu and Dr. Bishnupada Mandal.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: **Shampa Sen**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

CENTRE FOR THE ENVIRONMENT

CERTIFICATE

It is certified that the work described in this thesis entitled "***Studies on production and applications of alkaline protease from Bacillus pseudofirmus***" by Shampa Sen for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Centre for the Environment, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

Dr. V. Venkata Dasu
Associate Professor
(Thesis Supervisor)
Department of Biotechnology
Indian Institute of Technology Guwahati
Guwahati - 781 039, India

Dr. B. Mandal
Associate Professor
(Thesis Supervisor)
Department of Chemical Engineering
Indian Institute of Technology Guwahati
Guwahati - 781 039, India

ACKNOWLEDGEMENTS

I first wish to thank my loving parents, my sister and my niece, Neha, for being near to my heart and providing the support and encouragement. I would thank my in-laws for their moral support and blessings and all the good wishes.

It is with my deepest sense of appreciation that I express my heartiest acknowledgement to my research supervisors, Dr. V. Venkata Dasu and Dr. B. Mandal for their continuous care, support and encouragement throughout my research work. I must acknowledge the unconditional freedom to think, plan, execute and express, that I was given in every step of my research work, while keeping faith and confidence on my capabilities.

I am thankful to my doctoral committee members, Dr. K. Pakshirajan, Dr. P. Ghosh and Dr. G. K. Saini for their constructive criticism and precious suggestions.

I owe my gratitude to ex-Heads of the Centre for the Environment, IIT Guwahati, Dr. M. Jawed, Dr. M. Ray and current Head, Dr. C. Mahanta, for providing me with the necessary facilities. I wish to acknowledge the ex-staffs of the Centre for the Environment, Bhabenda, Dhrubojyoti and Monojitda, and present staff Partha, Deepmoni, Jyashree, Rupinder and Amjad for their cooperation.

I would express thank to my friends Sanjay, Vijay, Ravi Kiran, Avishek, Abhishek, Bedabrata and Atul for their valuable suggestions, Achlesh, Kasturi, Rachna, Kaushik, Naresh, Pallab, Aditi, Atreyi, Vigya, Priyanka, Sukhwinder, Suresh, Rajani and Anand for providing me moral support whenever I needed.

Naturally, I need not acknowledge my husband Dr. Biswanath Mahanty, who is always there in my life. However, I have to thank him for his constant encouragement

and moral support, listening to me and suggesting ways to solve problems whenever I needed, especially during the last tenure of my Ph.D. program, without which the present work would not have been successful. I wish thank him for supporting me in every aspect of life.

Last, but not the least I would thank God for the blessings I am always showered with in every step of my life.

Date: **Shampa Sen**

ABSTRACT

Microbial alkaline proteases are one of the most important hydrolytic enzymes and hence are being studied extensively since last two decades. These enzymes constitute 60% to 65% of the total enzymes sales in the world. These enzymes have versatile applications in many industries *viz*., tannery, dairy, food, pharmaceutical, silk, detergent, waste management and effluent treatment. Microbial alkaline proteases, especially from *Bacillus* sp. are most widely exploited industrially. In general, the majority of the commercially available enzymes are not stable in the presence of oxidizing agents, organic solvents and wide range of pH as well as temperature.

An efficient alkaline protease producer was isolated from a tannery waste soil, which grew in a wide range of pH. A new screening media was designed containing gelatin as a sole source of carbon and nitrogen. The culture broth (supernatant) was found to hydrolyze natural proteins *viz*., clotted blood, egg white and poultry feather. Very efficient dehairing was achieved using the culture broth (supernatant). The isolate was identified to be *Bacillus pseudofirmus* and designated as *B. pseudofirmus* SVB1 on the basis of 98% similarity in 16s rRNA sequence.

Physical parameters *viz*., initial pH of the medium, temperature and rpm of the shaking incubator were optimized using central composite experimental design to enhance the production of alkaline protease and cell growth simultaneously. The individual optimum levels of parameters were found to be 9.2, 27°C and 195 for initial pH of the medium, temperature and rpm of the shaking incubator, respectively for the production of alkaline protease (specific activity, U/mg of protein), and 11, 29°C and

210, respectively for cell growth. From multi-response analysis, the optimal levels were found to be 9.6, 28ºC and 191 rpm for initial pH of the medium, temperature and rpm of the shaking incubator, respectively for both production and cell growth. The values of cell growth (A_{600}), alkaline protease activity and specific activity obtained were 0.44, 40.03 U/ml, 6.37 U/mg, respectively, under these optimal conditions. Before optimization the values were 0.30, 30.56 U/ml, 4.7 U/mg, for cell growth (A_{600}), alkaline protease activity and specific activity, respectively. The production of alkaline protease and cell growth were enhanced by 36% and 44%, respectively under optimal levels of the physical parameters. The results obtained from optimization of individual responses and multi-response were compared.

Medium was developed to maximize the production of alkaline protease from *B. pseudofirmus* SVB1 in three steps. In the first step, various carbon/nitrogen source and salts were selected using one variable at a time (OVAT) method. Casein was found to be the most efficient source for both carbon and nitrogen for alkaline protease production along with $MgSO_4$, $CaCl_2$, NH_4NO_3, $FeCl_3$, K_2HPO_4, KH_2PO_4 and NaCl. In the second step, Plakett-Burman experimental design was applied to screen the significantly influencing medium components, and casein, $MgSO_4$, NH_4NO_3 and $CaCl_2$ were selected for optimization of their levels. These medium components were optimized using central composite experimental design in the third step to improve the production. The optimal levels of casein, $MgSO_4$, $CaCl_2$ and NH_4NO_3 were found to be 7.636 g/l, 0.811 g/l, 0.086 g/l and 0.576 g/l, respectively. An overall 4.9 fold increase in alkaline protease production (specific activity) was achieved in the optimized medium.

The alkaline protease produced from SVB1 was purified by acetone precipitation of culture supernatant followed by a cation exchange column chromatography (TOYOPERL®). Presence of a single band of 85 kDa on SDS-PAGE proved the homogeneity of the preparation and a clear zone of hydrolysis of casein in zymogram at pH 10.0 from SDS as well as native PAGE confirmed the band to be of alkaline protease. From characterization studies, the optimum pH and temperature were found to be 10.0 and 40°C, respectively. The enzyme was found to be serine alkaline protease. The alkaline protease exhibited wide substrate specificity (casein, gelatin and egg albumin). The enzyme had shown K_m of 1.83 mg/ml and V_{max} of 533.83 U. The thermodynamic properties (ΔH, ΔS, E and ΔG) were found to vary with pH.

The crude alkaline protease from *Bacillus pseudofirmus* SVB1 was found as much efficient as the partially purified counter part of it in removing burnt-on casein from the mica surface. Among the four procured enzymes, P-8038 cleaned the protein burnt-on residues as efficiently as our enzyme did. The other three commercial enzymes *viz*., P-4860, P-5459 and P-5380 performed the cleaning operation not significantly as compared to SVB1 alkaline protease both in crude and partially purified form. The burnt on effluents from dairy, abattoir house and poultry were also removed. SEM studies of goat skin revealed that the epidermis was completely removed and hair was uprooted leaving empty follicles in the skin using all the enzymes (crude, partially purified and commercial). Along with dehairing, the enzyme exhibited opening up of collagen bundles. Enzymatically removed intact hair will reduce the pollution load from leather manufacturing process.

Both the crude and partially purified alkaline proteases were found to be very useful in the treatment of effluents from tannery, dairy and domestic sewage. The reduction of TS, COD and BOD in all the effluents was achieved within 48 h using all the enzyme solutions and 72 h with the viable cells of SVB1. Poultry slaughterhouse waste was solubilized successfully with both crude and partially purified form of alkaline protease obtained in this study. Alkaline protease from *Bacillus pseudofirmus* SVB1 was found to solubilize abattoir solid wastes efficiently. Both the crude as well as the partially purified enzymes were found to reduce the microbial count in the domestic sewage. Also, the alkaline protease of *B. pseudofirmus* SVB1 was found to lyse the cells of both gram positive and gram negative species (*B. coagulans*, *P. carotovorum*, *S. marcescens* and *W. eutropha*).

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND NOTATIONS

AFM	Atomic force microscope
ANOVA	Analysis of variance
BH	Bushnell Hass
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
BSA	Bovine serum albumin
CCD	Central composite design
CIP	Clean in place
CM	Carboxymethyl
C/N	Carbon/nitrogen
DCl	Dichloroisocoumarin
DEAE	Diethylaminoethyl
DF	Degree of freedom
DFP	Diisopropylfluorophosphate
DO	Dissolved oxygen concentration
E-64	2,3-epoxypropyl-leucylamido (4-guanidine) butane
EC.	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
GA	Gelatin agar
ITCHY	Iterative truncation for the creation of hybrid enzymes
LB	Louria-Bertani broth
MS	Mean sum of squares
NA	Nutrient agar
NB	Nutrient broth
NCBI	National Center for Biotechnology Information

OD	Optical dnesity
OVAT	One variable at a time
PAGE	Polyacrylamide gel electrophoresis,
PB	Plackett-Burman
PEG	Polyethylene glycol
PMSF	Phenyl methyl sulfonyl fluoride
PPE	Partially purified enzyme
RACHITT	Random chimeragenesis on transient templates
RETT	Recombined extension on truncated templates
RSM	Response surface methodology
SBTI	Soybean trypsin inhibitor
SDS	Sodium n-dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SEM	Scanning electron microscopy
SS	Sum of squares
SSV	Settlable sludge volume
StEP	Staggered extension process
TCA	Trichloroacetic acid
TLCK	N-α-tosyl-L-lysinyl-Chloromethyl ketone
TNBS	Trinitrobenzene sulfonate
TOC	Total organic carbon
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TS	Total solid
UASB	Up-flow anaerobic sludge blanket
UF	Ultra filtration
YE	Yeast extract

K_m	Michaelis-Menten constant (mg/ml)
V_{max}	Maximum reaction rate for enzyme catalyzed reaction (U)
ΔA_{660}	Change in absorbance at 660 nm
C_t	OD equivalent tyrosine concentration
V_s	Volume of the reaction mixture (ml)
$\Delta A_{278.5}$	Change in absorbance at 278.5 nm
v	Volume of the enzyme source (ml)
V_m	Volume used in spectrophotometric determination (ml)
C	Optical density (OD)
ΔA_{600}	Absorbance at 600 nm
V	Initial rate of enzyme catalyzed reaction (U)
$[S]$	Initial substrate concentration (mg/ml)
E	Amount of enzyme in active state (U)
E_d	Amount of enzyme in inactive state (U)
$t_{\frac{1}{2}}$	Half life of enzyme (min)
k_d	First-order deactivation rate constant (min^{-1})
k_0	Frequency factor (min^{-1})
κ	Boltzmann's constant (J/K)
T	Absolute temperature (K)
h	Plank's constant (Js)
ΔS^*	Entropy change (J/mol K)
R	Universal gas constant (J/mol K)
ΔH^*	enthalpy change (kJ/mol)
ΔG^*	Gibb's free energy change (kJ/mol)
E_A	Activation energy (kJ/mol)

CHAPTER 1

INTRODUCTION, LITERATURE REVIEW AND OBJECTIVES

1.1 Introduction

Proteases are enzymes produced by microorganisms, plants and animal tissues, and catalyze the hydrolysis of peptide bonds in proteins. Proteases are classified based on site of action *viz*., endopeptidase and exopeptidase, activity in the wide range of pH (acid, neutral and alkaline protease) and 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). Alkaline proteases, active in neutral to alkaline pH range either have serine center (serine protease) or are of metallo-type (metalloprotease) (Ward, 1985) of which the alkaline serine proteases are the most important group of enzymes exploited commercially (Gupta *et al*., 2002a). Compared to animal and fungal proteases, bacterial alkaline proteases have more commercial importance due to their high production capacity and catalytic activity (Kumar and Takagi, 1999; Gupta *et al*., 2002a). Physical, biochemical, molecular and catalytic properties of alkaline proteases varies with the nature of the organism. Bacteria of the genus *Bacillus* are active producers of commercially available extracellular alkaline proteases (Kumar and Takagi, 1999; Gupta *et al*., 2002a; Haki and Rakshit, 2003). Although microbial alkaline proteases have been produced by solid-state and submerged fermentation, the latter has been more popular among researchers as found in literature. Alkaline proteases with high activity at different pH and at high temperatures have

potential applications in pharma, diagnostic, detergent, tannery, amino acid production, contact-lens cleaning agents, effluent treatment, enzymatic debridement and supporting the natural healing process in the skin ulcerations (Kumar and Takagi, 1999; Anwar and Saleemuddin, 2000; Gupta *et al*., 2002a; Sjodahl *et al*., 2002). The activity and stability are the most important parameters to understand utility of alkaline proteases in different sectors. In addition to robustness against solvents, surfactants and oxidants, the applications of alkaline proteases highly depends on their stability during isolation, purification and storage (Gupta *et al*., 2005; Najafi *et al*., 2005; El-Hadj-Ali *et al*., 2007; Sivasubramanian *et al*., 2008). Thus, in depth knowledge of kinetic and catalytic behavior of alkaline protease from any new strain is a prerequisite for evaluation of its biotechnological potential. Often, the wild type alkaline protease from any microbial strain is not suitable for industries and needs some improvement in their function in relation to activity and stability. Hence, there have been extensive studies on screening for new strains producing alkaline protease or/and protein engineering on the existing strains to obtain alkaline proteases suitable for commercial applications. Major industrial companies are continuously trying to identify enzymes that have potential industrial applications, either to use them directly or to create notified enzymes that have enhanced catalytic activity for well adapted large scale industrial processes (Glaser, 2000). However, these new enzymes would have to offer a competitive advantage over existing products. Details of the literature survey on different methods of protein engineering giving special emphasis on directed evolution to improve enzyme functions are presented elsewhere (Sen *et al*., 2007).

The present work focuses on screening and isolation of alkaline protease producing bacteria from tannery waste, identification of the most efficient producer among the isolates, maximization of the production of the alkaline protease from the chosen isolate and characterization of the alkaline protease to explore its potential for various industrial and environmental applications. The potential of the alkaline protease was tested for various applications *viz*., burnt-on protein removal from solid surface, goatskin dehairing, treatment of effluents from tannery, dairy, domestic sewage and slaughterhouse and the antibacterial activity of the enzyme. An attempt was made to test its efficiency in comparison to some commercially available alkaline proteases.

1.2 Literature Review

Alkaline proteases catalyze the hydrolysis and synthesis of proteins and peptides under alkaline condition. The many applications of alkaline proteases include detergent additives, dye removal and silk processing in textile industry, peptide syntheses in pharmaceutical industry, recovery of silver, hydrolysis of proteins and peptides, modification of proteins, test enhancement in food processing, resolution of racemic mixtures, and chemical analyses (Gupta *et al*., 2002a). The capability of suitable enzymes to catalyze reactions in a non conventional environment created considerable interest in the exploitation of proteases in organic solvents. Many researchers are continuously involved in efforts to isolate and identify novel microbial biosystems producing enzymes suitable for industrial as well as commercial applications directly and indirectly. A detailed literature survey on the production, purification, characterization and applications of microbial alkaline protease is presented in following sections to understand the state of the art development in this area.

1.2.1 Production of Alkaline Protease

1.2.1.1. Microorganisms producing alkaline protease: Isolation and screening

1.2.1.1.1 Isolation

Alkaline protease producing microorganisms have been isolated from various sources such as leather and dairy industries and natural environments like marine water hot spring water, soda lake and saline lake (Matta and Punj, 1998; Banerjee *et al*., 1999; Dayanandan *et al*., 2003; Joo *et al*., 2005; Mei *et al*., 2005; Amoozegar *et al*., 2007; Patel *et a*l., 2006a; Tari *et al*., 2006). Bacteria, fungi, yeasts, and actinomyces are found to produce alkaline protease (Kumar and Takagi, 1999). Bacteria are the most dominant group of alkaline protease producers. Among the bacteria, *Bacillus* (Kalisz, 1988; Kumar and Takagi, 1999; Rao *et al*., 1998; Gupta *et al*., 2002a) and *Pseudomonas* sp. are potential producers (Ogino *et al*., 1999; Bayoudh *et al*., 2000). Among actinomyces, *Streptomyces* are the preferred source, where as in fungi, *Aspergilli* is the most exploited group (Rajamani and Hilda, 1987; Petinate *et al*., 1999; Chakrabarti *et al*., 2000). *Conidiobolus* sp. and *Rhizopus* sp. also produce alkaline protease (Banerjee and Bhattacharyya, 1993; Bhosale *et al*., 1995). *Candida* sp. has been studied in detail as a potential alkaline protease producer among yeasts (Poza *et al*., 2001).

1.2.1.1.2 Screening of microorganisms producing alkaline protease

Horikoshi and Akiba (1982) described a simple and reliable method for detecting alkaline protease activity in microorganisms by using skim milk agar medium. In situ protease production from the potential isolates was identified by the clearing of opaque milk proteins in the area surrounding colonies growing on the surface. Nehra *et al*. (2004)

screened proteolytic fungi on skim milk agar medium and further confirmed the production of alkaline protease on modified Reese medium (pH 9.0).

1.2.1.2 Production, media development and assay methods for alkaline protease

1.2.1.2.1 Production and media development

Microbial alkaline proteases are produced both by submerged culture and solid state fermentation methods (Mukherjee *et al*., 2008; Nedra *et al*., 2007; Reddy *et al*., 2008). Immobilized cell cultures for microbial alkaline protease production have also been used (Adinarayana *et al*., 2004; Potumarthi *et al*., 2007). Optimal culture and nutritional requirements for alkaline protease production from different strains have been studied extensively (Potumarthi *et al*., 2007; Seyedeh *et al*., 2008). It has been postulated in earlier reports that the production of alkaline protease is influenced by the type and concentration of carbon and nitrogen sources, dissolved oxygen concentration, pH and temperature (Reddy *et al*., 2008; Seyedeh *et al*., 2008). Varela *et al*. (1996) reported that the medium components *viz*., variation in C/N ratio and metal ions had strongly influenced the production of alkaline protease from microorganisms. According to the study of Beg *et al*. (2002), the presence of some easily metabolizable sugars such as glucose influenced the production of alkaline protease significantly. Generally, high yield of alkaline protease have been achieved in the presence of both complex and inorganic carbon/nitrogen sources (Manachini *et al*., 1988; Banerjee *et al*., 1999; Ferrero *et al*., 1996; Johnvesly and Naik, 2001). Primarily, glucose or starch coupled with expensive nitrogen sources such as yeast extract, peptone, or casamino acids had been used to induce alkaline protease production (Hanlon *et al*., 1982; Kole *et al*., 1988; Mabrouk *et al*., 1999; Rahman *et al*., 2005). However, fewer attempts have also been made using low

cost carbon and nitrogen sources (Dey *et al*., 2001; Chauhan and Gupta, 2004). Enhanced growth and production of alkaline protease in the presence of complex carbon and organic nitrogen sources such as molasses, wheat flour and wheat bran have been reported (Sinha and Satyanarayana, 1991; Germano *et al*., 2003; Chauhan and Gupta, 2004; Shikha Saran and Darmwal, 2007). It was also reported that the production of protease was enhanced in the presence of ammonium sulfate and potassium nitrate by *Bacillus licheniformis* (Mabrouk *et al*., 1999). There are several reports on the repressive role of organic nitrogen sources, excessive amino acid and ammonium ions in alkaline protease production (Ferrero *et al*., 1996; Puri *et al*., 2002). Mehta *et al*. (2006) stated that the production of alkaline protease from *alkaliphilic actinomycete* was repressed above a critical limit of glucose, peptone, yeast extract, KH_2PO_4 and amino acids (tyrosine, tryptophan, lysine, and arginine) in the media.

1.2.1.2.2 Assay methods for alkaline protease

Alkaline protease activity can be measured by either qualitative or quantitative methods. The basic principle underlying both types of methods involves measurement of either the products of protein hydrolysis or of residual protein itself in alkaline condition. The commonly followed assays reported in large array of literature for the measurement of alkaline protease activity are described below.

1.2.1.2.2.1 Qualitative assays

Qualitative assays rely on the formation of a clear zone of proteolysis on agar plates as a result of protease production. The most commonly used qualitative assays include protein agar plate assay, radial diffusion and thin-layer enzyme assay.

Protein agar plate assay is commonly used for the initial screening of proteolytic activity, where depending upon the need and strategy of screening, different protein substrates are selected. The most commonly used protein substrates for selective screening are skim milk, casein, gelatin, bovine serum albumin (BSA) and keratin (Rajamani and Hilda, 1987; Vermelho *et al*., 1996; Friedrich *et al*., 1999). The plate assay can also be used to distinguish the type of protease as neutral or alkaline by manipulating the buffer system (Rajamani and Hilda, 1987).

Wikström *et al*. (1981) used radial diffusion or zone diffusion assay for semi-quantitative assessment of protease activity. The proteolytic activity was detected by observing the zone of hydrolysis around small wells cut in agar plates that contained the protein substrate. Hagen *et al*. (1997) described another method using the Coomassie prestained substrate agarose gel that allowed direct assessment of protease activity.

Wikström (1983) developed a convenient, low cost and sensitive technique, thin-layer enzyme assay to select specific microbial protease producers in a mixed sample. However, the major disadvantage of this assay was the requirement of more-or-less transparent culture agar medium supporting both optimal growth and enzyme production and simultaneously a high concentration of enzyme substrate in the agar gel.

1.2.1.2.2.2 Quantitative assays

Quantification of protease activity using spectrophotometry, fluorimetry, radiometry and enzyme-linked immunosorbent assay-based assays (ELISA) measures the extent of proteolytic potential of the enzyme by enumerating peptides in acid-soluble hydrolyzed product from natural or synthetic substrates (BSA, casein, hammerstein

casein, hemoglobin). Trivedi *et al*. (1999) used calf lens refolded β-crystallin aggregate as substrate for protease detection and assay in the pH range of 6-9.

Hatakeyama *et al*. (1992) developed a photometric assay for proteases with chemically succinylated casein as the substrate, where the extent of hydrolysis was determined using trinitrobenzene sulfonate (TNBS). The increase in absorbance due to reaction between TNBS and the newly formed amino groups in the substrate was determined using a microtiter plate reader (A_{405nm}). Unlike casein, succinyl casein is easily dissolved above pH 4 and serves as the substrate of choice for acidic proteases. However, colorimetric point assay (using TNBS) is tedious and cannot be used with enzymes with reducing agents such as dithiothreitol.

Gray *et al*. (1985) assayed alkaline protease HAP-PB92 from an alkalophilic *Bacillus* by monitoring release of p-nitroaniline from chromogenic tripeptide benzyloxy carbamoylglycyl-L-prolyl-L-citrulline-p-nitroanilide spectrophotometrically at 400 nm. Though, the assay was sensitive and required only a very short reaction time, the use of soluble chromolytic substrates necessitated the removal of nonhydrolyzed or partially hydrolyzed protein from the assay mixture before absorbance measurement. Insoluble chromolytic substrates, on the other hand, needed special care to ensure proper and adequate substrate distribution. In view of the above shortcomings, Wu and Abeles (1995) reported the use of magnetic beads with radio-labeled peptides replaced integration of substrate hydrolysis with separation of proteolytic products. Another, magnet-based protease assay was used by Safarikova and Safarik (1999) using dyed magnetic gelatin as an insoluble chromolytic substrate. Such substrates can be used to develop new automated protease assays based on the principle of flow injection analysis.

Fluorescence-based protease assays are simple, inexpensive and sensitive. Soluble fluorescein isothiocyanate (FITC)-labeled casein (Twining, 1984) or FTC-hemoglobin assays were used for acid proteases and trypsin (DeLumen and Tappel, 1970). The derivative is cleaved by various known proteases, *viz*., trypsin, chymotrypsin, elastase, subtilisin and thermolysin in a linear and time-dependent manner. The assay can be used to detect nanogram and sub-nanogram levels of these enzymes with reproducible results.

Ng and Auld (1989) developed fluorescent oligopeptide energy transfer assay. Fluorescent peptide substrate was designed to explore the protease specificity for the amino acids in the region of the cleavage site (C- and N-terminal). This assay was based on intramolecular quenching of indole fluorescence by an N-terminal dansyl group separated by six amino acid residues. Though this method was sensitive for detection and quantification of specific endoproteases but was not used much because of the high cost of the assay components.

Bedouet *et al*. (1998) used biotinylated-BSA as substrate for the enzyme-linked immunosorbent assay (ELISA) in polystyrene-coated microtiter plates. The absorbance was recorded at 405 nm using a microtiter ELISA reader. ELISA methods require three-dimensional structural detail of the test enzyme before using an antibody against it. Clements *et al*. (1990) used a very sensitive inhibition ELISA for detection and quantification of low levels (~0.24 ng/ml) of proteases as a framework for a test system in dairy industries. Koritsas and Atkinson (1995) developed a rapid, low cost and sensitive method for determining the proteolytic activity of different classes of endoproteases. Double-antibody-sandwich ELISA developed by Blair and McDowell (1995) could detect minute quantities of protease (~ 4 µg/ml).

The convenient, rapid and simple assay method of Cheung *et al*. (1991) was based on utilizing gelatin on the surface of an unprocessed Kodak X-Omat AR film as a proteolytic substrate. This could be used for a variety of proteases under a wide pH range of 5 to 8.5 and was useful as a general laboratory procedure.

Alhough a large array of assay protocols is available for protease estimation, in view of their reliability, ease of performance and cost effectiveness, the skim milk agar assay and casein-based spectrophotometric assays remain the methods of choice for routine laboratory analysis.

1.2.2 Statistical Techniques to Optimize the Production of Alkaline Protease

Optimizing, in general, refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. The term optimization has been commonly used in bioprocesses as a means identify the conditions that produce the best possible response(s). Traditionally, optimization in bioprocesses is carried out by monitoring the influence of one variable at a time (OVAT) on an experimental response (Trinetta *et al*., 2008; Jürgens *et al*., 2002; Seidel *et al*., 2002; Adinarayana *et al*., 2003). While only one parameter is changed, others are kept at predetermined level in this technique. This method fails to explain the interaction among the variables and to maximize the production levels. In one-factor-at-a-time technique, it is needed to conduct large number of experiments which is tedious and time consuming. In order to overcome this problem, the optimization of bioprocesses is carried out by using multivariate statistic techniques (Li *et al*., 2001; Krishna Prasad *et al*., 2005; Rao *et al*., 2004; Puri *et al*., 2002). Response surface methodology (RSM) is the most relevant multivariate technique among the statistical techniques used in analytical optimization.

Response surface methodology is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical predictions (Box and Hunter, 1975). It can be well applied when a response or a set of responses of interest are influenced by several variables. The objective is to simultaneously optimize the levels of these variables to attain the best performance of the system. Before applying the RSM, it is necessary to choose an experimental design that will define experiments needed to be carried out in the experimental region being studied. In reality, there may be a great number of factors influencing a process, but it does not mean that all the factors have significant effects on it. More often than not, the factors that influence the process significantly will have more attention than those that influence at low level, because the former are essential to the successful operation of the process. Thus, the first step to optimize a process is to identify the factors which have shown significant effect on the process.

Plackett–Burman design, which is a two-level fractional factorial design developed by Plackett and Burman, has been extensively used to screen significant factors for further investigation (Kennedy and Krouse, 1999; Srinivas *et al*., 1994; Ahuja *et al*., 2004; Reddy *et al*., 2008).

Experimental designs for first-order models (e.g., factorial designs) are used when the data set does not present curvature. However, to approximate a response function to experimental data that cannot be described by linear functions, experimental designs for quadratic response surfaces should be used, such as three level factorial, Box–Behnken, central composite and Doehlert designs. Among these, central composite design was

widely employed for alkaline protease production (Chauhan and Gupta, 2004; Dey *et al*., 2001; Li *et al*., 2001; Puri *et al*., 2002; Ahuja *et al*., 2004; Bandaru *et al*., 2006; Reddy *et al*., 2008; Tanyildizi *et al*., 2005).

The central composite design was presented by Box and Wilson (Box and Wilson, 1951). This design consists of the following parts: (1) a full factorial or fractional factorial design; (2) an additional design, often a star design in which experimental points are at a distance of α from its center; and (3) a central point. Central composite design was extensively applied in the optimization of bioprocesses (Chauhan and Gupta, 2004; Dey *et al*., 2001; Li *et al*., 2001; Puri *et al*., 2002; Ahuja *et al*., 2004; Bandaru *et al*., 2006; Reddy *et al*., 2008; Tanyildizi *et al*., 2005).

There are several commercial software packages, Design-Expert (Stat-Ease, Inc., USA), Minitab (Minitab, Inc., USA) and *etc*., are available to design experiments using Plackett–Burman, central composite and Box–Behnken designs and their analysis.

1.2.3 Purification and Characterization of Alkaline Protease

1.2.3.1. Purification strategies

A number of alkaline proteases from different sources have been purified and characterized. In general, for extra cellular enzymes, after separating the culture from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by means of ultrafiltration, salting out by ammonium sulfate, or solvent extraction methods using acetone and ethanol (Kobayashi *et al*., 1996; Thumar and Singh, 2007; Thangam and Rajkumar, 2002; Espósito *et al*., 2009). Additionally, other methods, such as PEG, activated charcoal, temperature-sensitive hydrogel, heat treatment of enzyme and

lyophilization (Porto *et al*., 2005; Aikat *et al*., 2001; Han *et al*., 1995; Manonmani and Joseph, 1993) are also used for concentrating alkaline proteases.

After concentration, further purification has been achieved in various chromatographic procedures such as ion exchange and hydrophobic interaction chromatography (CM-cellulose, DEAE-cellulose, phenyl-sepharose or DEAE-sepharose), affinity chromatography (bacitracin-sepharose column, sepharose bound to inhibitor/substrate or CNBr-activated sepharose) or gel filtration chromatography (such as sephadex) (Khosravi-Darani *et al*., 2008; Thumar and Singh, 2007; Gupta *et al*., 2005; Ma *et al*., 2007; Turkiewicz *et al*., 1999; Jahreis *et al*., 2001; Patel *et al*., 2006b). More sensitive and advanced procedures such as FPLC, converging-diverging foam fractionation, crystallization and preparative PAGE are also some times used for the purification of alkaline proteases (Abbas *et al*., 1989; Banerjee *et al*., 1993; Park *et al*., 1997; Phadatare *et al*., 1992).

1.2.3.2. Characterization of alkaline protease

1.2.3.2.1 Optimum pH and stability of alkaline proteases

Large number of acidic and basic amino acids located on surface of enzymes determines the net charge, charge distribution and reactivity of the catalytically active groups as acid dissociation constants of the amino acids varies with the pH of their environment. These effects are especially important in the neighborhood of the active sites, determines overall activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990).

The optimum pH range of alkaline proteases is generally between pH 9 and 11 (Ghorbel *et al*., 2003). Lee *et al*. (2002) reported that the optimum pH of the purified

protease was 8. The main commercially detergent enzymes for example Alcalase™ (Novozymes A/S) produced by *B. licheniformis*, Savinase™ (Novozymes A/S) produced by *B. clausii* and Purafect™ (Genencor International, Inc., USA) a genetic engineered donor *B. lentus* expressed in *Bacillus* sp. and Maxatase™ (Gist-Brocades) produced by *B. licheniformis* have maximal activity between pH 8.0-9.0 (Beg and Gupta, 2003), 8.0-10.0 (Maurer, 2004), 10.0 and 9.5-10.0, respectively. Nilegaonkar *et al*. (2007) reported protease having activity in a broad pH range from 6.0 to 12.0 having optimum activity at pH 9.0 associated with drastic reduction of activity on either side of optima. Alkaline proteases from *Vibrio fluvialis* VM10 (Venugopal *et al*., 2006), *Salinivibrio* sp. (Amoozegar *et al*., 2007), *gamma-Proteobacterium* (Sana *et al*., 2006), *A. clavatus* ES1 (Hajji *et al*., 2007) and some *Bacillus* sp., (Beg and Gupta, 2003; El-Hadj-Ali *et al*., 2007; Horikoshi, 1990) have been reported to show stability at pH between 8.0 and 10.5.

1.2.3.2.2 Optimum temperature and thermal stability of alkaline proteases

The heat stability of enzymes is largely dependent on its primary structure and specific additives present in solution or both. A high content of hydrophobic amino acids provides a compact structure, when the enzyme does not easily denature by change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Similarly, presence of specific components (polysaccharides and divalent cations) would stabilize the molecule (Öztürk *et al*., 2007).

Generally alkaline proteases produced from alkaliphilic *Bacillus* are known to be active over a wide range of temperature, with optimum temperature ranging from 40 to 80ºC. Lee *et al*. (2002) reported that the optimum temperature of purified protease was

ranged from 40 to 50ºC. A novel protease purified from the culture supernatant of *Yersinia ruckeri* (fish pathogen) was reported to remain active in the range of 25 to 42ºC with an optimum at 37ºC (Secades and Guijarro, 1999). Similarly, 45°C was the optimum temperature for extracellular proteinase (PSCP) produced by *Pseudomonas cepacia* (Mckevitt *et al*., 1989). The enzyme from an obligatory alkaliphilic *Bacillus* P-2 showed an exceptionally high optimum temperature of 90ºC. The protease from *Bacillus* P-2 has also good thermostability at high temperatures, being thermostable at 90ºC for more than 1 h and retained 95 and 37% of its activity at 99ºC (boiling) and 121ºC (autoclaving temperature), respectively. *Bacillus* P-2 was the only mesophile reported until 2001, which produced a proteolytic enzyme that was stable for so long even at autoclaving (121ºC) and boiling temperatures (Kaur *et al*., 2001). Various authors (Takami *et al*., 1989; Rahman *et al*., 2006) have correlated thermo-stability with addition of Ca^{2+}.

1.2.3.2.3 Kinetic and thermodynamic properties

Various substrates, *viz*., casein, azocasein and paranitroanilides esters are used to determine the kinetic parameters for alkaline proteases. The synthetic substrates are much more popular than complex substrates for defining K_m (Michaelis–Menten constant for enzyme catalyzed reactions) and V_{max} (maximum reaction rate for enzyme catalyzed reactions) (Kumar, 2002; Larcher *et al*., 1996). Kinetic properties, K_m, V_{max}, *etc*., are not only enzyme specific but also substrate and environment specific, and knowledge about them is essential for designing enzyme reactors or quantifying the applications of the enzyme in different conditions. As the reaction temperature was raised from 45 to 60ºC for an alkaline protease from *B. mojavensis* using casein as substrate, the K_m decreased with corresponding increase in V_{max} (Beg *et al*., 2002). In contrast, the K_m and V_{max} for an

alkaline protease from *Rhizopus oryzae* increased with an increase in temperature from 37ºC to 70ºC (Banerjee and Bhattacharyya, 1993). Kaur *et al*. (1998) reported K_m of 3.7 mg/ml in *B*. *polymyxa* protease, while Thangam and Rajkumar (2002) reported a K_m and V_{max} of 1.66 mg/ml and 526 U, respectively for alkaline protease of *Alcaligenes faecalis* using casein as a substrate.

1.2.3.2.4 Effect of metal ions

Salt tolerant proteases are highly significant from an industrial point of view (Joo *et al*., 2005). Kim (2004) reported a metalloprotease from a marine *Vibrio* whose activity was decreased by 16% in the presence of 0.5 M NaCl and also the production of this extracellular protease was severely inhibited. In another study, the alkaline protease retained more than 70% of its maximum activity after 18 h pre-incubation with 35% of NaCl (Sana *et al*., 2006).

Thermostability of alkaline protease from *Bacillus stearothermophilus* F1 was improved by the addition of Ca^{2+} ions, where the optimum temperature was raised to 85°C in the presence of 2.0 mM Ca^{2+} from 75°C without Ca^{2+} ions (Rahman *et al*., 2006). Similarly, protease from *Bacillus* species is active at 80°C (Kumar *et al*., 1993). Calcium chloride (5.0 mM) stabilized the activities of alkaline protease 1 (AP1) and alkaline protease 2 (AP2) at the pH range of 6.0–11.0 (Fukuda *et al*., 1997). Divalent cations like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these are required for alkaline proteases activity and thermal stability. It was observed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme even at high temperatures (Kumar and Takagi, 1999).

1.2.3.2.5 Effect of inhibitors

Inhibition studies give insight into the nature of the enzyme, requirement for cofactor and the nature of the active site. Alkaline proteases activities are known to be completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP) (Rao *et al*., 1998). PMSF contributing in sulfonation of the essential serine residue in the active site results in complete loss of activity is classified as serine hydrolases. PMSF and DFP are well-known inhibitors of serine proteases, which indicate involvement of serine residue in the catalytic activity. In addition, some of the alkaline proteases were found to be metal ion dependent reflected in their sensitivity to metal chelating agents such as EDTA. Thiol inhibitors have little effect on alkaline proteases of *Bacillus* sp., although they affect the alkaline enzymes produced by *Streptomyces* sp. (Kumar and Takagi, 1999).

Proteases can also be classified by their sensitivity to various inhibitors (Rao *et al.,* 1998). Inhibitors like N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and N-α-tosyl-L-Lysinyl-Chloromethylketone (TLCK) are chymotrypsin alkylating agent, benzamidine is a trypsin competitive reagent and SBTI is a soybean trypsin inhibitor (Rahman *et al*., 2006). Tsuchida *et al*. (1995) and Yamagata *et al*. (1989) reported that the protease was fully inhibited by PMSF. Alkaline protease from *Pseudomonas* species are dramatically inhibited by EDTA or EGTA and o-phenantroline (Rahman *et al*., 2006). EDTA and o-phenantroline were also reported to inhibit two novel extracellular alkaline metallo-endo-peptidases from *Vibrio* sp. NUF-BPP1 (Fukuda *et al*., 1997) and proteases from *S. corchorusii* ST36 (Yamagata *et al*., 1989).

1.2.3.2.6 Effect of surfactants

Tremacoldi *et al*. (2007) reported that the protease from *A. clavatus* CCT2759 was strongly inhibited by SDS and Tween 80. However, asparasiticus protease retained about 97% of its initial activity even after 1 h incubation with 2% SDS at room temperature (Tunga *et al*., 2003).

1.2.3.2.7 Substrate specificity

Alkaline proteases have broad substrate specificity and are active against a number of synthetic substrates (Shahrzad *et al*., 2005; Joo *et al*., 2001) and natural proteins (Gupta *et al*., 2005; Joo *et al*., 2001). However, the literature conclusively suggests that alkaline proteases are more active against casein than azocasein and hemoglobin or BSA (Gupta *et al*., 2002b). Moreover, there are specific types of alkaline proteases which are active against specific protein substrates (Friedrich *et al*., 1999), *viz*., collagenase (Ogino *et al*., 2008), elastase (Gupta *et al*., 2005), keratinase (Gessesse *et al*., 2003) and insect cuticle-degrading protease (Urtz and Rice, 2000).

1.2.4 Molecular Approaches for Improvement of Enzyme Functions

The efficient application of biocatalysts requires the availability of suitable enzymes with high activity and stability under process conditions, desired substrate selectivity and high enantio-selectivity. However, wild-type enzymes often need to be optimized to fulfill these requirements. The costs of alkaline protease production are expected to decrease as technology and techniques advance as along with exploration of low cost growth substrates for bacteria and fungi. Genetically modified organisms targeted to produce recombinant enzyme with desirable properties in fungal or bacterial

hosts have resulted in maximize expression of heterologous genes significantly above 40 g/l, where the wild type organisms produces a complex mixtures of enzymes at relatively low yields of less than 10 g/l (Cherry and Fidantsef, 2003). Directed evolution and rational protein design tools are used on molecular level to create tailor-made biocatalysts. Rational design usually requires both the availability of the structure of the enzyme and knowledge about the relationships between sequence, structure, and mechanism/function and is therefore, very information-intensive. The concept of directed evolution that mimic evolution on a laboratory timescale was introduced in 1967 (Mills *et al*., 1967). Application of directed evolution for engineering of enzymes with altered activity, specificity and stability has high potential. In vitro recombination techniques such as DNA shuffling, staggered extension process (StEP) (Zhao *et al*., 1998), random chimeragenesis on transient templates (RACHITT) (Coco, 2003), iterative truncation for the creation of hybrid enzymes (ITCHY) (Ostermeier *et al*., 1999) and recombined extension on truncated templates (RETT) (Lee *et al*., 2003) have been developed to mimic and accelerate nature's recombination strategy. In view of this, the cost of using alkaline protease in industries or as a tool to clean environment might be very less in near future making it a potential technique.

1.2.5 Applications

Alkaline proteases are one of the most important groups of enzymes, used in various industrial products/processes as detergents, pharmaceuticals, leather, meat tenderizers, protein hydrolyzates, food products and even in the waste processing. The alkaline protease produced from *Bacillus* sp. are the most widely exploited in industrial

applications as well as for bioremediation and as probiotic agent in aquaculture (Gupta *et al*., 2002a).

1.2.5.1. Industrial applications of alkaline proteases

Bacterial alkaline proteases find its various applications in diverse industrial sectors, where different companies worldwide have successfully launched numerous products (Gupta *et al*., 2002a), making it major player of the enzyme market all over the world (Kalisz, 1988). Alkazym (Novodan, Copenhagen, Denmark), Tergazyme (Alconox, New York, USA), Ultrasil (Henkel, Dusseldorf, Germany) and P3-pardigm (Henkel-Ecolab, Dusseldorf, Germany) used for cleaning of membrane systems; Pronod 153L for cleaning surgical instruments fouled by blood proteins and Subtilopeptidase A (Bausch and Lomb (India) Ltd.) marketed as optical cleaner (Kumar *et al*., 1999; Anwar and Saleemuddin, 2000) are few key examples of its potential. Protease solution has even found its way to clean packed columns of stainless steel particles fouled with gelatin (Sakiyama *et al*., 1999). In addition to these major applications, alkaline proteases are also reported to be used as contact lens cleaner (Nakagawa, 1994), in molecular biology for the isolation of nucleic acid (Kyon *et al*., 1994), for pest control (Kim *et al*., 1999), degumming of silk (Kanehisa, 2000) and for selective delignification of hemp (Dorado *et al*., 2001). The proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations by the efficient removal of the necrotic material (Sjodahl *et al*., 2002). However, these later applications though successful in a laboratory scale have yet not reached profitable sensation in terms of striking sales figures.

1.2.5.1.1 Detergent industry

The very first use of proteases as detergent from pancreatic extracts by Roehm in 1913 (Maurer, 2004) was in fact the first leap alkaline proteases toward its promising application, with respect to market volume and tonnage, in the giant detergent industry market. However, the use of enzyme was inefficient and uneconomical till identification of bacterial proteases in 1960s. The subtilisins from *Bacillus* species remains the sole source of proteases used in the detergent industry till date (Maurer, 2004). The fact that the amount of subtilisin produced and used in the European Union in 2002 was 900 tons, which flamboyantly reveals the importance of alkaline proteases (Human Health and Environmental Risk Assessment 'risk assessment of detergent raw materials': http://www.heraproject.com).

The proteases, which hydrolyse the proteinaceous residues of blood, egg, grass and sweat to form soluble peptides, are subsequently easily removed by detergent suds. All the enzymes, which are in present use, come from *Bacillus* sp. and that marketed mainly by just two companies. Novo Industry A/S produce and supply three proteases, Alcalase from *B. licheniformis*, Esperase from an alkalophilic strain of *B. licheniformis* and Savinase from an alkalophilic strain of *B. amyloliquefaciens* (often mistakenly attributed to *B. subtilis*). Gist Brocades produce and supply Maxatase from *B. licheniformis*. Alcalase and Maxatase (both mainly subtilisin) are recommended for use at 10-65°C and pH 7-10.5. Savinase and Esperase can be used at pH up to 11 and 12, respectively.

1.2.5.1.2 Food and feed industry

Traditionally, microbial proteases have been exploited in the food industries in the preparation of protein hydrolysates from various natural protein substrates, which play an important role in blood pressure regulation and are used in infant food formulations and specific therapeutic dietary products of high nutritional value, in the fortification of fruit juices and soft drinks (Neklyudov *et al*., 2000; Ward, 1985). The commercial protein hydrolysates are derived from casein (Miprodan; MD Foods, Viby, Germany), whey (Lacprodan; MD Foods) and soy protein (Proup;, Novo Nordisk, Bagsvaerd, Denmark) (Gupta *et al*., 2002a). Rebeca *et al*. (1991) reported that the fish hydrolysates of high nutritional value were produced using *B. subtilis* proteases. Protein hydrolysates of sardine muscle after treatment with *B. licheniformis* have shown inhibitory activity against angiotensin-I-converting enzyme (Matsui *et al*., 1993). Fujimaki *et al*. (1970) and Perea *et al*. (1993) used alkaline protease for the production of soy protein and whey protein hydrolysates. O'Meara and Munro (1984) reported that the upgrading of lean meat waste to edible products by alkaline protease hydrolysis of the meat waste, using commercial alkaline proteases. Tanimoto *et al*. (1991) reported that the use of alkaline protease in the enzymatic modification of zein to produce a non-bitter peptide fraction with high Fischer ratio for patients with hepatic encephalopathy. Keratinolytic activity of alkaline protease has also been exploited in the production of proteinaceous fodder from waste feathers or keratin-containing materials. Dalev (1994) and Cheng *et al*. (1995) reported the use of alkaline proteases (B72 from *B. subtilis* and *B. licheniformis* PWD-1) for the hydrolysis of feather keratin, to obtain a protein concentrate for fodder production.

1.2.5.1.3 Leather industry

Due to increased legislation geared towards environmental protection and preservation of water quality, tanneries are under constant pressure to implement "source reduction technologies" that reduce their organic and inorganic wastewater loading, where enzyme-based technologies are gaining importance for eco-friendly and cleaner technologies (Laxman *et al*., 2005). Due to specificity, and activity under harsh conditions, possibility of producing 'natural' products, non-polluting nature and biodegradability makes use of enzymes as one ways of solving the industrial pollution problems related to tannery effluent (Feigel, 1998). Proteolytic enzymes from a large number of *Bacillus* sp. and *Streptomyces* sp. have been used in dehairing of hides and skins (Huang *et al*., 2003, Riffle *et al*., 2003; Alexandre *et al*., 2005; Padmavathi *et al*., 1995). Lime and sulfide-free process of dehairing has been developed for the manufacturing of suede from goat skin and cow hides (Sivasubramanian *et al*., 2008). Enzymatic hair loosening processes play a role, wherever high-quality hair, wool or bristles are to be recovered (Khardenavis *et al*., 2009). Enzyme preparation from *B. subtilis* has successfully been used for bating pig skins generating bated skins with good physicochemical properties (Palanisamy *et al*., 2004).

1.2.5.2. Applications of alkaline proteases in waste and wastewater treatment

1.2.5.2.1 Applications of alkaline proteases in food industry waste processing

Proteases solubilize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era in waste management. Protein-based residues usually represent the

most significant potential foulants within food sectors, such as in milk- and meat-processing operations. Turner *et al*. (2005) envisaged that the replacement of acid/alkali washes from bioprocess cleaning operations with enzymatic alternative to reduce cleaning costs and potential hazard to bioprocess personnel and the environment as well as increased lifetime of equipment would therefore have significant potential advantages.

1.2.5.2.2 Applications of alkaline proteases in abattoir waste processing

Waste feathers, which make up approximately 5% of the poultry body weight, could be high protein source for food and feed if the rigid keratin structure could be destroyed. Dalev (1994) used alkaline protease from *B*. *subtilis* for the management of waste feathers from poultry slaughterhouses. Grazziotin *et al*. (2006) reported that the protease was used to hydrolyze food and feed industry waste and for degradation of keratinous material from poultry refuse. Zambare *et al*. (2007) used alkaline protease as depilatory agent to remove hair from the drains. A formulation containing proteolytic enzymes from *B*. *subtilis*, *B*. *amyloliquefaciens, Streptomyces* sp. and a disulfide reducing agent (thioglycolate) that enhances hair degradation and helps in clearing pipes clogged with hair-containing deposits is currently available in market (Gupta *et al*., 2002a).

1.2.5.2.3 Applications of alkaline proteases in silver recovery from photographic films

Used X-ray or photographic films contains 1.5-2.0% silver by weight in their gelatin layer can be used as a good source of silver for a variety of purposes if suitably recovered. Conventionally, this silver is recovered by burning the films, which causes undesirable environmental pollution and if the base film is made of polyester, silver cannot be recovered using this method. Since the silver is bound to gelatin, it is possible

to extract silver from the protein layer by proteolytic treatment using alkaline proteases. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but the polyester film base can also be recycled. Masui *et al*. (2004) used alkaline protease from *Bacillus* sp. B21-2 for the enzymatic hydrolysis of gelatin layers of X-ray films to release silver particles. The alkaline proteases of *Bacillus* sp. B18 (Fujiwara *et al*., 1991) and *B. coagulans* PB-77 (Gajju *et al*., 1996) were also efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered.

1.3 Objectives and Scope

Based on the extensive literature survey on the commercial importance of alkaline protease and the need to obtain new alkaline protease with better stability, activity and versatile applicability, the present investigation was focused on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1. In order to establish its potential applications in industries and for cleaning environment, experiments were conducted and compared with other commercially available alkaline proteases. Following are the detailed investigations performed in the present research work.

- Isolation, screening and identification of microorganisms for the production of alkaline protease.
- Optimization of the physical process parameters to maximize the production of alkaline protease.
- Development of medium to enhance the production of alkaline protease.
- Purification and characterization of alkaline protease.
- Exploring the suitability of the crude alkaline protease for potential industrial applications *viz*., goat skin dehairing (target industry: leather) and 'burnt-on'

protein residue removal from solid surface (target industry: food) and comparing the efficiency with the partially purified enzyme and commercially available alkaline proteases.

- Assessment of the applicability of the crude alkaline protease in different effluent treatment processes *viz*., tannery, dairy and domestic sewage effluents and evaluating its efficiency in comparison to the partially purified enzyme and commercially available alkaline proteases.

1.4 Organization of the Thesis

The presentation of the research work has been divided into eight chapters. The current **Chapter 1** presents a general introduction, relevant literature survey on the production, purification and characterization of microbial alkaline protease, objectives and scope of the present research work. Details on the isolation and screening of the microorganism producing alkaline protease from tannery waste, its identification and potential in degrading various natural proteins are described in **Chapter 2**. **Chapter 3** contains the optimization of physical process parameters to maximize the production of alkaline protease from the isolate using statistical design techniques. Selection of most significant C/N source, screening and optimization of concentration levels of medium components to maximize the production of alkaline protease from the isolate have been discussed in **Chapter 4**. In **Chapter 5**, the purification and characterization of the alkaline protease has been discussed thoroughly. The homogeneity of the alkaline protease preparation was established by the presence of single band in SDS-PAGE and its zymogram. The kinetic parameters and thermodynamic parameters were determined, and the effect of pH and temperature were emphasized in this chapter. It also describes the

effect of various chemical and physical parameters on the activity of the alkaline protease. **Chapter 6** explores the potential applications of alkaline protease in skin dehairing and removal of 'burnt-on' protein residue from solid surface. This chapter envisages the applicability of the enzyme in tannery (dehairing) and food industry (process equipment cleaning) in comparison with couple of commercial alkaline proteases. In **Chapter 7**, the possibility of applying the enzyme for different protein rich effluent (tannery, dairy and domestic sewage) treatment processes has been estimated. Comparison with commercial enzymes in this aspect has also been discussed. **Chapter 8** summarizes the inferences drawn from the present research work and its salient accomplishments. This chapter also provides the recommendations towards the future direction.

References

Abbas, C.A., Groves, S. and Gander, J.E. (1989) Isolation, purification and properties of *Penicillium charlesii* alkaline protease. *J. Bacteriol*., **171**: 5630-5637.

Adinarayana, K., Prabhakar, T., Srinivasulu, V., Anitha Rao, M., Jhansi Lakshmi, P. and Ellaiah, P. (2003) Optimization of process parameters for cephalosporin C production under solid state fermentation from *Acremonium chrysogenum*. *Process Biochem*., **39**: 171-177.

Adinarayana, K., Bapi-Raju, K.V.V.S.N., Ellaiah, P. (2004) Investigation on alkaline protease production with *B. subtilis* PE-11 immobilized in calcium alginate gel beads. *Process Biochem*., **39**: 1331-1339.

Ahuja, S.K., Ferreira, G.M., Moreira, A.R. (2004) Application of Plackett-Burman Design and Response Surface Methodology to achieve exponential growth for aggregated shipworm bacterium. *Biotechnol*. *Bioeng*., **85**: 666-675.

Aikat, K., Maiti, T.K., Bhattacharyya, B.C. (2001) Decolourization and purification of crude protease from *Rhizopus oryzae* by activated charcoal and its electrophoretic analysis. *Biotechnol*. *Lett*., **23**: 295-301.

Alexandre, J.M., Walter, O., Beys Da, S., Renata, G., David, D., Joao, A., Pegas, H. and Carlos, T. (2005) Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. *Appl*. *Environ*. *Microbiol*., **71**: 594-596.

Amoozegar, M.A., Fatemi, A.Z., Karbalaei-Heidari, H.R. and Razavi, M.R. (2007) Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004. *Microbiol*. *Res*., **162**: 369-377.

Anwar, A. and Saleemuddin, M. (2000) Alkaline protease from *Spilosoma obliqua*: potential application in bioformulations. *Biotechnol*. *Appl*. *Biochem*., **31**: 85-89.

Bandaru, V.V.R., Somalanka, S.R., Mendu, D.R., Madicherla, N.R. and Chityala, A. (2006) Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology. *Enzyme Microb*. *Technol*., **38**: 209-214.

Banerjee, R. and Bhattacharyya, B.C. (1993) Kinetic properties of extracellular alkaline proteases of *Rhizopus oryzae*. *J. Ferment*. *Bioeng*., **75**: 380-382.

Banerjee, R., Agnihotri, R. and Bhattacharya, B.C. (1993) Purification of alkaline protease of *Rhizopus oryzae* by foam fractionation. *Bioprocess Eng*., **9**(6): 245-248.

Banerjee, U.C., Sani, R.K., Azmi, W. and Soni, R. (1999) Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochem*., **35**: 213-219.

Bayoudh, A., Gharsallah, N., Chamkha, M., Dhouib, A., Ammar, S. and Nasri, M. (2000) Purification and characterization of an alkaline protease from *Pseudomonas aeruginosa* MN1. *J. Ind. Microbiol. Biotechnol*., **24**: 291-295.

Bedouet, L., Arnold, F., Batina, P. and Robreau, G. (1998) Potential application of the *Clostridium tyrobutyricum* flagellin as substrate for proteinase detection. *Biotechnol. Lett*., **20**: 1011-1016.

Beg, Q. and Gupta, R. (2003) Purification and characterization of an oxidation stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb. Technol*., **32:** 294-304.

Beg, Q.K., Saxena, R.K. and Gupta, R. (2002) Kinetic constants determination for an alkaline protease from *Bacillus mojavensis* using response surface methodology. *Biotechnol. Bioeng*., **78**: 289-295.

Bhosale, S.H., Rao, M.B., Deshpande, V.V. and Srinivasan, M.C. (1995) Thermostability of high-activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20). *Enzyme Microb. Technol*., **17**: 136-139.

Blair, I.S. and McDowell, D.A. (1995) Detection of extracellular proteinase of *Pseudomonas fragi* by enzyme-linked immunosorbent assay. *Curr. Microbiol*., **31**: 180-185.

Box, G.E. and Hunter, J.S. (1975) Multiple experimental designs for exploring response surfaces. *Ann. Math. Stat*., **28**: 195-241.

Box, G.E.P. and Wilson, K.B. (1951) Experimental attainment of optimum conditions. *J. Roy Stat. Soc*., **13**: 1-45.

Chakrabarti, S.K., Matsumura, N. and Ranu, R.S. (2000) Purification and characterization of an extracellular alkaline serine protease from *Aspergillus terreus* (IJIRA 6.2). *Curr. Microbiol*., **40**: 239-244.

Chaplin, M.F. and Bucke, C. (1990) *Enzyme Technology*. Cambridge: Cambridge University Press.

Chauhan, B. and Gupta, R. (2004) Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. *Process Biochem*., **39**: 2115-2122.

Cheng, S.W., Hu, M.N., Shen, S.W., Takagi, H., Asano, M. and Tsai, Y.C. (1995) Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. *Biosci*. *Biotech*. *Biochem*., **59**: 2239-2243.

Cherry, J.R., and Fidantsef, A.L. (2003) Directed evolution of industrial enzymes: an update. *Curr*. *Opin*. *Biotechnol*., **14**: 438-443.

Cheung, A.L., Ying, P. and Fischetti V.A. (1991) A method to detect proteinase activity using unprocessed X-ray films. *Anal*. *Biochem*., **193**: 20-23.

Clements, R.S., Wyatt, D.M., Symons, M.H. and Ewings, K.N. (1990) Inhibition enzyme-linked immunosorbent assay for detection of *Pseudomonas fluorescens* proteases in ultrahigh-temperature-treated milk. *Appl*. *Environ*. *Microbiol*., **56**: 1188-1190.

Coco, W.M. (2003) RACHITT: Gene family shuffling by Random Chimeragenesis on Transient Templates. *Methods Mol*. *Biol*., **231**: 111-127.

Dalev, P.G. (1994) Utilization of waste feathers from poultry slaughter for production of a protein concentrate. *Bioresour*. *Technol*., **48**: 265-267.

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

DeLumen, B.O. and Tappel, A.L. (1970) Fluorescein-hemoglobin as a substrate for cathepsin D and other proteases. *Anal*. *Biochem*., **26**: 22-29.

Dey, G., Mitra, A., Banerjee, R. and Maiti, B.R. (2001) Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. *Biochem*. *Eng*. *J*., **7**: 227-231.

Dorado, J., Field, J.A., Almendros, G. and Alvarez, R.S. (2001) Nitrogen-removal with protease as a method to improve the selective delignification of hemp stemwood by white-rot fungus *Bjerkandera* sp. strain BOS55. *Appl*. *Microbiol*. *Biotechnol*., **57**: 205-211.

El-Hadj-Ali, N., Agrebi, R., Ghorbel-Frikha, B., Sellami-Kamoun, A., Kanoun, S. and Nasri, M. (2007) Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. *Enzyme Microb*. *Technol*., **40**: 515-523.

Espósito, T.S., Amaral, I.P.G., Buarque, D.S., Oliveira, G.B., Carvalho, Jr L.B. and Bezerr, R.S. (2009) Fish processing waste as a source of alkaline proteases for laundry detergent. *Food Chem*., **112**: 125-130.

Feigel, T. (1998) The hair-save liming process: a tool for environment friendly *World Leather*, **11**: 31-32.

Ferrero, M.A., Castro, G.R., Abate, C.M., Baigori, M.D. and Sinerz, F. (1996) Thermostable alkaline protease of *Bacillus licheniformis* MIR 29: Isolation, production and characterization. *Appl*. *Microbiol*. *Biotechnol*., **45**: 327-332.

Friedrich, J., Gradisar, H., Mandin, D. and Chaumont, J.P. (1999) Screening fungi for synthesis of keratinolytic enzymes. *Lett*. *Appl*. *Microbio*l., **28**: 127-130.

Fujimaki, M., Yamashita, M., Okazawa, Y. and Arai, S. (1970) Applying proteolytic enzymes on soybean. 3. Diffusable bitter peptides and free amino acids in peptic hydrolyzate of soybean protein. *J*. *Food Sci*. **35**: 215-218.

Fujiwara, N., Yamamoto, K. and Masui, A. (1991) Utilization of a thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. *J*. *Ferment*. *Bioeng*., **72**: 306-308.

Fukuda, K., Hasuda, K., Oda, T., Yoshimura, H. and Muramatsu, T. (1997) Novel extracellular alkaline metalloendoproteases from *Vibrio* sp. NUF-BPP1: purification and characterization. *Biosci*. *Biotech*. *Biochem*., **61**: 96-101.

Gajju, H., Bhalla, T.C. and Agarwal, H.O. (1996) Thermostable alkaline protease from thermophilic *Bacillus coagulans* PB-77. *Indian J*. *Microbiol*., **36**: 153-155.

Germano, S., Pandey, A., Osaku, C.A., Rocha, S.N. and Soccol, C.R. (2003) Characterization and stability of protease from *Penicillium* sp. produced by solid-state fermentation. *Enzyme Microb*. *Technol*., **32**: 246-251.

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

Ghorbel, B., Sellami-Kamoun, A. and Nasri, M. (2003). Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microb*. *Technol*., **32**: 513-518.

Glaser, V. (2000) Steady growth for industrial enzyme market. *Genetic Eng*. *News*, **20**: 8-36.

Gray, C.J., Barker, S.A., Dhariwal, M.S. and Sullivan, J.M. (1985) Assay of the high-alkaline proteinase from alkalophilic *Bacillus* PB92 using a chromogenic tripeptide substrate. *Biotechnol*. *Bioeng*., **27**: 1717-1720.

Grazziotin, A., Pimentel, F.A., de Jong, E.V. and Brandelli, A. (2006) Nutritional improvement of feather protein by treatment with microbial keratinase. *Anim*. *Feed Sci*. *Technol*., **126**: 135-144.

Gupta, A., Roy, I., Khare, S.K. and Gupta, M.N. (2005) Purification and characterization of a solvent stable protease form *Pseudomonas aeruginosa* Pse A. *J. Chromatogr*., **1069**: 155-161.

Gupta, R., Beg, Q. and Lorenz, P. (2002a) Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl*. *Microbiol*. *Biotechnol*., **59**: 15-32.

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

Hagen, H.E., Klager, S.L., McKerrow, J.H. and Ham, P.J. (1997) A simple assay for the detection of native and recombinant protease activity. *Anal*. *Biochem*., **251**: 121-122.

Hajji, M., Kanoun, S., Nasri, M. and Gharsallah, N. (2007) Purification and characterization of an alkaline serine-protease produced by a new isolated *Aspergillus clavatus* ES1. *Process Biochem*., **72**: 791-797.

Haki, G.D. and Rakshit, S.K. (2003) Developments in industrially important thermostable enzymes: a review. *Bioresour*. *Technol*., **89**: 17-34.

Han, J., Park, C.H. and Ruan, R. (1995) Concentrating alkaline serine protease, subtilisin, using a temperature-sensitive hydrogel. *Biotechnol*. *Lett*., **17**: 851-852.

Hanlon, G.W., Hodges, N.A. and Russel, A.D. (1982) The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. *J*. *Gen*. *Microbiol*., **128**: 845-851.

Hatakeyama, T., Kohzaki, H. and Yamasaki, N. (1992) A microassay for proteases using succinylcasein as a substrate. *Anal*. *Biochem*., **204**: 181-184.

Horikoshi, K. and Akiba, T. (1982) Alkalophilic Microorganisms: A New Microbial World. Tokyo, Japan: *Japan Scientific Societies* Press and Berlin, Germany: Springer-Verlag.

Horikoshi, K. (1990) Enzymes of alkalophiles. In: *Microbial enzyme and biotechnology*, **2**nd ed., 275-294.

Huang, Q., Peng, Y., Li, X., Wang, H.F. and Zhang, Y.Z. (2003) Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*, *Curr*. *Microbiol*.**, 46**: 169-173.

Jahreis, G., Peters, K. and Kirschke, H. (2001) Purification of soluble and membrane-bound proteases with substrate-analogous inhibitors by affinity chromatography. *J. Biochem*. *Biophy*. *Meth*., **49**: 491-505.

Johnvesly, B. and Naik, G.R. (2001) Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. *Process Biochem*., **37**: 139-144.

Joo, H.S. and Chang, C.S. (2005) Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. *Process Biochem*., **40**: 1263-1270.

Joo, H.S., Park, G.C., Kim, K.M., Paik, S.R. and Chang, C.S. (2001) Novel alkaline protease from the polychaeta, *Periserrula leucophryna*: Purification and characterization. *Process Biochem*., **36**: 893-900.

Jürgens, M., Seidel, G. and Schügerl, K. (2002) Production of cephalosporin C by *Acremonium chrysogenum* in semisynthetic medium. *Process Biochem*., **38**: 263-272.

Kalisz, H.M. (1988) Microbial proteinases. *Adv*. *Biochem*. *Eng*. *Biotechnol*., **36**: 1-65.

Kanehisa, K. (2000) Woven or knit fabrics manufactured using yarn dyed raw silk. *US Patent*, **6080689**.

Kaur, M., Dhillon, S., Chaudhary, K. and Singh, R. (1998) Production, purification and characterization of thermostable alkaline protease from *Bacillus polymyxa*. *Indian*. *J*. *Microbiol*., **38**: 63-67.

Kaur, S., Vohra, R.M., Kapoor, M., Beg, Q.K. and Hoondal, G.S. (2001) Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus* sp. P-2. *World J*. *Microbiol*. *Biotechnol*., **17**: 125-129.

Kennedy, M. and Krouse, D. (1999) Strategies for improving fermentation medium performance: a review. *J*. *Ind*. *Microbiol*. *Biotechnol*., **23**: 456-475.

Khardenavis, A.A., Kapley, A. and Purohit, H.J. (2009) Processing of poultry feathers by alkaline keratin hydrolyzing enzyme from *Serratia* sp. HPC 1383. *Waste Manage*., **29**: 1409-1415.

Khosravi-Darani, K., Falahatpishe, H.R. and Jalali, M. (2008) Alkaline protease production on date waste by an alkalophilic *Bacillus* sp. 2-5 isolated from soil. African *J*. *Biotechnol*., **7** (10): 1536-1542.

Kim, H.K., Hoe, H.S., Suh, D.S., Kang, S.C., Hwang, C., Kwon, S.T. (1999) Gene structure and expression of the gene from *Beauveria basiana* encoding bassiasin I, an insect cuticle-degrading serine protease. *Biotechnol*. *Lett*., **21**: 777-783.

Kim, Y.J. (2004) The influence of NaCl and carbonylcyanide-m-chlorophenylhydrazone on the production of extracellular proteases in a marine *Vibrio* strain. *J*. *Microbio*l., **42**: 156-159.

Kobayashi, T., Hakamada, Y., Hitomi, J., Koike, K. and Ito, S. (1996) Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. *Appl*. *Microbial*. *Biotechnol*., **45**: 63-71.

Kole, M.M., Draper, I. and Gerson, D.F. (1988) Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. *J*. *Chem*. *Technol*. *Biotechnol*., **41**: 197-206.

Koritsas, V.M. and Atkinson, H.J. (1995) An assay for detecting nanogram levels of proteolytic enzymes. *Anal*. *Biochem*., **227**: 22-26.

Krishna Prasad, K., Venkata Mohan, S., Sreenivas Rao, R., Pati, B.R. and Sarma, P.N. (2005) Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. *Biochem*. *Eng*. *J*., **24**: 17-26.

Kumar, C.G. (2002) Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. *Lett*. *Appl*. *Microbiol*., **34**: 13-17.

Kumar, C.G. and Takagi, H. (1999) Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnol*. *Adv*., **17**: 561-594.

Kumar, C.G., Tiwari, M.P. and Jany, R.D. (1993) Novel alkaline proteases from alkalophilic *Bacillus* sp.: purification and some properties. *Process Biochem*., **34**: 441-449.

Kyon, Y.T., Kim, J.O., Moon, S.Y., Lee, H.H. and Rho, H.M. (1994) Extracellular alkaline proteases from alkalophilic *Vibrio metschnikovii* strain RH530. *Biotechnol*. *Lett*., **16**: 413-418.

Larcher, G., Cimon, B., Symoens, F., Tronchin, G., Chabasse, D. and Bouchara, JP. (1996) A 33 kDa serine proteinase from *Scedosporium apiospermum*. *Biochem*. *J*., **315**: 119-126.

Laxman, R.S., Sonawane, A.P., More, S.V., Rao, B.S., Rele, M.V., Jogdand, V.V., Deshpande, V.V. and Rao, M.B. (2005) Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus*. *Process Biochem*., **40**: 3152-3158.

Lee, C.Y., Cheng, M.F., Yu, M.S. and Pan, M.J. (2002) Purification and characterization of a putative virulence factor, serine protease, from *Vibrio parahaemolyticus*. *FEMS Microbiol*. *Lett*., **209**(1): 31-37.

Lee, S.H., Ryu, E.J., Kang, M.J., Wang, E-S, Piao, Z., Choi, Y.J., Jung, K.H., Jeon, J.Y.J. and Shin, Y.C. (2003) A new approach to directed gene evolution by recombined extension on truncated templates (RETT). *J*. *Mol*. *Catal*., *B Enzym*., **26**: 119-129.

Li, C., Bai, J., Cai, Z., Ouyang, F. (2001) Optimization of a cultural medium for bacteriocin production by Lactococcus lactis using response surface methodology. *J. Biotechnol*., **93**: 27-34.

Ma, C. Ni, X., Chi, Z., Ma, L.and Gao, L. (2007) Purification and characterization of an alkaline protease from the marine yeast *Aureobasidium pullulans* for bioactive peptide production from different sources. *Marine Biotechnol*., **9** (3): 343-351.

Mabrouk, S.S., Hashem, A.M., El-Shayeb, N.M.A., Ismail, A.S. and Abdel-Fattah A.F. (1999). Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415. *Bioresour. Technol*., **69**: 155-159.

Manachini, P.L., Fortina, M.G. and Parini, C. (1988) Thermostable alkaline protease produced by *Bacillus thermoruber*—a new species of *Bacillus*. *Appl. Microbial. Biotechnol*., **28**: 409-413.

Manonmani, H.K., Joseph, R. (1993) Purification and properties of an extracellular proteinase of *Trichoderma koningii*. *Enzyme Microb. Technol*., 15: 624-628.

Masui, A., Yasuda, M., Fujiwara, N. and Ishikawa, H. (2004) Enzymatic hydrolysis of gelatin layers on used lith film using thermostable alkaline protease for recovery of silver and PET film. *Biotechnol. Prog*., **20**(4): 1267-1269.

Matsui, T., Matsufuji, H., Seki, E., Osajima, K., Nakashima and M., Osajima, Y. (1993) Inhibition of angiotensin I-converting enzyme by *Bacillus licheniformis* alkaline protease hydrolyzates derived from sardine muscles. *Biosci. Biotechnol. Biochem*., **57**: 922-925.

Matta, H. and Punj, V. (1998) Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. *Int. J. Food Microbiol*., **42**: 139-145.

Maurer, K.H. (2004) Detergent proteases. *Curr. Opin. Biotechnol*., **15**: 330-334.

Mckevitt, A.I.., Bajaksouzian, S., Klinger, J.D. and Woods, D.E. (1989) Purification and characterization of an extracellular protease from *Pseudomonas cepacia*. *Infect. Immun*., **57**(3): 771-778.

Mehta, V.J., Thumar, J.T. and Singh S.P. (2006) Production of alkaline protease from an alkaliphilic actinomycete. *Bioresour. Technol*., **97**: 1650-1654.

Mei, C. and Jiang, X. (2005) A novel surfactant- and oxidation-stable alkaline protease from *Vibrio metschnikovii* DL 33-51. *Process Biochem.*, **40**: 2167-2172.

Mills, D.R. Peterson, R.L. and Spiegelman, S. (1967) An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule. *Proc. Natl. Acad. Sci. USA*., **58**(1): 217-224.

Mukherjee, A.K., Adhikari, H. and Rai, S.K. (2008) Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation. *Biochem. Eng. J.*, **39**: 353-361.

Najafi, M.F., Deobagkar, D. and Deobagkar, D. (2005) Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. *Elect. J. Biotechnol.*, **8**: 197-203.

Nakagawa, A. (1994) Method for cleaning a contact lens. *US Patent*, **5314823**.

Nedra, E.H.A., Rym, A., Basma, G.F., Alya, S.K., Safia, K. and Moncef, N. (2007) Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. *Enzyme Microb. Technol.*, **40**: 515-523.

Nehra, K.S., Singh, A., Sharma, J., Kumar, R. and Dhillon, S. (2004) Production and characterization of alkaline protease from *Aspergillus* species and its compatability with commercial detergents. *Asian J. Microbiol. Biotechnol. Environ. Sci.*, **6**: 67-72.

Neklyudov, A.D., Ivankin, A.N., Berdutina, A.V. (2000) Properties and uses of protein hydrolysates (review). *Appl. Biochem. Microbiol.*, **36**: 452-459.

Ng, M. and Auld, D.S. (1989) A fluorescent oligopeptide energy transfer assay with broad applications for neutral proteases. *Anal. Biochem.*, **183**: 50-56.

Nilegaonkar, S.S., Zambare, W.P., Kanekar, P.P., Dhakephalkar, P.K. and Sarnaik, S.S. (2007) Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. *Bioresour. Technol.*, **98**: 1238-1245.

Ogino, H., Otsubo, T. and Ishikawa, H. (2008) Screening, purification, and characterization of a leather-degrading protease. *Biochem. Eng. J.*, **38**: 234-240.

Ogino, H., Watanabe, F., Yamada, M., Nakagawa, S., Hirose, T., Noguchi, A., Yasuda, M. and Ishikawa, H. (1999) Purification and characterization of organic solvent-stable protease from organic solvent-tolerant *Pseudomonas aeruginosa* PST-01. *J. Biosci. Bioeng.*, **87**: 61-68.

O'Meara, G.M. and Munro, P.A. (1984) Selection of a proteolytic enzyme to solubilize lean beef tissue. *Enzyme Microb. Technol.*, **6**: 181-185.

Ostermeier, M., Shim, J.H. and Benkovic, S.J. (1999) A combinatorial approach to hybrid enzymes independent of DNA homology. *Nat. Biotechnol.*, **17**: 1205-1209.

Öztürk, N., Akgöl, S., Arısoy, M. and Denizli, A. (2007) Reversible adsorption of lipase on novel hydrophobic nanospheres. *Sep. Purif. Technol.*, **58**: 83-90.

Padmavathi, S., Chandrababu, N.K., Chitra, S. and Chandrakasan, G. (1995) Application of the extracellular proteases from the culture filtrate of *Streptomyces* spp. G_{157} in bating, *J. Soc. Leath. Technol. Chem.*, **79**: 88-90.

Palanisamy, T., Raghava Rao, J. Nair, B.U. and Ramasami, T. (2004) Progress and recent trends in biotechnological methods for leather processing. *Trends Biotechnol.*, **22**: 181-188.

Park, D.H., Lee, H.J. and Lee, E.K. (1997) Crystallization of alkaline protease as a means of purification process. *Korean J. Chem. Eng.*, **14** (1): 64-68.

Patel, R.K., Dodia, M.S., Joshi, R.H. and Singh, S.P. (2006a) Production of extracellular halo-alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. isolated from seawater in Western India. *World J. Microbiol. Biotechnol.*, **22**: 375-382.

Patel, R.K., Dodia, M.S., Joshi, R.H. and Sing,h S.P. (2006b) Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. *Process Biochem.*, **41**: 2002-2009.

Perea, A., Ugalde, U., Rodriguez, I. and Serra, J.L. (1993) Preparation and characterization of whey protein hydrolysates: applications in industrial whey bioconversion processes. *Enzyme Microb.* Technol., **15**: 418-423.

Petinate, S.D.G., Branquinha, M.H., Coelho, R.R.R., Vermelho, A.B. and DeSimone, S.G. (1999) Purification and partial characterization of an extracellular serine-proteinase of *Streptomyces cyaneus* isolated from Brazilian cerrado soil. *J. Appl. Microbiol.*, **87**: 557-563.

Phadatare, S.V., Srinivasan, M.C. and Deshpande, V.V. (1992) Evidence for controlled autoproteolysis of alkaline protease. A mechanism for physiological regulation of *Conidiobolus coronatus*. *Eur. J. Biochem.*, **205**: 679-686.

Porto, T.S., Monteiro, T.I.R., Moreira, K.A., Lima-Filho, J.L., Silva, M.P.C., Porto, A.L.F. and Carneiro-da-Cunha, M.G. (2005) Liquid–liquid extraction of an extracellular alkaline protease from fermentation broth using aqueous two-phase and reversed micelles systems. *World J. Microbiol. Biotechnol.*, **21**: 655-659.

Potumarthi, R. and Jetty, S.C.A. (2007) Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. *Biochem. Eng. J.*, **34:** 185-192.

Poza, M., de Miguel, T., Sieiro, C. and Villa, T.G. (2001) Characterization of a broad pH range protease of *Candida caseinolytica*. *J. Appl. Microbiol.*, **91**: 916-921.

Puri, S., Beg, Q.K. and Gupta, R. (2002) Optimization of alkaline protease production from *Bacillus* sp. using response surface methodology. *Curr. Microbiol.*, **44**: 286-290.

Rahman, R.N.Z.A., Geok, L.P., Basri, M. and Salleh, A.B. (2005) Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresour Technol*., **96**: 429-436.

Rahman, R.N.Z.A., Geok, L.P., Basri, M. and Salleh, A.B. (2006) An organic solvent-stable alkaline protease from *Pseudomonas aeruginosa* strain K: Enzyme purification and characterization. *Enzyme Microb*. *Technol*., **39**: 1484-1491.

Rajamani, S. and Hilda, A. (1987) Plate assay to screen fungi for proteolytic activity. *Curr*. *Sci*., **56**: 1179-1181.

Rao, M.B., Tanksale, M.A., Ghatge, S.M. and Deshpande, V.V. (1998) Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiol*. *Mol*. *Biol*. *Rev*., **62**(3): 597-635.

Rao, S.S., Prakasham, R.S., Krishna, K.P., Rajesham, S., Sarma, P.N. and Rao, L.V. (2004) Xylitol production by *Candida* sp.: parameter optimization using Taguchi approach, *Process Biochem*., **39**: 951-956.

Rebeca, B.D., Pena-Vera, M.T., Diaz-Castaneda, M. (1991) Production of fish protein hydrolysates with bacterial proteases; yield and nutritional value. *J*. *Food Sci*., **56**: 309-314.

Reddy, L.V.A., Wee, Y.J., Yun, J.S. and Ryu, H.W. (2008) Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett-Burman and response surface methodological approaches. *Bioresour*. *Technol*., **99**: 2242-2249.

Riffle, A., Ortolan, S. and Brandelli, A. (2003) Dehairing activity of extracellular proteases produced by keratinolytic bacteria. *J*. *Chem*. *Technol*. *Biotechnol*., **78**: 855-859.

Safarikova, M. and Safarik, I. (1999) Determination of proteolytic activity with magnetic dye-stained gelatine. *Biotechnol*. *Tech*., **13**: 621-623.

Sakiyama, T., Tanino, K., Urakawa, M., Imamura, K., Takahashi, T., Nagai, T. and Nakanishi, K. (1999) Adsorption characteristics of tryptic fragments of bovine β-lactoglobulin on a stainless steel surface. *J*. *Biosci*. *Bioeng*., **88**: 536-541.

Sana, B., Ghosh, D., Saha, M. and Mukherjee, J. (2006) Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new *gamma-Proteobacterium* isolated from the marine environment of the Sundarbans. *Process Biochem*., **41**: 208-215.

Secades, P. and Guijarro, J.A. (1999) Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. *Appl*. *Environ*. *Microbiol*., **65**(9): 3969-3975.

Seidel, G., Tollnick, C., Beyer, M., Fahimi, Y. and Schügerl, K. (2002) Process engineering aspects of the production of cephalosporin C by *Acremonium chrysogenum*. Part I. Application of complex media. *Process Biochem*., **38**: 229-239.

Sen, S., Venkata Dasu, V. and Mandal, B. (2007) Developments in Directed Evolution for Improving Enzyme Functions. *Appl*. *Biochem*. *Biotechnol*., **143**: 212-223.

Seyedeh, F.G.O., Tabandeh, F., Yakhchali, B. and Eftekhar, F. (2008) Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. *Biochem*. *Eng*. *J*., **39**: 37-42.

Shahrzad, B., Rui, J.E. and Rajni, H.K. (2005) Substrate specificity of alkaline protease from alkaliphilic feather-degrading *Nesterenkonia* sp. AL20. *Enzyme Microb*. *Technol*., **37**; 534-540.

Shikha Saran, A. and Darmwal, N.S. (2007) Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. *Bioresour*. *Technol*., **98**(4): 881-885.

Sinha, N. and Satyanarayana, T. (1991) Alkaline protease production by thermophilic *Bacillus licheniformis*. *Indian J*. *Microbiol*., **31**: 425-430.

Sivasubramanian, S., Murali Manohar, B., Rajaram, A. and Puvanakrishnan, R. (2008) Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. *Chemosphere*, **70**: 1015-1024.

Sjodahl, J., Emmer, A., Vincent, J. and Roeraade, J. (2002) Characterization of proteinases from *Antarctic krill (Euphausia superba)*. *Protein Expr*. *Purif*., **26**: 153-161.

Srinivas, M.R.S., Chand, N., Lonsane, B.K. (1994) Use of Plackett– Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation system. *Bioprocess Eng*., **10**: 139-144.

Takami, H., Akiba, T. and Horikoshi, K. (1989) Production of extremely thermostable alkaline protease from *Bacillus* sp. No. AH-101. *Appl*. *Microbiol*. *Biotechnol*., **30**: 120-124.

Tanimoto, S-Y., Tanabe, S., Watanabe, M. and Arai, S. (1991) Enzymatic modification of zein to produce a non-bitter peptide fraction with a very high Fischer ratio for patients with hepatic encephalopathy. *Agric*. *Biol*. *Chem*., **55**: 1119-1123.

Tanyildizi, M.S., Ozer, D. and Elibol, M. (2005) Optimization of a-amylase production by *Bacillus* sp. using response surface methodology. *Process Biochem*., **40**: 2291-2296.

Tari, C., Genckal, H. and Tokatli, F. (2006) Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. *Process Biochem*., **41**: 659-665.

Teplyakov, A.V., Kuranova, I.P., Harutyunyan, E.H., Vainshtein, B.K., Frommel, C., Hohne, W.E. and Wilson, K.S. (1990) Crystal structure of thermitase at 1.4 Å resolution. *J*. *Mol*. *Biol*., **214**: 261-279.

Thangam, E.B. and Rajkumar, G.S. (2002) Purification and characterization of alkaline protease from *Alcaligenes faecalis*. *Biotechnol*. *Appl*. *Biochem*., **35**: 149-154.

Thumar, J. and Singh, S.P. (2007) Two-step purification of a highly thermostable alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1. *J*. *Chromatogr*. *B*., **854**: 198-203.

Tremacoldi, C.R., Monti, R., Selistre-De-Araújo, H.S. and Carmona, E.C. (2007) Purification and properties of an alkaline protease of *Aspergillus clavatus*. *World J*. *Microbiol*. *Biotechnol*., **23**: 295-299.

Trinetta, V., Rollini, M. and Manzoni, M. (2008) Development of a low cost culture medium for sakacin A production by *L*. *sakei*. *Process Biochem*., **43**: 1275-1280.

Trivedi, V.D., Raman, B., Ramakrishna, T. and Rao, Ch.M. (1999) Detection and assay of proteases using calf lens β-crystallin aggregate as substrate. *J*. *Biochem*. *Biophys*. *Methods*, **40**: 49-55.

Tsuchida, O., Yamaga, Y., Ishizuka, T., Arai, T., Yamada, Y., Takeuchi, M. and Ichishima, E. (1995) An alkaline protease from alkalophilic *Bacillus* sp. KSM-K16. *Appl*. *Microbiol*. *Biotechnol*., **43**: 473-481.

Tunga, R., Shrivastava, B. and Banerjee, R. (2003) Purification and characterization of a protease from solid state cultures of *Aspergillus parasiticus*. *Process Biochem*., **38**: 1553-1558.

Turkiewicz, M., Gromek, E., Kalinowska, H. and Zielinska, M. (1999) Biosynthesis and properties of an extracellular metalloprotease from the Antarctic marine bacterium *Sphingomonas paucimobilis*. *J*. *Biotechnol*., **70**: 53-60.

Turner, K., Serantoni, M., Boyce, A. and Walsh, G. (2005) The use of proteases to remove protein-based residues from solid surfaces. *Process Biochem*., **40:** 3377-3382.

Twining, S.S. (1984) Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Anal*. *Biochem*., **143**: 30-34.

Urtz, B.E. and Rice, W.C. (2000) Purification and characterization of a novel extracellular protease from *Beauveria bassiana*. *Mycol*. *Res*., **104**: 180-186.

Varela, H., Ferrari, M.D., Belobradjic, L., Weyrauch, R. and Loperena, M.L. (1996) Effect of medium composition on the production by a new *Bacillus subtilis* isolate of protease with promising unhairing activity. *World J. Microbiol. Biotechnol.*, **12**: 643-645

Venugopal, M. and Saramma, A.V. (2006) Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. *Process Biochem.*, **41**: 1239-1243.

Vermelho, A.B., Meirelles, M.N.L., Lopes, A., Petinate, S.D.G., Chaia, A.A. and Branquinha, M.H. (1996) Detection of extracellular proteases from microorganisms on agar plates. *Mem. Inst. Oswaldo Cruz*, **91**: 755-760.

Ward, O.P. (1985) Proteolytic enzymes. In: Moo-Young M (ed) *Comprehensive biotechnology, the practice of biotechnology: current commodity products*, Pergamon Press, Oxford, **3**: 789-818.

Wikström, M.B. (1983) Detection of microbial proteolytic activity by a cultivation plate assay in which different proteins adsorbed to a hydrophobic surface are used as substrates. *Appl. Environ. Microbiol.*, **45**: 393-400.

Wikström, M.B., Elwing, H. and Linde, A. (1981) Determination of proteolytic activity: a sensitive and simple assay utilizing substrates adsorbed to a plastic surface and radial diffusion in gel. *Anal. Biochem.*, **118**: 240-246.

Wu, Y. and Abeles, R.H. (1995) Protease assay based on magnetic beads. *Anal. Biochem.*, **229**: 143-144.

Yamagata, Y. and Ichishima, E. (1989) A new alkaline proteinase with PI 2.8 from alkalophilic *Bacillus* sp. *Curr. Microbiol.*, **19**: 259-264.

Zambare, V.P., Nilegaonkar, S.S. and Kanekar, P.P. (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.

Zhao, H., Giver, L., Shao, Z., Affholter, J.A. and Arnold, F.H. (1998) Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat. Biotechnol.*, **16**: 258-261.

CHAPTER 2

ISOLATION, SCREENING AND IDENTIFICATION OF MICROORGANISM

2.1 Introduction

Worldwide research is being carried out to isolate and identify novel microbial species that produce enzymes suitable for industrial and commercial applications. As mentioned in Chapter 1, alkaline protease producing microorganisms have been found in diverse habitats such as wastes of leather industry (Tari *et al*., 2006), marine water (Patel *et al*., 2006), hot spring water (Banerjee *et al*., 1999), soda lake (Mei *et al*., 2005) and saline lake (Amoozegar *et al*., 2007).

Whenever a promising strain is isolated, the scientific community has paid special attention to the correct identification of bacteria. The phenotypic methods for identification are of low cost. Thus, those have stimulated the popular use of commercially available identification systems (API® or BIOLOG®). But, similar phenotypes displayed by strains do not always correspond to similar or even closely related species. Substantial amount of variability among strains belonging to the same species may not correspond to the database relying on an individual and subjective interpretation. Additionally, poor reproducibility, ambiguity, extensive logistics for large-scale investigations and poor discriminatory power of phenotypic method have resulted a shift towards the use of genotypic characterization as a robust classification and differentiation technique (McCartney, 2002). The molecular approach has been used for bacterial phylogeny and is of major importance for species definition and identification

(Clarridge, 2004; Fredricks and Relman, 1996; Raoult *et al*., 2004; Rossello-Mora and Amann, 2001). A very accurate method routinely used in identifying bacterial species from various environmental or clinical specimens and trace phylogenetic relationships is the 16S rDNA gene sequence determination. Universal existence of small subunit ribosomal RNA (SSU rRNA) among bacteria along with species-specific variability makes it possible to identify at the genus or species level by comparison with databases in the public domain with the 16S rRNA sequence (Vandamme *et al*., 1996).

This chapter discusses isolation, screening and identification of the microorganism producing alkaline protease from the tannery waste. It is also explored the potential application of this enzyme in degrading various natural proteins.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

All salts or media constituents *viz*., NaCl, NaOH and agar were purchased from CDH, India. borax was procured from Merck, India. Biological grade gelatin and nutrient broth were obtained from Himedia, India.

2.2.2 Microorganisms and Culture Conditions

The soil samples were collected in the premises of a tannery industry located at Park Circus, Kolkata, India and diluted in sterile saline solution (0.9% w/v). The diluted samples were plated onto nutrient agar (NA) plates (pH 10.0) containing (in g/l) beef extract 1.0, yeast extract 2.0, peptone 5.0, NaCl 5.0, agar 20.0 and were incubated at 30°C. After 36 h, the isolates grown on the plates were streaked on gelatin agar (GA) plate (pH 10.0) containing (in g/l) gelatin 5.0, NaCl 5.0, and agar 20.0 to screen potential

alkaline protease producers. Most efficient strain was selected based on its ability to form colony in very less time and large zone of clearance on gelatin agar plate. Cultures were regenerated every 2-3 weeks on a fresh nutrient agar plates from the frozen stock culture.

2.2.3 Digestion of Natural Proteins

The culture broth (supernatant) from log phase culture of SVB1 (2 ml) was incubated with slaughter house wastes *viz*. blood clot, coagulated egg or poultry feather in borax-NaOH buffer (pH 10.0) at 30ºC for 12 h. Borax-NaOH buffer (pH 10.0) was used to replace enzyme in the negative control experiment. Dehairing was carried out with 2 ml of the culture broth (supernatant) of SVB1 in 25 mM borax–NaOH buffer at pH 10.0. Hide of a freshly butchered goat was cut into pieces of approximately 5 cm × 5 cm and washed with distilled water repeatedly to remove all extraneous matter. After brief air drying, the hide was weighed (between 2 to 3 g) and transferred to 90 mm Petri plate. The culture broth (supernatant) was applied to the skin by using brush and paint method. The control used was 25 mM borax–NaOH buffer of pH 10.0 instead of culture broth (supernatant) to rule out any dehairing contributed by the alkaline condition solely. Incubations were carried out for 6 h after which hair was scraped off gently from the hides with fingers.

2.2.4 Isolation of DNA and Amplification of 16S rDNA

Amplification and sequencing of 16S rDNA (~ 1.5 kb) from the chromosomal DNAs of SVB1 was performed by Bangalore GeNei, India. Related sequences were retrieved from Nucleotide collection (nr/nt) database (NCBI) using blast program

(Appendix I, pp. 207). Phylogenetic tree was constructed with MEGA 3.1 software based on the 16S rDNA sequence of 10 closely related strains.

2.2.5 Analytical Technique

2.2.5.1 Cell growth measurement

After fermentation, the culture broth was centrifuged at 8000 rpm for 10 min at 4ºC. The cell density was determined by reading the optical densities of the culture broth at 600 nm using UV-visible spectrophotometer (Perkin Elmer, Lamda 45). The cell-free supernatant was used for the natural protein digestion experiments.

2.3 Results and Discussion

2.3.1 Screening and Isolation of Microorganisms Producing Alkaline Protease

A total of 67 strains were isolated from the soil collected in the premises of tannery industry. Among them, 23 isolates were screened for the production of extracellular alkaline protease based on the diameter of clear zone due to gelatin hydrolysis on the agar plates. Gelatin was used as the sole source of carbon and nitrogen in the agar plates at pH 10.0 to screen alkaline protease producers. Hydrolysis of gelatin at pH 10.0 has been performed by alkaline protease, which solubilize gelatin by digesting peptide bonds. Hence, it was confirmed that all the organisms grown on the gelatin agar plates were producing alkaline protease. The strain (designated as SVB1), which exhibited maximum zone of clearance shown in Fig. 2.1 was used in all the following experiments in the present research work. This microorganism had grown at a wide range of pH (6.2 - 11.0) and temperature (20 - 36°C) as well as shown ability to produce alkaline protease at similar conditions.

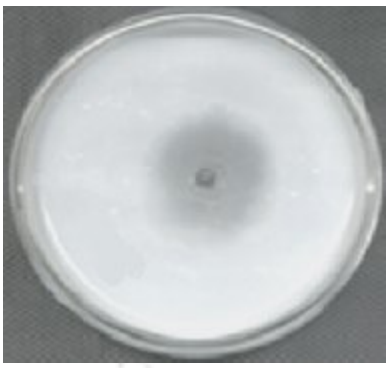


Figure 2.1 Alkaline protease activity of SVB1 on gelatin agar plate.

2.3.2 Digestion of Natural Proteins

Insoluble coagulated egg white and coagulated blood were converted to soluble form after incubation with the alkaline protease as apparent from Fig. 2.2. Poultry feather was also found to be solubilized with the culture broth (supernatant).

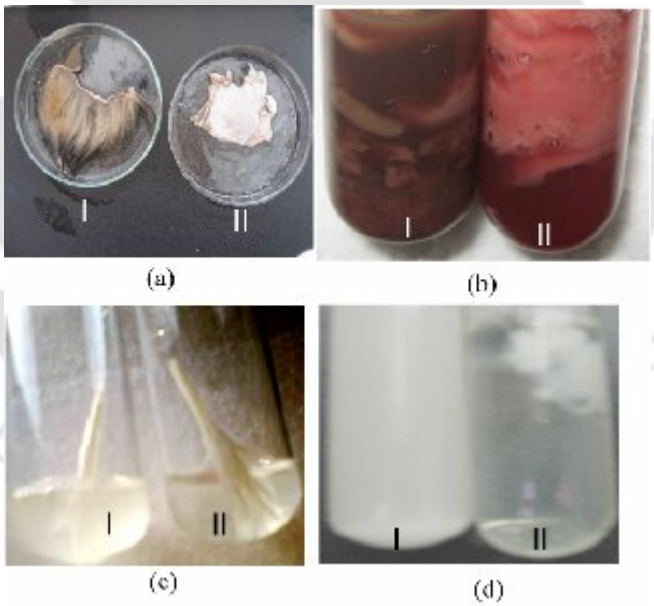


Figure 2.2 Alkaline protease activity on various natural proteins: (a) goat skin dehairing (I: control, II: test), (b) clotted blood (I: control, II: test), (c) poultry feather (I: test, II: control) and (d) egg white (I: test, II: control).

2.3.3 Identification of the SVB1

The partial 16S rRNA nucleotide sequence of SVB1 (~1.5 kb) was determined and submitted to GenBank (accession no. EU533950). The sequence was subjected to pair wise alignment with databases using BLAST. Sequences producing significant alignment result are presented in Table 2.1. The phylogenetic tree constructed by fast minimum evolution method on 16S rRNA sequences is shown in Fig. 2.3. It is evident from the phylogenetic tree that the isolate was related to the *Bacillus* lineage, closely clustering with *B. pseudofirmus* (GenBank accession no. AB029256.1) along with *Bacillus* sp. (Accession no. AB043857.1) being the nearest homolog. Having 98% similarity with its nearest neighbor *Bacillus pseudofirmus* (GenBank accession no. AB029256.1) in the phylogenetic tree, the isolate was tentatively identified to be *B. pseudofirmus* (designated as *B. pseudofirmus* SVB1).

Table 2.1 16S rRNA sequences producing significant alignments

Accession No.	Organism	Total score	Query coverage	Max identity
EU533950.1	*Bacillus pseudofirmus* strain SVB1	2752	100%	100%
AB029256.1	*Bacillus pseudofirmus*	2597	100%	98%
AB043857.1	*Bacillus* sp.	2591	100%	98%
AB043842.1	*Bacillus* sp.	2591	100%	98%
AB201795.1	*Bacillus pseudofirmus*	2582	100%	97%
NR_026139.1	*Bacillus pseudofirmus* strain DSM 8715	2579	99%	97%
FJ764777.1	*Bacillus* sp. E-272	2577	100%	97%
AB043845.1	*Bacillus* sp. 27-1	2575	100%	97%
AY553129.1	*Bacillus* sp. GSP77	2573	99%	97%
AF406790.1	*Bacillus pseudofirmus* strain FTU	2573	100%	97%
DQ852633.1	*Bacillus* sp. DK1122	2553	99%	97%
AB201799.1	*Bacillus pseudofirmus*	2542	98%	97%
EU652482.1	*Bacillus* sp. JPB-3.06b	2495	96%	97%
AF326122.1	*Bacillus* sp. XE22-4-1	2492	99%	97%
X92158.1	Bacterial sp.	2464	99%	96%
FJ764776.1	*Bacillus* sp. E-257	2459	100%	96%

Table 2.1 Continued

Accession No.	Organism	Total score	Query coverage	Max identity
FJ764772.1	*Bacillus* sp. E-127	2453	100%	96%
FJ764768.1	*Bacillus* sp. E-006	2453	100%	96%
EU315248.1	*Bacillus pseudofirmus* strain Mn6	2429	95%	97%
GQ202203.1	*Bacillus* sp. ZL223	2398	97%	96%
X92160.1	Bacterial sp.	2398	99%	95%
X92159.1	Bacterial sp.	2377	99%	95%

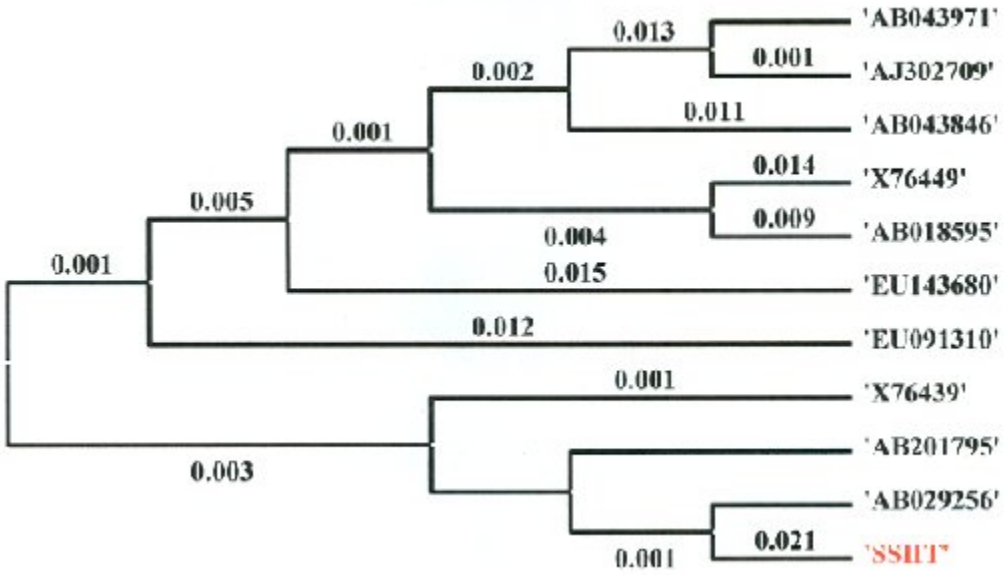


Figure 2.3 Fast minimum evolution method phylogenetic distance tree: SSIIT is the designation for SVB1.

2.4 Conclusions

Based on 16S rRNA sequence the isolate that showed maximum alkaline protease activity was identified to be *B. pseudofirmus* and was designated as *B. pseudofirmus* SVB1. Its ability to grow and produce alkaline protease in a wide range of pH may be of great use in different effluent treatment processes as well as in tannery and detergent industries.

References

Amoozegar, M.A., Fatemi, A.Z., Karbalaei-Heidari, H.R. and Razavi, M.R. (2007) Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004. *Microbiol*. *Res*., **162**: 369-377.

Banerjee, U.C., Sani, R.K., Azmi, W. and Soni, R. (1999) Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochem*., **35**: 213-219.

Clarridge, J.E. III. (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease. *Clin*. *Microbiol*. *Rev*., **17**: 840-862.

Fredricks, D.N. and Relman, D.A. (1996) Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin*. *Microbiol*. *Rev*., **9**: 18-33.

McCartney, A.L. (2002) Application of molecular biological methods for studying probiotics and the gut flora. *Br*. *J*. *Nutr*., **88**: S29-S37.

Mei, C. and Jiang, X. (2005) A novel surfactant- and oxidation-stable alkaline protease from *Vibrio metschnikovii* DL 33-51. *Process Biochem*., **40**: 2167-2172.

Patel, R.K., Dodia, M.S., Joshi, R.H. and Singh, S.P. (2006) Production of extracellular halo-alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. isolated from seawater in Western India. *World J*. *Microbiol*. *Biotechnol*., **22**(4): 375-382.

Raoult, D., Fournier, P.E. and Drancourt, M. (2004) What does the future hold for clinical microbiology. *Nat*. *Rev*., **2**: 151-159.

Rossello-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. *FEMS Microbiol*. *Rev*., **25**: 39-67.

Tari, C., Genckal, H. and Tokatli, F. (2006) Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. *Process Biochem*., **41**: 659-665.

Vandamme, P., Pot, B., Gillis, M., DeVos, P., Kersters, K. and Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol*. *Rev*., **60**: 407-438.

CHAPTER 3

OPTIMIZATION OF PHYSICAL PARAMETERS TO ENHANCE THE PRODUCTION OF ALKALINE PROTEASE

3.1 Introduction

Production of extracellular alkaline protease from microorganisms is known to be influenced significantly by different physical process parameter *viz*., pH, temperature, dissolved oxygen, incubation time, inoculum volume, aeration and agitation (Calic *et al*., 2002; 2003; Potumarthi *et al*., 2007; Hanlon *et al*., 1982; Kole *et al*., 1988; McKeller and Cholette, 1984). However, to access the effects of various parameters and their optimum levels for desirable responses, it is essential to apply statistical techniques both in experimental design as well as in devising valid models (Myers and Montgomery, 2002). Response surface methodology (RSM) has been employed successfully in many areas of biotechnology such as bioconversion (Lee *et al*., 2003), enzyme production (Bocchini *et al*., 2002), enzyme kinetics (Beg *et al*., 2002), bacteriocin production (Li *et al*., 2001) and production of protease from *Bacillus* species (Puri *et al*., 2002; Beg *et al*., 2003).

The physical process parameters *viz*., initial pH, temperature and rpm of the shaking incubator were optimized by central composite design technique using single and multi-response analysis to enhance the alkaline protease production from *Bacillus pseudofirmus* SVB1. The methodology and results for the same are discussed in detail in the present chapter.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

All salts or media constituents *viz*., NaCl, NaOH and agar were purchased from CDH, India. TCA, acetic acid and borax were procured from Merck, India. Biological grade casein, gelatin and Louria-Bertani broth was obtained from Himedia, India.

3.2.2 Microorganisms and Culture Conditions

Bacillus pseudofirmus SVB1 was isolated from the premises of tannery industry as described in Chapter 2. Cultures were regenerated every 2-3 weeks on a freshly prepared nutrient agar plate from the frozen stock culture.

3.2.3 Inoculum Preparation and Alkaline Protease Production

The inoculum was prepared by adding a loop full of pure culture into 50 ml of sterile Luria-Bertani broth (LB) medium (pH 10.0) containing (in g/l) casein enzymatic hydrolysate 10.0, yeast extract 5.0, and NaCl 5.0 in a 250 ml Erlenmeyer flask and incubated at 30°C on a shaking incubator for 10 h. Inoculum (1%) from the seed culture ($A_{600} \approx 1.0$) was added to 100 ml of the medium in 500 ml shake flasks at different initial pH of the medium and incubated at different temperatures and rpm of the shaking incubator as per the central composite experimental design presented in Table 3.1. Samples were withdrawn at regular interval of time for measurement of growth and alkaline protease production.

3.2.4 Experimental Design

3.2.4.1 Individual response optimization

The initial pH of medium, temperature and rpm of the shaking incubator were considered as independent parameters to enhance the production of alkaline protease. The production of alkaline protease (in terms of specific activity and enzyme activity) and cell growth of *Bacillus pseudofirmus* SVB1 were taken as the responses in the present optimization process. The physical process parameters were optimized using the response surface methodology (Box and Hunter, 1957). Three variables each at three levels were employed in the central composite design (CCD) (Box and Wilson, 1951) using statistical software package MINITAB® Release 15.1, PA, US. According to CCD, the total number of treatment combinations was 20 ($=2^k + 2k + 6$), where k is the number of independent variables (Araujo *et al*., 1996). Fourteen experiments were augmented with six replications at the center points to evaluate the pure error (Table 3.1). The fermentation was carried out in 500 ml Erlenmeyer flasks with a working volume of 100 ml for 24 h on a shaking incubator.

The relationship among the variables, *i.e*., initial pH of medium, temperature and rpm of the shaking incubator on the various responses were expressed mathematically in the form of a quadratic equation (Eq. 3.1).

$$Y = \beta_0 + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (3.1)$$

where Y is the response (enzyme production or cell growth), β_0 the constant coefficient, X_i (i = 1–3) are non-coded variables (X_1: initial pH of medium, X_2: temperature and X_3:

rpm of the shaking incubator), β_is are the linear, β_{ii}s are the quadratic, and β_{ij}s (i and j = 1–3) are the second-order interaction coefficients.

Table 3.1 Central composite design for optimizing physical parameters for cell growth, specific activity and enzyme activity during the production of alkaline protease from *Bacillus pseudofirmus* SVB1. Values in the parenthesis indicate the un-coded values

Run number	Variables*		
	x_1 (≡ X_1), pH	x_2 (≡X_2), ºC	x_3 (≡X_3), rpm
1	1.68 (≡ 9.8)	–1.68 (≡ 23.2)	1.68 (≡ 209)
2	1.68 (≡ 9.8)	1.68 (≡ 32.8)	1.68 (≡ 209)
3	0 (≡ 8)	0 (≡ 28)	1 (≡ 250)
4	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
5	–1.68 (≡ 6.2)	1.68 (≡ 32.8)	1.68 (≡ 209)
6	0 (≡ 8)	0 (≡ 28)	–1 (≡ 50)
7	0 (≡ 8)	–1 (≡ 20)	0 (≡ 150)
8	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
9	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
10	1.68 (≡ 9.8)	1.68 (≡ 32.8)	–1.68 (≡ 91)
11	1 (≡ 11)	0 (≡ 28)	0 (≡ 150)
12	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
13	1.68 (≡ 9.8)	–1.68 (≡ 23.2)	–1.68 (≡ 91)
14	–1.68 (≡ 6.2)	–1.68 (≡ 23.2)	1.68 (≡ 209)
15	–1.68 (≡ 6.2)	1.68 (≡ 32.8)	–1.68 (≡ 91)
16	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
17	0 (≡ 8)	1 (≡36)	0 (≡ 150)
18	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
19	–1.68 (≡ 6.2)	–1.68 (≡ 23.2)	–1.68 (≡ 91)
20	–1 (≡ 5)	0 (≡ 28)	0 (≡ 150)

*X_1: pH, X_2: temperature (ºC) and X_3: rpm of the shaking incubator.

3.2.4.2 Multiple response optimization

An analysis in which a number of responses (output variables) are measured simultaneously for each setting of a group of parameters (input variables) is called multi

response analysis. In systems having a large number of input variables and responses, the single response analysis has serious limitations as the optimum conditions for one response may not be suitable or practical for other responses and thus the meaning of optimum becomes unrealistic. The optimal conditions evaluated by this analysis are sometimes called 'near' optimal for all responses. This is well understood fact that response is enhanced more in case of single response optimization as compared to multiple response optimization. The optimization methodology based on the individual desirability using a desirability function (also called utility transfer function) evaluates how the settings optimize a single response. The composite desirability evaluates how the settings optimize a set of responses overall. Desirability has a range of zero to one. One represents the ideal case; zero indicates that one or more responses are outside their acceptable limits. Optimal settings for input variables were determined by maximizing the composite desirability. These values are combined to determine the composite or overall desirability of the multi-response system. An optimal solution occurs where composite desirability reaches its maximum.

3.2.5 Analytical Techniques

3.2.5.1 Cell growth measurement

Cell growth was measured as described in Chapter 2 (Section 2.2.5.1, pp. 45).

3.2.5.2 Assay for proteolytic activity

Alkaline protease activity of the culture supernatant of SVB1 was measured by a modified method of Anson–Hagihara (Hagihara *et al*., 1958). The culture supernatant (35 µl) was added to 840 µl of 0.6% w/v casein solution (in 25 mM Borax–NaOH buffer, pH

10.0). The reaction mixture was incubated at 30ºC for 10 min and the reaction was terminated by the addition of 896 µl of TCA mixture (0.11 M trichloro acetic acid, 0.22 M sodium acetate and 0.33 M acetic acid). The terminated reaction mixture was again incubated for 30 min at room temperature, centrifuged at 13500 rpm and absorbance of the supernatant was measured at 278.5 nm. One unit of alkaline protease activity (U/ml) was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under assay conditions. Specific enzyme activity was obtained by dividing the enzyme units by total protein content.

The alkaline protease activity was calculated as per Eq. 3.2.

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A_{278.5} \times C_t \times V_s}{V_m \times t \times v} \quad (3.2)$$

where $\Delta A_{278.5}$ is the change in optical density (OD) at 278.5 nm, C_t is OD equivalent tyrosine concentration (µg /ml) from standard plot, V_s is the volume of the reaction mixture (ml), t is the time of reaction (min), v is the volume of the enzyme source (ml) for enzyme activity determination and V_m is the volume (ml) used in spectrophotometric determination.

3.2.5.3 Protein concentration measurement

The total protein contents of the samples were determined according to the method described by Lowry *et al*. (1951) using bovine serum albumin (BSA) (Sigma chemicals, India) as standard in a range of concentration from 0-200 µg/ml to plot a standard curve.

Reagent A: Sodium hydroxide (0.4 g) and sodium carbonate (2.0 g) were dissolved in water and the volume made up to 100 ml.

Reagent B1: 2% sodium potassium tartarate.

Reagent B2: 1% copper sulfate.

Reagent C: Prepared fresh by mixing 1.0 ml of reagent B1 and 100 ml of reagent A followed by addition of 1.0 ml of reagent B2.

Phenol reagent: 1 N Folin-Ciocalteu reagent

0.2 ml of sample containing protein or BSA was added to 1 ml of reagent C. After 10 min, 0.1 ml of phenol reagent was added and mixed and the optical density (OD) was measured after 30 min at 660 nm against a blank.

The concentration of protein was calculated as per Eq. 3.2.

$$\text{Protein Concentration (μg/ml)} = \frac{\Delta A_{660} \times C}{V_s} \quad (3.3)$$

where C is Optical density (OD) equivalent of BSA from standard plot, ΔA_{660} is the change in absorbance of the sample and V_s is the volume of the sample.

3.3 Results and Discussion

3.3.1 Individual Response Optimization

The production of the alkaline protease from *Bacillus pseudofirmus* SVB1 exhibited a characteristic relationship with its growth as shown in Fig. 3.1. Alkaline protease production reached its highest at the late log phase as it is evident from Fig. 3.1. Finally, the enzyme production has been decreased as stationary phase was reached. Hence, it seems from the plot that the alkaline protease may be one of the primary metabolite and might be growth associated and probably not a secondary metabolite.

Similar profile for alkaline protease production has been reported earlier (Singh *et al*., 2004; Silva *et al*., 2007).

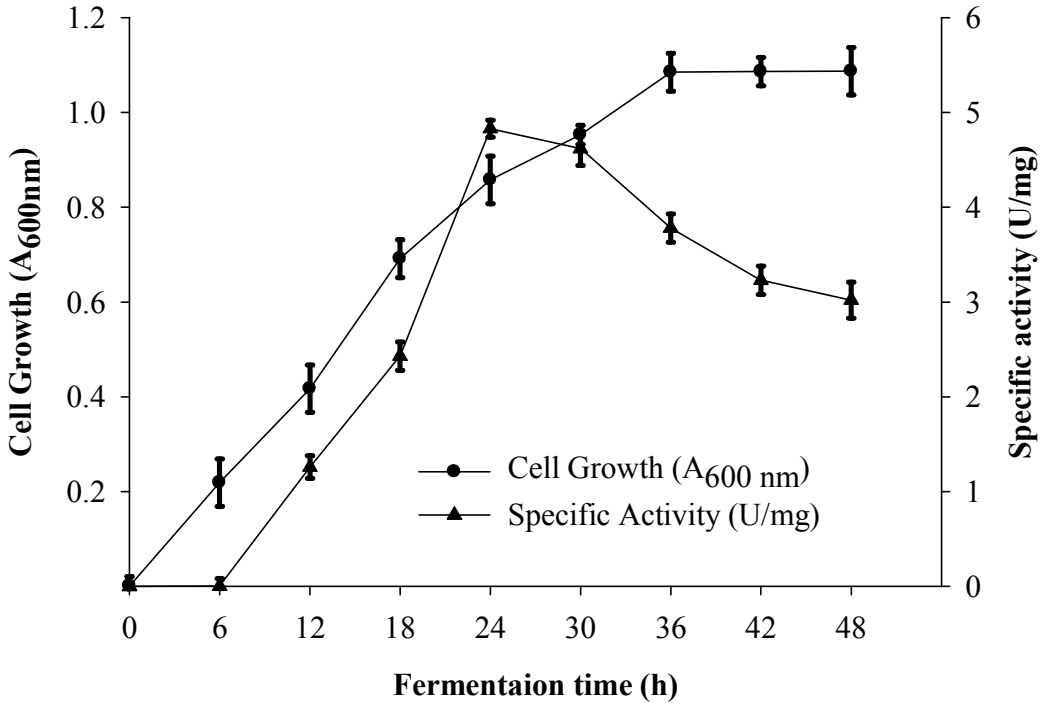


Figure 3.1 Cell growth and alkaline protease production profile under unoptimized conditions (initial medium pH : 8, temperature : 28ºC and agitation : 250 rpm).

As the growth environment was expected to have some influence on the alkaline protease synthesis, the effect of physical parameters on the production of alkaline protease (enzyme activity, U/ml and specific activity, U/mg of protein) and growth of *Bacillus pseudofirmus* SVB1 was studied. Experiments were performed according to the central composite design given in Table 3.1. Experimental and predicted responses *viz.,* enzyme production in terms of specific activity and enzyme activity of culture supernatant and growth of microorganism are presented in Table 3.2.

Table 3.2 Experimental and predicted values of specific enzyme activity and cell growth obtained at various combinations of pH, temperature and rpm of shaking incubator as per the design given in Table 3.1

Run No.	Enzyme production				Cell Growth, A_{600nm}	
	Specific activity, U/mg		Enzyme activity, U/ml			
	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	6.0	6.02	35.08	37.31	0.42	0.41
2	6.29	5.77	40.90	37.40	0.43	0.43
3	4.82	4.76	33.80	31.92	0.34	0.32
4	5.52	5.42	36.59	39.03	0.30	0.31
5	1.83	2.0	14.45	16.48	0.07	0.10
6	0.93	1.08	7.35	9.10	0.22	0.21
7	4.88	4.45	34.94	31.08	0.22	0.21
8	5.32	5.43	36.52	39.03	0.30	0.31
9	5.62	5.43	42.25	39.03	0.31	0.31
10	3.91	3.45	27.45	24.93	0.39	0.38
11	3.63	4.32	23.77	27.25	0.45	0.48
12	5.22	5.43	38.07	39.03	0.31	0.31
13	2.78	2.56	21.32	19.44	0.34	0.33
14	2.12	2.53	15.64	18.32	0.08	0.11
15	1.17	1.09	9.29	7.21	0.02	0.05
16	5.34	5.43	40.33	39.03	0.31	0.31
17	4.25	4.76	30.42	34.15	0.25	0.24
18	5.52	5.43	40.31	39.03	0.31	0.31
19	0	0.47	0	3.65	0.00	0.02
20	0	0.61	0	3.62	0.00	0.05

Experimental values are average of triplicates within ±5% standard error.

The levels of the production and the cell growth as a function of initial pH of medium, temperature and rpm of the shaking incubator can be represented by second order regression equations as given below:

$$Y_1 = -44.0 + 6.44X_1 + 0.83X_2 + 0.10X_3 - 0.40X_1^2 - 0.01X_2^2 - 0.0003X_3^2 + 0.01X_1X_2 + 0.0033X_1X_3 - 0.0010X_2X_3 \quad (3.4)$$

$$Y_2 = -336.74 + 50.81X_1 + 6.07X_2 + 0.742X_3 - 3.02X_1^2 - 0.10X_2^2 - 0.002X_3^2 + 0.06X_1X_2 + 0.01X_1X_3 - 0.005X_2X_3 \quad (3.5)$$

$$Y_3 = -2.32 + 0.24X_1 + 0.07X_2 + 0.003X_3 - 0.01X_1^2 - 0.0013X_2^2 + 0.0008X_1X_2 \quad (3.6)$$

where Y_1, Y_2 and Y_3 represent responses *viz*., specific activity, enzyme activity (production) and cell growth, respectively. X_1, X_2 and X_3 are initial pH of the medium, temperature and rpm of the shaking incubator, respectively.

The analysis of variance (ANOVA) for both production (specific activity and enzyme activity) and growth demonstrates that the model is highly significant at $P<0.0005$ as presented in the Tables 3.3, 3.4 and 3.5, respectively. The F values corresponding to production of the alkaline protease (specific activity, 36.79; enzyme activity, 27.95) and cell growth (54.16) are much greater than the tabulated F value (25.27) at 0.001% level of significance rejecting the null hypothesis for all the two responses. This shows that squared regression was significant at the level of 100% for the two responses. The value of R^2 (coefficient of determination) is the measure of the total variation of the observed values of responses about the mean explained by the fitted model, which is often expressed in percentage. In other words, R^2 describes the goodness of fit of the model. The values of R^2 corresponding to production of the alkaline protease specific activity, enzyme activity and cell growth are 97%, 96% and 98%, respectively. This indicates 97%, 96% and 98% of the total variation in the experimental and predicted values for specific activity, enzyme activity (production) and cell growth, respectively, are explained by the model.

The optimum level of each variable along with their interactions on the production of the alkaline protease and cell growth were studied by plotting three dimensional response surface curves against any two independent variables, while keeping the other variable at its respective middle level. Response surface plots for specific enzyme activity and enzyme activity are represented in Figs. 3.2a and 3.3a,

respectively. According to the response surface plots, production increases sharply with initial pH of the medium, reaches maxima at pH 9 and finally starts decreasing.

Table 3.3 ANOVA for specific activity of alkaline protease: effect of physical process parameters

Source	Degrees of Freedom	Sequential Sum of Squares	Adjusted Sequential Sum of Squares	Adjusted Mean of Sum of Squares	F	P
Regression	9	78.93	78.93	8.77	36.79	0.000
Linear	3	45.70	14.11	4.71	19.74	0.000
Square	3	31.54	31.54	10.51	44.11	0.000
Interaction	3	1.69	1.69	0.56	2.36	0.133
Residual Error	10	2.38	2.38	0.24	–	–
Lack-of-Fit	5	2.27	2.27	0.45	19.49	0.003
Pure Error	5	0.12	0.12	0.02	–	–
Total	19	81.31	–	–	–	–

R^2 = 97%, R^2 (predicted) = 79%, R^2 (adjusted) = 94%

Table 3.4 ANOVA for alkaline protease activity: effect of pH, temperature and rpm of shaking incubator

Source	Degrees of Freedom	Sequential Sum of Squares	Adjusted Sequential Sum of Squares	Adjusted Mean of Sum of Squares	F	P
Regression	9	3609.18	3609.18	401.02	27.95	0.000
Linear	3	1788.25	876.59	292.20	20.36	0.000
Square	3	1799.37	1799.37	599.79	41.80	0.000
Interaction	3	21.55	21.55	7.18	0.50	0.690
Residual Error	10	143.48	143.48	14.35	–	–
Lack-of-Fit	5	116.63	116.63	23.33	4.34	0.07
Pure Error	5	26.85	26.85	5.37	–	–
Total	19	3752.66	–	–	–	–

R^2 = 96%, R^2 (predicted) = 75%, R^2 (adjusted) = 93%

Table 3.5 ANOVA for growth of SVB1 during production of alkaline protease: effect of pH, temperature and rpm of shaking incubator

Source	Degrees of Freedom	Sequential Sum of Squares	Adjusted Sequential Sum of Squares	Adjusted Mean of Sum of Squares	F	P
Regression	9	0.39	0.39	0.044	54.16	0.000
Linear	3	0.36	0.02	0.007	8.45	0.004
Square	3	0.03	0.03	0.01	11.26	0.002
Interaction	3	0.001	0.001	0.0003	0.39	0.76
Residual Error	10	0.01	0.01	0.001	–	–
Lack-of-Fit	5	0.01	0.01	0.002	114.31	0.000
Pure Error	5	0.00007	0.00007	0.00001	–	–
Total	19	0.40	–	–	–	–

R^2 = 98%, R^2 (predicted) = 85%, R^2 (adjusted) = 96%

Similarly, with temperature, production increases up to 30ºC and then decreases. However, the range of change of production of alkaline protease is negligible when the shaking incubator was kept at 150 rpm. When temperature was kept constant at 28ºC, production increased with initial pH of the medium up to a maximum value at 9.5 and then decreased so also with rpm of the shaking incubator (Fig. 3.2b and 3.3b). However, when initial pH of medium was kept constant at 8, production increased very slowly with temperature up to 30ºC and then decreased. But, under the same condition, the production increased with rpm sharply and then started decreasing in the design space after it reached 220 rpm (Fig. 3.2c and 3.3c). This is very clear from the response surface plots that temperature has very little effect on the production, whereas initial pH of medium and rpm influences it very strongly in the design space.

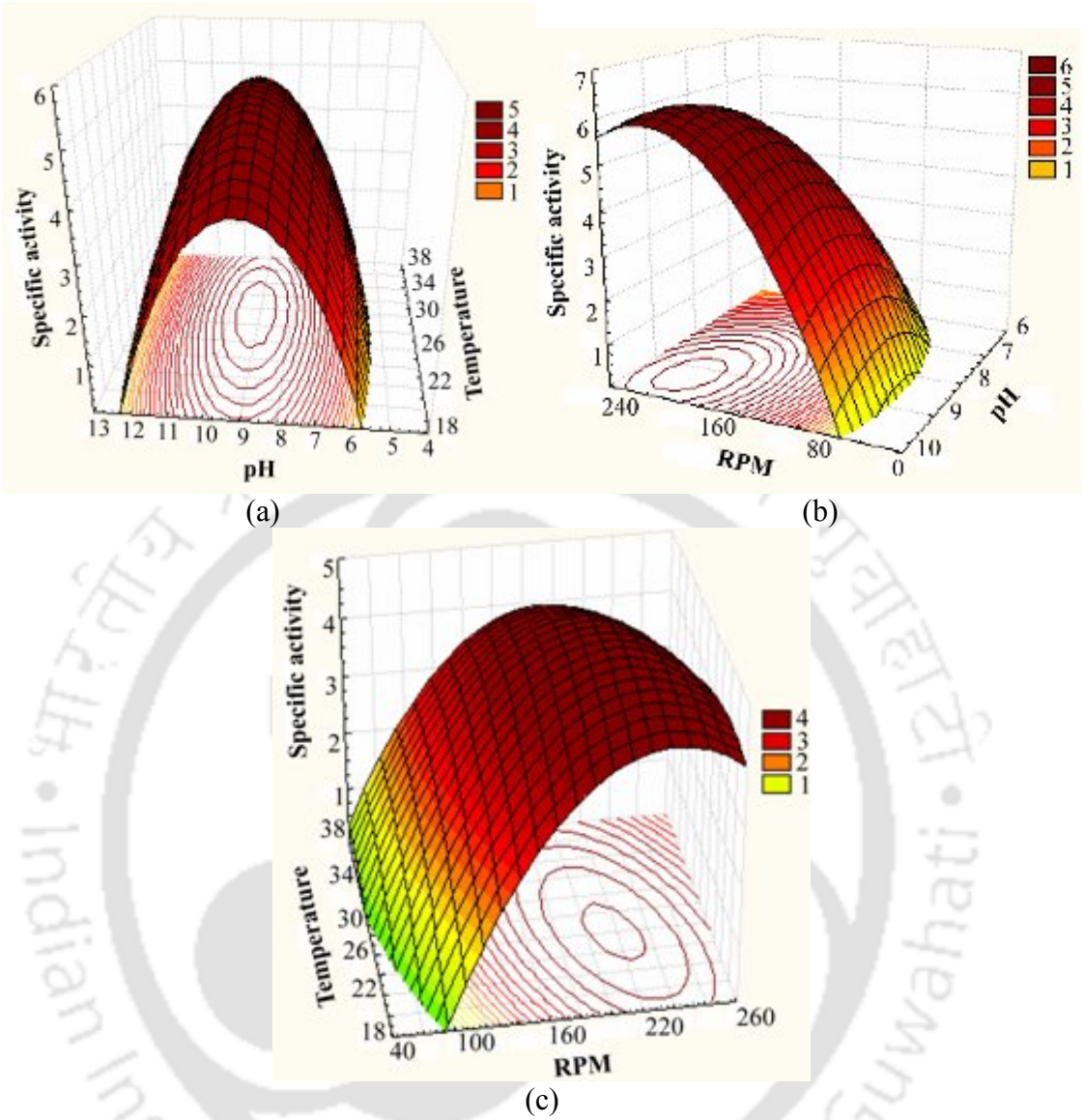


Figure 3.2 Three-dimensional response surface plot for specific activity (production) of alkaline protease from SVB1 showing the interactive effects of (a) pH and temperature (ºC) (constant rpm: 150), (b) rpm and pH (constant temperature: 28ºC) and (c) temperature (ºC) and rpm (constant pH: 8.0).

The optimum levels of initial pH of medium, temperature and rpm of the shaking incubator were determined by maximizing the regression (Eq. 3.4) and were found to be 9.2, 27ºC and 195 rpm, respectively for specific activity (Table 3.6). In term of enzyme activity, the optimum levels of initial pH of medium, temperature and rpm of the shaking

incubator were found to be 8.9, 28ºC and 181 rpm, respectively, by maximizing the regression (Eq. 3.5).

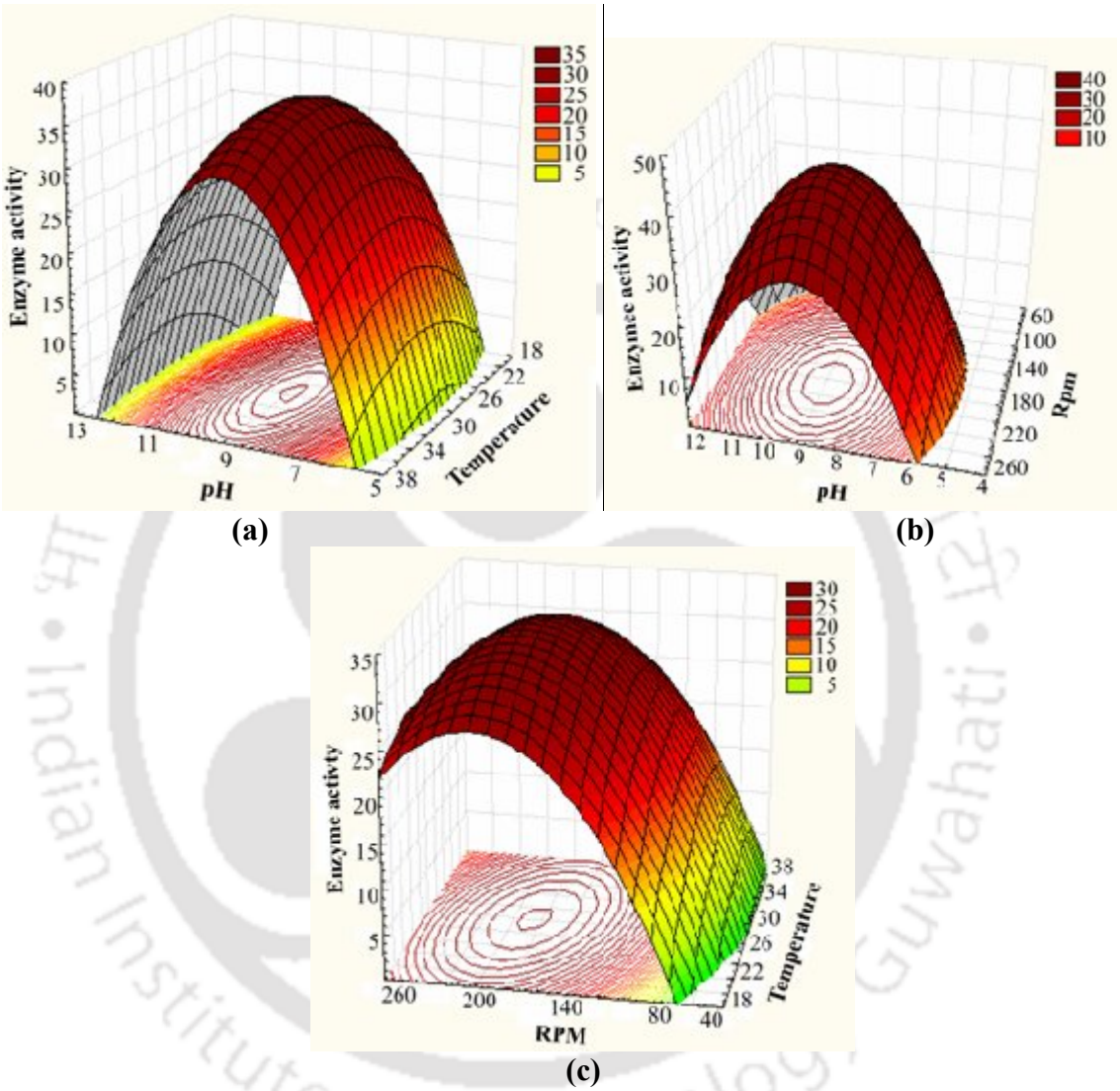


Figure 3.3 Three-dimensional response surface plots for enzyme activity (production) of alkaline protease from SVB1 showing the interactive effects of (a) pH and temperature (ºC) (constant rpm: 150), (b) rpm and pH (constant temperature: 28ºC) and (c) temperature (ºC) and rpm (constant pH: 8.0).

Experiments were performed at these optimal levels of parameters and specific activity and enzyme activity was found to be 6.58 U/mg and 44.25 U/ml, respectively. An enhancement of 36% in specific activity and 31% in enzyme activity was achieved under

these optimal levels of parameters. Alkaline protease from *Bacillus subtilis* NCIM 2713 (Mane and Bapat, 2001) was maximally active at pH 8 and from *Halomonas campisalis* at pH 8–11 (Alva and Peyton, 2003). Patel *et al*. (2006) reported similar trend with another alkaline protease producer. The optimum pH range between 9 and 10 for growth and protease production is common among alkaliphilic and haloalkaliphilic organisms (Patel *et al*., 2006; Kaur *et al*., 2001; Fujiwara and Yamamota, 1987; Johnvesly and Naik, 2001; Studdert *et al*., 2001).

According to Figs. 3.4a and b, cell growth increased with initial pH of the medium and temperature when rpm of the shaking incubator was kept constant at 150 rpm in the design space. Similarly, cell growth increased with initial pH of the medium and rpm of the shaking incubator when temperature was kept constant at 28ºC. But, Fig. 3.4c inferred that, maximum cell growth was achieved at higher values of temperature and moderate values of rpm of the shaking incubator when the initial pH of the medium was kept constant at 8. The optimum values of initial pH of the medium, temperature and rpm of the shaking incubator were found to be 11, 29ºC and 210 rpm, respectively for cell growth by maximizing the Eq. 3.6. Experiment was carried out under optimum levels of variables and the cell growth was observed to be 0.50 at 600nm. The cell growth under unoptimized condition was found to be 0.34 for initial pH of medium being 8, temperature 28ºC and rpm of the shaking incubator 250 rpm. Hence, an enhancement of 44% was achieved. A similar type of response has been observed on the growth of some haloalkaliphilic archaea such as a *Natronoincula* (Zhilina *et al*., 1998) and *Natronorubrum bangense* (Xu *et al*., 1999), where the optimum pH for growth was 9–9.5. At low temperatures (up to optimal level), an increase in temperature would enhance the

growth of the microorganism and therefore improvement in the enzyme activity was observed. At high temperatures, above optimal level, the volatilization of water in the medium restrains the growth and production of enzyme (Hongzhang *et al*., 2006).

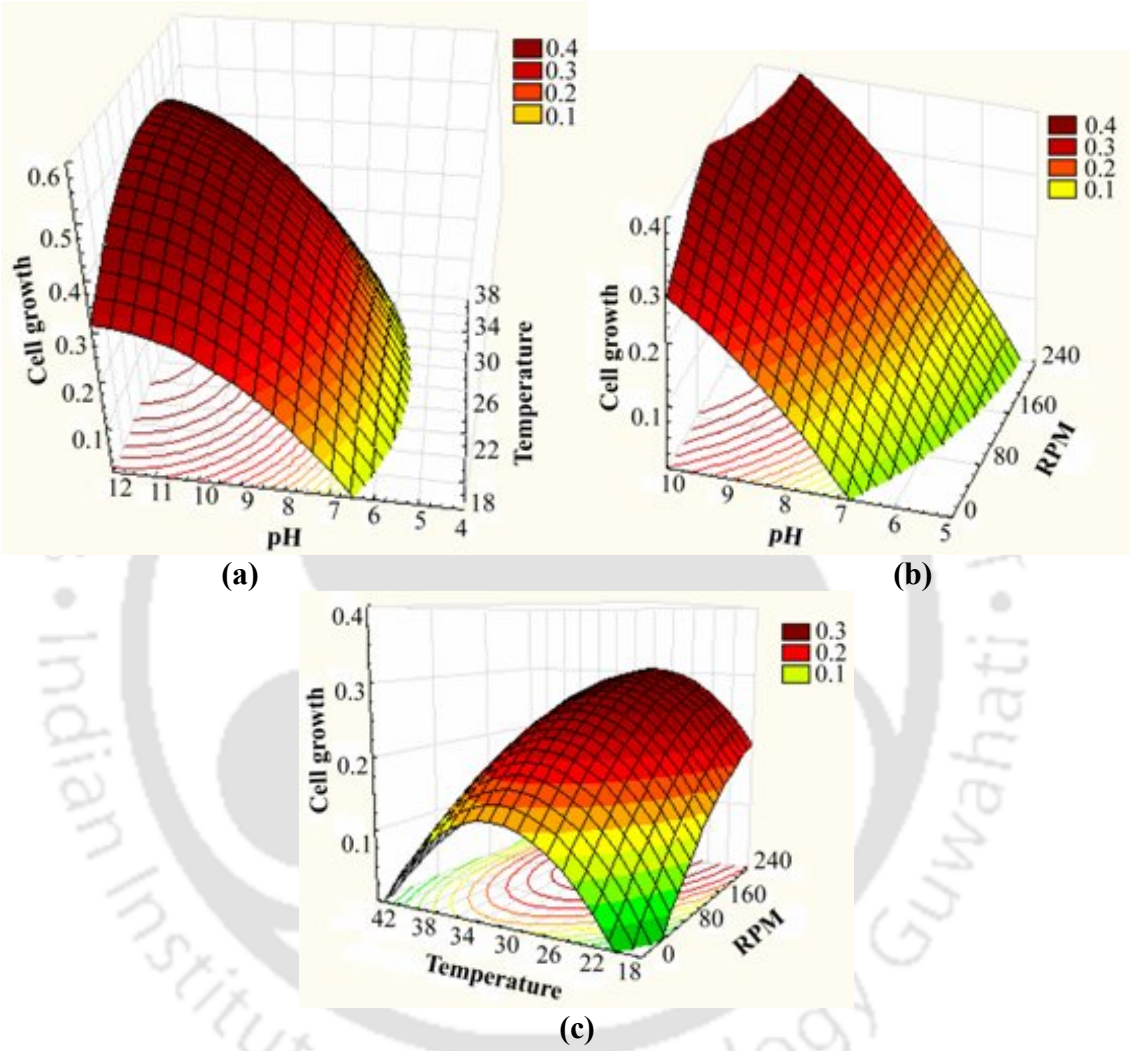


Figure 3.4 Three-dimensional response surface plots for cell growth of SVB1 showing the interactive effects of (a) pH and temperature (ºC) (constant rpm: 150), (b) rpm and pH (constant temperature: 28ºC) and (c) temperature (ºC) and rpm (constant pH: 8.0).

3.3.2 Multiple Response Optimization

The optimum level of each variable predicted by multi-response optimization for all the responses (specific activity, enzyme activity and cell growth) is shown in Table 3.6. The optimal levels of initial pH of the medium, temperature and rpm of the shaking

incubator were found to be 9.6, 28ºC and 191 rpm, respectively using multi response optimization. The predicted values of specific activity, alkaline protease activity and cell growth (A_{600}) were 6.29 U/mg, 41.81 U/ml, 0.44, respectively, under these optimized conditions of parameters.

The main difficulty observed in multi-response analysis is optimization of variables simultaneously to obtain maximum output for multiple responses (Muralidhar *et al*., 2003). Similarly, we observed that the final response value obtained in single response optimization is always higher than the value obtained in multiple response optimization (Table 3.6), though the difference in values obtained are very small. Moreover with single response analysis we could not have an idea about the nature of the variation of other responses.

A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for enzyme production. Similarly, an improved production was reported in other RSM experiments, most notably in the case of protease production using *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004). Response surface methodology was used to optimization of protease production, particularly by *Bacillus spp*. (Puri *et al*., 2002; Beg *et al*., 2003). The production of protease from *Bacillus* sp. PE-11 was enhanced by 2.6 fold after optimization using response surface methodology (Oh *et al*., 1995). Similarly, an overall 4.2 fold increase in protease production by *Bacillus* sp. RGR-14 was achieved (Puri *et al*., 2002).

Table 3.6 The individual and simultaneous maxima, location of individual optima for optimization of physical parameters

Individual / Multiple response optimized		Optimal level of variables			Values of optimized response					
		pH	Temp.	rpm	Observed			Predicted		
					Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Individual Response	Specific activity (Y_1)	9.2	27	195	6.58	-	-	6.34	-	-
	Alkaline protease activity (Y_2)	8.9	28	181	-	44.25	-	43.21	-	-
	Cell growth (Y_3)	11	29	210	-	-	0.49	-	-	0.50
Multiple response	Cell growth, Alkaline protease activity and Specific activity	9.6	28	191	6.37	40.03	0.44	6.29	41.81	0.44

X_1: pH, X_2: temperature (ºC), X_3: rpm (rpm) of the shaking incubator, Y_1: specific activity (U/mg) and Y_2: enzyme activity (U/mg) and Y_3: cell growth in terms of A_{600nm}.

3.3.3 Validation of the Experimental Model for Multiple Response Analysis

The model was validated for all three variables set at the optimal values as obtained from multi-response analysis. The experimentally determined production values were in agreement with the statistically predicted ones (Table 3.6) confirming the model's authenticity. Experiments were performed at those optimum values of parameters, the values of cell growth (A_{600}), alkaline protease activity and specific activity obtained were 0.44, 40.03 U/ml, 6.37 U/mg respectively. Hence, an enhancement of 30%, 18% and 32% was achieved in cell growth, alkaline protease activity and specific activity, respectively. Production of alkaline protease and cell growth obtained in this run was higher than in any other experimental run in the design.

3.4 Conclusions

Bacillus pseudofirmus SVB1 was found to grow and produce alkaline protease in a wide range of pH, which may be of great use in different effluent treatment processes dealing with very high protein content. Response surface methodology (RSM) was successfully applied to determine the optimum levels of physical parameters for enhanced production of this enzyme and cell growth. The optimum levels of initial pH of the medium, temperature and rpm of the shaking incubator were found to be 9.6, 28ºC and 191 rpm, respectively. This is the first report on the comparison of results obtained from individual responses and multi-response optimization for the production of alkaline protease.

References

Alva, V.A. and Peyton, B.M. (2003) Phenol and catechol biodegradation by the haloalkaliphile *Halomonas campisalis*: influence of pH and salinity. *Environ*. *Sci*. *Technol*., **37**: 4397-4402.

Araujo, P.W. and Brereton, R.G. (1996) Experimental design II. Optimization. *Trends Analyt*. *Chem*., **15**(2): 63-70.

Beg, Q.K., Saxena, R.K. and Gupta, R. (2002) Kinetic constants determination for an alkaline protease from *Bacillus mojavensis* using response surface methodology. *Biotechnol*. *Bioeng*., **78**: 289-295.

Beg, Q. and Gupta, R. (2003) Purification and characterization of an oxidation stable, thiol- dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb*. *Technol*., **32**: 294-304.

Bocchini, D.A., Alves-Prado, H.F., Baida, L.C., Roberto, I.C., Gomes, E. and Da-Silva, R. (2002) Optimization of xylanase production by *Bacillus circulans* D1 in submerged fermentation using response surface methodology. *Process Biochem*., **38**: 727-731.

Box, G.E.P. and Wilson, K.B. (1951) On the experimental attainment of the optimum conditions. *J*. *Roy*. *Stat*. *Soc*., **13**: 1-45.

Box, G.E.P. and Hunter, J.S. (1957) Multifactor experimental designs for exploring response surfaces. *Ann*. *Math*. *Stat*., **28**: 195-241

Calik, P., Bilir, E., Calik, G. and Ozdamar, T.H. (2002) Influence of pH conditions on metabolic regulations in serine alkaline protease production. *Enzyme Microb*. *Technol*., **31**(5): 685-697.

Calik, P., Bilir, E., Calik, G. and Ozdamar, T.H. (2003) Bioreactor operation parameters as tools for metabolic regulations in fermentation processes: influence of pH conditions. *Chem*. *Eng*. *Sci*., **58**(3): 759-766.

Chauhan, B. and Gupta, R. (2004) Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. *Process Biochem*., **39**: 2115-2122.

Fujiwara, N. and Yamamota, K. (1987) Production of alkaline protease in a low-cost medium by alkalophilic *Baicillus* sp. and properties of the enzyme. *J*. *Ferment*. *Technol*., **65**: 345-348.

Hagihara, B., Matsubara, H., Nakai, M. and Okunuki, K. (1958) Crystalline bacterial proteinase. I. Preparation of crystalline proteinase of *Bacillus subtilis*. *J. Biochem*., **45**: 185-194.

Hanlon, G.W., Hodges, N.A. and Russel, A.D. (1982) The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. *J. Gen. Microbiol*., **128**: 845-851.

Hongzhang, C., Hui, W., Aijun, Z. and Zuohu, L. (2006) Alkaline protease production by solid state fermentation on polyurethane foam. *Chem. Biochem. Eng. Q*., **20**(1): 93-97.

Johnvesly, B. and Naik, G.R. (2001) Studies on production of novel thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB99 in a chemically defined medium. *Process Biochem*., **37**: 139-144.

Kaur, S., Vohra, R.M., Kapoor, M., Beg, Q.K. and Hoondal, G.S. (2001) Enhanced production of a highly thermostable alkaline protease from *Bacillus* sp. *World J. Microbiol. Biotechnol*., **17**: 125-129.

Kole, M.M., Draper, I. and Gerson, D.F. (1988) Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. *J. Chem. Technol. Biotechnol*., **41**: 197-206.

Lee, H., Song, M. and Hwang, S. (2003) Optimizing bioconversion of deproteinated cheese whey to mycelia of *Ganoderma lucidum*. *Process Biochem*., **38**: 1685-1693.

Li, C., Bai, J., Cai, Z. and Ouyang, F. (2001) Optimization of a cultural medium for bacteriocin production by *Lact*ococc*us lactis* using response surface methodology. *J. Biotechnol*., **93**: 27-34.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J.R. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem*., **193**: 265-275.

Mane, R. and Bapat, M. (2001) A study of extracellular alkaline protease from *Bacillus subtilis* NCIM 2713. *Indian J. Exp. Biol*., **39**: 578-583.

McKeller, R.C. and Cholette, H. (1984) Synthesis of extracellular proteinase by *Pseudomonas fluorescens* under conditions of limiting carbon, nitrogen and phosphate. *Appl. Environ. Microbiol*., **47**: 1224-1227.

Muralidhar, R., Gummadi, S.N., Dasu, V.V. and Panda, T. (2003) Statistical analysis on some critical parameters affecting the formation of protoplasts from the mycelium of *Penicillium griseofulvum*. *Biochem. Eng. J*., **16**: 229-235.

Myers, R.H. and Montgomery, D.C. (2002) Response surface methodology: process and product optimization using designed experiments. *Wiley*, New York.

Oh, S., Rheem, S., Sim, J., Kim, S. and Baek, Y. (1995) Optimizing conditions for the growth of *Lactobacillus casei* YIT 9018 in tryptone-yeast extract-glucose medium by using response surface methodology. *Appl*. *Environ*. *Microbiol*., **61**: 3809-3814.

Patel, R.K., Dodia, M.S., Joshi, R.H. and Singh, S.P. (2006) Production of extracellular halo-alkaline Protease from a newly isolated haloalkaliphilic *Bacillus* sp. isolated from seawater in western India. *World J*. *Microbiol*. *Biotechnol*., **22**(4): 375-382.

Potumarthi, R., Ch., S. and Jetty, A. (2007) Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. *Biochem*. *Eng*. *J*., **34**(2): 185-192.

Puri, S., Khali, O. and Gupta, R. (2002) Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. *Curr*. *Microbiol*., **44**: 286-290.

Silva, C.R., Delatorre, A.B. and Martins, M.L. (2007) Effect of the culture conditions on the production of an extracellular protease by thermophilic *Bacillus* sp. and some properties of the enzymatic activity. *Braz*. *J*. *Microbiol*., **38**(2): 253-258.

Singh, J., Vohra, R.M. and Sahoo, D.K. (2004) Enhanced production of alkaline proteases by *Bacillus sphaericus* using fedbatch culture. *Process Biochem*., **39**(9): 1093-1101.

Studdert, C.A., Seitz, M.K.H., Gilv, M.I.P., Sanchez, J.J. and DeCastro, R.E. (2001) Purification and biochemical characterization of the Haloalkaliphilic archaeon *Natronococcus occultus* extracellular serine protease. *J*. *Basic Microbiol*., **41**(6): 375-383.

Xu, Y., Zhou, P. and Tian, X. (1999) Characterization of two novel haloalkaliphilic archaea *Natronorubrum bangense gen*. *nov., sp*. *nov*. and *Natronorubrum tibetense gen*. *nov., sp*. *nov*. *Int*. *J*. *Syst*. *Bacteriol*., **49**: 261-266.

Zhilina, T.N., Detkova, E.N., Rainey, F.A., Osipov, G.A., Lysenko, A.M., Kostrikina, N.A. and Zavarzin, G.A. (1998) *Natronoincola histidinovorans gen*. *nov*. *sp*. *nov*., a new alkaliphilic acetogenic anaerobe. *Curr*. *Microbiol*., **37**: 177-185.

CHAPTER 4

DEVELOPMENT OF MEDIUM TO MAXIMIZE THE PRODUCTION OF ALKALINE PROTEASE

4.1 Introduction

Production of extracellular alkaline protease is known to be significantly influenced by optimal levels of media components, especially carbon and nitrogen sources and metal ions (Kaur *et al*., 2001; Oberoi *et al*., 2001). Medium development plays a vital role to enhance the production, in which even small improvements can be decisive for commercial success. Development of an economically feasible production medium requires proper selection of carbon, nitrogen, phosphorus, potassium and trace element sources. Statistical methodologies are generally preferred over classical techniques (Krishna Prasad *et al*., 2005; Li *et al*., 2001), because of their better capability to explain the interaction among the variables and to determine the optimal levels of variables with minimum number of experimental runs. There are many reports on biological process optimization using experimental design techniques (Krishna Prasad *et al*., 2005; Li *et al*., 2001; Puri *et al*., 2002; Rao *et al*., 2004). Plackett-Burman experimental designs are widely applied for screening and selection of significant variables from large pool of candidate variables in a small number of trials. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimal levels of factors (Myers and Montgomery, 2002).

This chapter describes the development of medium to improve the production of alkaline protease from *Bacillus pseudofirmus* SVB1. Selection of most efficient C/N source, screening and optimization of concentration levels of medium components to maximize the production of alkaline protease from the isolate are presented in this chapter.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

All salts and media constituents *viz*., NaCl, NaOH, $MgSO_4.7H_2O$, $CaCl_2$, NH_4NO_3, KH_2PO_4, K_2HPO_4, $FeCl_3$, lactose, glycerol, glucose and agar were purchased from CDH, India. TCA, acetic acid and borax were procured from Merck, India. Biological grade casein, gelatin and Louria-Bertani broth, tryptone, peptone, yeast extract, starch and Bushnell Hass (BH) media were obtained from Himedia, India.

4.2.2 Microorganism and Culture Conditions

Bacillus pseudofirmus SVB1 was isolated from the premises of tannery industry and subcultured under the same conditions as described in Chapter 2 (Section 2.2.2, pp. 43-44). Cultures were regenerated every 2-3 weeks on a freshly prepared nutrient agar plates from the frozen stock culture.

4.2.3 Inoculum Preparation and Alkaline Protease Production

The inoculum was prepared as described in Chapter 3. An 1% v/v of inoculum from the culture ($A_{600} \approx 1.0$) was added into 500 ml Erlenmeyer flasks containing 100 ml of the sterile medium (at previously optimized initial pH of 9.6, Chapter 3) constituted of

different carbon or nitrogen sources each at 1% w/v concentrations for finding the most suitable C and N source to maximize the production of alkaline protease from SVB1. 1% of inoculum from seed culture ($A_{600} \approx 1.0$) was added to 100 ml of the medium in 500 ml shake flasks containing casein as sole source of carbon and nitrogen, which was selected from the one variable at a time experiment and other components as per the Plackett–Burman design (Table 4.1) for screening of significant medium components and as per the CCD (Table 4.2) for optimization of their levels. For all the experiments the culture flasks were incubated in orbital shaking incubator at 28ºC and 191 rpm (previously optimized conditions, Chapter 3). Samples were withdrawn at regular interval of time for measurement of alkaline protease production.

4.2.4 Experimental Design

4.2.4.1 Selection of most suitable carbon and nitrogen sources

Different carbon or nitrogen sources (tryptone, casein, yeast extract, peptone, gelatin, glycerol, glucose, starch, and lactose each at 1% w/v) either solely or in combinations along with or without NaCl and Bushnell Hass (BH) media having the composition (g/l): $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $FeCl_3$, 0.05 were used for each experimental run. Samples were withdrawn after 24 h of incubation and growth of microorganism were spectro-photometrically measured at 600 nm (A_{600}).

4.2.4.2 Screening of the most significant medium constituents by Plackett–Burman experimental design

For the selection of significant medium components for protease production, a variety of factors (carbon source, nitrogen source, inorganic salts) were tested and identified via the Plackett–Burman design using statistical software package MINITAB® Release 15.1, PA, USA (Appendix II, pp. 208-210). A total of eight parameters, *i.e.*, casein, NaCl, $MgSO_4$, $CaCl_2$, NH_4NO_3, K_2HPO_4, KH_2PO_4 and $FeCl_3$ were included in the design each at three different levels, high (+), low (−) and centre point (0). A total of 13 experiments were performed in duplicates. The principal effects of each variable on production of alkaline protease were estimated as the difference between both averages of measurements made at the higher level and at the lower level. The effect of each variable was determined by the following equation:

$$E_{(x_i)} = \frac{2\left(\sum M_{i+} - M_{i-}\right)}{N} \tag{4.1}$$

where $E_{(x_i)}$ is the concentration effect of the tested variable. M_{i+} and M_{i-} are the alkaline protease activities from the trials in which the concerned variable (X_i) was present at high and low levels, respectively in the different N number of trials.

For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$x_i = \frac{X_i - X_0}{\delta X} \tag{4.2}$$

The name of the variables, symbol and their actual level in the experimental design is shown in Table 4.1. The significance of each variable was determined via Student's t-test.

4.2.4.3 Optimization of screened medium components using Central Composite Design (CCD)

The most significant medium components were selected according to Plackett-Burman experimental design and further optimized using central composite design (CCD). 31 ($=2^k + 2k + 7$) treatment combinations (Table 4.2) were considered in the CCD, where k is the number of independent variables. Twenty four experiments were augmented with seven replications at the center points to evaluate the pure error. The levels of the statistically non-significant medium components (as reflected from the Plackett-Burman design analysis) were maintained at their mid values throughout the central composite design (Table 4.2) *viz.,* NaCl (5 g/l), K_2HPO_4 (2.75 g/l), KH_2PO_4 (2.75 g/l) and $FeCl_3$ (0.045 g/l). The average of duplicates in each trial was taken as the corresponding response values (Y_i). The minimum and maximum ranges of the variables were used, and the full experimental plan with regard to their values in actual and coded form is provided in Table 4.2.

The data obtained from CCD on alkaline protease production were subjected to analysis of variance (ANOVA). The experimental results were fitted to the following second order polynomial equation:

$$Y_i = \beta_0 + \sum \beta_{ii} X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (4.3)$$

where Y_i is the predicted response, X_i *and* X_j are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient.

The statistical significance of the model terms in the model equation were valuated through the Fisher's F-test. The quality of the fit of polynomial model was expressed by the coefficient of determination R^2, and its statistical significance was

Table 4.1 Plackett–Burman design matrix for eight variables showing their actual and coded values along with the observed and predicted protease activity

Run Order	Variables								Alkaline protease specific activity(U/mg)	
	Casein, $X_1(x_1)$	$MgSO_4$, $X_2(x_2)$	$CaCl_2$, $X_3(x_3)$	NH_4NO_3, $X_4(x_4)$	NaCl, $X_5(x_5)$	K_2HPO_4, $X_6(x_6)$	KH_2PO_4, $X_7(x_7)$	$FeCl_3$, $X_8(x_8)$	Observed	Predicted
1	12 (+)	0.5 (+)	0.01 (-)	2 (+)	2 (-)	0.5 (-)	0.5 (-)	0.07 (+)	0.07±0.01	0.125
2	2 (-)	0.5 (+)	0.05 (+)	2 (+)	2 (-)	5 (+)	0.5 (-)	0.07 (+)	6.13±0.44	5.626
3	12 (+)	0.5 (+)	0.05 (+)	0.5 (-)	2 (-)	0.5 (-)	5 (+)	0.02 (-)	0.05±0.01	0.150
4	2 (-)	0.5 (+)	0.01 (-)	0.5 (-)	8 (+)	5 (+)	0.5 (-)	0.02 (-)	0.68±0.22	0.3350
5	12 (+)	0.1 (-)	0.01 (-)	2 (+)	2 (-)	5 (+)	5 (+)	0.02 (-)	0.66±0.06	0.513
6	7 (0)	0.3 (0)	0.03 (0)	1.25 (0)	5 (0)	2.75 (0)	2.75 (0)	0.045 (0)	0.44±0.06	2.679
7	12 (+)	0.1 (-)	0.05 (+)	0.5 (-)	8 (+)	0.5 (-)	0.5 (-)	0.07 (+)	0.06±0.01	0.127
8	2 (-)	0.5 (+)	0.05 (+)	2 (+)	8 (+)	0.5 (-)	5 (+)	0.02 (-)	1.64±0.07	1.401
9	2 (-)	0.1 (-)	0.01 (-)	0.5 (-)	2 (-)	0.5 (-)	0.5 (-)	0.02 (-)	9.09±0.21	0.02 (-)
10	12 (+)	0.5 (+)	0.01 (-)	0.5 (-)	8 (+)	5 (+)	5 (+)	0.07 (+)	0.06±0.08	0.07 (+)
11	2 (-)	0.1 (-)	0.05 (+)	0.5 (-)	2 (-)	5 (+)	5 (+)	0.07 (+)	16.36±0.02	0.07 (+)
12	12 (+)	0.1 (-)	0.05 (+)	2 (+)	8 (+)	5 (+)	0.5 (-)	0.02 (-)	0.04±0.01	0.02 (-)
13	2 (-)	0.1 (-)	0.01 (-)	2 (+)	8 (+)	0.5 (-)	5 (+)	0.07 (+)	0.14±0	0.07 (+)

(concentrations are in g/l)

Table 4.2 Central composite design matrix for the experimental runs along with observed and predicted responses

Run Order	Variables (Uncoded levels)				Alkaline protease specific activity (U/mg)	
	Casein $X_1(x_1)$	$MgSO_4$ $X_2(x_2)$	$CaCl_2$ $X_3(x_3)$	NH_4NO_3 $X_4(x_4)$	Observed	Predicted
1	7.75 (+1)	0.3 (-1)	0.0775 (+1)	1.1 (+1)	33.05 ± 0.043	31.39
2	5.5 (0)	0.5 (0)	0.1 (+α)	0.8 (0)	32.33±0.780	30.41
3	3.25 (-1)	0.7 (+1)	0.0325 (-1)	0.5 (-1)	19.79±1.220	21.12
4	5.5 (0)	0.9 (+α)	0.055 (0)	0.8 (0)	31.62±0.023	30.54
5	3.25 (-1)	0.7 (+1)	0.0775 (+1)	1.1 (+1)	14.32±0.770	14.32
6	3.25 (-1)	0.3 (-1)	0.0325 (-1)	1.1 (+1)	16.27±1.090	14.94
7	5.5 (0)	0.5 (0)	0.055 (0)	0.8 (0)	32.04±0.510	31.18
8	1 (-α)	0.5 (0)	0.055 (0)	0.8 (0)	3.81±2.030	1.73
9	7.75 (+1)	0.7 (+1)	0.0325 (-1)	1.1 (+1)	31.08±1.180	30.25
10	5.5 (0)	0.1 (-α)	0.055 (0)	0.8 (0)	29.23±0.237	29.48
11	5.5 (0)	0.5 (0)	0.055 (0)	0.8 (0)	32.04±0.831	31.18
12	3.25 (-1)	0.3 (-1)	0.0775 (+1)	0.5 (-1)	19.65±0.056	20.15
13	5.5 (0)	0.5 (0)	0.01 (-α)	0.8 (0)	28.11±0.008	29.19
14	7.75 (+1)	0.3 (-1)	0.0325 (-1)	1.1 (+1)	32.22±1.338	31.31
15	3.25 (-1)	0.7 (+1)	0.0325 (-1)	1.1 (+1)	14.31±2.322	14.06
16	3.25 (-1)	0.3 (-1)	0.0775 (+1)	1.1 (+1)	12.91±0.513	14.33
17	7.75 (+1)	0.7 (+1)	0.0325 (-1)	0.5 (-1)	34.11±0.013	33.86
18	5.5 (0)	0.5 (0)	0.055 (0)	0.8 (0)	31.04±1.098	31.18
19	5.5 (0)	0.5 (0)	0.055 (0)	0.8 (0)	28.04±1.113	31.18
20	5.5 (0)	0.5 (0)	0.055 (0)	0.2 (-α)	31.69±0.033	28.60
21	5.5 (0)	0.5 (0)	0.055 (0)	0.8 (0)	31.04±1.233	31.18
22	5.5 (0)	0.5 (0)	0.055 (0)	0.8 (0)	33.04±0.047	31.18
23	5.5 (0)	0.5 (0)	0.055 (0)	0.8 (0)	31.04±0.019	31.18
24	7.75 (+1)	0.3 (-1)	0.0775 (+1)	0.5 (-1)	32.34±0.009	33.76
25	3.25 (-1)	0.3 (-1)	0.0325 (-1)	0.5 (-1)	18.62±1.003	19.87
26	7.75 (+1)	0.7 (+1)	0.0775 (+1)	0.5 (-1)	34.69±0.017	35.69
27	5.5 (0)	0.5 (0)	0.055 (0)	1.4 (+α)	16.92±0.037	19.17
28	7.75 (+1)	0.7 (+1)	0.0775 (+1)	1.1 (+1)	31.27±0.357	31.19
29	7.75 (+1)	0.3 (-1)	0.0325 (-1)	0.5 (+α)	33.13±0.279	32.79
30	3.25 (-1)	0.7 (+1)	0.0775 (+1)	0.5 (+α)	20.18±0.313	22.26
31	10 (+α)	0.5 (0)	0.055 (0)	0.8 (0)	30.28±0.037	31.53

α =2.0

checked by the *F* statistics. An *F*- test that is larger than the critical value from the *F*-distribution, using the appropriate confidence level and degrees of freedom, supports rejecting the null hypothesis that the means are equal, where as *P*-value determines the appropriateness of rejecting the null hypothesis in a hypothesis test. In order to illustrate the main and interactive effects of the independent variables on the dependent ones, the fitted polynomial equation was then represented in the form of three-dimensional surface plots. The point optimization method was employed to optimize the level of each variable in order to maximize the protease production. The combination of different optimized variables, which yielded the maximum response were experimentally verified against the proposed regression model.

4.2.4.4 Validation of the model

To validate the model, experiments were carried out under optimal levels of the variables as predicted by the CCD results. Under the optimized medium composition (casein, 7.64 g/l; $MgSO_4.7H_2O$, 0.81 g/l; $CaCl_2$, 0.09 g/l; NaCl, 4.5 g/l; $FeCl_3$, 0.36 g/l; NH_4NO_3, 0.58 g/l; K_2HPO_4, 2.75 g/l and KH_2PO_4, 2.75 g/l) experiments were carried out for alkaline protease production.

4.2.5 Analytical Techniques

Cell growth was measured as described in Chapter 2 (Section 2.2.5.1, pp.45). Alkaline protease activity and specific activity of the culture supernatant of SVB1 were measured as described earlier (Chapter 3, Section 3.2.5.2, pp. 54). The total protein contents of the samples were determined as described in Chapter 3 (Section 3.2.5.3., pp. 55).

4.3 Results and Discussion

4.3.1 Effect of Carbon and Nitrogen Sources on Alkaline Protease Production

Effect of different carbon and nitrogen sources on the production of alkaline protease (specific activity) is illustrated in Fig. 4.1. The maximum production of alkaline protease (34.57 U/mg) from SVB1 has been observed in the presence of casein which is 4.4 fold higher as compared to in the unoptimized medium (6.37 U/mg).

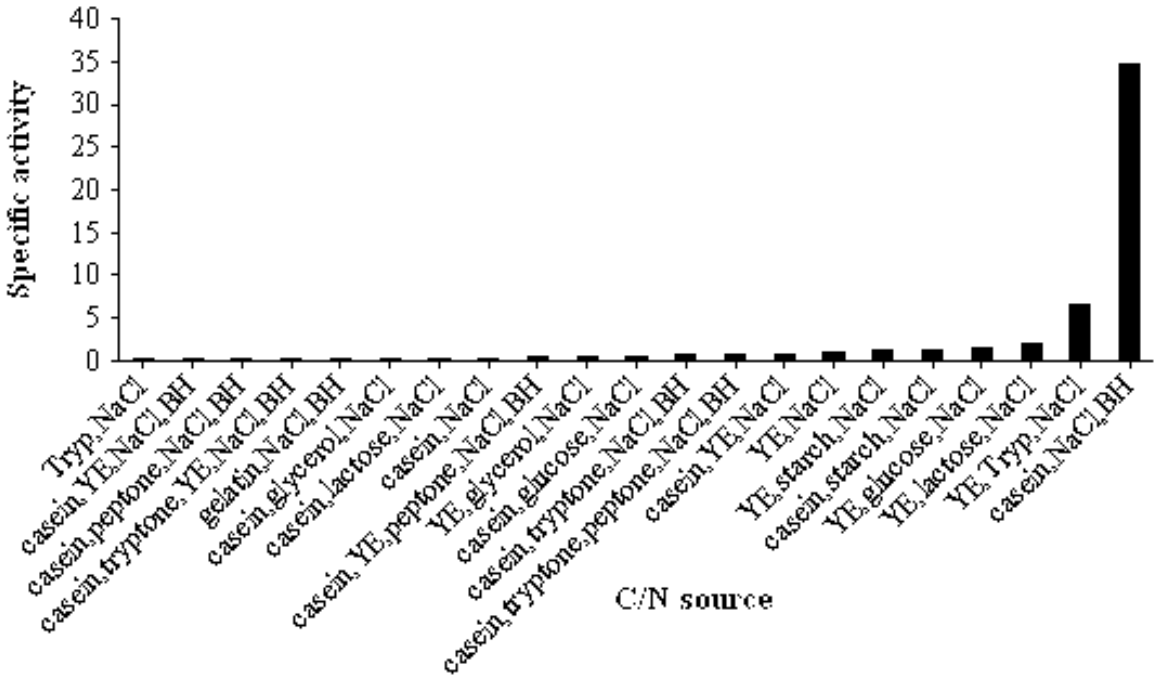


Figure 4.1 Effect of different C/N sources on **a**lkaline protease production (initial medium pH: 9.6, temperature: 28ºC and agitation: 191 rpm).

Though complex organic nitrogen sources enhanced the alkaline protease production (Fig. 4.1) but the simple inorganic nitrogen sources (namely, $(NH_4)_2SO_4$, NH_4NO_3 NH_4Cl, KNO_3 and urea) failed to support the growth of SBV1 and alkaline protease production.

It was evident from Fig. 4.1 that the supplementation of additional carbon and nitrogen sources along with casein not only failed to improve the enzyme production rather showed diminished efficiency when compared to medium containing casein as sole essential carbon and nitrogen source. Casein had also earlier been reported to be an inducer for the production of alkaline protease (Saran *et al*., 2007). This observation could be attributed to the fact that when casein is the only C/N source, the microorganism is forced to produce extracellular alkaline protease in sufficient amount to hydrolyse casein to be utilised for cellular metabolism. However, when any other C/N source as yeast extract, peptone or tryptone are present in the media, some smaller peptides already present along with them can easily be taken up by the bacterial cell without the need of protease for hydrolyzing the portentous nutrients. This type of reduction in protease production in presence of easier substrates like yeast extract, peptone and tryptone has been reported earlier (Ferrero *et al*., 1996; Manivannan and Karthiresan, 2007; Saran *et al*., 2007). Sinha and Satyanarayana (1991) have reported the use of soya meal along with glucose, corn starch and wheat as carbon sources for the production of protease using thermophilic *Bacillus licheniformis*. In a similar study soyacake - another protein rich component was used as the sole source of carbon and nitrogen for production of protease (Kanekar *et al*., 2002). Joshi and Ball (1993) have reported protease activity of 0.65, 1.55 and 1.85 U/ml by alkaliphilic *Bacillus sphaericus*, *Bacillus brevis* and *Bacillus lactosporus* respectively, using nutrient broth medium containing 1% gelatin. There are similar reports of enhanced alkaline protease production in the presence of complex carbon (Hubner *et al*., 1993; Gusek *et al*., 1988; Ferrero *et al*., 1996) and organic nitrogen sources (Hubner *et al*., 1993; Ferrero *et al*., 1996). Contrasting reports are there where

high yield of protease have been observed in presence of ammonium sulfate and potassium nitrate in *B. licheniformis* (Sinha and Satyanarayana, 1991).

4.3.2. Screening of the Most Significant Medium Constituents by Plackett–Burman Experimental Design

Plackett–Burman design was applied to evaluate the significant effects of eight different medium components on the production of alkaline protease. The experiments were performed according to the Plackett–Burman experimental design matrix (Table 4.1). The experimental and predicted responses (production of alkaline protease) are shown in Table 4.1. The adequacy of the model was evaluated and the significant variables were screened using Student's t-test (Table 4.3). Factors evidencing *P*-values of less than 0.00005 were considered to be significant and were therefore selected for further optimization studies. On the analysis of the regression coefficients of the medium components, only calcium chloride has shown positive effect whereas, casein, magnesium sulfate and ammonium nitrate have shown negative effects.

Table 4.3 Estimated effect, regression coefficient and corresponding *T* and *P* values for alkaline protease activity in eight variable Plackett–Burman design experiment

Term	Effect	Coef.	SE Coef.	*T*	*P*	Confidence level (%)
Constant		2.679	0.1789	14.97	0.000[b]	100
$X_1(x_1)$	-5.394	-2.697	0.1862	-14.48	0.000[c]	100
$X_2(x_2)$	-5.373	-2.686	0.2944	-9.12	0.000[c]	100
$X_3(x_3)$	4.237	2.119	0.2944	7.20	0.000[b]	100
$X_4(x_4)$	-3.655	-1.828	0.2634	-6.94	0.000[c]	100
$X_5(x_5)$	-1.254	-0.627	0.2944	-2.13	0.051[a]	94.9
$X_6(x_6)$	2.116	1.058	0.2634	4.02	0.001[a]	99.9
$X_7(x_7)$	-3.417	-1.709	0.3724	-4.59	0.000[c]	100
$X_8(x_8)$	-1.517	-0.758	0.2634	-2.88	0.012[a]	98.8

[a]Non-significant at $P > 0.00005$; [b]Significant positive effect; [c]Significant negative effect.

The absolute values of effect in Table 4.3 were used to indicate the relative contribution of the variable on the response. When a higher-level variable setting (+) results in a higher response compared to its lower-level setting (-) the absolute value of the effect is termed positive or else negative. Experiments performed by sequential elimination of negatively significant components resulted in decreased alkaline protease production. The *P*-value of components NaCl, K_2HPO_4, KH_2PO_4 and $FeCl_3$ were above 0.00005 for the production of alkaline protease and hence these were considered as insignificant ones at a confidence level of 99.995%. The remaining components, casein, magnesium sulfate, calcium chloride and ammonium nitrate have shown *P*-value below 0.00005 and were considered to be significant ones (Fig. 4.2). The levels of these screened components were optimized using central composite experimental design to maximize the production of alkaline protease.

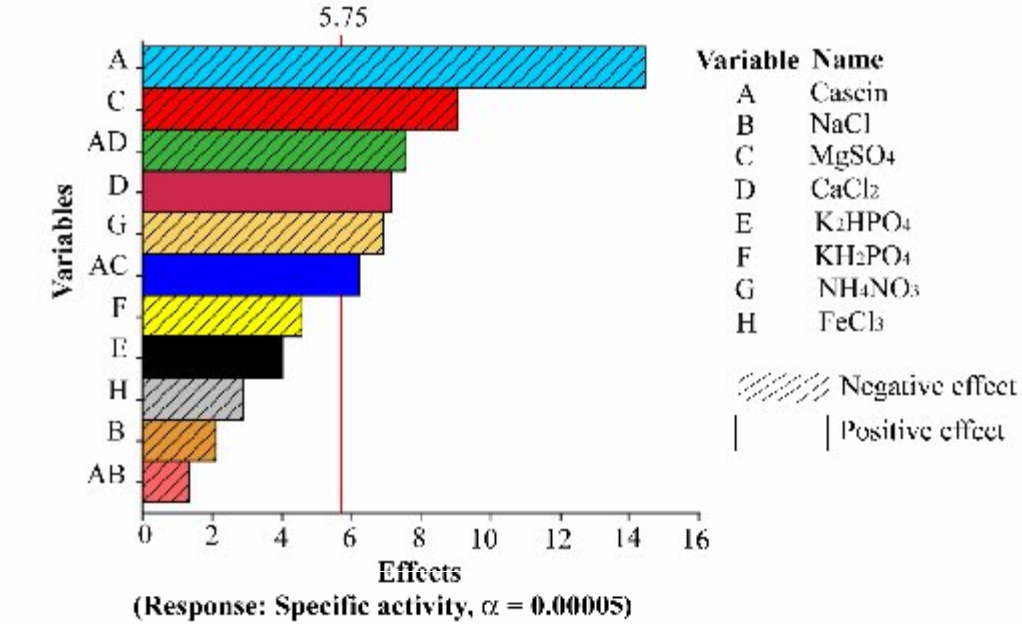


Figure 4.2 Pareto chart of standardized effects of the factors on alkaline protease production.

The inducing effect of casein on the production of alkaline protease was reported (Beg *et al*., 2003; Ferrero *et al*., 1996). Stimulating effect of ammonium sulfate and potassium nitrate, $MgSO_4$ or $CaCl_2$ on alkaline protease production have been reported in various studies (Sinha and Satyanarayana, 1991; Shikha Sharan and Darmwal, 2007; Tari *et al*., 2006; Banerjee *et al*., 1999). Stimulating effect of $CaCl_2$ was also reported by Mabrouk *et al*. (1999). They explained that this may be due to the stabilizing effect of $CaCl_2$ on the alkaline protease. However, in the present investigation $CaCl_2$ have shown positive effect but the other factors have shown negative effect on the production of alkaline protease (Table 4.3).

4.3.3. Optimization of Levels of Medium Components

A large variation in the production of alkaline protease was 0.035 –16.392 U/mg as obtained in the Plackett–Burman design experiments suggested the need for further optimization. Central composite design with 31 experiments conducted in duplicates is shown in Table 4.2 along with the experimental and predicted responses (specific activity). The analysis of variance (ANOVA) of the optimization study as shown in Table 4.4 indicated that the linear effects of casein ($P < 0.001$) and ammonium nitrates ($P < 0.1$) were significant. These results indicate that the concentration of casein source bears a direct relationship to protease production. Moreover, the interactions between casein and ammonium nitrate were significant, as shown by the low *P*-value (<0.05) for the interactive terms. Attempt to fit the experimental data into the second order polynomial model revealed high correlation coefficient ($R^2 = 0.96$) and the coefficients of the model terms were calculated (Table 4.4).

The model *F*-value was 36.31 and the *F*-value for lack of fit was 1.85 (Table 4.5). The high F-value and non-significant lack of fit indicated that the model is a good fit. The *P*-values for the model (<0.0001) and for lack of fit (0.233) also suggested that, the obtained experimental data was a good fit with the model.

Table 4.4 Estimated effect, regression coefficient and corresponding T and P values for alkaline protease activity in eight variable CCD experiment in coded levels

Term	Coef	SE Coef	T	P	Confidence level (%)
Constant	-18.075	9.092	-1.988	0.064[c]	93.6
Casein	10.061	1.250	8.047	0.000[b]	100
$MgSO_4$	13.681	14.136	0.968	0.348[a]	65.2
$CaCl_2$	72.288	125.018	0.578	0.571[a]	42.9
NH_4NO_3	23.795	9.572	2.486	0.024[b]	97.6
Casein*Casein	-0.719	0.072	-9.991	0.000[c]	100
$MgSO_4$*$MgSO_4$	-7.342	9.105	-0.806	0.432[a]	56.8
$CaCl_2$*$CaCl_2$	-682.868	719.416	-0.949	0.357[a]	64.3
NH_4NO_3*NH_4NO_3	-20.263	4.047	-5.007	0.000[c]	100
Casein*$MgSO_4$	-0.100	1.082	-0.092	0.927[a]	7.3
Casein*$CaCl_2$	3.394	9.618	0.353	0.729[a]	27.1
Casein*NH_4NO_3	1.275	0.721	1.768	0.096[b]	90.4
$MgSO_4$*$CaCl_2$	47.964	108.199	0.443	0.663[a]	33.7
$MgSO_4$*NH_4NO_3	-8.883	8.115	-1.095	0.290[a]	71
$CaCl_2$*NH_4NO_3	-32.837	72.133	-0.455	0.655[a]	34.5

[a]Non-significant at P > 0.1; [b]Significant positive effect; [c]Significant negative effect.

Table 4.5 ANOVA for the parameters of response surface methodology fitted to second-order polynomial equation

Source	DF	SS	MS	F	P
Model	14	1928.27	137.734	36.31	0.000
Residual	16	60.69	3.793		
Lack-of-Fit	10	45.83	4.583	1.85	0.233
Pure Error	6	14.86	2.476		
Total	30	1988.96			

Alkaline protease production (*Yi*) by SVB1 was expressed in terms of the following regression equation.

$$Y = -18.075 + 10.061X_1 + 13.681X_2 + 72.288X_3 + 23.795X_4 - 0.719X_1^2 - 7.342X_2^2 - 682.868X_3^2 - 20.263X_4^2 - 0.100X_1X_2 + 3.394X_1X_3 + 1.275X_1X_4 + 47.964X_2X_3 - 8.883X_2X_4 - 32.837X_3X_4 \quad (4.4)$$

where X_1 is casein, X_2 is magnesium sulfate, X_3 is calcium chloride and X_4 is ammonium nitrate. The R^2 (correlation coefficient) of regression equation was 0.97. This is an estimate of the overall variation in the data accounted by the model and thus the model is capable of explaining 97% of the variation in response.

Three-dimensional response surface plots were constructed by plotting the response (alkaline protease production) on the Z-axis against any two independent variables, while maintaining other variables at their mid values. As shown in Fig. 4.3 (a), a curvature in the response surface indicated that alkaline protease production increased up to a maximum level of casein and magnesium sulfate after which production decreased. A similar profile was observed in Fig. 4.3 (b) with casein and calcium chloride concentration, Fig. 4.3 (c) with casein and ammonium nitrate concentration, Fig. 4.3 (e) with magnesium sulfate and ammonium nitrate concentration and Fig. 4.3 (f) with calcium chloride and ammonium nitrate concentration. In case of Fig. 4.3 (d) the

curvature of the response surface was almost flattened. There was an optimum point at the centre of the contour diagram, but no sharp change apparent in the response with magnesium sulfate and calcium chloride concentration.

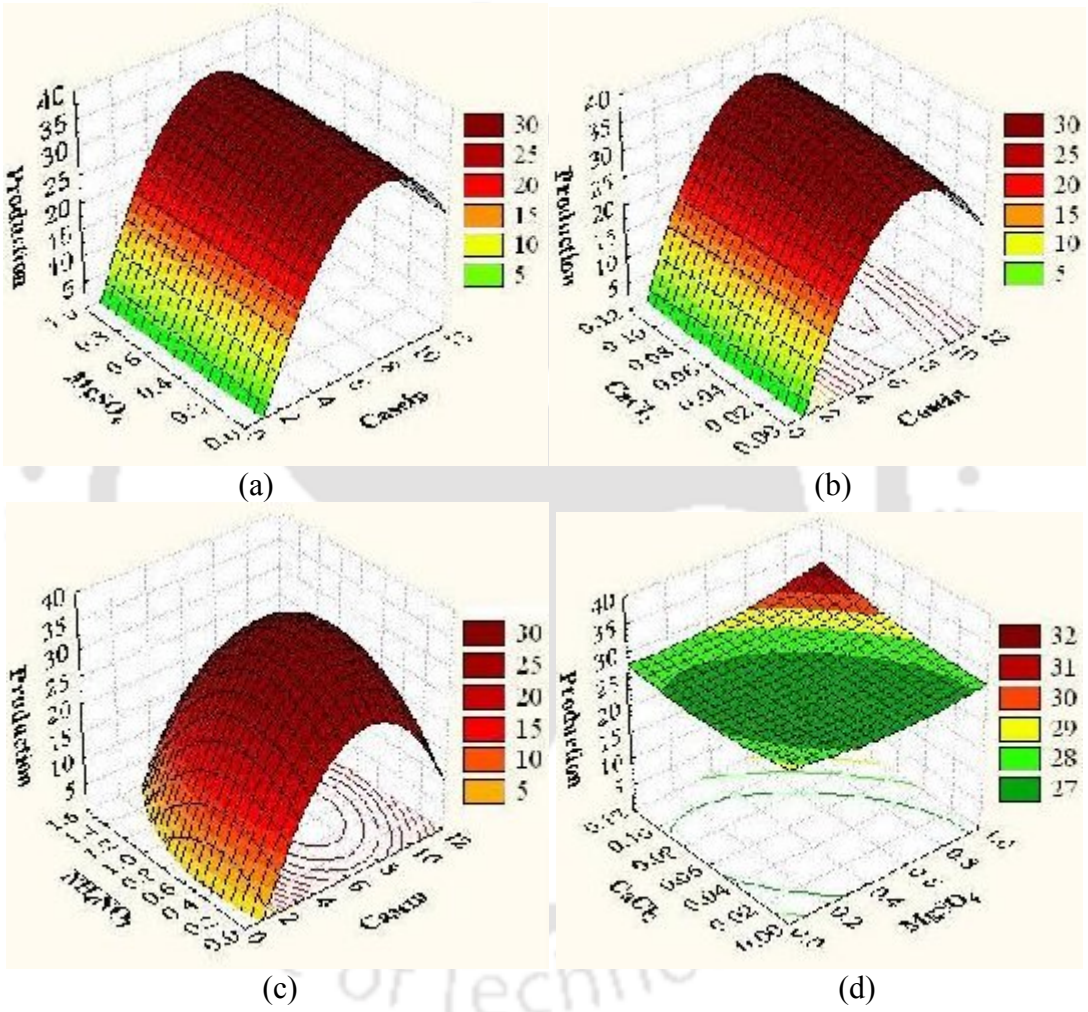
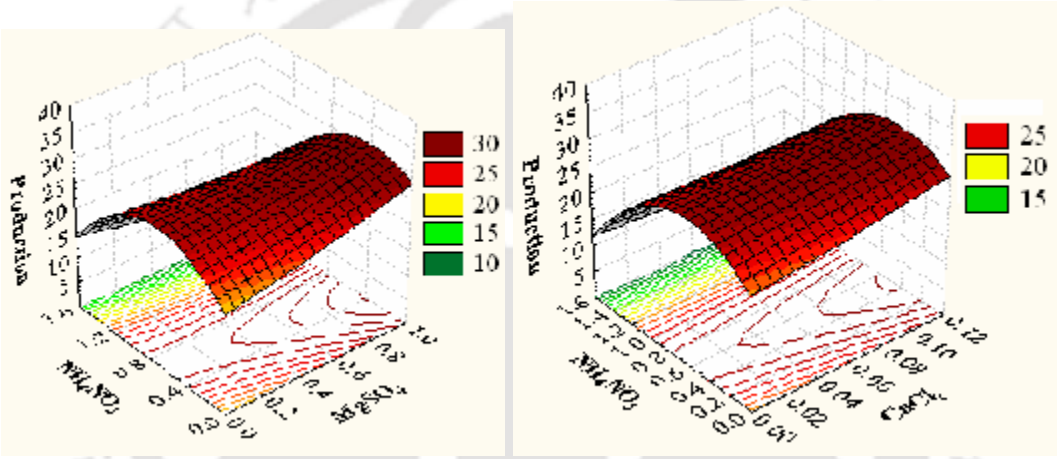


Figure 4.3 Three-dimensional response surface plot for alkaline protease production showing the interactive effects of medium components in g/l: (a) casein and magnesium sulfate, (b) casein and calcium chloride, (c) casein and ammonium nitrate, (d) magnesium sulfate and calcium chloride

However, as shown in Fig. 4.3, the nature of curve and optimum production levels varies with different levels indicating that there are significant interaction effects of the variables on alkaline protease production that was reflected in Table 4.4 as well. The optimum levels of each variable were determined by fitting the experimental data into the second order polynomial equation (Eq. 4.4), and solving the same. The optimal levels of casein, $MgSO_4$, $CaCl_2$ and NH_4NO_3 were found to be 7.636 g/l, 0.811 g/l, 0.086 g/l and 0.576 g/l (w/v), respectively.



(e) (f)

Figure 4.3 Three-dimensional response surface plot for alkaline protease production showing the interactive effects of medium components in g/l: (e) magnesium sulfate and ammonium nitrate and (f) calcium chloride and ammonium nitrate concentrations.

CCD design plan exploited in the present work enabled the exploration the culture conditions that would support a 6.8 fold increase in protease production. The close relationship between the predicted and experimental response values conferred that the goodness of fit of the model for the optimization of the medium components. In the validation run, the predicted response for alkaline protease production was 36.03 U/mg, and the observed experimental value was 37.59 U/mg. The high degree of similarity was

observed between the predicted and experimental value in the validation run reflected the accuracy and applicability of RSM to optimize the process for enzyme production.

Tanyildizi *et al*. (2005) also found similar dependency of response and variables in RSM experiments involving α-amylase production by *Bacillus* sp. IMG 22 as for alkaline protease production in this study. Alkaline protease production was heavily dependent on the availability of carbon as well as nitrogen sources within the medium and both exert regulatory effects on the enzyme synthesis (Chu *et al*., 1992). The nitrogen source, casamino acid has been reported to function as inducers for protease production in a marine microorganism (Chauhan and Gupta, 2004).

The majority of the alkaline protease was secreted within the first 24 h, and this result might be an indication that the alkaline protease production was related to the exponential growth phase of the bacteria (Chapter 3, Section 3.3.1, Fig. 3.1, pp. 56). Previous reports are there citing the alkaline protease production in the exponential growth phase (Kaur *et al*., 2001; Uyar and Baysal, 2004). A significant enhancement of 4.9 fold in alkaline protease production (specific activity) was achieved under optimal levels of medium components. This enhancement is higher than that obtained in previous studies. Reddy *et al*. (2008) reported an improvement of 2.3 fold in alkaline protease production from *Bacillus* sp. in the optimized media. Another study performed by Beg *et al*. (2003) showed that alkaline protease production by *Bacillus mojavensis* was improved up to 4.2 fold using response surface method. Further more, the specific activity achieved in this study is much higher than the pure enzymes available commercially like proteinase from *Bacillus licheniformis* (Sigma P5380) (7-15 units/mg), protease from

Bacillus licheniformis (Sigma P4860) (2.4 U/g), Proteinase bacterial Type XXIV (Sigma P8038) (7-14 units/mg).

4.4 Conclusions

Alkaline protease production (specific activity) from *Bacillus pseudofirmus* SVB1 was found to be affected significantly by the medium components *viz*., casein, $MgSO_4$, $CaCl_2$ and NH_4NO_3 as evident from the Plackett-Burman experimental design analysis. Optimization of these screened medium components was performed by employing central composite design (CCD). The optimal levels of casein, $MgSO_4$, $CaCl_2$ and NH_4NO_3 were found to be 7.636 g/l, 0.811 g/l, 0.086 g/l and 0.576 g/l, respectively. Under the optimal conditions, a significant improvement (4.9 fold) in the production of alkaline protease by SVB1 was accomplished using casein as sole source of carbon and nitrogen. Moreover, dairy industry waste, which is enriched of casein may replace its pure commercial variants to reduce the production cost of this enzyme.

References

Banerjee, U.C., Sani, R.K., Azmi, W. and Soni, R. (1999) Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochem*., **35**: 213-219.

Beg, Q.K., Sahai, V. and Gupta, R. (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochem*., **39**: 203-209.

Chauhan, B. and Gupta, R. (2004) Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. *Process Biochem*., **39**: 2115-2122.

Chu, I.M., Lee, C. and Li, T.S. (1992) Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. *Enzyme Microb. Technol*., **14**: 755-761.

Ferrero, M.A., Carto, G.R., Abate, C.M., Baigori, M.D. and Sineriz, F. (1996) Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: isolation, production and characterization. *Appl*. *Microbiol*. *Biotechnol*., **45**: 327-332.

Gusek, T.W., Wilson, D.B. and Kinselle, J.E. (1988) Influences of carbon source on production of heat stable protease from *Thermomonospora fusca* YX. *Appl*. *Microbiol*. *Biotechnol*., **28**: 80-84.

Hubner, U., Block, U. and Schugerl, K. (1993) Production of alkaline serine protease subtilisin Carlsberg by *Bacillus licheniformis* on complex medium in a stirred tank reactor. *Appl*. *Microbiol*. *Biotechnol*., **40**: 182-188.

Joshi, A. and Ball, B. (1993) Extracellular alkaline enzymes of facultative bacteria of $CaCO_3$ kilns of Jabalpur. *Indian J*. *Microbiol*., **33**: 179-183.

Kanekar, P.P., Nilegaonkar, S.S., Sarnaik, S.S. and Kelkar, A.S. (2002) Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. *Bioresour*. *Technol*., **85**: 87-93.

Kaur, S., Vohra, R.M., Kapoor, M., Beg, Q.K. and Hoondal, G.S. (2001) Enhanced production of a highly thermostable alkaline protease from *Bacillus* sp. *World J. Microbiol*. *Biotechnol*., **17**: 125-129.

Krishna Prasad, K., Venkata Mohan, S., Sreenivas Rao, R., Pati, B.R. and Sarma, P.N. (2005) Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. *Biochem*. *Eng*. *J*., **24:** 17-26.

Li, C., Bai, J., Cai, Z. and Ouyang, F. (2001) Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using response surface methodology. *J. Biotechnol*., **93**: 27-34.

Mabrouk, S.S., Hashem, A.M., El-Shayeb, N.M.A., Ismail, A.S. and Abdel-Fattah, A.F. (1999) Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415. *Bioresour. Technol*., **69**: 155-159.

Manivannan, S. and Karthiresan, K. (2007) Alkaline protease production by *Penicillium fellutanum* isolated from mangrove sediment. *Int. J. Biol. Chem*., **1**(2): 98-103.

Myers, R.H. and Montgomery, D.C. (2002) Response surface methodology: process and product optimization using designed experiments, *Wiley*, New York.

Oberoi, R., Beg, Q.K., Puri, S., Saxena, R.K. and Gupta, R. (2001) Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp. *World J. Microbiol. Biotechnol*., **17:** 493–497.

Puri, S., Khali, O. and Gupta, R. (2002) Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. *Curr. Microbiol*., **44**: 286-290.

Rao, S.S., Prakasham, R.S., Krishna, K.P., Rajesham, S., Sarma, P.N. and Rao, L.V. (2004) Xylitol production by *Candida* sp.: parameter optimization using Taguchi approach. *Process Biochem*., **39**: 951-956.

Reddy, L.V.A., Wee, Y-J, Yun, J-S and Ryu, H-W. (2008) Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett–Burman and response surface methodological approaches. *Bioresour. Technol*., **99**: 2242-2249.

Saran, S., Isar, J., Gupta, P. and Saxena, R.K. (2007) Enhanced productivity of serine alkaline protease by *Bacillus* sp. using soybean as substrate. *Mal. J. Microbiol*., **3**(1): 1-6.

Shikha Sharan, A. and Darmwal, N.S. (2007) Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. *Bioresour. Technol*., **98**: 881-885.

Sinha, N. and Satyanarayana, T. (1991) Alkaline protease production by thermophilic *Bacillus licheniformis*. *Indian J. Microbiol*., **31**: 425-430.

Tanyildizi, M.S., Oze, D. and Elibol, M. (2005) Optimization of α-amylase production by *Bacillus* sp. using response surface methodology. *Process Biochem*., **40**: 2291-2296.

Tari, C., Genckal, H. and Tokatli, F. (2006) Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. *Process Biochem*., **41**: 659-665.

Uyar, F. and Baysal, Z. (2004) Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. *Process Biochem*. **39**: 1893-1898.

CHAPTER 5
PURIFICATION AND CHARACTERIZATION

5.1 Introduction

The biotechnological promise of alkaline proteases makes them an important class of industrial enzyme and therefore their efficient purification in downstream processing remains the essential prerequisite for subsequent commercial exploitation. Majority of the purification procedures for extracellular enzymes involve concentration of the culture filtrate either with salt/solvent precipitation or by ultrafiltration followed by purification in a combination of chromatographic procedures. Previous reports on purification of alkaline protease describe various column chromatography, *viz*., ion exchange as well as hydrophobic interaction chromatography (CM-cellulose, DEAE-cellulose, phenyl-sepharose or DEAE-sepharose) (Khosravi-Darani *et al*., 2008; Thumar and Singh, 2007; Gupta *et al*., 2005a; Ma *et al*., 2007), affinity chromatography (bacitracin-sepharose column or sepharose bound to inhibitor/substrate or CNBr-activated sepharose) (Turkiewicz *et al*., 1999; Jahreis *et al*., 2001) or gel filtration chromatography (sephadex) (Patel *et al*., 2006). Apart from these conventional procedures, more sensitive and advanced procedures such as FPLC (Abbas *et al*., 1989), converging-diverging foam fractionation (Banerjee *et al*., 1993), crystallization (Park *et al*., 1997) and preparative PAGE (Phadatare *et al*., 1992) have been used for the purification of alkaline proteases. After the purification process, the homogeneity of the enzyme preparation is checked by either polyacrylamide gel electrophoresis or gel-filtration or iso-electric focusing (IEF) before further characterization of the enzyme.

Commercialization of alkaline protease largely depends on their stability during isolation, purification and storage as well as on their robustness against solvents, surfactants and oxidants (Gupta *et al*., 2005b; El-Hadj-Ali *et al*., 2007; Najafi *et al*., 2005; Sivasubramanian *et al*., 2008). These properties are known to vary with the nature of the organism from which the enzyme is produced (Gupta *et al*., 2002; Geok *et al*., 2003; Ghorbel *et al*., 2003). Thus, study of kinetics and catalytic behavior of enzyme isolated from any new strain is indispensable (Rao *et al*., 2009).

In these contexts, purification of the alkaline protease produced from *B. pseudofirmus* SVB1 to obtain a homogeneous preparation was carried out. Detail study of the biochemical and themodynamic properties of the purified enzyme was undertaken. The purification and characterization of the enzyme is discussed elaborately in the following sections.

5.2 Materials and Methods

5.2.1 Chemicals and Reagents

All salts and media constituents *viz*., NaCl, NaOH, $MgSO_4.7H_2O$, $CaCl_2$, NH_4NO_3, KH_2PO_4, K_2HPO_4 and $FeCl_3$ were purchased from CDH, India. TCA, acetic acid, Tris, HCl and borax were procured from Merck, India. Biological grade casein, gelatin and agar were obtained from Himedia, India. Protein molecular weight markers and Ezeeblue gel stainer® were procured from Bangalore Genei, India. BSA and egg albumin were purchased from Sigma chemicals, India.

5.2.2 Microorganisms and Culture Conditions

Bacillus pseudofirmus SVB1 was isolated from the premises of tannery industry as described in Chapter 2. Cultures were regenerated every 2-3 weeks on a freshly prepared nutrient agar plates from the frozen stock culture.

5.2.3 Inoculum Preparation and Alkaline Protease Production

The inoculum was prepared as described in Chapter 3. 1% v/v of inoculum from the culture ($A_{600} \approx 1.0$) was added into 500 ml Erlenmeyer flasks containing 100 ml of the sterile medium (at previously optimized initial pH of 9.6) constituted of (in g/l) casein (7.64), $MgSO_4.7H_2O$ (0.81), $CaCl_2$ (0.09), NaCl (4.5), $FeCl_3$ (0.36), NH_4NO_3 (0.58), K_2HPO_4 (2.75) and KH_2PO_4 (2.75) for alkaline protease production from SVB1 (as described in Chapter 4). The culture flasks were incubated in an orbital shaking incubator at 28ºC and 191 rpm (previously optimized conditions, Chapter 3). The cultures were centrifuged at 8000 rpm for 10 minutes at 4ºC and the cell free extract was used as crude alkaline protease preparation for further purification.

5.2.4 Purification

5.2.4.1 Acetone precipitation

The culture supernatant containing the extracellular enzyme was first subjected to acetone precipitation, where acetone fractions of 0–20%, 20–40%, 40–60% and 60–80% (v/v) were collected by centrifugation at 8000 rpm and the pellet obtained were air-dried at 4ºC and suspended in a minimal volume of 25 mM borax–NaOH buffer (pH 10.0).

5.2.4.2 Ion exchange chromatography

The 40–60% acetone fraction was applied to a 2 cm × 25 cm CM-650 TOYOPEARL® column (Tosho Corporation, Tokyo, Japan). This column served as a weak cation exchanger for biomolecule purification. The column was pre-equilibrated with 25 mM Tris–HCl, pH 8.5. After washing with the same buffer, bound proteins were eluted with a linear gradient of pH 8.0 (with 25 mM Tris–HCl) to pH 10.0 (with 25 mM borax–NaOH buffer). At a constant flow rate of 60 ml/h, fractions (4 ml each) were collected and analyzed for protease activity and protein concentration. Active fractions were pooled and stored at 4ºC for further analysis. All the purification steps were conducted at 4ºC.

5.2.4.3 Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the previously purified enzyme fractions were carried out to check the homogeneity of the enzyme preparation and to determine its molecular weight. SDS-polyacrylamide gel electrophoresis was performed in Mini PROTEAN® Tetra Cell system (BIO-RAD, USA) using 1.5 mm thick gels, following the method of Laemmli (1970). 10% (w/v) of acrylamide for resolving gel and 5% (w/v) of acrylamide for stacking gel were used. The protein samples were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2.3% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue. The sample buffer contained SDS (the anionic detergent) for movement of samples under charged conditions. The samples for loading on to the gel were prepared by mixing 1 part of 5x sample buffer to 4 parts of the enzyme sample. This

mixture was incubated at 95°C for 5 min to denature the enzyme for performing the SDS-PAGE. For running the enzyme samples under non denaturing conditions the sample buffer (5x) used did not contain β-mercaptoethanol and the enzyme sample mixed with sample buffer was not subjected to heat denaturation. To maintain the non denaturing condition the native PAGE gels were devoid of SDS. Crude and Purified alkaline protease from different steps of purification was loaded on 10% acrylamide gel (without the stacking gel) and was run under SDS-non denaturing condition. Electrophoresis was carried out using running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl pH: 10.0) with a current of 2 mA per lane (SDS was absent in the running buffer for native page). Silver staining was performed to visualize protein bands after SDS-PAGE. The molecular weight of the enzyme was estimated using ready-to-use protein molecular weight marker (Bangalore Genei, India). The protein marker contained myosin, rabbit muscle (205 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa); ovalbumin (43 kDa), carbonic anhydrase (29 kDa); soyabean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa) and insulin α and β chains (3 kDa). Ezeeblue® staining was used to detect the protein bands on the nondenaturing PAGE (native PAGE).

5.2.4.4 Zymography

Zymography on SDS-PAGE was performed according to the method of Garciacarreno *et al*. (1993). After electrophoresis, the gel was immersed in 100 mM Tris–HCl buffer (pH 8.5) containing 2.5% Triton X-100 at 4ºC, at shaking condition for 30 min to remove SDS. Subsequently, Triton X-100 was removed by washing the gel three times with 100 mM Tris–HCl buffer (pH 8.5). The gel was then incubated with 1% (w/v) casein in 25 mM borax–NaOH buffer (pH 10.0) at 37ºC for 1 h and finally was stained

with Ezeeblue®. For native gel Zymography, casein was copolymerized with polyacrylamide before electrophoresis at 1% final concentration. After electrophoresis as mentioned earlier, the gel was incubated in 25 mM borax–NaOH buffer (pH 10.0) at 37ºC for 1 h. Finally, the gel was stained with Ezeeblue® where clear zone on the blue background indicated the presence of protease.

5.2.5 Characterization

5.2.5.1 Determination of the optimum pH for activity and stability

Determination of the optimum pH for maximum activity of purified protease was performed by analyzing its activity using casein as a substrate in the pH range of 5.0–12.0 with different buffer systems (0.05 M), *viz*., phosphate buffer (for pH 5.0–7.5), Tris–HCl (for pH 8.0–9.0), borax–NaOH (for pH 9.5–11.0) and sodium phosphate (for pH 11.5–12.0). Enzyme activity at pH 10 was considered to be 100%. Stability studies were performed by pre-incubating 0.5 ml of purified enzyme in 3.5 ml of selected buffer at 30°C for 0-60 min at an interval of 10 min, which were subsequently analyzed of their residual activities measured at pH 10.0 and 30°C. The activity of the enzyme before incubation was taken as 100%.

5.2.5.2 Determination of the optimum temperature and thermal stability

Optimum temperature of the enzyme activity was measured by incubating the enzyme reaction mixture at different temperatures (15–50°C) during assay in 25 mM borax–NaOH buffer (pH 10.0). The activity of the enzyme at 30°C was taken as 100%. For determining thermal stability, the enzyme preparation was pre-incubated for 1 h at different temperatures (30–50°C) before assay and the residual activity was measured

under standard assay conditions. The original activity at pre-incubation was taken as 100%.

5.2.5.3 Estimation of kinetic and thermodynamic parameters

5.2.5.3.1 Determination of V_{max} and K_m values

The kinetic parameters, V_{max} and K_m, of the purified protease were determined by measuring the enzyme activity at different substrate concentrations (0–15 mg/ml). The K_m and V_{max} values were determined using Michaelis–Menten equation (Eq. 5.1) (Michaelis and Menten, 1913) and from a double reciprocal plot of Lineweaver and Burk (1934).

$$V = \frac{K_m V_{\max}}{K_m + [S]} \tag{5.1}$$

where V is initial rate of reaction, $V_{\max}$: maximum rate of reaction, K_{m}: Michaelis-Menten constant and [S] is initial substrate concentration.

5.2.5.3.2 Estimation of deactivation rate constant (k_d) and half-life time ($t_{1/2}$)

The deactivation of alkaline protease was assumed to follow first-order rate kinetics and a single step two-stage mechanism (Sadana, 1995) as shown below:

$$E \rightarrow E_{\mathrm{d}} \tag{5.2}$$

The assumption in the mechanism was that the active enzyme state E directly converts to inactive state E_{d} without any intermediate conformations, which can be represented as:

$$\frac{dE}{dt} = -k_d [E] \tag{5.3}$$

Integration of Eq. 5.3 leads to:

$$\alpha = \exp(-k_d t) \tag{5.4}$$

where

$$\alpha = \frac{E_d}{E} \tag{5.5}$$

From the plot of ln (α) versus t, the slope gives the value of deactivation rate constant k_d.

The half-life of an enzyme was defined as the time required by the enzyme to loose half of its initial activity, which was given by:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_d} \tag{5.6}$$

5.2.5.3.3 Estimation of thermodynamic parameters

The energies and entropies of deactivated enzyme were estimated by making use of absolute reaction rates (Eyring, 1935). The temperature dependency of deactivation rate constant can be expressed as:

$$k_d = \frac{\kappa T}{h} \exp\left(\frac{\Delta S^*}{R}\right) \exp\left(\frac{-\Delta H^*}{RT}\right) \tag{5.7}$$

or,

$$\ln\left(\frac{k_d}{T}\right) = \ln\left(\frac{\kappa}{h}\right) + \left(\frac{\Delta S^*}{R}\right) - \left(\frac{-\Delta H^*}{R}\right)\left(\frac{1}{T}\right) \tag{5.8}$$

The values of ΔH^* and ΔS^* were calculated from the slope and intercept of the plot of ln (k_d/T) versus $1/T$, respectively. Values of ΔG^* were further estimated by the following relationship:

$$\Delta G^* = \Delta H^* - T\Delta S^* \tag{5.9}$$

The activation energy was calculated from the Arrhenius equation as:

$$k_d = k_0 \exp\left(-\frac{E_A}{RT}\right) \tag{5.10}$$

or,

$$\ln k_d = \ln k_0 - \left(\frac{E_A}{R}\right)\left(\frac{1}{T}\right) \tag{5.11}$$

The values of E_A and k_0 were estimated from the slope and intercept of the plot of ln (k_d) versus $1/T$, respectively.

5.2.5.4 Effect of various metal ions

Impact of various metal ions (Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+} and Fe^{3+}) on performance of the enzyme was studied by pre-incubating it at room temperature

in the presence of a specified ion (10 mM) as described by Rao *et al*. (2009) dissolved in the borax-NaOH buffer prior to assay. After 1 h of incubation, casein was added to the mixture and residual activity of the enzyme was measured under standard assay conditions. Activity correspondent to 0.5 U of enzyme in absence of any metal ion was considered to be 100%.

5.2.5.5 Effect of protease inhibitors

The influence of different protease inhibitors, *viz.*, phenyl methyl sulphonyl fluoride (PMSF), (serine protease inhibitor), ethylene diamine tetra acetic acid (EDTA) (metalloprotease inhibitor), iodoacetamide (cysteine protease inhibitor) and Pepstatin A (aspartic protease inhibitor) was investigated by incubating the enzyme for 30 min at 30°C in the selected protease inhibitor contained in the assay mixture at a final concentration of either 1.0 or 5.0 mM. The residual proteolytic activity of the enzyme was determined under standard assay conditions. Activity of the enzyme in the absence of any inhibitor was considered as 100%.

5.2.5.6 Effect of surfactants/detergents and oxidizing agents

The effect of 1% final concentration of different surfactants (SDS in w/v, Tween-80, Triton X-100 in v/v) and oxidants (H_2O_2 in v/v) on proteolytic activity of the purified alkaline protease was studied by pre-incubating it for 4 h in the above solutions at 30°C before testing for protease activity. A parallel control was kept with enzyme and buffer with substrate in the absence of any surfactant or oxidizing agents.

5.2.5.7 Effect of various organic solvents

In the organic solvent stability test, 1.0 ml of organic solvent was added to 3.0 ml of the purified enzyme solution in a 5 ml screw-cap vial and incubated at 30°C in shaking condition at 150 rpm for 30 min. The remaining proteolytic activity using casein as substrate was measured. Stability was expressed as the remaining proteolytic activity relative to the solvent-free controls.

5.2.5.8 Substrate specificity

Proteolytic activity with various protein substrates including BSA, casein, egg albumin and gelatin was assayed by mixing 0.5 U of the enzyme with 840 µl of assay buffer containing the protein substrates (0.6% w/v). After incubation at 30°C for 30 min, the reaction was stopped by addition of 896 µl of TCA mixture (0.11 M TCA, 0.22 M sodium acetate and 0.33 M acetic acid) followed by 30 min incubation at room temperature. Undigested protein was removed by centrifugation (13500 rpm) and concentrations of released peptides were measured by reading the absorbance at 280 nm. Activity with casein as substrate was considered to be 100%.

5.2.6 Analytical Techniques

Cell growth was measured as described in Chapter 2 (Section 2.2.5.1, pp. 45). Alkaline protease activity and specific activity of the culture supernatant of SVB1 were measured as described earlier (Chapter 3, Section 3.2.5.2., pp. 54). The total protein contents of the samples were determined as described in Chapter 3 (Section 3.2.5.3., pp. 55).

5.3. Results and Discussion

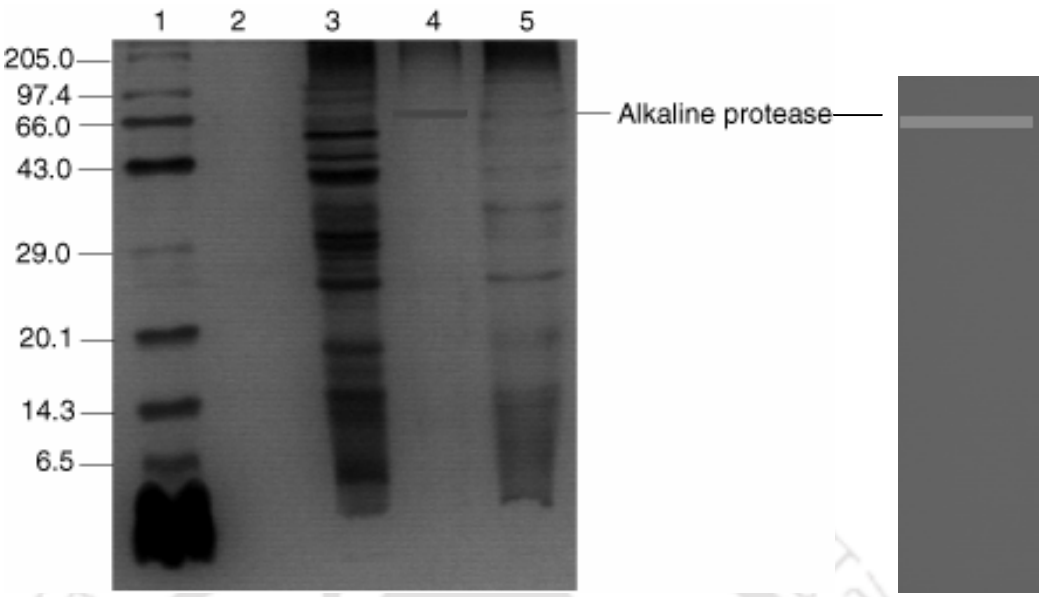
5.3.1. Enzyme Purification and Molecular Weight

About 18.8-fold purification and near homogeneity of the enzyme, starting from the culture filtrate was achieved by acetone precipitation (40-60%) followed by cation-exchange chromatography in CM-650 TOYOPEARL® column presented in Table 5.1. The specific activity and % recovery of the purified enzyme was 711.43 U/mg protein and 52.3%, respectively.

Table 5.1 Purification table of *Bacillus pseudofirmus* SVB1 alkaline protease

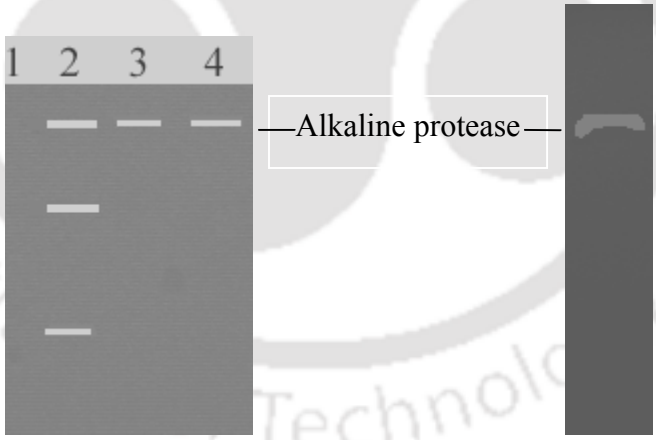
Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Fold Purification
Crude extract	370000	9868	37.49	100	1
Acetone precipitation (40–60%)	290820	2439	119.24	78.6	3.18
Ion exchange (Toyopearl)	193510	272	711.43	52.3	18.77

Appearance of single band in SDS and native PAGE as well as zymography indicated that the purified alkaline protease was a monomeric protein with molecular mass of 85 kDa (Figs. 5.1 a, b, c and d). These results were in accordance with few literature reports, where the molecular masses of alkaline proteases from *Bacillus* genus have been found to be above 70 kDa (75kDa: Jung *et al*., 2007; 80 kDa: Ghosh *et al*., 2008; 90 kDa: Kato *et al*., 1992; 86.29 kDa: Arulmani *et al*., 2007). Moreover, alkaline proteases from other species having molecular weight above 70 kDa were also reported (86 kDa: Vidyasagar *et al*., 2006; 80 kDa: Singh and Venkata Ramana, 1998).



(a) (b)

Figure 5.1 (a) Silver stained SDS-PAGE of the purified protease from *B. pseudofirmus* SVB1. Lane 1: molecular mass markers (sizes in kDa), lane 2: gel loading buffer, lane 3: crude supernatant, lane 4: purified protease after CM-650 TOYOPEARL® cation-exchange column, lane 5: 40-60% acetone fraction of the crude supernatant; (b) Ezeeblue® stained zymography on SDS-PAGE of the purified protease after CM-650 TOYOPEARL® cation-exchange column.



(c) (d)

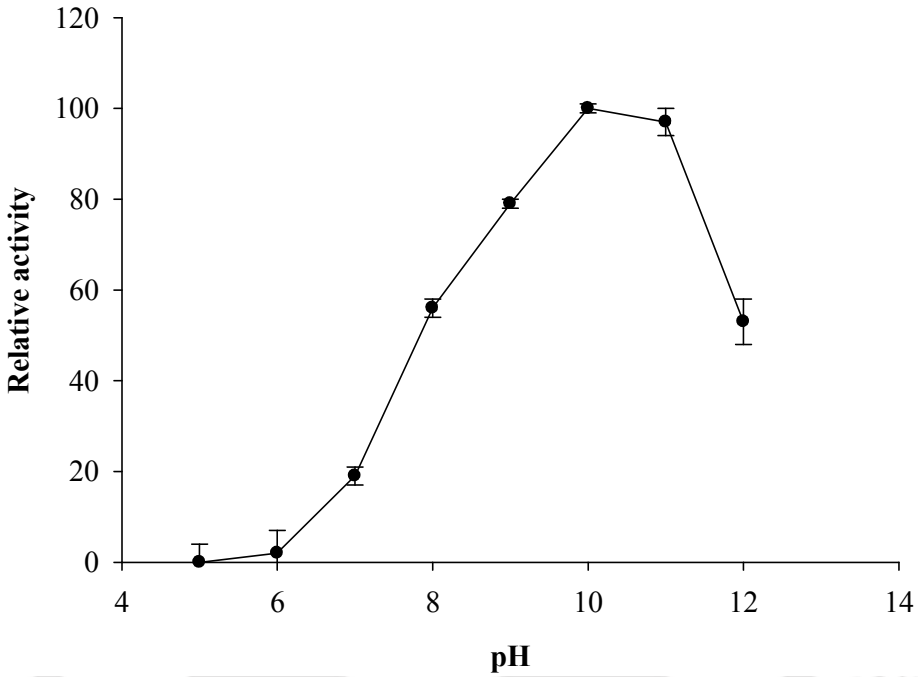
Figure 5.1 (c) Ezeeblue® stained native PAGE of the purified protease from *B. pseudofirmus* SVB1. Lane 1: gel loading buffer, lane 2: crude supernatant, lane 3: 40-60% acetone fraction of the crude supernatant, lane 4: purified protease after CM-650 TOYOPEARL® cation-exchange column, (d) Ezeeblue® stained zymography on native PAGE of the purified protease after CM-650 TOYOPEARL® cation-exchange column.

5.3.2 Characterization

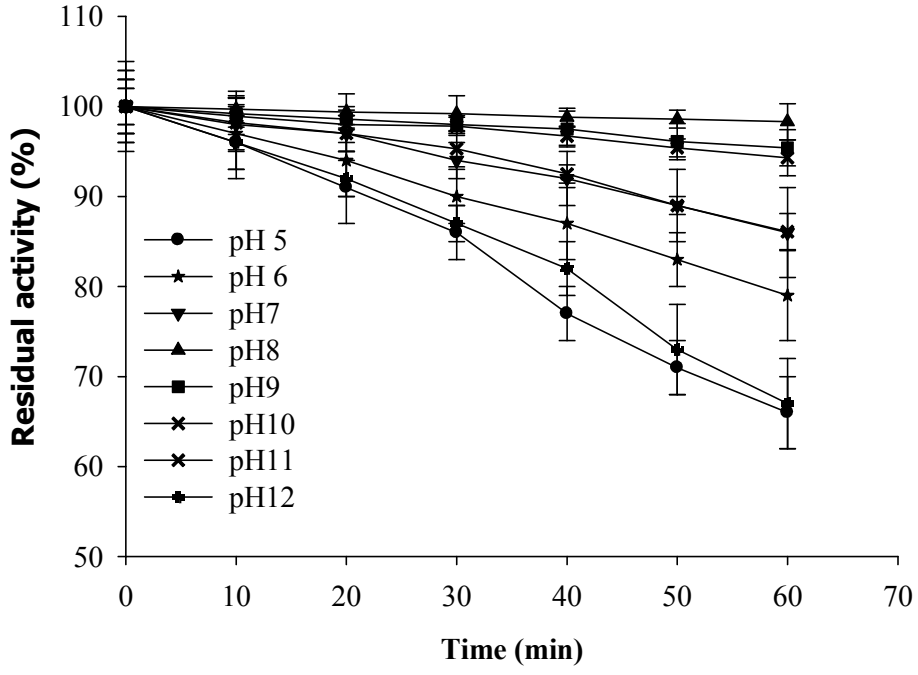
5.3.2.1 Determination of the pH optimum and stability

In general, bacteria belonging to *Bacillus* genus are known to secrete mostly two types of extracellular proteases, neutral or metalloprotease which exhibits optimum activity at pH 7.0 and alkaline protease having pH optima between 9.0 and 11.0 (Ghorbel *et al*., 2003). The enzyme produced by *Bacillus pseudofirmus* SVB1 showed its optimum activity at pH 10.0. This indicated that the enzyme belonged to alkaline protease group. Any further variation of the pH of the reaction mixture resulted in reduced catalytic activity as shown in Fig. 5.2a and even more drastically towards alkalinity. However, the purified enzyme retained 50% relative activity at pH as high as 12.0 or as low as pH 7 that pointed to its robust nature in pH range of 7.0–12.0 (Fig. 5.2a). Nilegaonkar *et al*. (2007) reported that the protease being active in a broad pH range of 6.0 -12.0 showed optimum activity at pH 9.0 and was associated with drastic reduction of activity on either side of pH optima.

Enzyme stability studies at the broad pH range of 5.0 to 12.0 for 10-60 min indicated that the enzyme activity varied with incubation time and pH as evident from Fig. 5.2b. Incubation of this enzyme for 1 h at pH of 8.0–10.0 did not show any reduction in the activity, whereas merely 15% reduction in activity was noticed when incubated at pH of 7.0 or 11.0. This revealed that the high stability of the protease under these conditions as shown in Fig. 5.2b. Although there was no activity observed at pH 5.0, but in stability study about 85% activity was retained when incubated at pH 5.0. These findings were in accordance with several earlier reports showing pH optima between 8.0 and 10.5 for proteases from *Vibrio fluvialis* VM10 (Venugopal *et al*., 2006), *Salinivibrio*



(a)



(b)

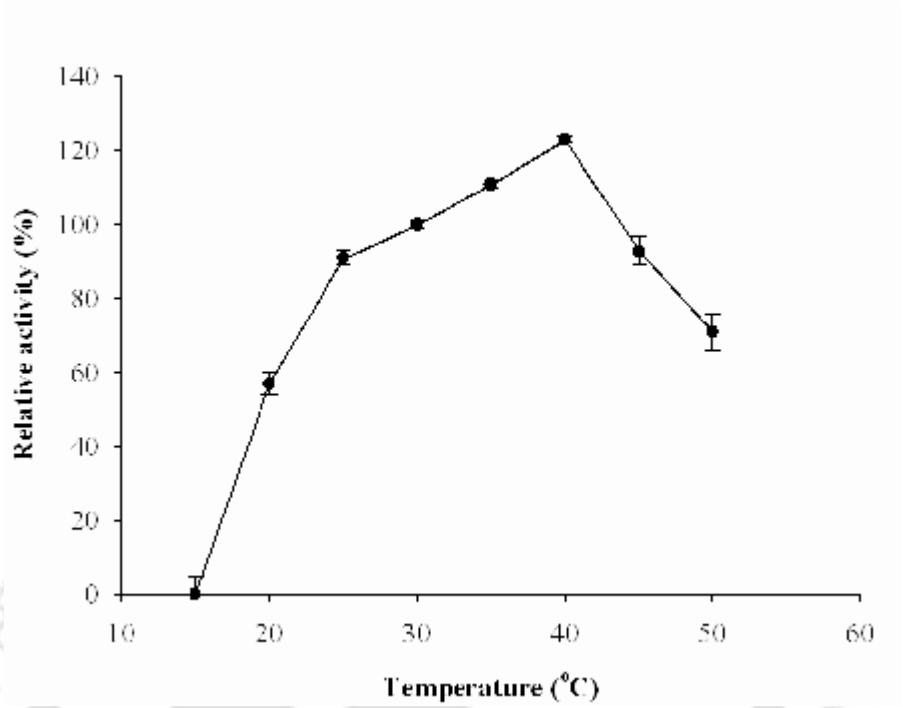
Figure 5.2 Effect of pH on activity (a) and (b) stability of the purified alkaline protease from *Bacillus pseudofirmus* SVB1 assayed at 30°C under standard assay conditions at different pH mentioned in Section 5.2.5.1.

sp. (Amoozegar *et al*., 2007), *gamma-Proteobacterium* (Sana *et al*., 2006), *A. clavatus* ES1 (Hajji *et al*., 2007) and some *Bacillus* sp. (Beg and Gupta, 2003; El-Hadj-Ali *et al*., 2007; Horikoshi, 1990).

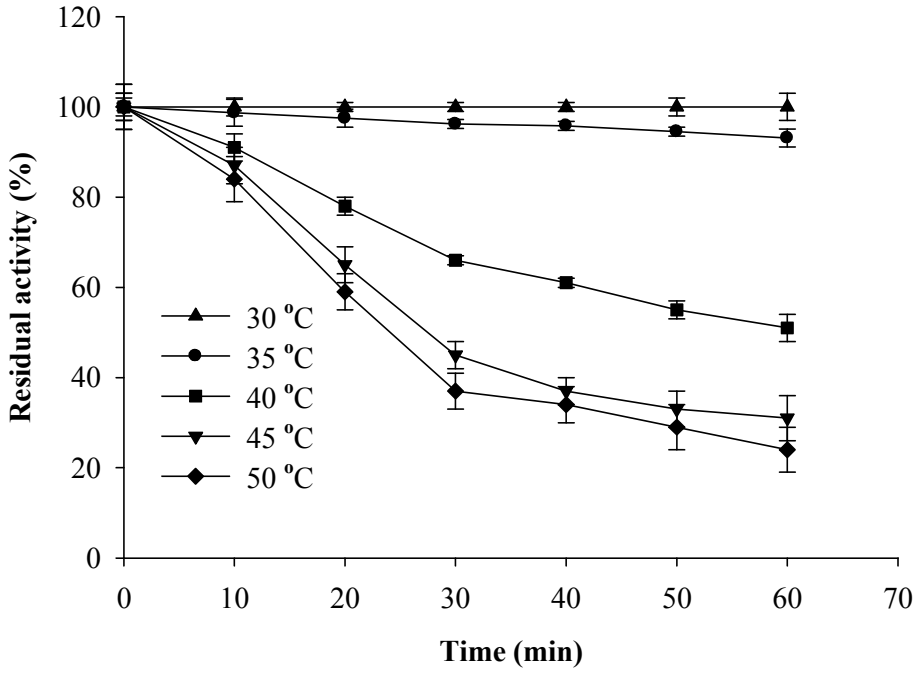
5.3.2.2 Determination of the optimum temperature and thermal stability

The protease activity of the purified enzyme was measured at different temperatures that ranged from 15 to 50°C as shown in Fig. 5.3a. Optimum activity of the purified enzyme was found at 40°C. About 17% of the activity was lost after incubation at 50°C. The result showed that alkaline protease from strain SVB1 was active over a wide range of temperature (from 20 to 50°C) due to mesophilic nature of the enzyme. The profiles from stability study carried out at a temperature range from 30 to 50°C are presented in Fig. 5.3b. The purified enzyme retains its full activity for 30 min in the temperature range of 30 to 35°C and the loss of activity was nominal even after 60 min of incubation. However, the loss of enzyme activity was more prominent and drastic when incubated at 45 or 50°C (retaining only 25% residual activity after 1 h).

Results indicated that the better stability of alkaline protease from SVB1 than that reported by Mitra and Chakrabartty (2005). Proteolytic activity in this study decreased dramatically when the temperature increased above 40°C which retained only 30% and 25% activity at 45 and 50°C, respectively (Fig. 5.3b). This was similar to the results reported by Pawar *et al*. (2009), where about 95% and 70% activity of *Bacillus* protease were retained at 40 and 50°C, respectively compared to that of its optimum temperature 30°C. The proteases obtained from *B. pumilus* (Feng *et al*., 2001; Huang *et al*., 2003), which lost its 53% activity at 50°C from its optimal temperature of 45°C and thus was inferior to protease from SVB1 in this study. Another serine protease was reported to



(a)



(b)

Figure 5.3 Effect of temperature on (a) activity: temperature range 15-50°C and (b) stability: temperature range 10-60°C of the purified alkaline protease from *Bacillus pseudofirmus* SVB1.

retain the activity at the pH range of 7.5 to 9.5 and temperature range of 30 to 45°C, resulting in the relative activity of higher than 80% (Yossan *et al*., 2006).

5.3.2.3 Estimation of kinetic and thermodynamic parameters

5.3.2.3.1 Determination of V_{max} and K_m values

A plot was drawn between the rate of alkaline protease catalyzed reaction (V) versus the initial casein (substrate) concentration ([S]) (Fig. 5.4a). V_{max} and K_m values were evaluated from Lineweaver and Burk double reciprocal plot as presented in Fig. 5.4b. The K_m and V_{max} were determined to be 1.83 mg/ml and 533.83 U, respectively. The low K_m and high V_{max} values inferred that the high affinity and efficient catalytic role of the enzyme. These values were comparable to the literature reported values of alkaline proteases, haloalkaliphilic *Bacillus* sp. (K_m of 2 mg/ml and V_{max} of 289 mg/min) (Gupta *et al*., 2005a), *Haloalkaliphilic bacterium* sp. AH-6 (K_m of 2.5 mg/ml and V_{max} of 625 U/min) (Dodia *et al*., 2008) and *Pseudomonas aeruginosa* PseA (K_m of 2.69 mg/ml and V_{max} of 3.03 mmol/min) (Gupta *et al*., 2005b).

The nature of the Lineweaver and Burk double reciprocal plot as evident from Fig. 5.4b obtained in this study was very much similar to that reported by Padron-Pereira *et al*. (2009) except that their R^2 value (0.85) was much lower than that obtained in this study (0.93). Similarly, R^2 obtained for calculating K_m and V_{max} for different enzymes in the literature are lower than that achieved in the present study (0.70: Evans *et al*., 2009; 0.62: Linton and Greenaway, 2004). Thus, a good fitting of the equation suggested that Michaelis-Menten kinetics was followed by this alkaline protease for protein hydrolysis reaction.

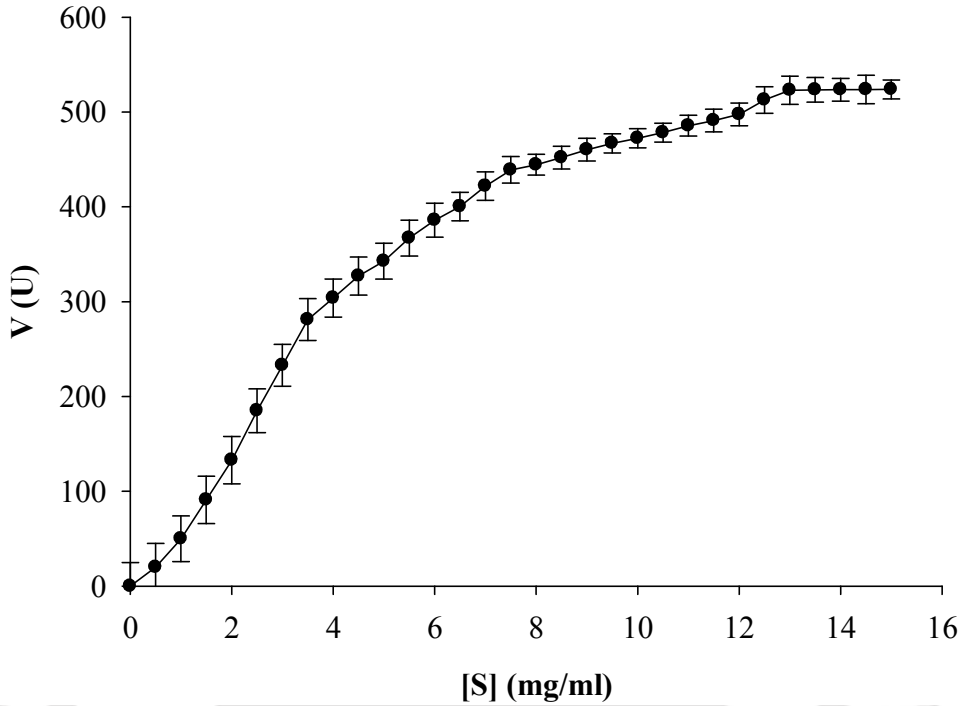


Figure 5.4 (a) Plot of initial velocity of reaction (*V*) vs. initial substrate concentration [*S*].

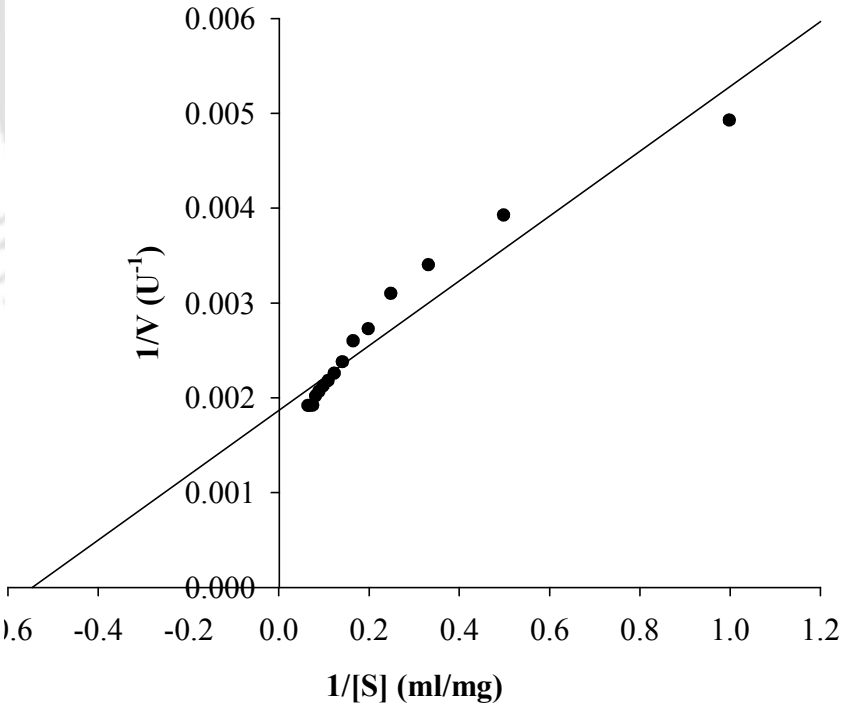


Figure 5.4 (b) Lineweaver–Burk double reciprocal plot of 1/*V* vs. 1/[*S*].

5.3.2.3.2 Estimation of deactivation rate constant (k_d) and half-life time ($t_{1/2}$)

Information about the half life and deactivation constants at different pH and temperature is presented in Table 5.2. The half-life of the alkaline protease at 50°C and pH 9 was observed to be 27 min, which had strongly suggested its thermal stability at 50°C. In general, it was found that at lower temperature (30ºC) half life was greater at higher pH. Whereas, at higher temperatures (35-50ºC), enzyme was more stable at lower pH. Exception was found with pH 9.0 at 45ºC and 50ºC. Moreover, half-life of alkaline protease from SVB1 at these temperatures when compared to that from *Neurospora crassa* NH1 (Hanson and Marzluf, 1975) and *Bacillus* sp. (Stellwag *et al*., 1988) indicated its superiority and robustness. Kapat and Panda (1997) reported similar temperature dependency of rate constant for thermal deactivation of chitinase from *Trichoderma harzianum*.

Table 5.2 Effect of temperature on half life time ($t_{1/2}$) and deactivation constant (k_d) of the purified alkaline protease from SVB1

Temperature (ºC)	k_d (min^{-1})			$t_{1/2}$ (min)			R^{2a}		
	pH 7.0	pH 9.0	pH 10.0	pH 7.0	pH 9.0	pH 10.0	pH 7.0	pH 9.0	pH 10.0
30	0.0444	0.0344	0.0318	15.60	20.15	21.80	0.91	0.98	0.97
35	0.0234	0.0380	0.0362	29.65	18.26	19.14	0.75	0.95	0.91
40	0.0312	0.0351	0.0348	22.21	19.75	19.94	0.86	0.98	0.83
45	0.0285	0.0251	0.0298	24.36	27.61	23.26	0.88	0.99	0.74
50	0.0273	0.0257	0.0271	25.37	27.02	25.58	0.89	0.97	0.73

[a]Appendix III, pp. 211-212

5.3.2.3.3 Estimation of thermodynamic parameters

The change in enthalpy and entropy were calculated by transition state theory according to Eqs. 5.7, 5.8 and results are shown in Table 5.3. Solvent and structural effects are the two major factors which influence the numerical values of ΔH^* and ΔS^*. The entropy and enthalpy values were greater at pH 7.0 and 9.0 as compared to those at pH 10.0. The increase in ΔS^* at pH 7.0 and 9.0 indicated an increase in the number of protein molecules in a transition activated state. The values of ΔG^* as calculated from Eq. 5.9, are also included in Table 5.3. It was apparent from the result that the entropy and enthalpy values were the minimum at pH 10.0. This could be attributed to the fact that at higher pH, the stable three dimensional structure of enzyme probably gets compressed resulting in lower residual activity. The temperature dependency of first-order deactivation rate constant was calculated by Arrhenius equation (Eq. 5.10). The activation energy E and frequency factor k_0 were estimated from Eq. 5.11. The values of all these thermodynamic parameters at different pH are given in Table 5.3. The high correlation coefficient (0.96) obtained in the thermodynamic parameter studies suggested that first order kinetics was followed in the temperature range of 30–50°C.

It was postulated that the entropy values provide information regarding the degree of solvation and very likely the degree of compactness of protein molecule (Naidu and Panda, 2003). Increase in the entropy and deactivation energy was observed as pH was shifted from 7.0 to 9.0 and decreased as pH was shifted from 9.0 to 10.0 (Table 5.3). Naidu and Panda (2003) also found that the deactivation rate constant was dependent on pH. The standard free energy change increased with the increase in pH. However, at higher pH, the activation energy was found to be the lowest. This was in accordance with

the observations reported by Kapat and Panda (1997). More recently, Rao *et al*. (2009) reported similar observation on serine alkaline protease from *B*. *circulans*. However, the alkaline protease obtained from SVB1 in this study may be used even in room temperature with unaltered activity in contrast to the alkaline protease from *B*. *circulans*, which needs higher temperature for its optimum activity.

Table 5.3 Estimated thermodynamic parameters during the thermal deactivation of alkaline protease from SVB1

pH	ΔH (KJ/mol)	ΔS (J/mol.K)	E_A (KJ/mol)	k_0 (min^{-1})	ΔG (KJ/mol)
7.0	223.60	68.47	12855.41	4637.004	20522.54-21891.92
9.0	271.51	69.71	16166.91	16050.24	20850.84-22245.05
10.0	156.66	66.64	8228.41	744.8021	20035.38-21368.19

R^2 of plot of ln (k_d/T) verus $1/T$ was 0.96 (Appendix IV, pp. 213); R^2 of plot of ln (k_d) verus $1/T$ was 0.94. The temperature range was 30-50°C

5.3.2.4 Effect of various metal ions

Influence of various metal ions on the activity of alkaline protease from SVB1 is presented in Table 5.4. It is quite evident that though Ca^{2+}, Mg^{2+} and Mn^{2+} ions regulated the enzyme activity positively but, other tested metal ions did not show much influence except Cu^{2+} and Fe^{3+}. The Ca^{2+} ion dependent activity improvement might be attributed to its involvement in stabilization of the enzyme molecular structure, which has also been reported for some other proteases derived from *Bacillus* sp. (Towatana *et al*., 1997; Takii *et al*., 1990; Kobayashi *et al*., 1996; Sana *et al*., 2006; North, 1982).

Table 5.4 Influence of different metal ions on activity of alkaline protease from SVB1

Metal ion	Concentration (mM)	Relative activity (%)
Control	--	100±2
Na^{+} (NaCl)	10	102±1
Mg^{2+} ($MgCl_2$)	10	113±5
Ca^{2+} ($CaCl_2$)	10	132±5
Mn^{2+} ($MnCl_2$)	10	117±3
Zn^{2+} (ZnCl)	10	92±1
Cu^{2+} ($CuCl_2$)	10	78±4
Cd^{2+}($CdCl_2$)	10	91±1
Hg^{2+}($HgCl_2$)	10	86±4
Fe^{3+} ($FeCl_3$)	10	43±5

100% of activity correspondent to 0.5 U of enzyme in absence of any metal ion

5.3.2.5 Effect of protease inhibitors

Proteases are classified based on their sensitivity to various inhibitors (North, 1982). To know the nature of the alkaline protease produced by *B. pseudofirmus*, the enzyme activity in the presence of different protease inhibitors (1.0 and 5.0 mM) were analyzed.

Table 5.5 Influence of different inhibitors on activity of alkaline protease from SVB1

Inhibitors	Relative activity at inhibitor concentration of		Type
	1 mM	5 mM	
Control	100±5	100±5	-----
PMSF	3±1	0±0	Serine protease inhibitor
Iodoacetamide	98±4	94±4	Cysteine protease inhibitor
EDTA	100±4	96±4	Metallo-protease inhibitor
Pepstatin A	97±2	91±3	Aspartic protease inhibitor

100% of activity correspondent to 0.5 U of enzyme in absence of any inhibitor

The results in Table 5.5 revealed that EDTA (metalloprotease inhibitor), iodoacetamide (cysteine protease inhibitor) and pepstatin A (aspartic protease inhibitor) showed no or minimal effect on alkaline protease activity. However, PMSF (serine

protease inhibitor) almost completely inhibited the enzyme activity even at very low concentration (1.0 mM) and completely at 5.0 mM concentration, which suggested that the protease produced by *B. pseudofirmus* belongs to serine proteases group.

5.3.2.6 Effect of surfactants/detergents and oxidizing agents

The purified enzyme showed stability in the presence of all compounds selected in this study as shown in Table 5.6. In fact the non-ionic detergents, Triton X-100 and Tween-20 enhanced its activity by 13 and 25%, respectively. In the presence of strong anionic surfactant SDS (1%), the enzyme retained 71% of its initial activity. In general and as per the literature, the proteases belonging to *Bacillus* genus are known to be unstable against the oxidants and bleaching agents (Anwar and Saleemuddin, 2000). However, the enzyme under investigation showed very little inhibition in presence of 1% hydrogen peroxide and retained 89% of its activity. This experimental observation suggested that the purified enzyme was stable to all tested cationic, anionic, non-ionic and to the different commercially available detergents.

Table 5.6 Influence of different surfactants/detergents on the activity of alkaline protease from SVB1

Surfactants/detergents	Relative activity (%)
Control	100±1
Triton X-100	113±2
Tween-20	125±2
SDS	71±5
H_2O_2	89±4
Nirma	91±3
Surf excel	95±2
Rin	98±2
Ariel	87±5

100% of activity correspondent to 0.5 U of enzyme with casein as substrate in absence of any surfactants/detergents

5.3.2.7 Effect of various organic solvents

The enzyme was stable in presence of all the organic solvents tested after 30 min incubation as given in Table 5.7. In organic solvents such as isooctane, n-decane, n-dodecane and acetone (25%, v/v) retained much of the activity of purified alkaline protease by 51%, 45%, 39% and 63%, respectively. Hydrophobic solvents are usually superior to hydrophilic solvents because the latter have a greater tendency to strip tightly bound water from the enzyme molecules essential for catalytic activity. If organic solvents are used as media for enzymatic reactions, the reaction equilibrium of hydrolytic enzymes can be shifted towards completion of the reverse of hydrolysis, the synthetic reaction. Therefore, proteases, which are naturally stable in the presence of organic solvents could be very useful for synthetic reactions. There are very few reports available on organic solvent tolerant alkaline protease. The alkaline protease from SVB1 is superior to other alkaline proteases reported in the literature (Thumar and Singh, 2008; Abd Rahman *et al*., 2006).

Table 5.7 Influence of different organic solvents on activity of alkaline protease from SVB1

Organic solvents	Relative activity (%)
Control	100±3
Isooctane	51±4
Decane	45±4
Dodecane	39±5
Acetone	63±3

100% of activity correspondent to 0.5 U of enzyme with casein as substrate in absence of any organic solvent

5.3.2.8 Substrate specificity

Substrate specificity of the purified protease was examined using various natural protein substrates. The alkaline protease showed a high level of hydrolytic activity

against casein and moderate hydrolysis of gelatin and egg albumin, and very low affinity towards BSA (Table 5.8).

Table 5.8 Substrate specificity of alkaline protease from SVB1

Substrate added	Relative activity (%)
Casein	100±2
Gelatin	68±3
BSA	17±5
Egg albumin	43±4

100% of activity correspondent to 0.5 U of enzyme with casein as substrate

5.4 Conclusions

The purification and characterization of an 85 kDa serine alkaline protease from *B. pseudofirmus*, which exhibited a broad spectrum of physical and chemical stresses, are described in this investigation. The enzyme exhibited high activity at alkaline pH and moderate temperature (below 50°C). The Stability of the purified enzyme in the presence of surfactants and bleach (Triton X-100, SDS and H_2O_2), commercial detergents (Nirma®, Surf excel®, Rin® and Ariel®), heavy metals (Cd and Hg) and organic solvents (isooctane, decane and dodecane) inferred that the purified enzyme seems to be a potential candidate for industrial applications. The enzyme had shown broad substrate specificity.

References

Abbas, C.A., Groves, S. and Gander, J.E. (1989) Isolation, purification, and properties of *Penicillium charlesii* alkaline protease. *J. Bacteriol*., **171**: 5630-5637.

Abd Rahman, R.N.Z.R., Geok, L.P., Basri, M. and Salleh, A.B. (2006) An organic solvent-stable alkaline protease from *Pseudomonas aeruginosa* strain K: Enzyme purification and characterization. *Enzyme Microb. Technol*., **39**(7): 1484-1491.

Amoozegar, M.A., Fatemi, A.Z., Karbalaei-Heidari, H.R. and Razavi, M.R. (2007) Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004. *Microbiol. Res*., **162**: 369-377.

Anwar, A. and Saleemuddin, M. (2000) Alkaline protease from *Spilosoma obliqua*: potential application in bioformulations. *Biotechnol. Appl. Biochem*., **31**: 85-89.

Arulmani, M., Aparanjini, K., Vasanthi, K., Arumugam, P., Arivuchelvi, M., Kalaichelvan, P.T. (2007) Purification and partial characterization of serine protease from thermostable alkalophilic *Bacillus laterosporus* AK1. *World J. Microbiol. Biotechnol*., **23**: 475-481.

Banerjee, R., Agnihotri, R. and Bhattacharya, B. C. (1993) Purification of alkaline protease of *Rhizopus oryzae* by foam fractionation. *Bioprocess Eng*., **9**(6): 245-248.

Beg, Q.K. and Gupta, R. (2003). Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microb. Technol*., **32**: 294-304.

Dodia, M.S., Rawal, C.M., Bhimani, H.G., Joshi, R.H., Khare, S.K., Singh, S.P. (2008) Purification and stability characteristics of an alkaline serine protease from a newly isolated *Haloalkaliphilic bacterium* sp. AH-6. *J. Ind. Microbiol. Biotechno*l., **35**: 121-131.

El-Hadj-Ali, N., Agrebi, R., Ghorbel-Frikha, B., Sellami-Kamoun, A., Kanoun, S. and Nasri, M. (2007) Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. *Enzyme Microb. Technol*., **40**: 515-523.

Evans, C.E., Yisa, J. and Egwim, P.O. (2009) Kinetics studies of protease in fermenting locust beans (*Parkia biglobosa*) and melon seed (*Citrullus vulgaris*). *African J. Biochem*. Res., **3**(4): 145-149.

Eyring, H. (1935) The activated complex in chemical reactions. *J. Chem. Phys*., **3**: 107-115.

Feng, Y.Y., Yang, W.B., Ong, S.L., Hu, J.Y. and Ng, W.J. (2001) Fermentation of starch for enhanced alkaline protease production by constructing an alkalophilic *Bacillus pumilus* strain. *Appl*. *Microbiol*. *Biotechnol*., **57**: 153-160.

Garciacarreno, F.L., Dimes, L.E. and Haard, N.F. (1993) Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal*. *Biochem*., **214**: 65-69.

Geok, L.P., Razak, C.N.A., Rahman, R.N.Z.A., Basri, M. and Salleh, A.B. (2003) Isolation and screening of an extracellular organic solvent-tolerant protease producer. *Biochem*. *Eng*. *J*., **13**: 73-77.

Ghorbel, B., Sellami-Kamoun, A. and Nasri, M. (2003) Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microb*. *Technol*., **32**: 513-518.

Ghosh, A., Chakrabarti, K. and Chattopadhyay, D. (2008) Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. *J*. *Ind*. *Microbiol*. *Biotechnol*., **35**: 825-834.

Gupta, A., Roy, I., Patel, R.K., Singh, S.P., Khare, S.K. and Gupta, M.N. (2005a) One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. *J*. *Chromatogr*. *A*, **1075**: 103-108.

Gupta, A., Roy, I., Khare, S.K. and Gupta, M.N. (2005b) Purification and characterization of a solvent stable protease form *Pseudomonas aeruginosa* Pse A. *J*. *Chromatogr*., **1069**: 155-161.

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

Hajji, M., Kanoun, S., Nasri, M. and Gharsallah, N. (2007) Purification and characterization of an alkaline serine-protease produced by a new isolated *Aspergillus clavatus* ES1. *Process Biochem*., **72**: 791-797.

Hanson, M.A. and Marzluf, G.A. (1975) Control of the synthesis of a single enzyme by multiple regulatory circuits in *Neurospora crassa*. *Proc*. *Nat*. *Acad*. *Sci*. *USA*, **72**(4): 1240-1244.

Horikoshi, K. (1990) Enzymes of alkalophiles. In: *Microbial enzyme and biotechnology*, **2**nd ed., 275-294.

Huang, Q., Peng, Y., Li, X., Wang, H. and Zhang, Y. (2003) Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. *Curr*. *Microbiol*., **46**: 169-173.

Jahreis, G., Peters, K. and Kirschke, H. (2001) Purification of soluble and membrane-bound proteases with substrate-analogous inhibitors by affinity chromatography. *J. Biochem. Biophy. Meth.*, **49**: 491-505.

Jung, S.C., Paik, H-R., Kim, M.S., Baik, K.S., Lee, W-Y., Seong, C.N. and Choi, S.K. (2007) InhA-like protease secreted by *Bacillus* sp. S17110 inhabited in Turban shell. *J. Microbiol.*, **45**(5): 402-408.

Kapat, A. and Panda, T. (1997) pH and thermal stability studies of chitinase from *Trichoderma harzianum*: a thermodynamic consideration. *Bioprocess Eng.*, **16**: 269-272.

Kato, T., Yamagata, Y., Arai, T. and Ichishima, E. (1992) Purification of a new extracellular 90-kDa serine proteinase with isoelectric point of 3.9 from *Bacillus subtilis* (natto) and elucidation of its distinct mode of action. *Biosci. Biotechnol. Biochem.*, **56**: 1166-1168.

Khosravi-Darani, K., Falahatpishe, H.R. and Jalali, M. (2008) Alkaline protease production on date waste by an alkalophilic *Bacillus* sp. 2-5 isolated from soil. African *J. Biotechnol.*, **7**(10): 1536-1542.

Kobayashi, T., Hakamada, Y., Hitomi, J., Koike, K. and Ito, S. (1996) Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. *Appl. Microbial Biotechnol.*, **45**: 63-71.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature*, **227**: 680-685.

Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. *J. American Chem. Soc.*, **5**: 658-666.

Linton, S.M. and Greenaway, P. (2004) Presence and properties of cellulase and hemicellulase enzymes of the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes*. *J. Experimental Biol.*, **207**: 4095-4104.

Ma, C. Ni, X., Chi, Z., Ma, L. and Gao, L. (2007) Purification and characterization of an alkaline protease from the marine yeast *Aureobasidium pullulans* for bioactive peptide production from different sources. *Marine Biotechnol.*, **9**(3): 343-351.

Michaelis, L. and Menten, M. L. (1913) Die kinetik der invertinwirkung. *Biochem. Z.*, **49**: 333-369.

Mitra, P. and Chakrabartty, P.K. (2005) An extracellular protease with depilation activity from *Streptomyces nogolator*. *J. Sci. Ind. Res.*, **64**: 978-983.

Naidu, G.S.N. and Panda, T. (2003) Studies on pH and thermal deactivation of pectolytic enzymes from *Aspergillus niger*. *Biochem. Eng. J.*, **16**: 57-67.

Najafi, M.F., Deobagkar, D. and Deobagkar, D. (2005) Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. *Elect. J. Biotechnol.*, **8**: 197-203.

Nilegaonkar, S.S., Zambare, W.P., Kanekar, P.P., Dhakephalkar, P.K. and Sarnaik, S.S. (2007) Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. *Bioresour. Technol.*, **98**: 1238-1245.

North, M.J. (1982) Comparative biochemistry of the proteinases of eukaryotic microorganisms. *Microbiol Rev.*, **46**: 308-340.

Padrón-Pereira, C.A., Moreno-Álvarez, M.J., Montes-Hernández, A.I., Oropeza-González, R.A. (2009) Obtention of enzymatically hydrolyzed flour from epiphytes cactus phyllocladia (*Epiphyllum hookeri* (Link and Otto) Kimn.). *African J. Food Sci.*, **3**(9): 262-269.

Park, D.H., Lee, H.J. and Lee, E.K. (1997) Crystallization of alkaline protease as a means of purification process. *Korean J. Chem. Eng.*, **14(**1): 64-68.

Patel, R.K., Dodia, M.S., Joshi, R.H. and Singh, S.P. (2006) Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. *Process Biochem.*, **41**: 2002-2009.

Pawar, R., Zambare, V., Barve, S. and Paratkar, G. (2009) Application of protease isolated from *Bacillus* sp. 158 in enzymatic cleansing of contact lenses. *Biotechnol.*, **8**(2): 276-280.

Phadatare, S.U., Srinivasan, M.C. and Deshpande, V.V. (1992) Evidence for controlled autoproteolysis of alkaline protease. A mechanism for physiological regulation of *Conidiobolus coronatus*. *Eur. J. Biochem.*, **205**: 679-686.

Rao, Ch.S., Sathish, T., Ravichandra, P. and Prakasham, R.S. (2009) Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. *Process Biochem.*, **44**: 262-268.

Sadana, A. (1995) Biocatalysis: fundamentals of deactivation kinetics. Prentice Hall, New Jersey, USA.

Sana, B., Ghosh, D., Saha, M. and Mukherjee, J. (2006) Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new *gamma-Proteobacterium* isolated from the marine environment of the Sundarbans. *Process Biochem.*, **41**: 208-215.

Singh, L. and Venkata Ramana, K. (1998) Isolation and characterization of psychrotrophic antarctic bacteria from blue-green algal mats and their hydrolytic enzymes. *Fourteenth Indian Expedition to Antarctica*, *Scientific Report*, Department of

Ocean Development, Technical Publication, **12**: 199-206.

Sivasubramanian, S., Murali Manohar, B., Rajaram, A. and Puvanakrishna, R. (2008) Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. *Chemosphere*, **70**: 1015-1024.

Stellwag, E.J., Durham, D.R., Swann, W.E., Nolf, C.A. and Stewart, D.B. (1988) Heat stable alkaline proteases produced by a *Bacillus*. US Patent **4771003**.

Takii, Y., Kurlyama, N. and Suzuki, Y. (1990) Alkaline serine protease produced-from citric acid by *Bacillus alcalophilus* subsp. *halodurans* KP 1239. *Appl*. *Microbial Biotechnol*., **34**: 57-62.

Thumar, J. and Singh, S.P. (2007) Two-step purification of a highly thermostable alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1. *J*. *Chromatogr*. *B*., **854**: 198-203.

Thumar, J. and Singh, S.P. (2008) Organic solvent tolerance of an alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1. *J*. *Ind*. *Microbiol*. *Biotechnol*., **36**(2): 211-218.

Towatana, N.H., Painupong, A. and Suntinanalert, P. (1997) Purification and characterization of an extracellular protease from alkaliphilic and thermophilic *Bacillus* sp. PS7 19. *J*. *Biosci*. *Bioeng*., **87**: 581-587.

Turkiewicz, M., Gromek, E., Kalinowska, H., and Zielinska, M. (1999) Biosynthesis and properties of an extracellular metalloprotease from the Antarctic marine bacterium *Sphingomonas paucimobilis*. *J*. *Biotechnol*., **70**: 53-60.

Venugopal, M. and Saramma, A.V. (2006) Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. *Process Biochem*., **41**: 1239-1243.

Vidyasagar, M., Prakash, S., Litchfield, C. and Sreeramulu, K. (2006) Purification and characterization of a thermostable, haloalkaliphilic extracellular serine protease from the extreme halophilic archaeon *Halogeometricum borinquense* strain TSS101. *Archaea*, **2**: 51-57.

Yossan, S., Reungsang, A. and Yasuda, M. (2006) Purification and characterization of alkaline protease from *Bacillus megaterium* isolated from Thai fish sauce fermentation process. *Sci*. *Asia*, **32**(4): 379-385.

CHAPTER 6

POTENTIAL APPLICATIONS IN FOOD AND TANNERY INDUSTRIES

6.1 Introduction

Alkaline protease has been exploited commercially for cleaning purposes in detergent industries since last decade. But the application of alkaline protease, in cleaning the process equipments in food industries is yet to be explored.

Safety and quality of food products as a major concern in food industry requires maintenance of strict hygienic standards of the processes. This can be achieved by implementing successful cleaning and proper sanitation procedures particularly when the process equipments come in direct contact with the food products. Milk products require extra effort during manual washing as it is known to harbor the highest bacterial load when compared with other types of foods (Lee *et al*., 2007). The most significant potential foulants within food process sectors are of protein-based residues (Allie *et al*., 2003; Jeurnink *et al*., 1996), which can be removed using hot alkali and/or acid detergent washes followed by heat or chemical sanitization and final rinsing (Jeurnink *et al*., 1996). Cleaning operations are crucial in the dairy industry to obtain microbiologically safe products. Hence, c1eaning-in-place (CIP) systems were developed (International Dairy Federation, 1979; Romney, 1990). CIP is defined as "the cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator. The process involves jetting or spraying of surfaces or circulation of cleaning solutions through the plant under

conditions of increased turbulence and flow velocity" (Romney, 1990). In the dairy industry CIP systems generally involve the sequential use of caustic (sodium hydroxide) and acid (nitric acid) wash steps (Chisti, 1999). The most common and aggressive caustic cleaner is sodium hydroxide (NaOH), which is typically used in 1–5% concentrations for plate-type and tubular heat exchangers, and other heavily soiled surfaces and 1–2 % for general use (Flint *et al*., 1997). The primary role of the caustic (alkali) wash step is the removal of proteins and carbohydrates (Chisti, 1999). This results in high dissolved solids levels, especially sodium levels, being discharged in effluent streams from factories. Along with the pollution load, this increases the cost of cleaning and harms the personnel involved in the cleaning (Turner *et al*., 2005). Thus, it will be of potential advantage if such harsh chemical washing could be substituted with enzymatic one. The cleaning efficiency of enzymes on ultrafiltration membranes fouled with protein-based residue from milk- or meat-processing environments has previously been reported (Allie *et al*., 2003; Arguello *et al*., 2003; Munoz-Aguando *et al*., 1996). Previously the efficiency of a commercial proteolytic preparation 'Alcalase' was studied in removing model proteins (casein, haemoglobin and bovine serum albumin) from a model solid surface (glass) (Turner *et al*., 2005). Few studies have focused upon the cleaning effect of proteases on stainless steel particles experimentally fouled with protein. The fouled stainless steel particles were packed into glass columns during cleaning and protein release was assayed by Lowry assay (Nagata *et al*., 1995; Sakiyama *et al*., 1998). Grasshoff (1999; 2002) reported the use of proteases for cleaning milk pasteurizers and heaters, which was solely assessed by visual inspection. The extent of cleaning of the process equipment is examined by using on-line total organic carbon (TOC) analyzer which assays the carbon

content of the effluent coming from washing or rinsing of the equipment. However, to get into the detailed surface topology change upon such enzymatic treatment, more powerful analytical tool such as Atomic Force Microscopy (AFM) is known to produce high resolution surface topography of organic/inorganic films and biological materials and able to detect the presence of even a single protein on the surface (Herrmann *et al*., 2004; Hodge and Rousseau, 2002; Morris *et al*., 2001; Morris, 2004; Raghavan *et al*., 2001). Thus AFM could be highly promising for detection of presence of protein on fouled surface. TOC analyzers are not able to detect the presence of foulant until and unless the foulant is scraped off the surface or released in the washing solution.

Right enzyme or sensitive detection tool to monitor the cleaning efficiency are not sufficient for commercialization of enzymatic cleaning preparations, rather cleaning efficiency of the enzyme should also be evaluated in context with real industrial effluents (dairy effluent, abattoir effluent, etc.) containing proteins and others components (lipids, salts etc.) known to hinder proteolytic activity. Enzymatic treatment using protease or lipase has been reported to reduce the adhesive forces between dairy effluent and solid surfaces (Handojo *et al*., 2009). Earlier reports have identified proteins and lipids as the major membrane fouling constituents present in abattoir effluent, where enzymes and enzyme-detergent mixtures could effectively be used as cleaning agents (Maartens *et al*., 1996a; 1996b). These findings prompted further studies on the use of biological cleaning protocols for UF membranes operating on abattoir effluent (Allie *et al*., 2003).

The other most important potential field of application of alkaline protease is in leather processing. The use of microbial alkaline proteases has become popular since the late nineties (Varela *et al*., 1997). The process employed in the manufacturing of leather

in several developing countries still remains traditional (Germann, 1999). In this traditional process, the chemicals used in pre-tanning and tanning processes contribute > 90% of the total pollution load from leather processing causing serious environmental problems and/or health hazards. Major pollutants from the leather industry that may have significant environmental impact include lime, sulfide and chromium (Alessandro *et al*., 2003). Huge amounts of lime sludge and total solids formation are the main drawbacks of lime (Thanikaivelan *et al*., 2003). Untreated sulfide can cause major problem in sewers. Sulfide also reduces the strength of hair, which directly hampers the recovery of this value-added byproduct. Dehairing (liming) and fiber opening (re-liming) alone contribute to 60–70% of the total pollution load in leather processing are inevitable in the present scenario. In spite of the fact that leather is one of the largest foreign exchange earners for India, it has gained a negative impact in the society. This is due to its ecological impact and therefore is facing severe challenge. Tanners are showing their interest in adopting several clean technologies, which would minimize the negative contribution to the environment (Maia, 1998). Alternative to the conventional lime-sulfide process, enzymatic dehairing is becoming increasingly popular as eco-friendly and cleaner technologies (Seeta Laxman *et al*., 2005). Enzymes speed up the dehairing process by swelling of hair roots in alkaline conditions and attacking on the hair follicle protein for its easy removal. There are many advantages of enzymatic dehairing. Firstly, total elimination of lime and sulfide from the effluent, secondly, recovery of hair as a byproduct which may be used for production of synthetic fibers, biogas, and foaming agent for fire extinguishers and lastly, elimination of the bating process required during deliming. Moreover, the hydrolyzed hair is used as agricultural fertilizer, soil conditioner,

compost, additive in chrome tanning or retanning processes, animal/poultry feed and also for production of cosmetics, pharmaceuticals and cysteine amino acid (Thanikaivelan *et al*., 2004a),

Recent advances in enzyme technology provided an approach in replacing lime and sodium sulfide using enzymes such as protease and α-amylase through a two step process (Aravindhan *et al*., 2004; Saravanabhavan *et al*., 2003; Thanikaivelan *et al*., 2002; 2004a; 2004b). An attempt to integrate the two-step enzymatic process into a single-step process by successive or simultaneous application of protease and α-amylase revealed better dehairing and fiber opening in the simultaneous application of the enzymes (Thanikaivelan *et al*., 2005; 2006). This has lead to the development of commercial product containing both protease and α-amylase in enzymatic beam house process (Thanikaivelan *et al*., 2005). Earlier studies using commercial enzymes for sheep skin dehairing revealed a high correlation between dehairing activity and proteolytic activity (Qing *et al*., 2003). Some microorganisms, for example *Streptomyces* sp. isolated from soil, have been shown to produce extra cellular enzyme with dehairing activity (Mukhopadhyay and Chandra, 1993). They reported degradation of human hair, chicken feather, silk, wool, and unhaired goatskin with the enzyme. Among bacteria, strains of *B. subtilis* and *B. amylolequifaciens* have been characterized for dehairing purposes (Nashy *et al*., 2005; Varela *et al*., 1997). Thus, the substitution of chemical dehairing agents in the leather industry by proteolytic enzymes produced by microorganisms will have an important economic and environmental impact.

The efficiency of crude alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1 in removing casein (model protein) from mica (model solid surface)

and in dehairing of goat skin has been studied in the present research work using AFM and SEM, respectively. The efficiency has also been compared with its partially purified counterpart and other commercially available alkaline proteases. Also, in protein removal from solid surface, experiments were carried out by replacing the model protein with various protein rich industrial effluents from dairy, abattoir and poultry.

6.2 Materials and Methods

6.2.1 Chemicals and Reagents

All salts and media constituents *viz*., NaCl, NaOH, $MgSO_4.7H_2O$, $CaCl_2$, NH_4NO_3, KH_2PO_4, K_2HPO_4, and $FeCl_3$, were purchased from CDH, India. TCA, acetic acid, Tris, HCl and borax were procured from Merck, India. Biological grade casein and agar were obtained from Himedia, India. All commercial protease enzymes (P5380: Proteinase from *Bacillus licheniformis* Type VIII, P4860: Protease from *Bacillus licheniformis* Subtilisin® A, P8038: Proteinase from bacterial Type XXIV, P5459: Protease from *Bacillus licheniformis*) were procured from Sigma-Aldrich Chemicals, India. Goat skin was purchased from the local market.

6.2.2 Microorganism and Culture Conditions

Bacillus pseudofirmus SVB1 was isolated from the premises of tannery industry as described in Chapter 2. Cultures were regenerated every 2-3 weeks on a freshly prepared nutrient agar plates from the frozen stock culture.

6.2.3 Inoculum Preparation and Alkaline Protease Production

The inoculum was prepared as described in Chapter 3 (Section 3.2.2, pp. 51) and alkaline protease was produced as per the methodology described in Chapter 5 (Section 5.2.3, pp. 95). Partially purified alkaline protease (PPE) was obtained from acetone fractionation as described in Chapter 5 (Section 5.2.4.1, pp. 95).

6.2.4 Removal of Protein from Solid Surface

6.2.4.1 Staining of mica surface with proteins

Clean new mica slides (100 mm × 100 mm) were extensively rinsed with deionised water and ethanol. The cleaned new mica slides were dried in oven at 100°C for 20 min. 1.0 ml of any one of the appropriate test substrate (casein (10% w/v), dairy, abattoir or poultry effluents) was pipette onto the slide, spread over its entire surface and was subsequently *burned on* at 100°C in an oven for 30 min.

6.2.4.2 Washing of stained surfaces

Pre-stained mica slides were thoroughly rinsed thrice with distilled water to wash out any loosely bounded protein. Then the casein coated slides were fully immersed into 20 ml of 25 mM borax–NaOH buffer (pH 10.0), which either contained crude alkaline protease (culture filtrate), partially purified enzyme or each of the commercial enzyme (P5380, P4860, P8038, P5459) at 20 U of their final concentration in 40 ml glass screw-cap vials. Then the vials were incubated in an orbital shaking incubator for 20 min at 30°C and 250 rpm. Casein coated slide immersed in buffer without the presence of any enzyme preparation served as experimental control. This control was used to ascertain whether the alkaline pH condition of the buffer itself or the enzyme inside the buffer was

actually contributing to the casein removal. Unstained mica slides incubated with the crude and partially purified enzymes were taken as additional negative controls. These controls were used to determine the contribution of surface bound enzymes, which get released along with casein into the wash. After removing from glass vials, all the slides were air dried at 37°C. The effluent coated slides were either treated with crude alkaline protease subsequently washed in the same manner as described for casein coated slide or left untreated. This step was necessary to check whether the crude enzyme is efficient enough for the complex liquor of industries as for model protein.

Detection of residual enzyme activity of the washing effluent from the mica surfaces after enzyme wash was undertaken by repeated (3 cycles) pipette rinsing of the air-dried, enzyme cleaned mica slide surface with 1.0 ml of borax–NaOH buffer, followed by assay of this wash solution.

6.2.4.3 AFM studies

Atomic force microscope (AFM) images of the uncoated, coated and coated-enzyme treated mica slides were obtained with the help of a PicoPlus® AFM (Agilent Technologies, USA). The AFM was operated in non-contact mode. A silicon tip of 20 nm radius was used. The silicon tip was attached to a cantilever resonated at a selected frequency in the range of 354–409 kHz. The raw image information was further processed using horizontal and vertical leveling with Picoscan 5.3.3 software® (Agilent Technologies, USA).

6.2.5 Goat Skin Dehairing

6.2.5.1 Enzymatic dehairing of goat skin

Dehairing was carried out with the following agents namely, crude and partially purified alkaline protease from *Bacillus pseudofirmus* SVB1, log phase (A_{600nm}~1.0) culture of *Bacillus pseudofirmus* SVB1 and commercial alkaline proteases (P5380, P4860, P8038 and P5459) in 25 mM borax–NaOH buffer, pH 10.0. Hide of a freshly butchered goat was cut into pieces of approximately 5 cm × 5 cm and washed with distilled water repeatedly to remove all extraneous matter. After brief air drying, the hide was weighed (between 2 to 3 g) and transferred to 90 mm Petri plate. The enzyme solutions (2 units) or cell suspension (1 ml; A_{600}~1.0) were applied to the skins by using brush and paint method. On each piece of hide, a portion is left untouched as control. Another control used was 25 mM borax–NaOH buffer, pH 10.0 instead of enzyme solution or cell suspension to rule out any dehairing contributed by the alkaline condition solely. After 6h incubation with the deharing agents or controls, hair was scraped off gently from the hides with fingers and hides were observed using a stereo zoom microscope (Nikon, USA) at 300x magnification.

6.2.5.2 SEM studies

The dehaired hides were observed using a scanning electron microscope (Leo 1430 VP, UK) at 500x magnification. Samples of size 5 mm × 2 mm were cut with fresh stainless steel blades from the dehaired hides. Then the hide pieces were dehydrated slowly by rinsing it subsequently with ethanol concentration from 0-100% and subjected to SEM analysis. The hides were mounted on aluminium stubs and then coated with gold

using sputter coater (Edward, UK). The stubs were then introduced into the specimen chamber of LEO 1430vp scanning electron microscope for analysis.

6.2.6 Analytical Techniques

6.2.6.1 Cell growth, proteolytic activity and protein concentration measurement

Cell growth was measured as described in Chapter 2 (Section 2.2.5.1, pp. 45). Alkaline protease activity and specific activity of the culture supernatant of SVB1 were measured as described earlier (Chapter 3, Section 3.2.5.2., pp. 54). The total protein contents of the samples were determined as described in Chapter 3 (Section 3.2.5.3., pp. 55).

6.2.6.2 Quantification of post-cleaning soluble protein in washing solution in protein removal experiments

To quantify the protein removed from the surface, the protein content in the washing solution was determined according to the method described by Lowry *et al*. (1951) using BSA standard as described in Chapter 3 (Section 3.2.5.3., pp. 55).

6.2.6.3 Enzyme inactivation studies in protein removal experiments

It was necessary to check whether any residual enzyme activity retained from cleaning operation even after subsequent washing/treatments (acid/alkali or heat treatments) often used in food industries in process equipment cleaning operations. The objective of this study was to access whether the residual crude alkaline protease could be successfully deactivated after the enzymatic cleaning in such washing/treatment steps, so that the result could well reasonably be extrapolated to real field situation. For this, 2.0 ml aliquots of crude alkaline protease diluted with 20 ml of 25 mM borax–NaOH buffer,

pH 10.0 were incubated with equal volumes of 0.5, 1.0 or 2.0 M HCl or NaOH for 5, 10 or 15 min, which was finally readjusted to pH 10 before enzyme assay. Thermal inactivation studies were undertaken by measuring the residual enzyme activity after incubation of suitably diluted crude alkaline protease solution for 5 min at 95°C or by autoclaving it at 121°C for 15 min, taking an enzyme sample at 4°C as control.

6.2.6.4 Physical testing of the dehaired leathers

The finished leathers were conditioned at 26 ± 2°C and 65 ± 2% relative humidity for 3 d before the physical tests were carried out. Then leather samples both at their longitudinal and latitudinal orientations (henceforth termed as "perpendicular" and "parallel" to distinguish between the two alternate orientations from each other) were tested for tensile strength, percentage elongation at break and tear strength. Tensile strength was measured with a Microtest 5000 (UK) tensile testing module with a uniform speed of jaws separation (0.005-1.5 mm/min). A section around the central 50-mm portion of a piece of leather (110 mm long), 10 mm from the centre and 25 mm from the outer margins, was tested. The average thickness of the piece was determined and the load at break noted giving the tensile strength (kg/cm^2). Percentage elongation at break was measured on the tensile machine using a similar-size sample. The jaws of the machine were set 50 mm apart and the percentage elongation at break measured. The tear strength of the leather (50 mm × 25 mm of measured thickness) with a 20-mm long cut in the centre was assessed by running the tensile machine until the leather tore apart. The highest load (kg/cm) at break was recorded.

6.2.6.5 Analysis of effluent from dehairing process

Eco-friendly nature of the enzymatic dehairing process of skins and hides was assessed in terms of pollution control parameters such as total solids (TS), biological oxygen demand (BOD), chemical oxygen demand (COD) and pH. Adhering contents of depilatory agents and other skin components that were removed during the process were suspended by washing with known amount of water. The effluent, thus collected, was subjected to analysis of the above environmental parameters. The effluents from the dehairing process of skins were analyzed for various parameters such as TS, BOD, COD and pH. The values were compared with the conventional processes from available literature. To determine TS, well -mixed sample (2 ml) was centrifuged at 7000 g for 10 min. After discarding the supernatant, the residue was washed with 2 ml of water and centrifuged again for 10 min. Solids were transferred to a pre -weighed petridish and kept in an oven maintained at 105±2ºC till complete drying. BOD and COD of the sludge samples were determined using standard techniques (APHA, 1985).

6.3. Results and Discussion

6.3.1 Protein Removal from Solid Surface

6.3.1.1 Atomic force microscopy

The effect of the enzyme-based cleaning of casein coated slides was investigated by atomic force microscopy. AFM analysis of an uncoated mica slide shown in Fig. 6.1, clearly illustrates that at the nanometer level, the glass slide surface topography is almost flat (Fig. 6.1a). This is again either represented three dimensionally in Fig. 6.1b or in Fig. 6.1c graphically, where the Z axis is represented as distance along the slide.

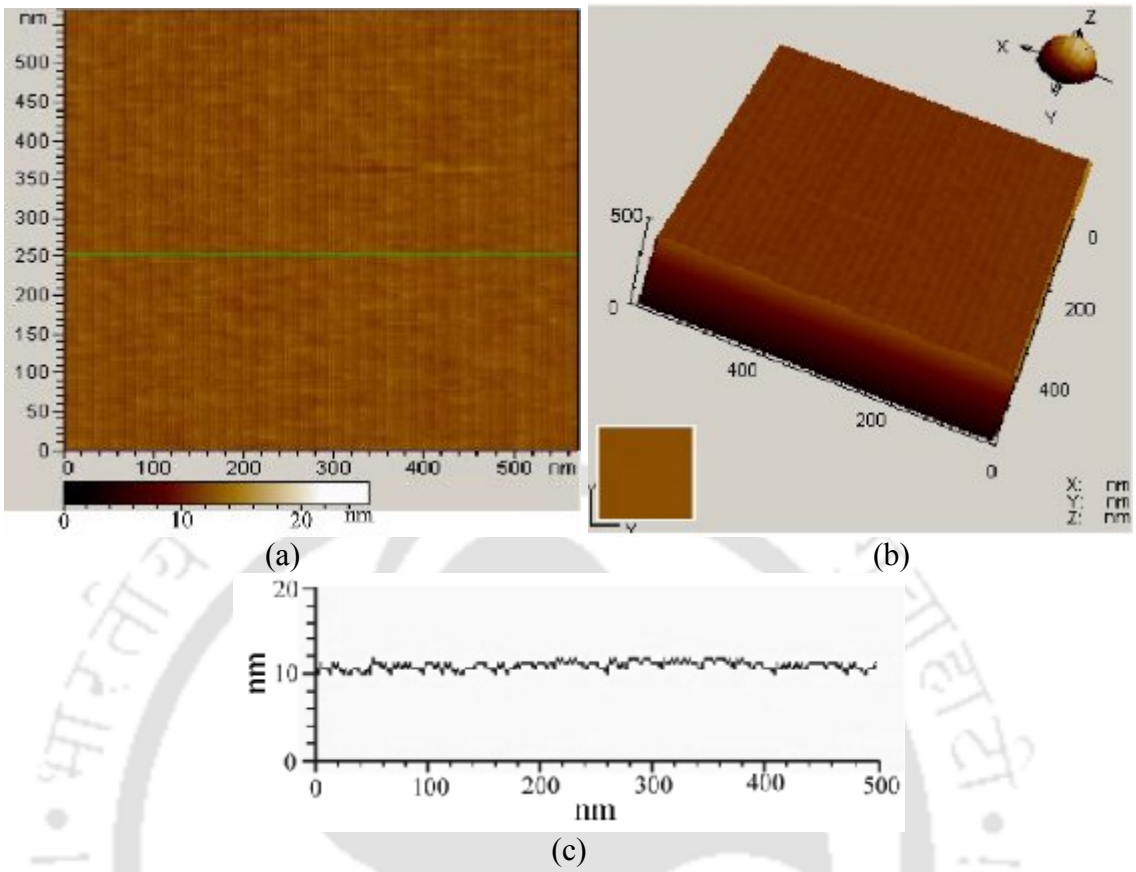


Figure 6.1 AFM image of a new mica slide: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

On the other hand, AFM images of casein-coated slides (Fig. 6.2) clearly indicate markedly uneven overall surface topography compared to the uncoated one. It was observed in the three dimensional AFM scan of the buffer treated control slide (Figs. 6.3 a, b, c) that though some portion of the loosely bound casein film has been removed by washing but majority of the protein film remained intact as was obvious from the surface topology. However, crude alkaline protease treatment has an obvious effect upon burnt-on surface topography (Fig. 6.4a), where mean burnt-on depth has been reduced (Figs. 6.4b, c). This closely resembled the original uncoated slide surface. This inferred that the protein removal from mica surface was due to enzymatic hydrolysis of the burnt-on protein and not due to the alkaline environment provided by the buffer.

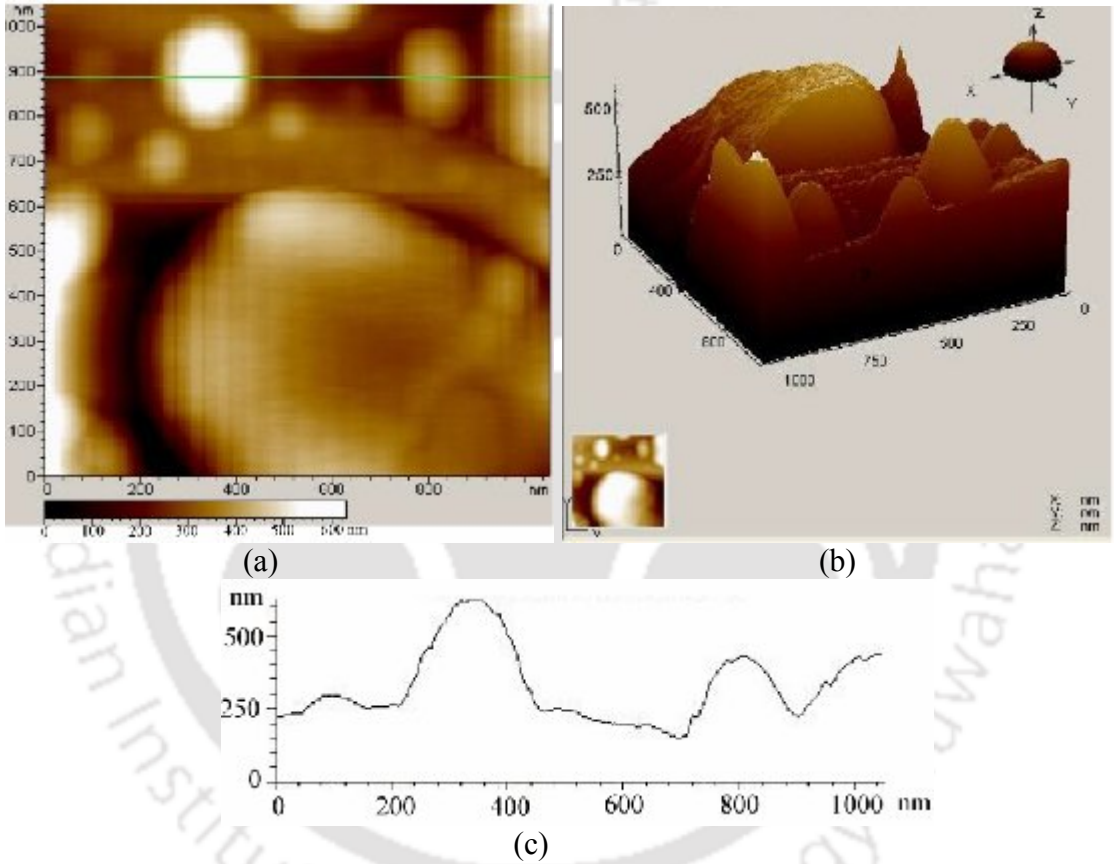


Figure 6.2 AFM image of casein coated mica slide: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

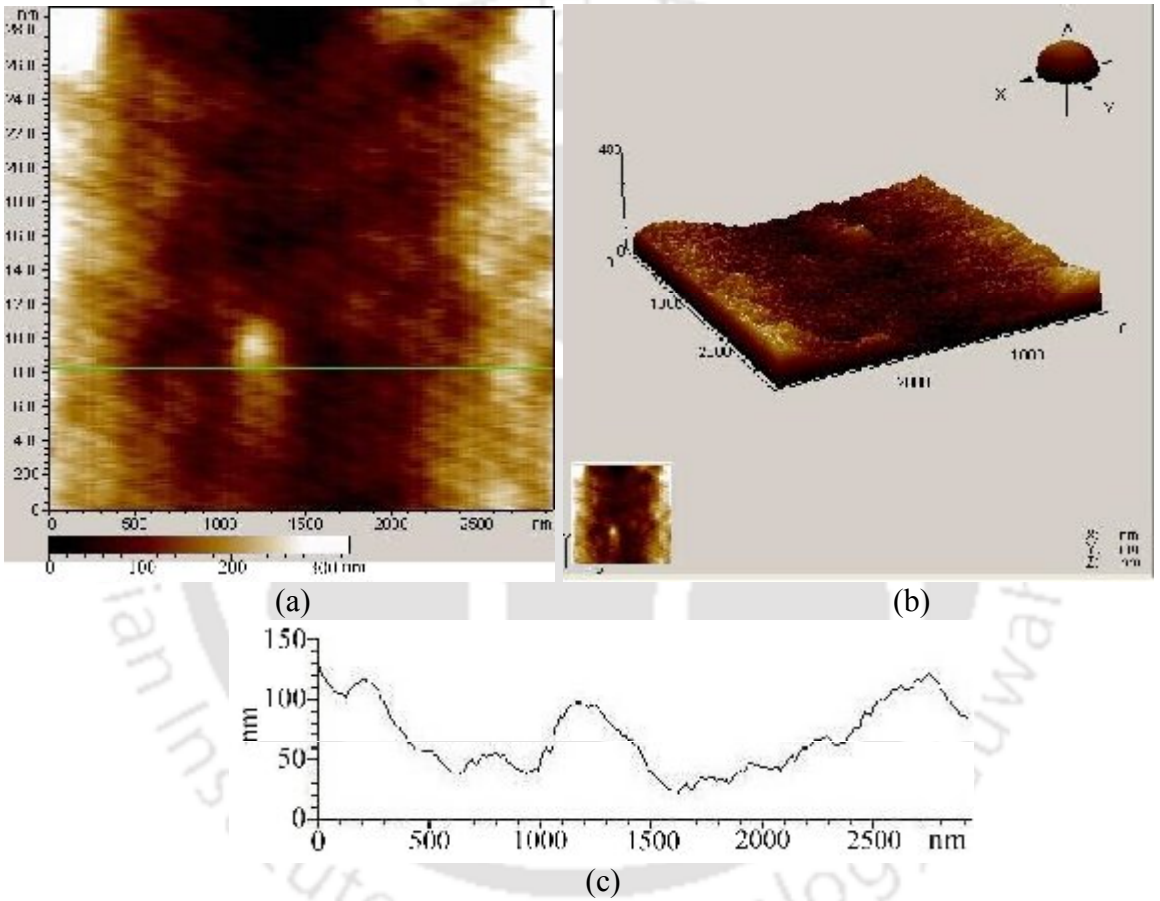


Figure 6.3 AFM image of a casein coated mica slide treated with buffer: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

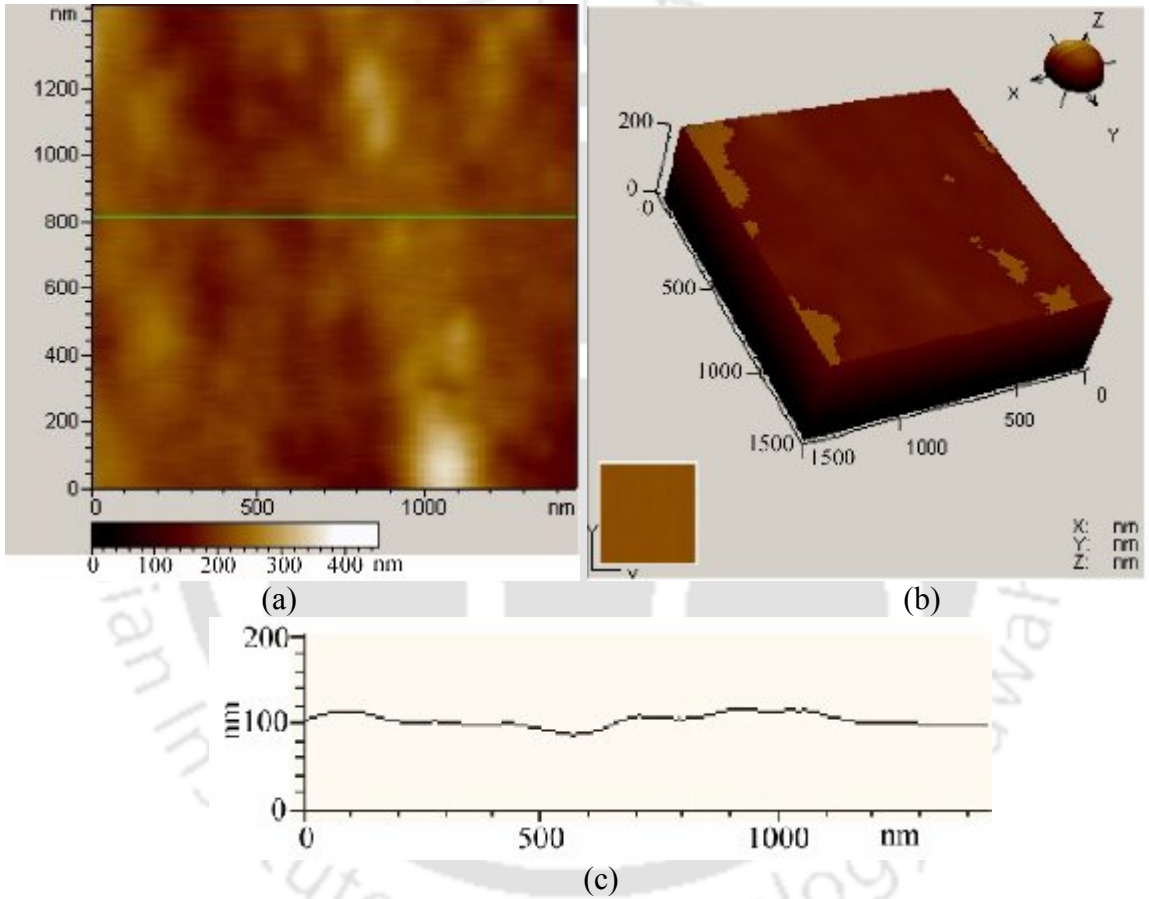


Figure 6.4 AFM image of casein coated mica slide treated with crude alkaline protease (from SVB1): (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

Even efficiency of this crude alkaline protease is much more than the other three commercially available alkaline proteases *viz*., P-4860 (Figs. 6.5 a, b, c), P-5459 (Figs. 6.6 a, b, c), P-5380 (Figs. 6.7 a, b, c). Although, one of the commercially available alkaline proteases, P-8038, removes the protein film efficiently (Figs. 6.8 a, b, c), but it was not much efficient as compared to crude alkaline protease from SVB1.

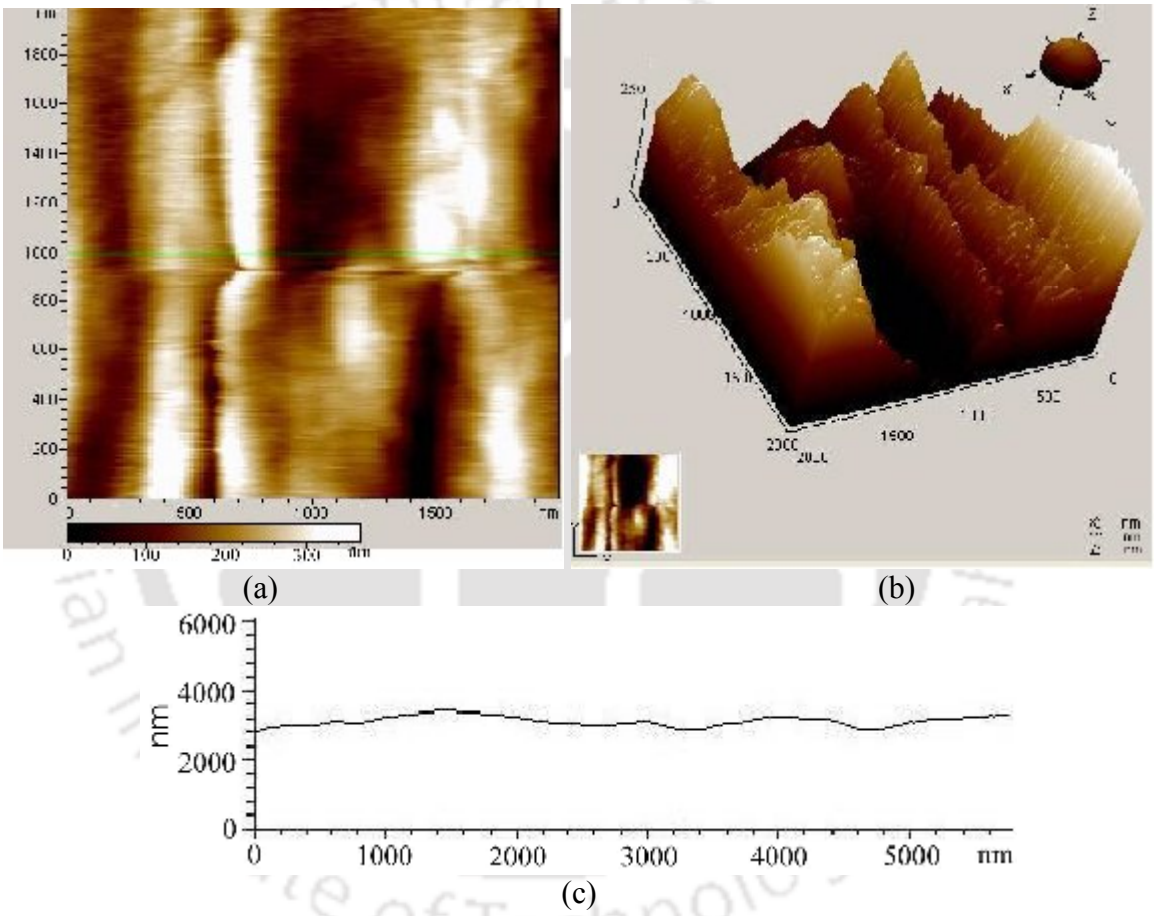


Figure 6.5 AFM image of casein coated mica slide treated with commercial alkaline protease P-4860treated: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

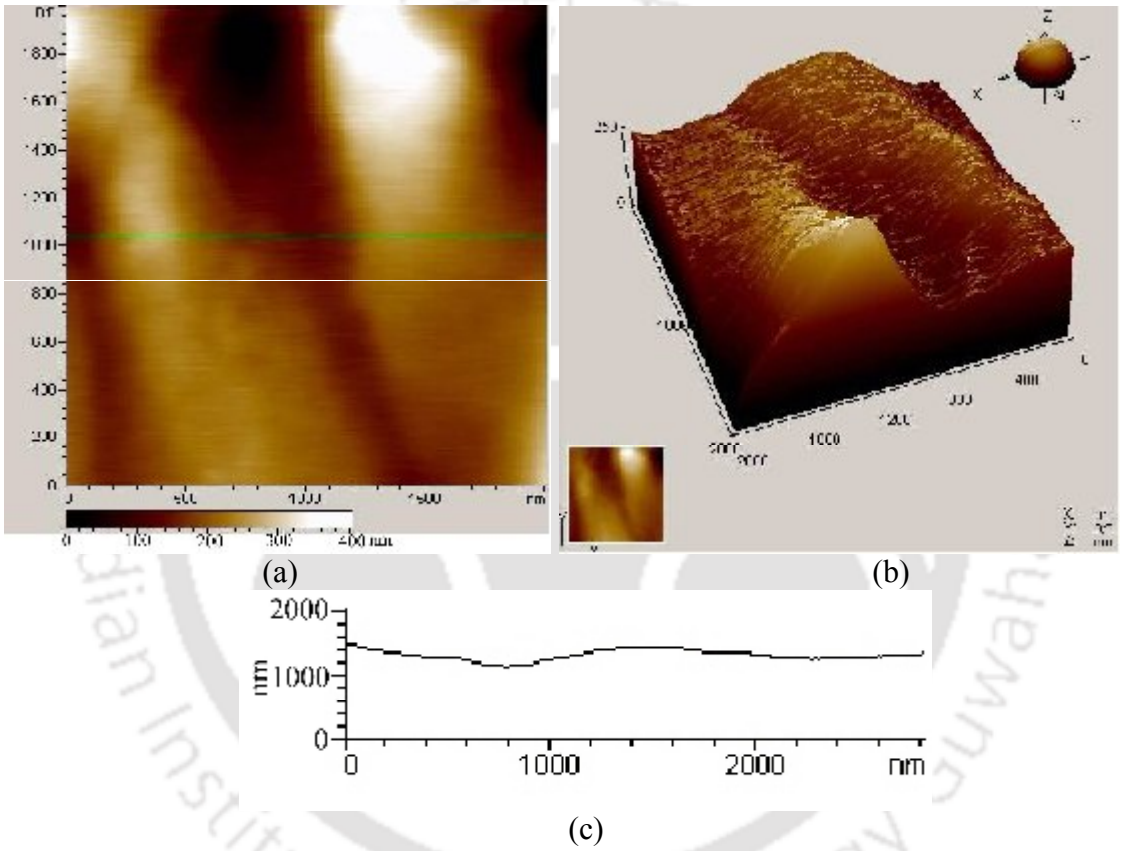


Figure 6.6 AFM image of casein coated mica slide treated with commercial alkaline protease P-5459: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

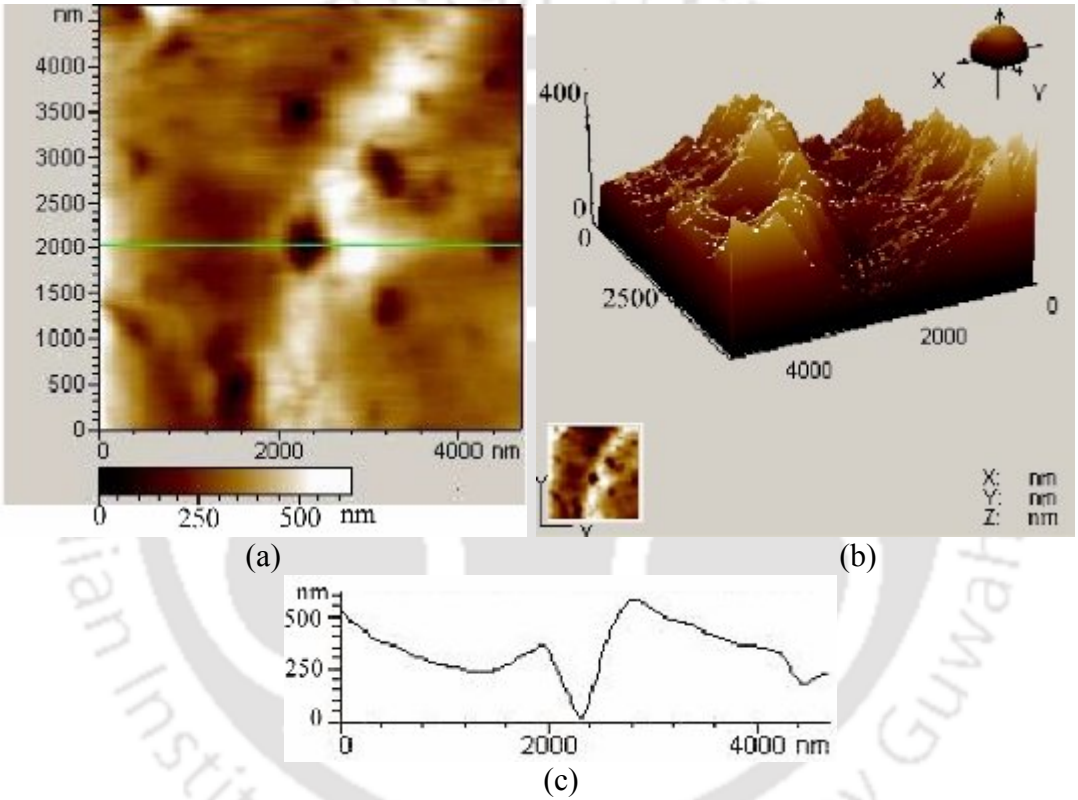


Figure 6.7 AFM image of casein coated mica slide treated with commercial alkaline protease P-5380: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

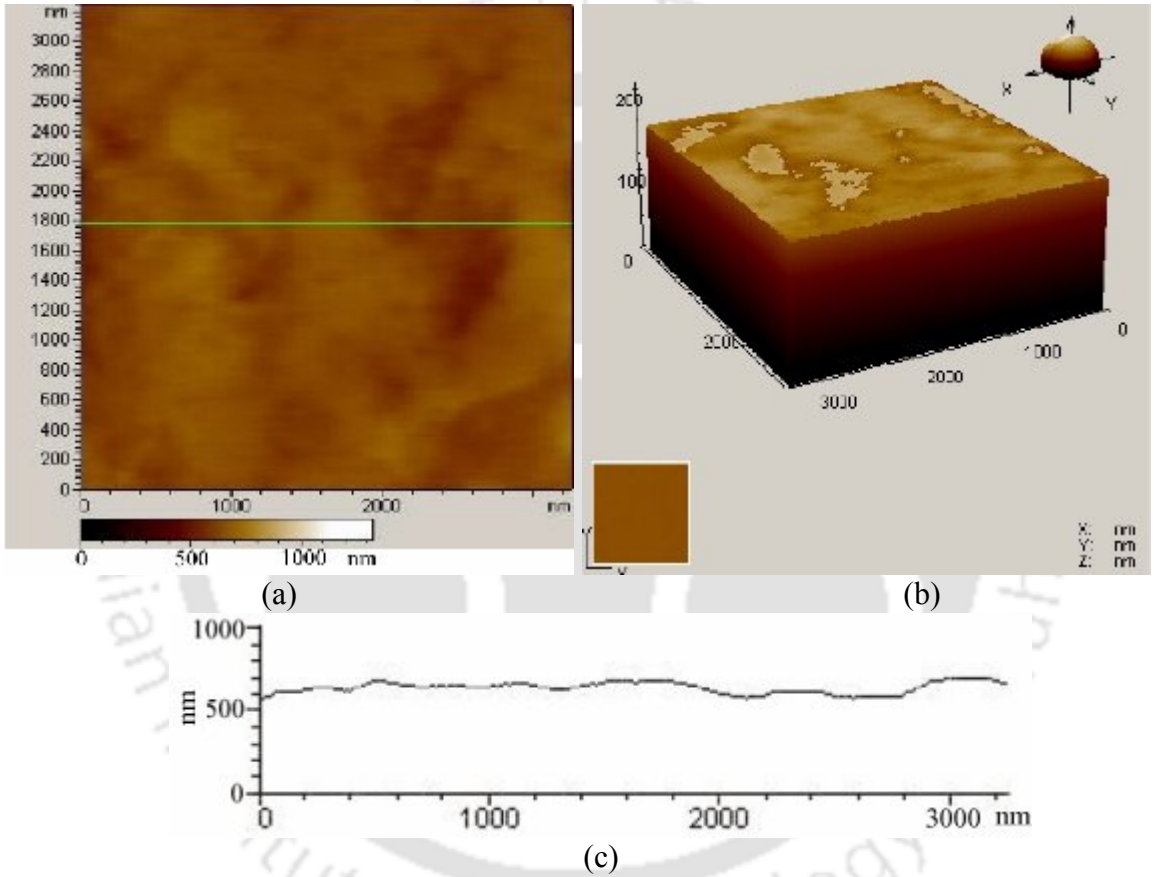


Figure 6.8 AFM image of casein coated mica slide treated with commercial alkaline protease P-8038: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

When the casein coated mica slide were treated with the partially purified alkaline protease from SVB1 (Figs. 6.9 a, b, c), the treated slide surface appeared almost similar to the uncoated one. This indicated the superior efficiency of the partially purified enzyme as compared to that of the crude enzyme.

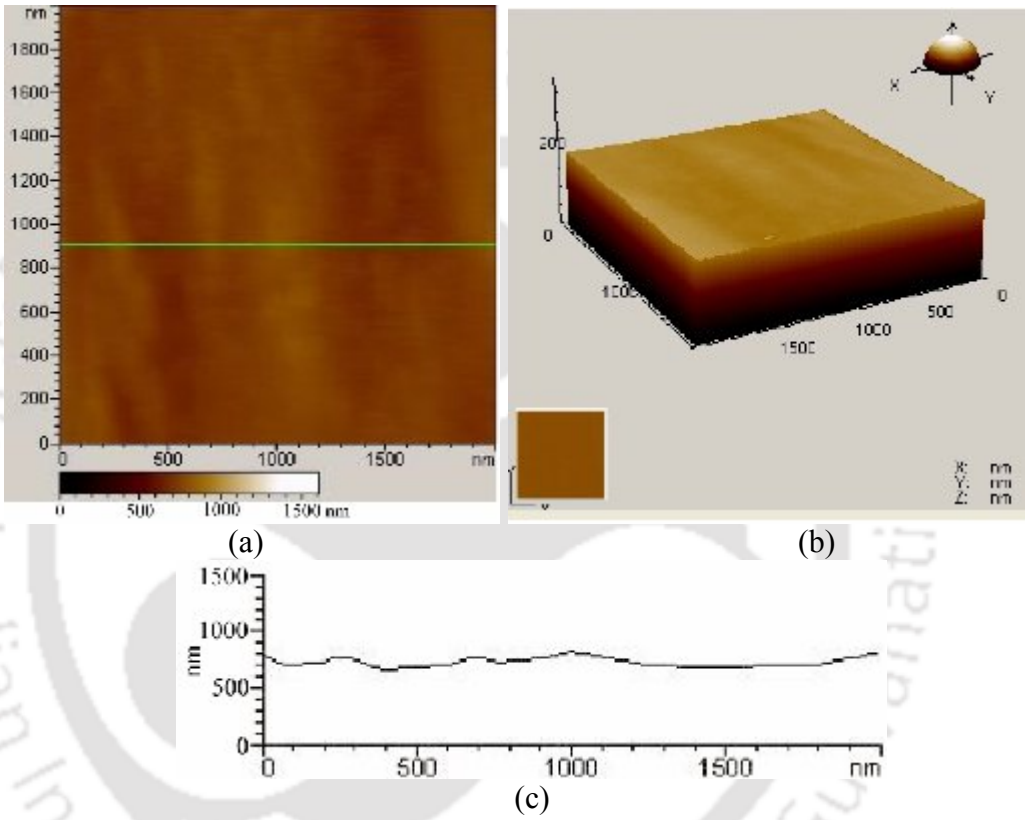


Figure 6.9 AFM image of casein coated mica slide treated with partially purified alkaline protease (from SVB1): (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

The protease mediated surface cleaning results obtained from this study with the three different protein rich effluents are also highly interesting. As, the dairy effluent is very rich in casein, the AFM images of the dairy effluent burnt slides (Figs. 6.10 a, b, c) were very similar to that of the casein coated one. After the crude alkaline protease treatment the film was efficiently removed (Figs. 6.11 a, b, c).

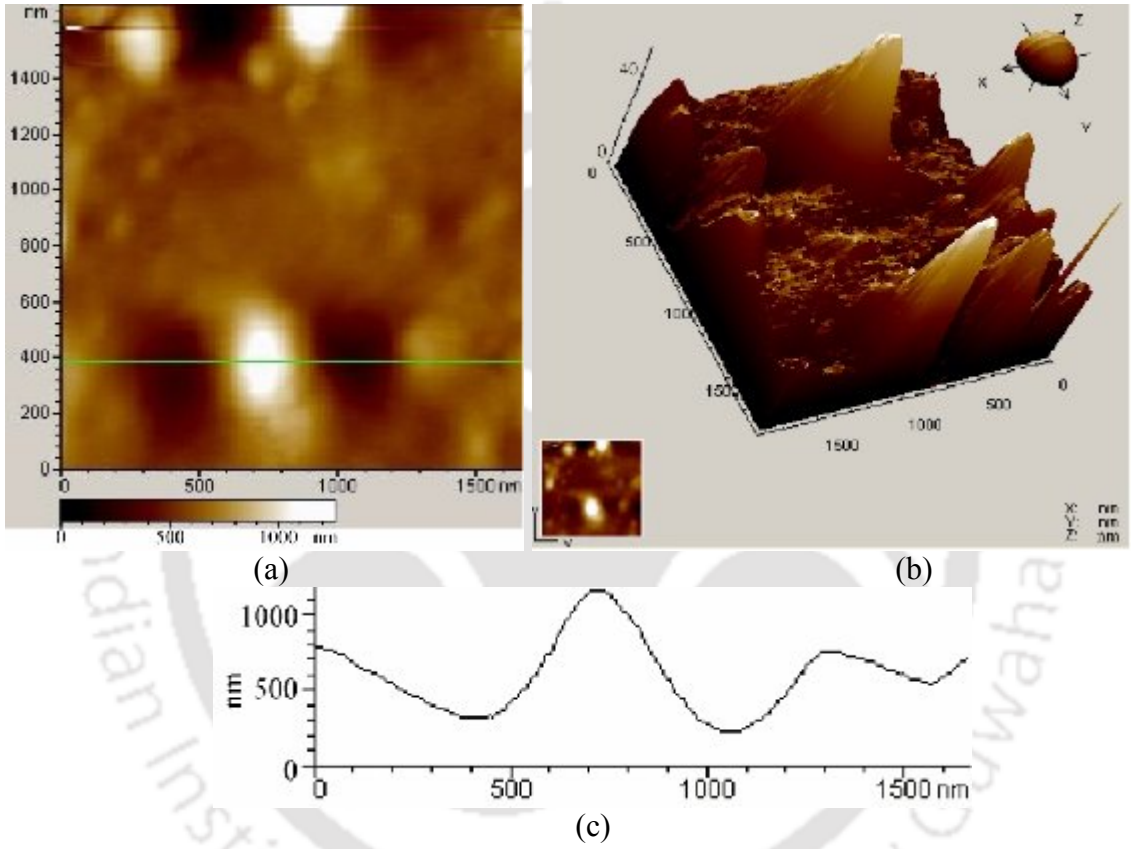


Figure 6.10 AFM image of dairy effluent coated mica slide: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

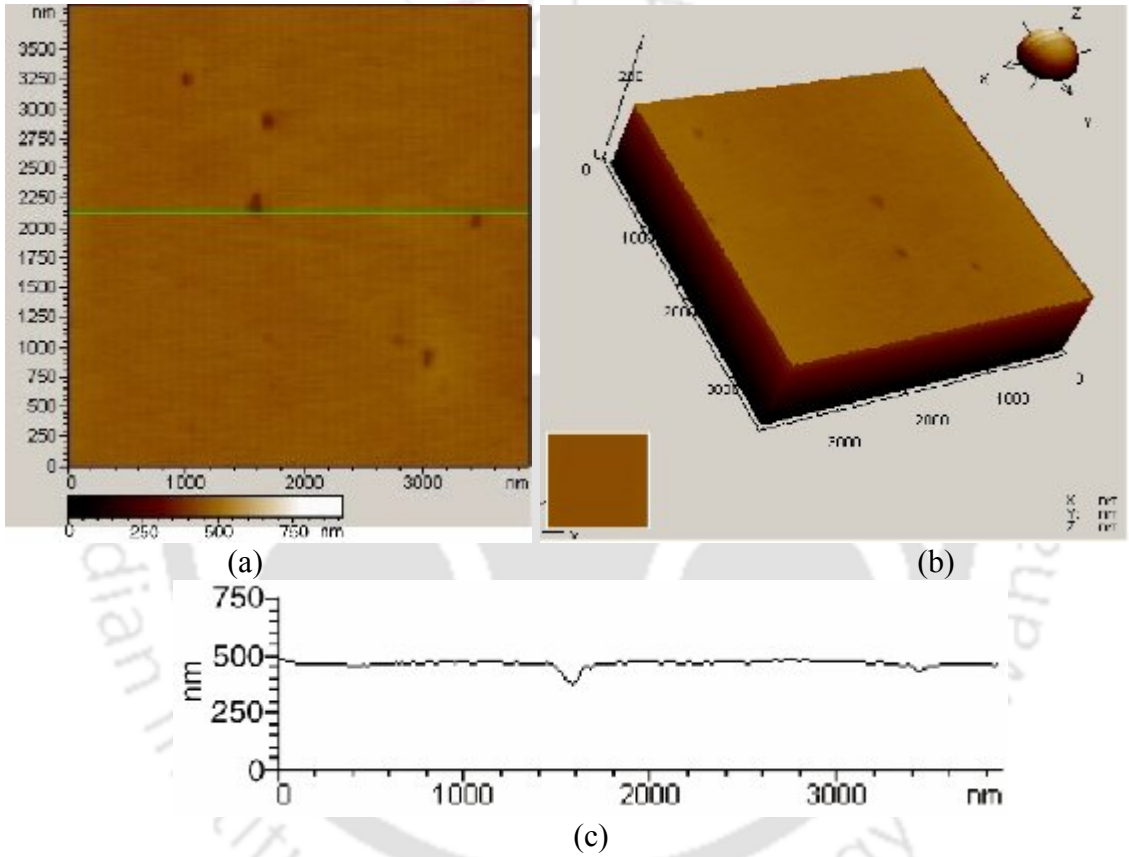


Figure 6.11 AFM image of dairy effluent coated mica slide treated with crude alkaline protease (from SVB1): (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

Similar result was observed with abattoir effluent. The film obtained with this effluent (Figs. 6.12 a, b, c) was markedly different from that of casein or dairy effluent, having higher degree of surface roughness. However, after enzyme treatment it was very similar to a new mica slide which indicated that the complete removal of the film was achieved (Figs. 6.13 a, b, c).

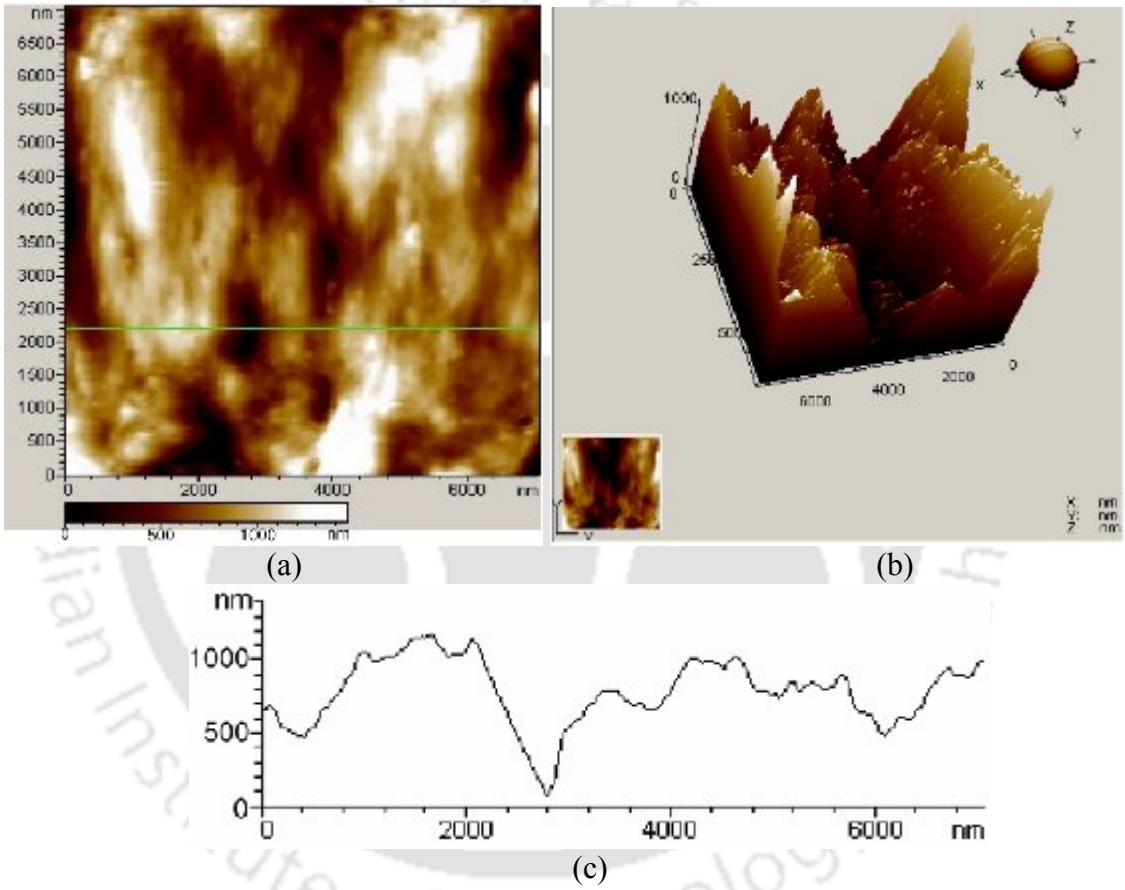


Figure 6.12 AFM image of abattoir effluent coated mica slide: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

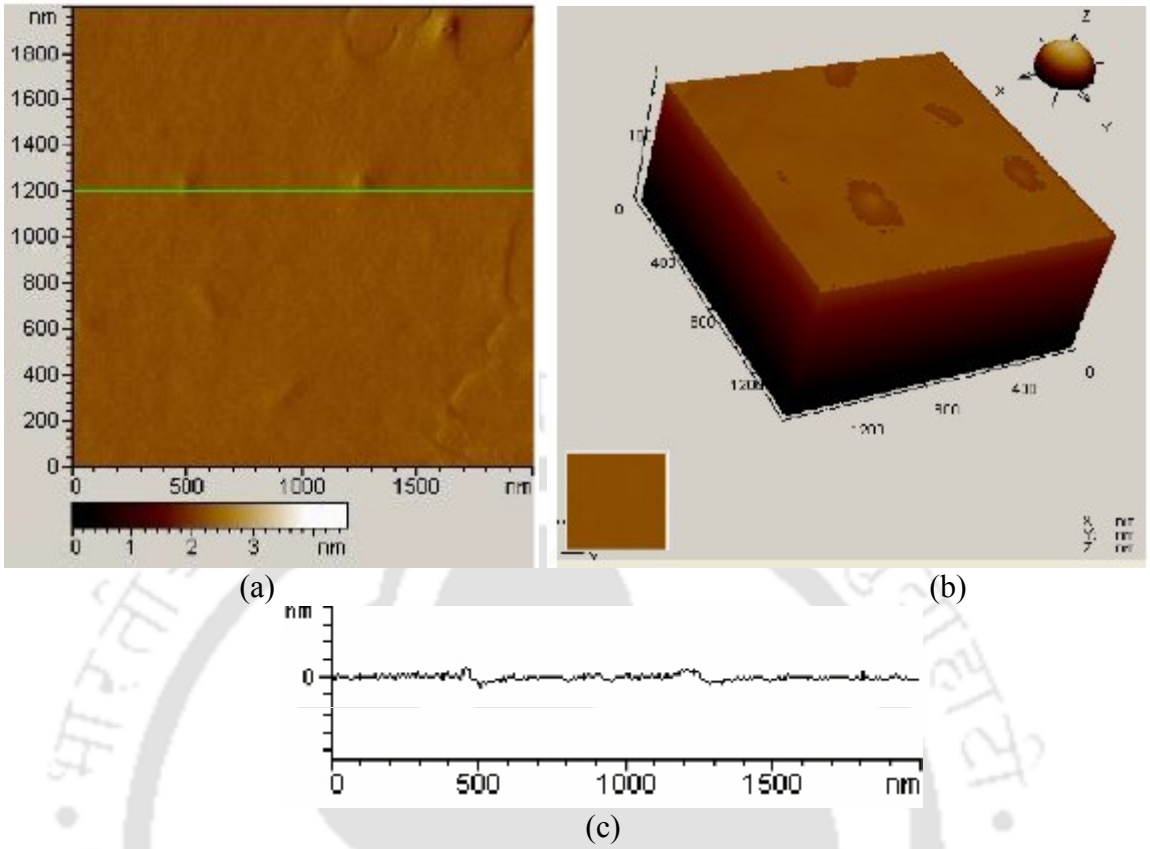


Figure 6.13 AFM image of abattoir effluent coated mica slide treated with crude alkaline protease (from SVB1): (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

The film obtained from the poultry effluent is neither similar to casein or dairy effluent nor to the abattoir effluent as observed in Figs. 6.14 a, b, c. In case of the poultry effluent, though the enzyme was able to remove most of the film (Figs. 6.15 a, b, c), but was not as much efficient as in all other cases. This may be due to some other ingredients present in the poultry waste, which might be inhibiting the enzyme activity.

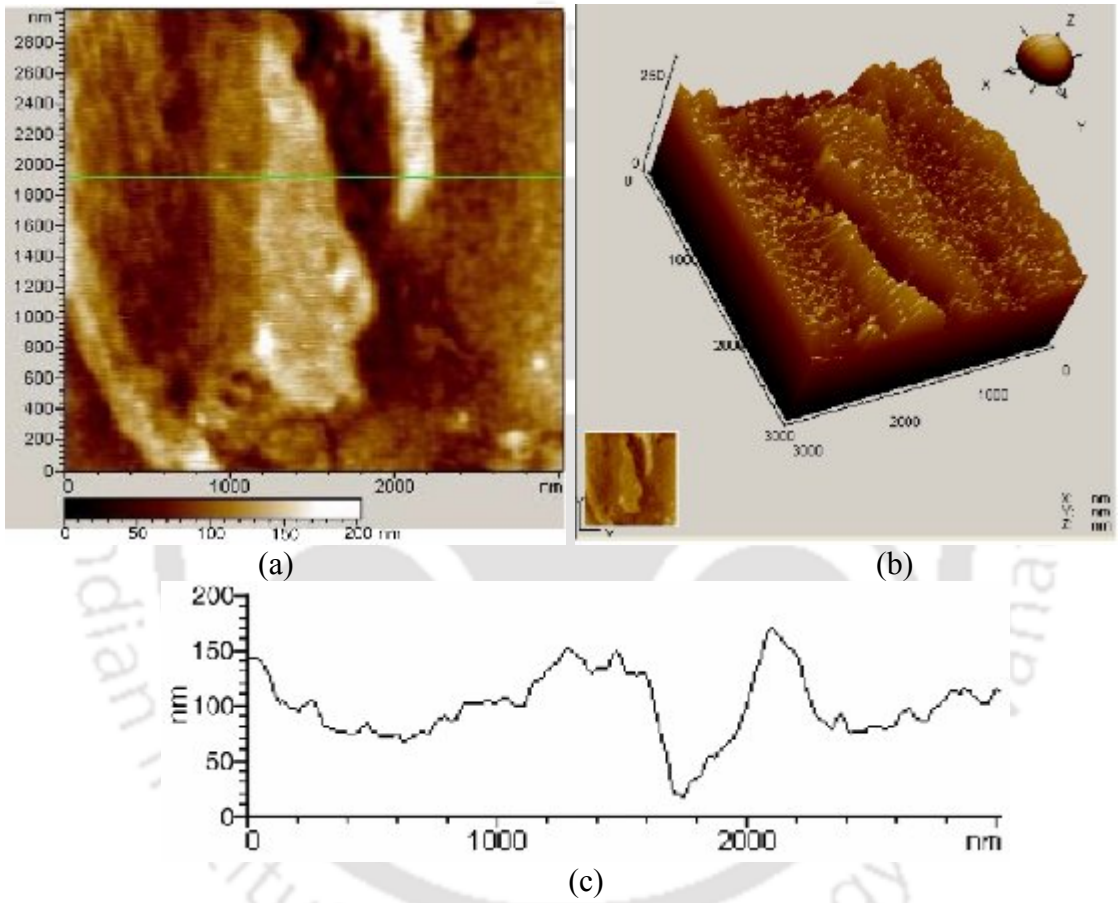


Figure 6.14 AFM image of poultry effluent coated mica slide: (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

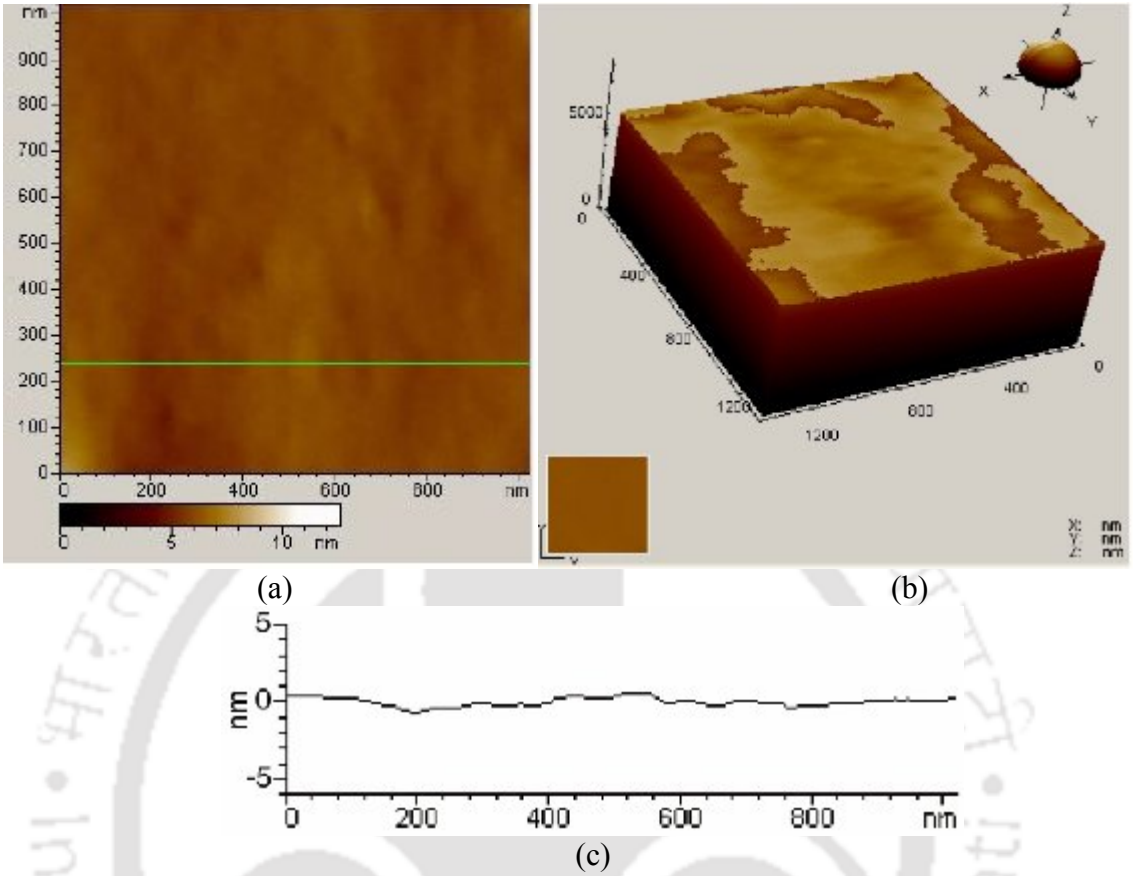


Figure 6.15 AFM image of poultry effluent coated mica slide treated with crude alkaline protease (from SVB1): (a) 2-D surface topography, (b) 3-D surface topography, (c) Y axis (distance along the slide) showing surface topography.

Turner *et al*. (2005) used 'Alkalase' to remove protein from solid surface. In another study, during cleaning trials, *Pseudomonas* lipase removed lipid alone as well as in combination with proteases (Allie *et al*., 2003). Though, AFM had been used to investigate protein adsorption onto solid surfaces (Foose *et al*., 2007; Kim *et al*., 2004; Ying *et al*., 2003), the present study appears to be the first report using crude alkaline protease for cleaning protein coated surface and reporting the efficiency in perceptible AFM images. Mica was chosen as the model surface in this study. However, it may be envisaged that the alkaline protease would equally promote the removal of protein-based burnt-on from other metalic/non-metalic surfaces. Salts of manganese, iron, nickel and

chromium (metals present in 316L stainless steel, widely used to build process equipments in food industry) have been shown not to inhibit alkaline protease activity if present at low concentrations (1.0 mM), although inhibition is evident at higher concentrations (10 mM) (Chapter 5, Section 5.3.2.4., pp. 114-115) significantly. Till date, there is no report in the literature on negative effect of stainless steel, plastic or other bioprocess-relevant surfaces on proteolytic activity. Given the relatively mild operational conditions and the use of high grade, polished stainless steel in process equipments, leachate levels from such systems would almost certainly be insignificant in the context of alkaline protease based cleaning activity. Moreover, the enzyme used in the cleaning process would not come into contact with the underlining solid substratum until cleaning was virtually complete.

6.3.1.2 Quantification of post-cleaning soluble protein in washing solution

The effluents from the first rinse of the mica slides after enzymatic cleaning were assayed for protein content. Fig. 6.16 gives a graphical quantitative estimation of the amount of protein removed from the 'burnt-on' surfaces by different enzymes. After washing, buffer treated negative control for casein and untreated negative control for all the processes releases negligible amount of protein in the solution. This indicates a strong adhesive nature of the burnt-on protein residues on the mica surfaces, which is difficult to remove by manual washing. On the other hand, enzyme treated slides released a large amount of protein in the washing solution giving an inference that the burnt on residue were either partially or fully removed. Uncoated empty slides after incubation with enzymes were considered as additional negative controls, which indicated that the amount of enzyme stuck to the surface during incubation were negligible and were not

contributing to the protein content in the washing solution from those sample/effluent coated slides. From Fig. 6.16, it is established quantitatively that the amount of protein removed from the surfaces in case of the crude alkaline protease from SVB1 was the higher as compared to its partially purified counterpart or the other commercially available alkaline proteases. The efficiency obtained in this study is comparable and even better than a similar study with the commercially available enzyme 'Alcalase' (Turner *et al*., 2005).

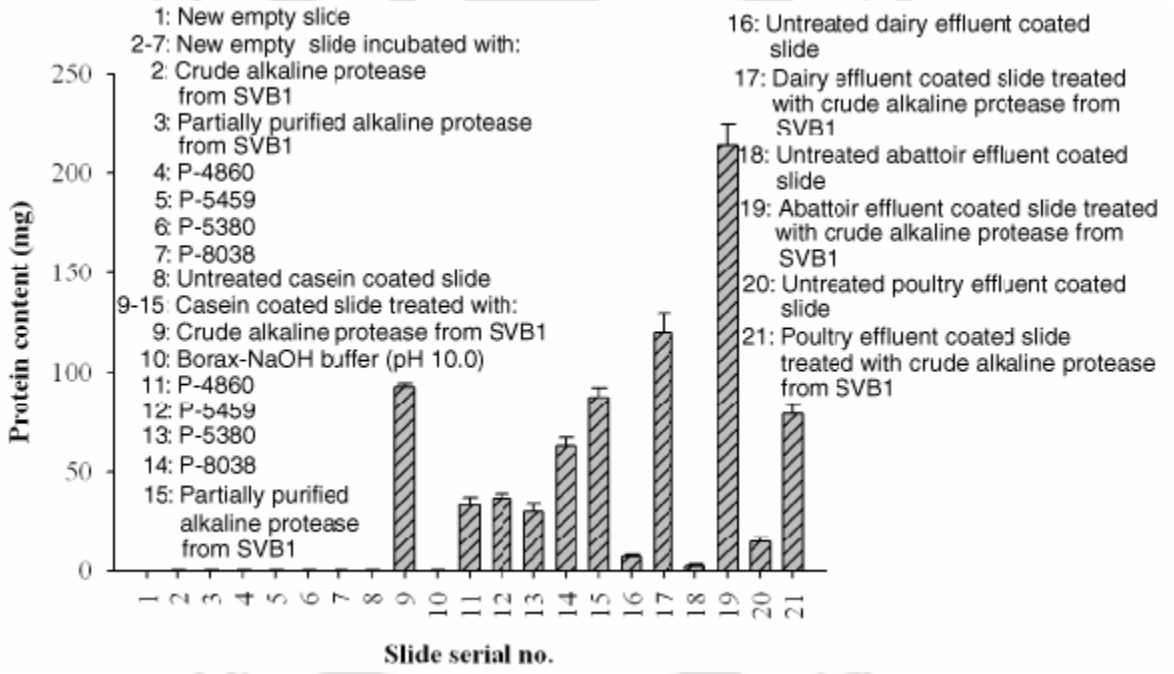


Figure 6.16 Total protein concentration of the washing solution after protein removal as described in section 6.2.4.3.

6.3.1.3 Quantification of post-cleaning residual proteolytic activity

Enzyme-based cleaning will find practical application in bioprocess operations especially in food industry, only if no residual activity remains on the surface post-cleaning operation. Otherwise, the presence of residual enzyme activity would contaminate subsequent production runs. In this study, direct assay of post-cleaning rinse

inferred that the residual protease activity was of very low-level, ranging from 0.025 to 0.05 U, representing only 0.125–0.25% of original activity (20.0 U) in the wash solution. No residual activity was observed in subsequent second rinse indicates that any residual activity present on the post-cleaning surface could be removed simply by rinsing with water. Complete inactivation of the enzymes was achieved after incubation with an equal volume of 0.5 M HCl or alkali for 4 min and 20 min, respectively. This extra resistance to alkali wash may be explained by the stability of alkaline protease from SVB1 at alkaline pH (7-11) (Chapter 5, Section 5.3.2.1, pp. 106-108). In industries, after cleaning the process equipment is often sanitized or sterilized by exposure to steam, boiling water or by autoclaving. Similar to a standard autoclave cycle, incubation of all the enzymes in water at 95°C for 5 min was enough to inactivate the enzymes completely. It is already shown in Chapter 5 (Section 5.3.1, Fig. 5.1, pp. 105) that only a single alkaline protease is present in the partially purified alkaline protease preparation from SVB1. It is active and stable in the range of temperature and pH used in this study and gets inactivated in the post-cleaning processes used in industries for sanitization. These results indicate that the subsequent steps typical of bioprocess cleaning regimes would almost certainly inactivate any residual enzyme remaining on the test surface. Only one report highlighted this issue upon enzyme based surface cleaning (Turner *et al*., 2005) and reported similar kind of results.

6.3.2 Goat Skin Dehairing

6.3.2.1 Dehairing studies

The effect of the enzyme-based dehairing shown in Fig. 6.17a clearly illustrates that the partially purified alkaline protease (PPE) from *Bacillus pseudofirmus* was as

much efficient as compared to the other commercial enzymes (Fig 6.17 b-e). The result from borax–NaOH buffer (25 mM, pH 10.0) treated control, where no dehairing was observed further confirmed that the dehairing was not due to alkaline treatment condition.

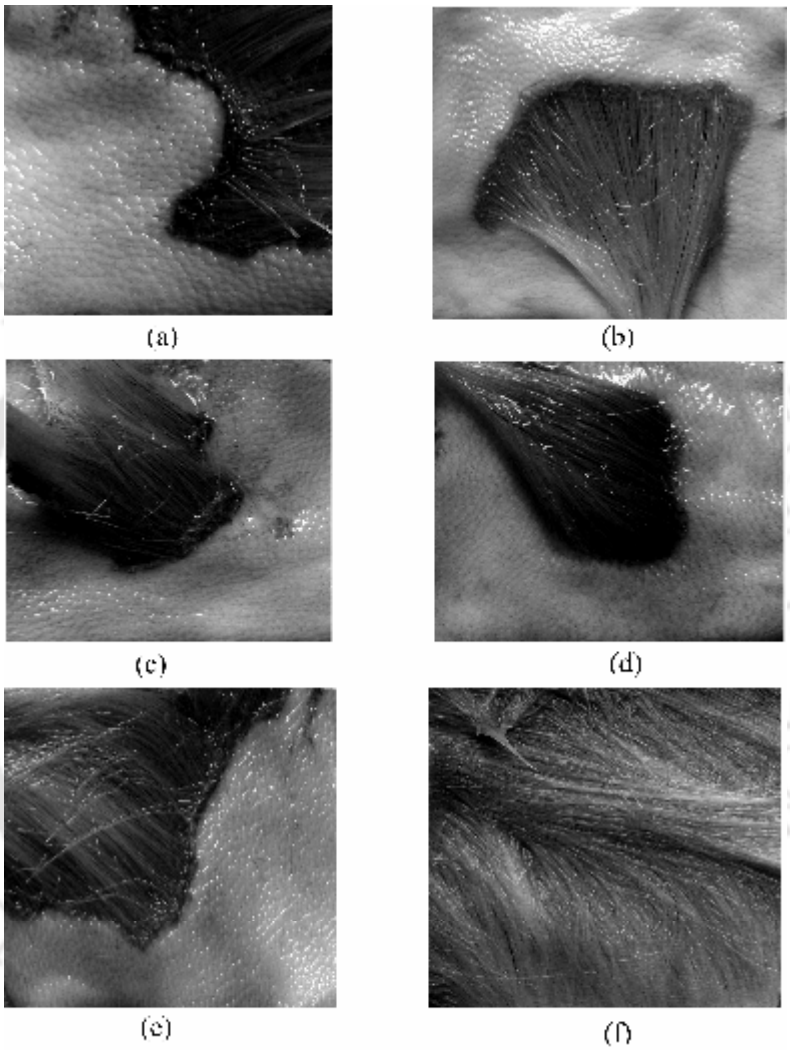


Figure 6.17 Optical micrograph of goat skin treated with (a) partially purified alkaline protease from SVB1 (PPE), (b) P-4860, (c) P-5380, (d) P-5459, (e) P-8038 and (f) buffer (control).

However, it was observed that very efficient dehairing was performed by the partially purified alkaline protease from *Bacillus pseudofirmus* (Fig. 6.18a) followed by the procured enzyme P-4860 (Fig. 6.18b) using stereo zoom microscope at 300x

magnification. With other alkaline proteases some trace hairs were left over after dehairing was done (Figs. 6.18c, d and e).

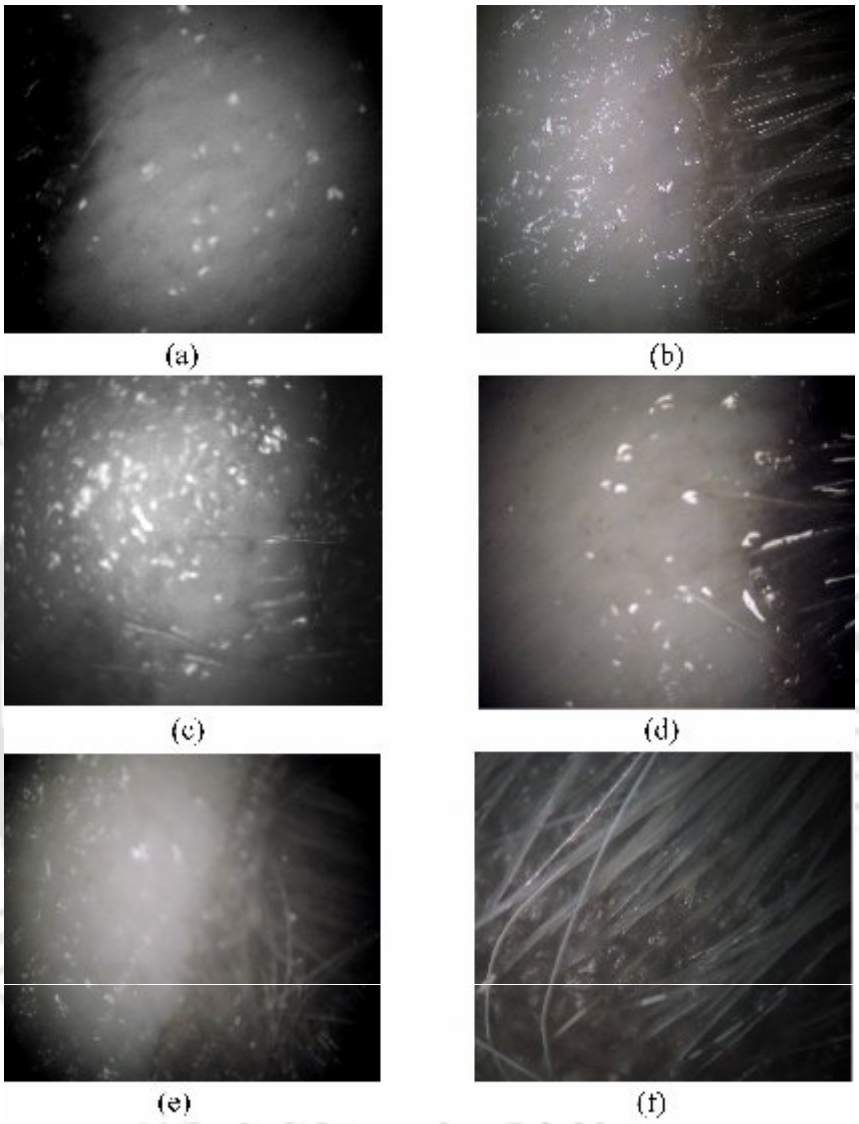


Figure 6.18 Stereo zoom 3-D microscopic images of Goat skin treated with (a) partially purified alkaline protease from SVB1 (PPE), (b) P-4860, (c) P-5380, (d) P-5459, (e) P-8038 and (f) buffer (control).

For better comparison and closure view, parts from all the treated hides were again investigated under scanning electron microscopy (SEM) at 500x magnification. This SEM figures also illustrate that the most efficient dehairing was performed by the

partially purified alkaline protease from *Bacillus pseudofirmus* (Fig.6.19 a) followed by the procured enzyme P-4860 (Fig. 6.19 b).

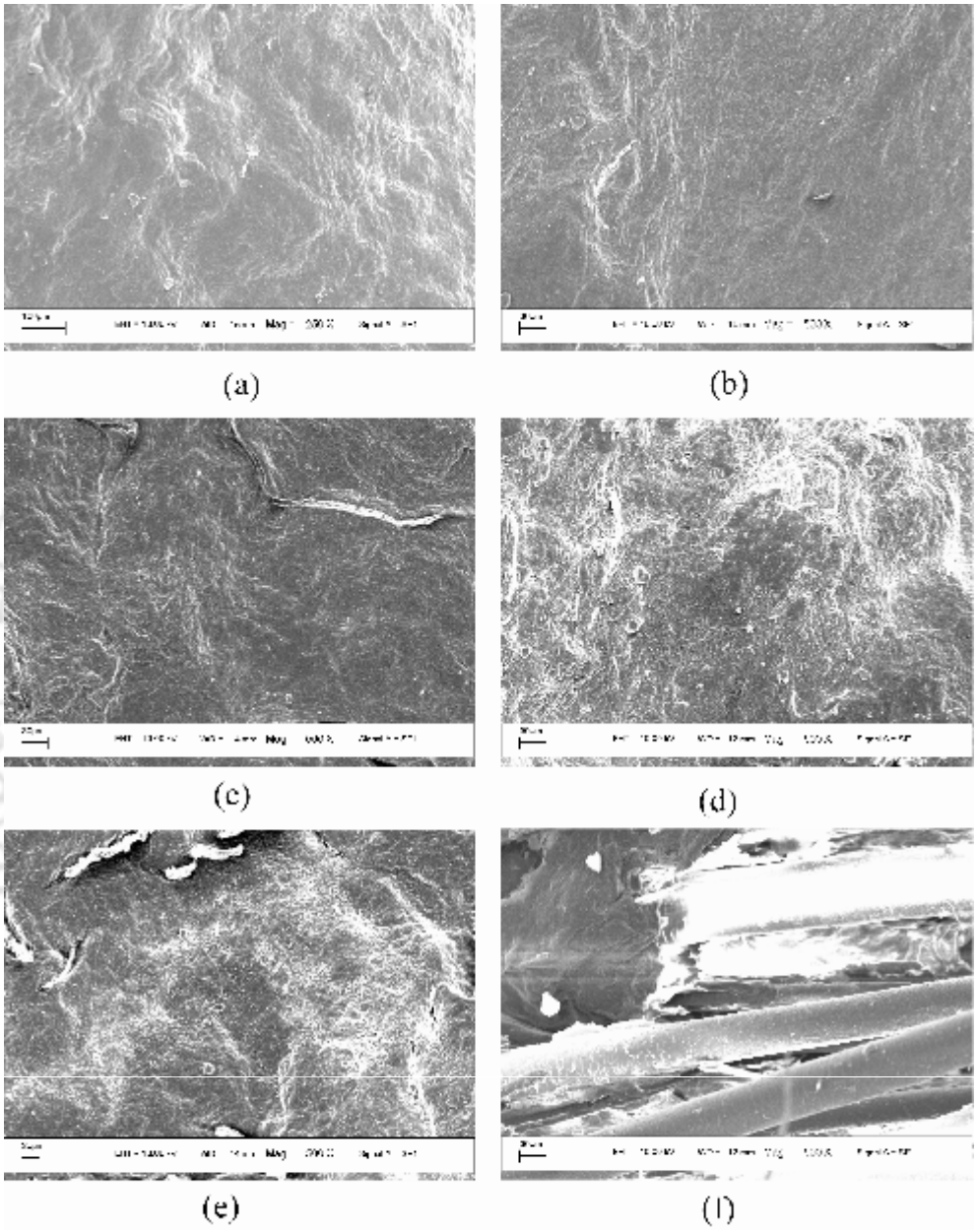


Figure 6.19 SEM images of Goat skin treated with (a) partially purified alkaline protease from SVB1 (PPE), (b) P-4860, (c) P-5380, (d) P-5459, (e) P-8038 and (f) buffer (control).

With the other commercial alkaline proteases, there are some trace hairs after dehairing process was performed (Fig. 6.19 c, d and e). Even there are differences in the

smoothness and texture of the leather surface after dehairing, the partially purified alkaline protease from *Bacillus pseudofirmus* SVB1 and P-4860 were the better dehairing agents compared to others used in this study.

To bring down the cost of dehairing further, it was tested with culture filtrate of *Bacillus pseudofirmus* SVB1 as crude alkaline protease preparation can result in the comparable extent of dehairing or not as of its partially purified counterpart or commercial alkaline proteases.

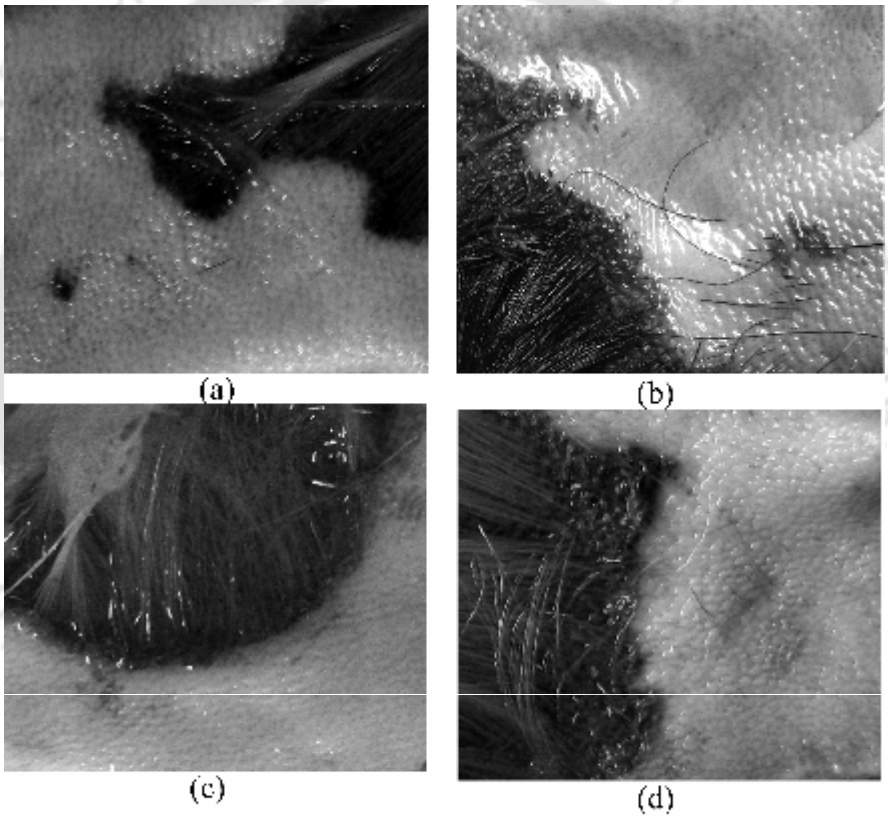


Figure 6.20 Goat skin treated with crude alkaline protease from SVB1 at temperatures (a) 30ºC, (b) 37ºC, (c) 26ºC, (d) 45 ºC.

Fig. 6.20 shows that the dehairing effect of the crude alkaline protease from *Bacillus pseudofirmus* SVB1 at different incubation temperatures *viz*., 26, 30, 37, 45ºC and room temperature (varying from 10ºC to 18ºC). The dehairing was far better at

comparatively lower temperatures (26ºC, 30ºC and at room temperature) than at 37ºC and 45ºC. The smoothness and texture of the surface was hampered when dehairing was carried out at 45ºC as observed from the figure.

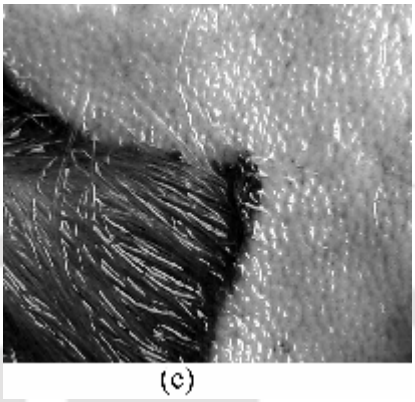


Figure 6.20 (e) Goat skin treated with crude alkaline protease from SVB1 at room temperature (varying from 10ºC to 18ºC).

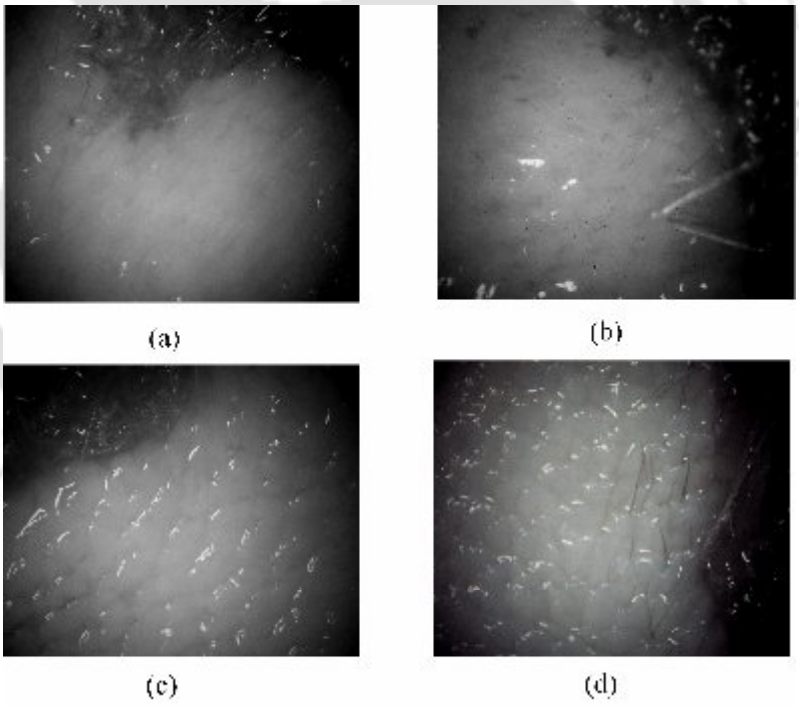
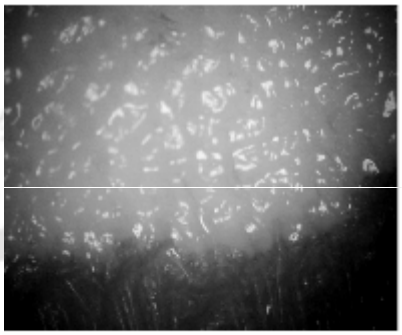


Figure 6.21 Stereo zoom 3-D microscopic images of Goat skin treated with crude alkaline protease from SVB1 at different temperatures (a) 30ºC, (b) 37ºC, (c) 26ºC, (d) 45 ºC.

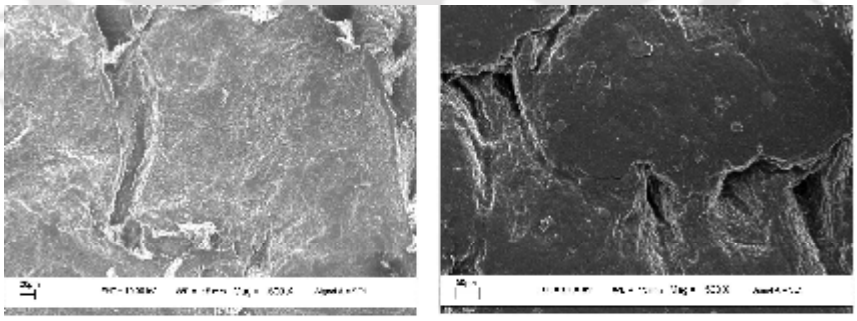
From the stereo-zoom optical microscopy studies it was confirmed that the lesser extent of dehairing was achieved at higher temperatures as shown in Fig. 6.21. There was no hair traces observed on the test surface at 26ºC, 30ºC and room temperature.



(c)

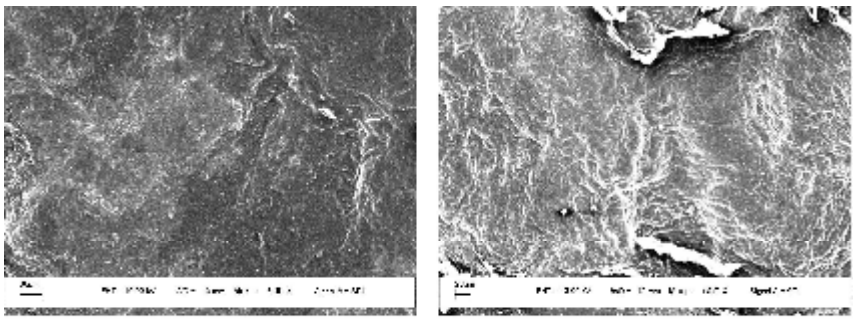
Figure 6.21 (e) Stereo zoom 3-D microscopic images of Goat skin treated with crude alkaline protease from SVB1 at room temperature (varying from 10ºC to 18ºC).

The SEM images (Fig. 6.22) gave clear idea about the degradation of surface at higher incubation temperatures, 37ºC and 45ºC, whereas, the better texture and smoothness of the surface obtained after dehairing with the crude alkaline protease at lower temperatures *viz*., 30ºC, 26ºC and at room temperature.



(a) (b)

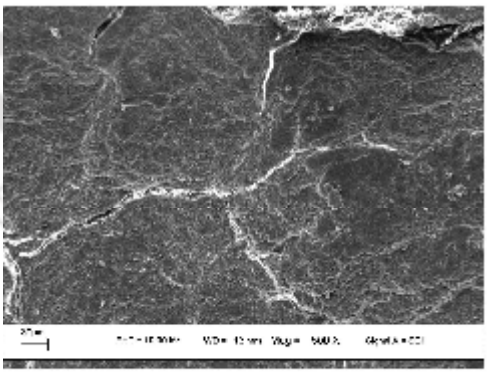
Figure 6.22 SEM images of Goat skin treated with crude alkaline protease from SVB1 at temperatures (a) 30ºC, (b) 37ºC.



(c) (d)

Figure 6.22 SEM images of Goat skin treated with crude alkaline protease from SVB1 at temperatures (c) 26ºC and (d) 45 ºC

Further, experiments were carried out to check whether viable cells of *B. pseudofirmus* SVB1 can dehair goat skin with similar efficiency as that of the crude alkaline protease. The results illustrated poor performance of viable cells of *B. pseudofirmus* SVB1 as the smooth texture of dehaired skin was absent (Fig. 6.23). It was unlike to that with the enzymatic treatment (Figs. 6.17). Moreover, the trace hair was not removed neatly in the case of dehairing with the viable cells. These shortcomings are more clearly demonstrated in the SEM images (Figs. 6.23 e and f). However, viable cells gave better result when incubated at 30ºC than at 37ºC.



(e)

Figure 6.22 (e) SEM images of Goat skin treated with crude alkaline protease from SVB1 at room temperature (varying from 10ºC to 18ºC).

Visual observation studies on the experimental dehaired pelts from goatskins apparently revealed that the complete absence of fine hair, which has earlier been reported to be the greatest challenge in development of enzymatic hair shaving process (Paul *et al*., 2001). Recently efficient removal of trace hair by enzymatic dehairing using protease from *B. subtilis* has been reported (Sivasubramanian *et al*., 2008). The enzyme treated hide showed visible dehairing activity after 2 h of incubation and complete dehairing

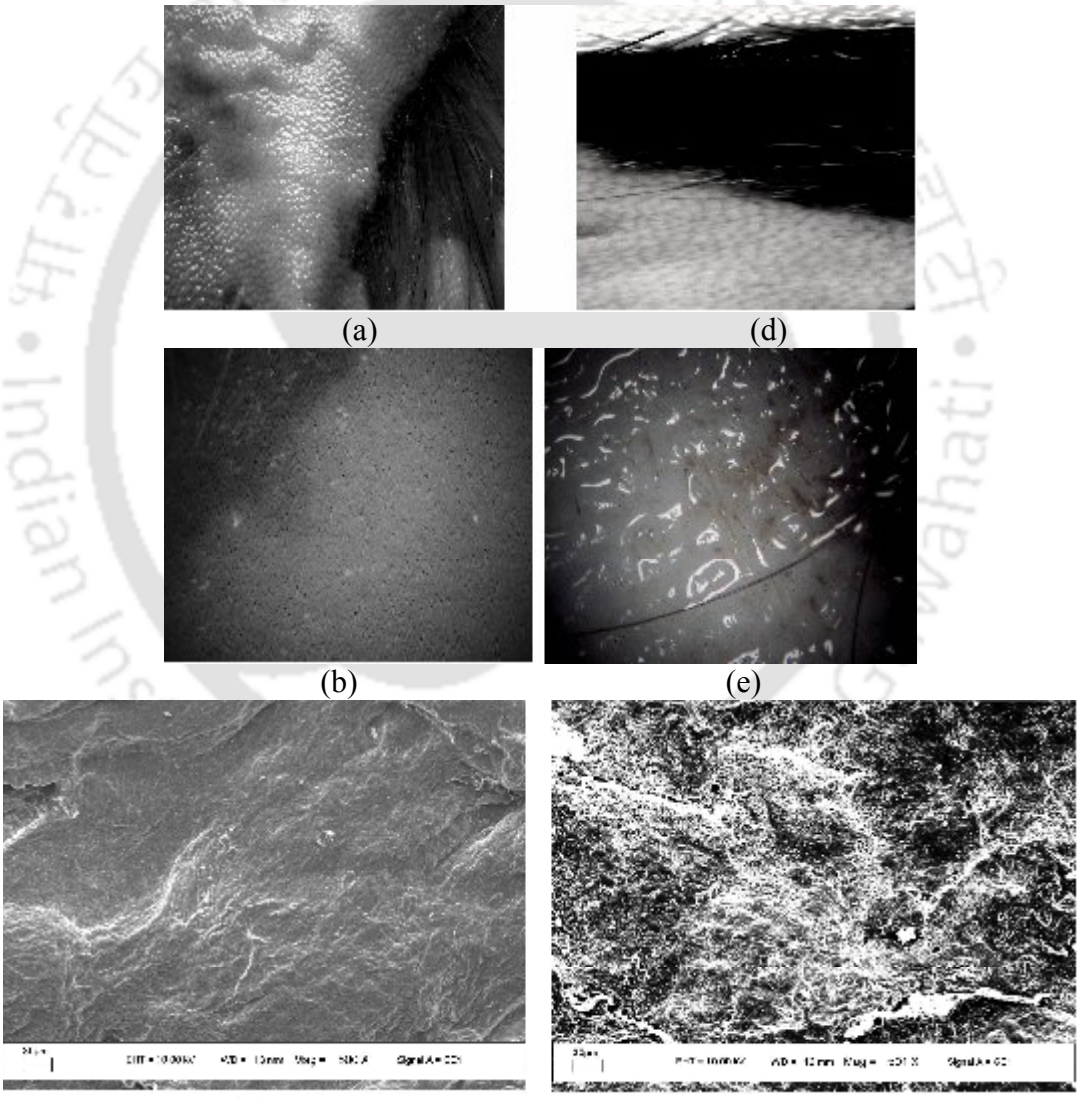


Figure 6.23 Microscopy image of goat skin treated with viable cells of SVB1 cultivated at their mid log phase at 30ºC: (a) optical, (b) stereo zoom, (c) SEM image and 37ºC: (d) optical, (e) stereo zoom, (f) SEM image.

at 6 h. In the control sample, hair loosening was not observed, even by mechanical means such as plucking by forceps (Fig. 6.16). Microscopic examination of the pelt treated with enzyme showed complete removal of epidermis and presence of empty hair follicles (Fig. 6.24) suggesting that the removal of hair from hair root follicle.

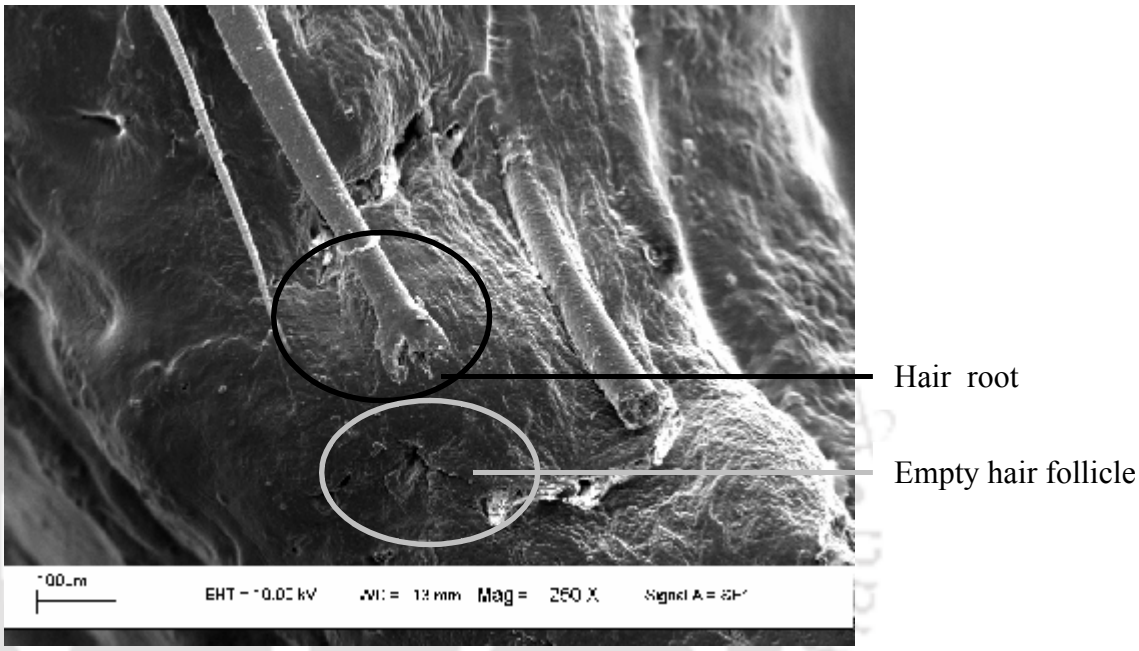


Figure 6.24 Uprooted hair after dehairing with crude alkaline protease from *B. pseudofirmus* SVB1.

Enzymatic dehairing did not damage the collagen layer as it is evident from the skin surface texture. Microscopic examination of the pelt after dehairing did not show the presence of any part of disintegrated shaft and bulb in the follicles, as observed in using multiple proteinase concentrate on goat skin (Chandrasekaran and Dhar 1985). Enzyme treated hair was intact, with hair root, shaft, and sheath (Fig. 6.24). In previous reports, the epidermis, hair shaft and bulbs were completely removed with better splitting of fibre bundles by the action of multiple protease concentrate (Chandrasekaran and Dhar 1985). Intact hair recovered from skins and hides are used in the manufacture of felt, organic fertilizer and poultry feed stuff.

Also, the recovered hair when subjected to hydrolysis by thermal, biological and chemical means has an array of applications ranging from biogas generation, regeneration of keratins, recovery of melanin for the preparation of suntan lotions and cosmetics, hair conditioner, pharmaceutics, re-tanning agent as well as chrome exhaustion in leather manufacture and in the manufacture of synthetic products such as nylon (Cantera and Buljan, 1997). In our studies, it was also observed that alkaline protease treated pelt swells moderately with adequate opening up of collagen fibre.

6.3.2.2 Physical testing of the dehaired leathers

Physical characteristics of the enzymatically dehaired skins were found to be quite superior as compared to that dehaired in conventional process from available literature data (Sivasubramanian *et al*., 2008; Saravanabhavan *et al*., 2006; Thanikaivelan *et al*., 2003). However, the tensile strength and the degree of elongation at break were found to be slightly higher in skins treated with the alkaline protease from SVB1 (especially crude) when compared to the commercial alkaline proteases (P-4860, P-5459, P-5380 and P-8038) (Table 6.1).

Table 6.1 Physical characteristics of dehaired goatskins

Treating agent	Tensile Strength (N/mm^2)		Elongation at break (%)		Tear Strength (N/mm	
	Parallel	Perpendicular	Parallel	Perpendicular	Parallel	Perpendicular
Conventional[1-3]	22-26	17-23	47-67	58-69	42-59	36-59
Enzyme assisted[1]	22-23	18-19	48-50	73-76	42-44	39-41
Enzymatic[1,3]	22-24	18-24	48-64	48-74	42-58	37-58
Crude enzyme*(30ºC)	25.56±2	19.79±7	68.53±5	72.27±3	56.84±2	59.67±5
Crude enzyme* (26ºC)	26.35±8	21.21±2	71.37±5	78±3	60.54±5	62.33±6
Crude enzyme* (room temperature)	26.1±5	20.19±8	69.33±2	76.74±4	58.65±8	61.36±3
Crude enzyme* (37ºC)	24.22±3	18.75±2	66.82±7	70.45±5	52.93±4	58.19±8
Crude enzyme* (45ºC)	20.13±7	16.25±1	62.21±6	67.77±2	49.46±3	52.38±5
Partially purified enzyme**	24.75±2	19.54±3	67.94±5	71.18±1	55.47±7	58.55±6
P-4860	22.87±7	20.77±1	62.15±2	68.24±5	54.67±7	56.39±2
P-5459	21.23±2	16.48±5	62.36±8	65.56±2	49.71±3	53.41±4
P-5380	19.34±4	15.27±3	60.11±5	62.35±7	50.13±2	52.78±4
P-8038	20.53±5	15.77±9	60.22±3	64.38±5	48.33±2	52.85±1
Viable cells$ (30ºC)	16.39±7	12.25±1	50.21±6	57.77±2	39.46±3	42.38±5
Viable cells$ (37ºC)	20.26±3	18.79±2	59.82±7	68.45±5	53.93±4	53.19±8

[1]Sivasubramanian *et al*., 2008; [2]Saravanabhavan *et al*., 2006; [3]Thanikaivelan *et al*., 2003

*Crude alkaline protease from SVB1; **Partially purified alkaline protease from SVB1; $Viable cells of SVB1 at mid log phase

6.3.2.3 Analysis of effluent from dehairing process

Although the brush and paint method of dehairing of skins did not generate effluent in the form of liquor, disposal of their contents into stream would significantly contribute to pollution problems besides toxicity as they contained high content of sulfide and lime during the conventional processes. Table 6.2 showed that total (TS) of enzymatic dehairing effluent was greatly reduced compared to dehairing with lime and sulfide, which was even lower in this study using the alkaline protease from SVB1. This could be mainly due to the elimination of sludge forming, sparingly soluble lime in the process. In traditional dehairing pulped hair and keratinous substances contributed to high BOD and COD in the effluent from lime–sulfide process. A large reduction of BOD and COD by the enzymatic dehairing effluent was observed in this study. This is due to the absence of pulped hair in effluent from the enzymatic dehairing process. The proportional reduction of BOD and COD was rather more in the enzymatic dehairing process involved the alkaline protease (crude or partially purified) from SVB1. Pretanning processes generally accounted for 70–80% of the total COD of effluent from all leather making processes (Marsal *et al*., 1999). About 75% of the organic waste from a tannery was from pretanning processes and 70% of this waste was from hair rich in nitrogen (Kamini *et al*., 1999). Elimination of hair destructive sulfide in the process led to the complete recovery of undamaged intact hair thereby resulting in huge reduction in COD. The values obtained in the purely enzymatic processes employed in this study are better than those obtained in previous enzyme assisted study (Sivasubramanian *et al*., 2008). Moreover, the analysis of effluent from enzymatic dehairing of skins devoid of lime and sulfide showed that TS, BOD and COD and pH were not only reduced to a greater extent but

also toxicity due to sulfide (Davies, 1997) was completely eliminated. Contrary to conventional dehairing, the enzymatic process did not generate high alkaline effluent as lime was eliminated in the process (Sivasubramanian *et al*., 2008). As the pH of the effluent from the experimental system for skins was near neutral, it was less toxic and less expensive than that having a high alkaline pH.

Table 6.2 Characteristic of effluent from different dehairing processes

Effluent parameters	TS (mg/l)	BOD (mg/l)	COD (mg/l)	pH
Conventional[1,2,3,4] (Literature)	10000-33000	5200	3000-14900	10-13
Enzyme assisted[1] (Literature)	3680	2810	9400	11.2
Enzymatic[1,3,4]	1430-28000	1120	2000-3500	7-9
Crude enzyme[*] (30ºC)	1244±21	995	1822	7.2
Crude enzyme[*] (26ºC)	1273±19	987	1844	7.2
Crude enzyme[*] (room temp.:10ºC - 18ºC)	1225±11	952	1802	7.3
Crude enzyme[*] (37ºC)	1298±17	998	1879	7.3
Crude enzyme[*] (45ºC)	1311	1014	1903	7.1
Partially purified enzyme[**]	1357±12	1003	1749	7.8
P-4860	2948±23	2243	5433	6.92
P-5459	4082±14	3221	6854	6.7
P-5380	7937±25	6422	9232	11.3
P-8038	1943±11	1811	3408	10.3
Viable cells[$] (30ºC)	12078±34	457	1043	8.1
Viable cells[$] (37ºC)	11673±12	762	1603	8.5

[1]Sivasubramanian *et al*., 2008; [2]Saravanabhavan *et al*., 2006; [3]Thanikaivelan *et al*., 2003; [4]Saravanabhavan *et al*., 2008;
[*]Crude alkaline protease from SVB1; [**]Partially purified alkaline protease from SVB1;
[$]viable cells of SVB1 at mid log phase

6.4 Conclusions

The conclusions drawn from this chapter are summarized below. The introduction of an enzyme-based cleaning step is likely to reduce the need for subsequent acid and/or alkali treatment. The cleaning efficacy of the alkaline protease was dictated by its

catalytic activity towards individual burnt-on substrate molecules. The crude alkaline protease from *Bacillus pseudofirmus* SVB1 was as much efficient as the partially purified counter part of it. Among the four procured enzymes, P-8038 cleaned the protein burnt-on residues as efficiently as our enzyme did. The other three commercial enzymes *viz.*, P-4860, P-5459 and P-5380 were performed the cleaning operation very poorly as compared to SVB1 alkaline protease both in crude and partially purified form. The better cleaning efficiency of alkaline protease from SVB1 was evident both from the surface topology studies using AFM and protein content of the washing effluent, which gave maximum protein released in the washing effluent of the slides treated with alkaline protease from SVB1.

No residual activity of the enzyme was observed after the post cleaning operations. This overcomes selected safety and regulatory-related concerns of practical importance within the food industry. Although successful in the context of the model system employed, the application of alkaline protease-based cleaning to specific bioprocess operations would require testing and optimization at pilot/process scale, particularly in the cases, where surface debris is of complex composition and perhaps containing additional non-protein-based elements (e.g. whole milk, raw meat). Additionally, the cost-to-benefit ratio of enzyme use would be of practical importance. Application of crude enzyme without any downstream processing suggests economical feasibility of the alternative cleaning operations. Though, one-step purification would be preferable in view of additional safety and its environmental benefits. If the CIP is performed with the partially purified alkaline protease from this study replacing the

caustic wash, the problems associated with alkali or acid washing may be reduced or removed completely.

The alkaline protease produced by *B. pseudofirmus* SVB1 or the procured enzymes efficiently dehaired goat skin. From the physical tests of the dehaired hides it was confirmed that the alkaline protease from *B. pseudofirmus* SVB1 produce the best quality of leather having tensile strength, elongation at break and tear strength of 24.75 N/mm^2 (parallel) and 19.54 N/mm^2 (perpendicular), 67.94% (parallel), 71.18% (perpendicular), 55.47 N/mm (parallel), 58.55 N/mm (perpendicular), which are far better than those of the dehaired hides from other alkaline proteases used in the present research work and the data available in literature as shown in Table 6.1.

The dehairing performed by alkaline protease from SVB1 was also superior with respect to the effluent from this process had less pollution load with TS, BOD and COD of 1357 mg/l, 1003 mg/l and 1749 mg/l, respectively, which are much less than the data available in the literature (Table 6.2).

References

Alessandro, R., Silvia, O. and Adriano, B. (2003) Dehairing activity of extracellular proteases produced by keratinolytic bacteria. *J. Chem. Technol. Biotechnol.*, **78**: 855-859.

Allie, Z., Jacobs, E.P., Maartens, A. and Swart, P. (2003) Enzymatic cleaning of ultrafiltration membranes fouled by abattoir effluent. *J. Memb. Sci.*, **218**: 107-116.

American Public Health Association. (1985) Standard Methods for Examination of Water and WasteWaters**,** ed. **16**, *APHA***,** Washington DC**.**

Aravindhan, R., Saravanabhavan, S., Thanikaivelan, P., Rao, J.R., Chandrasekaran, B. and Nair, B.U. (2004) A biodriven lime and pickle free tanning paves way for greener garment leather production. *J. Am. Leather Chem. Ass.*, **99**: 53-66.

Arguello, M.A., Alvarez, S., Riera, F.A. and Alvarez, R. (2003) Enzymatic cleaning of inorganic ultrafiltration membranes used for whey protein fractionation. *J. Memb. Sci.* **216**: 121-134.

Cantera, C.S. and Buljan, J. (1997) Hair - a new raw material - Overview. *World Leather*, **10**: 51-56.

Chandrasekaran, S. and Dhar, S.C. (1985) Studies on the development of a multiple proteinase concentrate and its application in the depilation of skin. *Leather Sci.*, **32**: 297-304.

Chisti, Y. (1999) Modern systems of plant cleaning. In: Robinson, R., Batt, C., Patel, P. eds., *Encyclopedia of Food Microbiology*, Academic Press, London, pp. 1086-1815.

Davies, R.M. (1997) Setting of consent limits for tanning industry trade effluents. *J. Soc. Leather. Technol.*, **81**: 32-36.

Flint, S.H., Bremer, P.J. and Brooks, J.D. (1997) Biofilms in dairy manufacturing plant—description, current concerns and methods of control. *Biofouling*, **11**: 81-97.

Foose, L.L., Blanch, H.W. and Radke, C.J. (2007) Immobilized protein films for assessing surface proteolysis kinetics. *J. Biotechnol*. **132**: 32-37.

Germann, H.P. (1999) The ecology of leather production-present state and development trends. Proceedings of the XXV IULTCS Congress. Tata McGraw-Hill publishing company Ltd, New Delhi.

Grasshoff, A. (1999) Laboratory studies on cleaning milk heaters with enzymatic cleaning solutions. *Kiel. Milchwirtsch. Forschungsber.*, **51**(4): 295–318.

Grasshoff, A. (2002). Enzymatic cleaning of milk pasteurizers. *Food Bioprod. Process.*, **80**(C4):247-252.

Handojo, A., Zhai, Y., Frankel, G. and Pascall, M.A. (2009) Measurement of adhesion strengths between various milk products on glass surfaces using contact angle measurement and atomic force microscopy. *J. Food Eng.*, **92**(3): 305-311.

Herrmann, P.S.P., Yoshida, C.M.P., Antunes, A.J. and Marcondes, J.A. (2004) Surface evaluation of whey protein films by atomic force microscopy and water vapor permeability analysis. *Packag. Technol. Sci.*, **17**: 267-273.

Hodge, S.M. and Rousseau, D. (2002) Fat bloom formation and characterization in milk chocolate observed by atomic force microscopy. *J. Am. Oil Chem. Soc.*, **79**: 1115-1121.

International Dairy Federation. (1979) Design and use of CIP systems in the dairy industry. *Bull. Int. Dairy Fed.* 117 1-76.

Jeurnink, T.J.M., Walstra, C.G. and DeKruif, C.G. (1996) Mechanisms of fouling in dairy processing. *Neth. Milk Dairy J.*, **50**: 407-426.

Kamini, N.R., Hemachander, C., Mala, J.G.S. and Puvanakrishnan, R. (1999) Microbial enzyme technology as an alternative to conventional chemicals in leather industry. *Curr. Sci.*, **77**: 80-86.

Kim, J., Yamaski, R., Park, J., Jung, H., Lee, H. and Kawai, T. (2004) Highly dense protein layers confirmed by atomic force microscopy and quartz crystal microbalance. *J. Biosci. Bioen.*, **97**(2): 138-140.

Lee, J., Cartwright, R., Grueser, T. and Pascall, M.A. (2007) Efficiency of manual dishwashing conditions on bacterial survival on eating utensils. *J. Food Eng.*, **80**: 885-891.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J.R. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.*, **193**: 265-275.

Maartens, A., Swart, P. and Jacobs, E.P. (1996a) Characterization techniques for organic foulants adsorbed onto flat-sheet UF membranes used in abattoir effluent. *J. Memb. Sci.*, **119**: 1-8.

Maartens, A., Swart, P. and Jacobs, E.P. (1996b) An enzymatic approach to the cleaning of UF membranes fouled in abattoir effluent. *J. Memb. Sci.*, **119**: 9-16.

Maia, R.A.M. (1998) Clean technologies, targets already achieved and trends for coming years. *J. Soc. Leather Technol. Chem.*, **82**:.111-113.

Marsal, A., Cot, J., Boza, E.G., Celma, P.J. and Manich, A.M. (1999) Oxidizing unhairing process with hair recovery. Part I. Experiments on the prior hair immunization. *J. Soc. Leather Technol.*, **83**: 310-315.

Morris, V.J. (2004) Probing molecular interactions in foods. *Trend Food Sci. Technol.*, **15**: 291-297.

Morris, V.J., Mackie, A.R, Wilde, P.J., Kirby, AR, Mills, E.C.N. and Gunning, A.P. (2001) Atomic force microscopy as a tool for interpreting the rheology of food biopolymers at the molecular level. *Lebensm. Wiss. Technol.*, **34**: 3-10.

Mukhopadhyay, R.P. and Chandra, A. (1993) Protease of a keratinolytic Streptomycete to unhair goat skin. *Indian J. Exp. Biol.*, **1**: 557-558.

Munoz-Aguando, M.J., Wiley, D.E. and Fane, A.G. (1996) Enzymatic and detergent cleaning of polysulfone ultrafiltration membrane fouled with BSA and whey. *J. Memb. Sci.*, **117**: 175-187.

Nagata, A., Sakiyama, T., Itoh, H., Toyomasu, T., Enomoto, E., Nagai, T., Saeki, T. and Nakanishi, K. (1995) Comparative studies on caustic and enzymatic cleanings of stainless steel surface fouled with beta-lactoglobulin. *Biosci. Biotechnol. Biochem.*, **59**(12): 2277-2281.

Nashy, E.H.A., Ismail, S.A., Ahmady, A.M., Fadaly, H.E. and Sayed, N.H. (2005) Enzymatic bacterial dehairing of bovine hide by a locally isolated strain of *Bacillus licheniformis*. *J. Soc. Leather Technol. Chem.*, **89**: 242-249.

Paul, R.G., Mohamed, I., Davighi, D., Covington, A.D. and Addy, V.L. (2001) The use of neutral protease in enzymatic unhairing, *J. Am. Leather Chem. As.*, **96**: 180-185.

Qing H., Yong P., Xin, L., Haifeng, W. and Yizheng, Z. (2003) Purification and characterization of an extracellular alkaline serine protease with dehairing function from Bacillus pumilus. *Curr. Microbiol.*, **46**: 169-173.

Raghavan, D., Gu, X., Nguyen, T. and Vanlandingham, M. (2001) Characterization of chemical heterogeneity in polymer systems using hydrolysis and tapping-mode atomic force microscopy. *J. Polym. Sci.*, **39**:1460-1470.

Romney, A.J.D. (1990) CIP: cleaning in place, 2nd edn., *The Society of Dairy Technology*, Cambridge, Shire.

Sakiyama, T., Toyomasu, T., Nagata, A., Imamura, K., Takahashi, T., Nagai, T. and Nakanishi, K. (1998) Performance of a protease as a cleaning agent for stainless steel surfaces fouled with protein. *J. Ferment. Bioeng.*, **85**(3): 297-301.

Saravanabhavan, S., Aravindhan, R., Thanikaivelan, P., Rao, J.R., Nair, B.U. (2003) Green solution for tannery pollution: effect of enzyme based lime-free unhairing and fiber opening in combination with pickle-free chrome tanning. *Green Chem*., **5**: 707-714.

Saravanabhavan, S., Rao, J.R. and Nair, B.U. (2008) A potential new commercial method for processing leather to reduce environmental impact. *Environ*. *Sci*. *Pollut*. *Res*., **15**: 293-295.

Saravanabhavan, S., Thanikaivelan, P., Raghava, J.R., Nair, B.U. and Ramasami, T. (2006) Reversing the Conventional Leather Processing Sequence for Cleaner Leather Production. *Environ*. *Sci*. *Technol*., **40**: 1069-1075.

Seeta Laxman, R., Sonawane, A.P., More, S.V., Seetarama Rao, B., Rele, M.V., Jogdand, V.V., Deshpande, V.V. and Rao, M.B. (2005) Optimization and scale up of production of alkaline protease from Conidiobolus coronatus. *Process Biochem*., **40**(9): 3152-3158.

Sivasubramanian, S., Murali Manohar, B., Rajaram, A. and Puvanakrishnan, R. (2008) Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. *Chemosphere*, **70**: 1015-1024.

Thanikaivelan, P., Bharath, C.K., Saravanabhavan, S., Anandhi, C., Chandrasekaran, B., Rao, J.R. and Nair, B.U. (2006) Single step hair removal and fiber opening process: simultaneous and successive addition of protease and α-amylase. *J*. *Am*. *Leather Chem*. *Assoc*., **101**: 388-398.

Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T. (2002) Zero discharge tanning: a shift from chemical to biocatalytic leather processing. Environ. Sci. Technol., 36: 4187- 4194.

Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T. (2004a) Progress and recent trends in biotechnological methods for leather processing. Trends Biotechnol., 22: 181-188.

Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T. (2004b) Ecofriendly bio-process for leather processing. US Patent **6708531**.

Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T. (2005) Recent trends in leather making: processes, problems, and pathways. Crit. Rev. *Environ*. *Sci*. *Technol*., 35: 37-79.

Thanikaivelan, P., Rao, J.R., Nair, U. and Ramasami, T. (2003). Approach towards zero discharge tanning: role of concentration on the development of eco-friendly liming-reliming processes. *J*. *Clean*. *Product*., 11.79-90.

Turner, K., Serantoni, M., Boyce, A. and Walsh, G. (2005) The use of proteases to remove protein-based residues from solid surfaces. *Process Biochem*., **40**: 3377-3382.

Varela, H., Ferrari, M.D., Belobrajdic, L., Vazquez, A. and Loperena, M.L. (1997) Skin unhairing proteases of Bacillus subtilis: production and partial characterization. *Biotechnol Lett*., 19 (8): 755-758.

Ying, P., Yu, Y., Jin, G. and Tao, Z. (2003) Competitive protein adsorption studies with atomic force microscopy and imaging ellipsometry. Colloids and Surfaces B: Biointerfaces **32** (1): 1-10.

CHAPTER 7

POTENTIAL APPLICATIONS IN EFFLUENT TREATMENT

7.1 Introduction

In leather industry, the various processing steps of soaking, liming, deliming, bating, pickling, skin degreasing, tanning, *etc*., as clubbed under the 'beam house' operations largely contribute in the pollution load. Around 70,000 tonnes of hides and skins are processed every year in India, which release liquid effluent of 75000 m^3/day (Rao *et al*., 2003).

In slaughterhouses, the consumption of water per slaughtered animal varies according to the animal and the industry-specific process employed and is discarded as wastewater (Caixeta *et al*., 2002). Wastewater from dairy industries (Cammarota *et al*., 2001; Danalewich *et al*., 1998; Jung *et al*., 2001; Marshall and Harper, 1988; Omil *et al*., 2003) and slaughterhouses (Batstone *et al*., 1997; Martınez *et al*., 1995; Masse´ *et al*., 2001; 2003) are rich in organic molecules and nutrients usually containing high levels of fats and proteins having low biodegradability coefficient. They cause gross pollution of land and water with their high biochemical oxygen demand (BOD) and chemical oxygen demand (COD). A large number of pretreatment systems are employed to remove oil and grease to prevent an array of problems. The efficiency of the treatment including reduction in the cell-aqueous phase transfer rates, sedimentation hindrance due to the development of filamentous microorganisms, development and flotation of sludge with poor activity, clogging and the emergence of unpleasant odors. The application of

pretreatment to hydrolyze and dissolve lipids may improve the biological degradation of fatty wastewaters and thereby accelerate the process efficiency. Conventional biological wastewater treatment processes are not suitable to treat such complex effluents (Ludzack and Noran, 1965), because of its high salinity, high levels of organic load and suspended solids (sand, lime, hair, flesh, dung, etc). Enzymatic treatment by alkaline protease, which is quite stable and active under high alkalinity, salt and metal ion concentrations, could be an viable alternative. However thus far, only a few studies describing the degradation of fats and oils by enzymatic hydrolysis have been reported. Alkaline protease from *B*. *subtilis* was used for the management of waste feathers from poultry slaughterhouses (Dalev, 1994). A formulation containing proteolytic enzymes from *B*. *subtilis*, *B*. *amyloliquefaciens, Streptomyces* sp. and a disulfide reducing agent (thioglycolate) is available in market since 1996 (Gajju *et al*.,1996), which helps in clearing pipes clogged with hair-containing deposits.

The biochemical processes for sludge digestion generally require long sludge retention times and show low removal efficiencies of organic carbon. The first step (hydrolysis) is the rate limiting step in anaerobic digestion (Watson *et al*., 2004). To minimize the effect of this rate-limiting step, enzyme treatment can accelerate the solubilisation of the sludge solids, thus improving anaerobic digestion (Tiehm *et al*., 2001). Breakdown of large, organic particulate matter into smaller particles increases the surface area available for contact with the bacteria responsible for degradation. In addition, disruption of the floc structure of the sludge itself releases associated dissolved organic material entrapped in the floc matrix and renders it bio-available. Thus, the utilization of the organic matter as the substrate for the anaerobic bacteria is enhanced

(Roman *et al*., 2006). Reduced particle size using physical disintegration of sludge solids resulted in improved digestion as indicated by increased biogas production and COD removal (Onyeche *et al*., 2002).

Extracellular enzymes *viz*., protease, lipase and cellulase are capable of killing pathogens and other bacteria (Ruggaber and Talley, 2006). Parmar *et al*. (2001) observed that the addition of alkaline protease to wastewater greatly decreased its coliform and *Salmonella* counts. In another study, cell lysis of pathogens by protease from *Bacillus proteolyticus* demonstrated the antibacterial activities of alkaline protease (Bhaskar *et al*., 2007). Solids reduction in sewage decreases the volume of sludge that requires dewatering and disposal. This reduction in solid also reduces a portion of COD in sludge liquor.

In view of all these, the potential of the alkaline protease from *B*. *pseudofirmus* SVB1 for the treatment of tannery, dairy and domestic effluents was tested with repect to the removal of COD, BOD and total solids (TS). The effect of this alkaline protease on human and plant pathogens was also studied.

7.2 Materials and Methods

7.2.1 Chemicals and Reagents

All salts and media constituents *viz*., NaCl, NaOH, $MgSO_4.7H_2O$, $CaCl_2$, NH_4NO_3, KH_2PO_4, K_2HPO_4, and $FeCl_3$, were purchased from CDH, India. TCA, acetic acid, Tris, HCl and borax were procured from Merck, India. Biological grade casein and agar were obtained from Himedia, India. All commercial protease enzymes (P5380: Proteinase from *Bacillus licheniformis* Type VIII, P4860: Protease from *Bacillus licheniformis* Subtilisin® A, P8038: Proteinase from bacterial Type XXIV, P5459:

Protease from *Bacillus licheniformis*) were procured from Sigma-Aldrich Chemicals, India.

Tannery effluent was collected from Alam Tannery, Leather Complex, Bantala, West Bengal, India. Dairy effluent was obtained from Central Dairy, Guwahati, Assam. Domestic sewage used in this study was from IIT Guwahati sewage treatment plant. Slaughterhouse waste was collected from butcher shop in local market.

7.2.2 Microorganisms and Culture Conditions

Bacillus pseudofirmus SVB1 was isolated from the premises of tannery industry as described in Chapter 2. Cultures were regenerated every 2-3 weeks on a freshly prepared nutrient agar plates from the frozen stock culture. *Bacillus coagulans* NRRL NRS 609 (Gram-positive) and *Pectobacterium carotovorum* MTCC1428 (Gram-negative), *Serratia marcescens* MTCC 97 (Gram-negative) and *Wautersia eutropha* NRRL B-2804 (Gram-negative) were used to test the antimicrobial activity of SVB1. All the species except SVB1 were grown in nutrient broth/agar at 28°C temperature and pH 7.0.

7.2.3 Inoculum Preparation and Alkaline Protease Production

The inoculum was prepared as described in Chapter 3 (Section 3.2.2, pp. 51) and alkaline protease was produced as per the methodology described in Chapter 5 (Section 5.2.3, pp. 95). Partially purified alkaline protease (PPE) was obtained from acetone fractionation as detailed in Chapter 5 (Section 5.2.4.1, pp. 95).

7.2.4 Effluent Treatment

The three different effluents were allowed to settle for 12 h. After this simple pretreatment, 98 ml of each effluent was added to 2 ml of either crude/partially purified alkaline protease from SVB1, commercial protease P-2380/P-8038 (20 U) or active cells of *Bacillus pseudofirmus* SVB1 in log phase (A_{600}~1.0). Distilled water/borax-NaOH buffer (pH 10.0) was used as negative controls. Volume in experimental flasks or control flasks was adjusted to 100 ml with sterile distilled water and was incubated in a shaking incubator at 30°C. Samples were collected for determination of total solid (TS), chemical oxygen demand (COD) and biological oxygen demand (BOD) before incubation and after 90 h of incubation.

7.2.5 Domestic Sewage Sludge Solubilization

Well mixed sludge (35 ml) collected from the domestic sewage treatment plant located at IIT Guwahati, Guwahati was taken in 250 ml Erlenmeyer flasks. Initial total suspended solids (TS) in each flask were adjusted to equal concentration by measuring the TS. In addition to the sludge, 10 ml of crude alkaline protease (185 U/ml, specific activity 36.9 U/mg) or distilled water were added and volume was finally adjusted to 100 ml with sterile distilled water before incubating in a shaking incubator at 30°C. Control flask containing distilled water was incubated in static condition to check the change in sludge volume with time.

7.2.6 Slaughter House Waste Degradation

The crude enzyme (2U) was incubated with slaughter house wastes *viz*., blood clot or coagulated egg white in Borax-NaOH buffer (pH 10.0) at 30ºC for 12 h.

Additional sets of experimental flasks were included which contained any one of the partially purified alkaline protease from *B. pseudofirmus* SVB1, P-5380 or P-8038, in place of crude alkaline protease from SVB1. Borax-NaOH buffer (pH 10.0) was used to replace enzyme in the negative control tube for these experiments.

7.2.7 Antibacterial Activity

7.2.7.1 Colony forming units count

Apart from sludge solubilisation, alkaline protease could have potential role in reduction of the pathogen load during domestic sewage treatment. To determine the effect of alkaline protease treatment, microbial count was performed by enumeration of the colony forming unit (cfu) per ml of the effluent before and after the treatment. In order to get the total viable cell counts in terms of the colony forming units per ml (cfu/ml), 100 µl of serially diluted domestic sewage samples from each experimental flask were plated on triplicate nutrient agar plate for each tested dilution and incubated at 37°C for 24 h to obtain fully developed colonies.

7.2.7.2 Plate studies on zone of inhibition

Antibacterial activity of crude alkaline protease was tested against *Pectobacterium carotovorum* MTCC 1428, *Serratia marcescens* MTCC 97 and *Wautersia eutropha* NRRL B-2804 by the disc diffusion method. The microbial suspensions at their mid log phase (A_{600}≈0.5) were streaked over the agar surface using a sterile cotton swab to ensure confluent growth. 10 µl aliquot of crude alkaline protease from SVB1 was spotted on Whatman No. 1 paper discs (6 mm) and then aseptically placed on the agar plate surface, and incubated at 30°C for 12 h.

7.2.7.3 Scanning electron microscopy (SEM) studies

After treatment with the various alkaline proteases used in this study, the bacterial cells were studied under SEM to get a view of the morphological changes caused by the enzymatic treatment. 100 µl crude alkaline protease from SVB1 was added to serially diluted (10^{-4}) mid log phase (A_{600}≈0.5) culture of *Bacillus coagulans* (NRRL NRS 609), *Pectobacterium carotovorum* MTCC 1428, *Serratia marcescens* MTCC 97 and *Wautersia eutropha* NRRL B-2804. These were then incubated at 30°C for 24 h. After incubation, the cells were harvested by centrifugation at 5000 rpm for 15 min followed by washing twice with sterile distilled water. The cell pellets thus obtained were transferred onto the SEM stub. The pellets were dehydrated in water–alcohol solutions at various alcohol concentrations (30%, 50%, 70%, 80%, 90% and 100%) for 10 min and dried in a desiccator. The samples were analyzed using a scanning electron microscope (LEO 1430VP).

7.2.8 Analytical Techniques

7.2.8.1 Cell growth, proteolytic activity and protein concentration measurement

Cell growth was measured as described in Chapter 2 (Section 2.2.5.1, pp. 45). Alkaline protease activity and specific activity of the culture supernatant of SVB1 were measured as described earlier (Chapter 3, Section 3.2.5.2., pp. 54). The total protein contents of the samples were determined as described in Chapter 3 (Section 3.2.5.3., pp. 55).

7.2.7.2 Determination of TS, BOD, COD and settleability

TS, BOD and COD were determined as described in Chapter 6 (Section 6.2.6.5, pp. 135). Settle ability of the domestic sewage was determined by placing 100 ml of sludge sample in a measuring cylinder to settle at room temperature for 2 h and the volume of settled solids was recorded and presented as milliliters of settled solids per 100 ml total sample.

7.3. Results and Discussion

7.3.1. Characteristics of Effluents

The comparison of physicochemical analysis of untreated effluents from tannery, dairy and domestic sewage are presented in Table 7.1. The tannery effluent was characterized to contain high TS and COD. Maximum BOD was observed in dairy effluents. Domestic wastewater was polluted with organic load plus dissolved and suspended matter though very less as compared to the above two industrial effluents. TS, COD and BOD reduction by using various treating agents (as mentioned in section 7.2.4) were studied in the present work.

Table 7.1 Characteristic of effluents from tannery, dairy and domestic sewage

Parameter	Tannery effluent	Dairy effluent	Domestic sewage
pH	11.6 ± 0.4	7.1 ± 0.2	7.1 ± 0.3
TS (mg/l)	135000 ± 2000	5000 ± 400	181 ± 9
COD (mg/l)	4270 ± 150	1850 ± 40	154 ± 8
BOD (mg/L)	570 ± 30	860 ± 31	88 ± 4

7.3.2 TS, COD, BOD and pH Reduction in Tannery Effluent

The reduction of TS, COD, BOD and pH in tannery effluent caused by each of different treating agents (live cells, partially purified enzyme and commercially available enzymes) were plotted to obtain a comparative view as presented in Fig. 7.1.

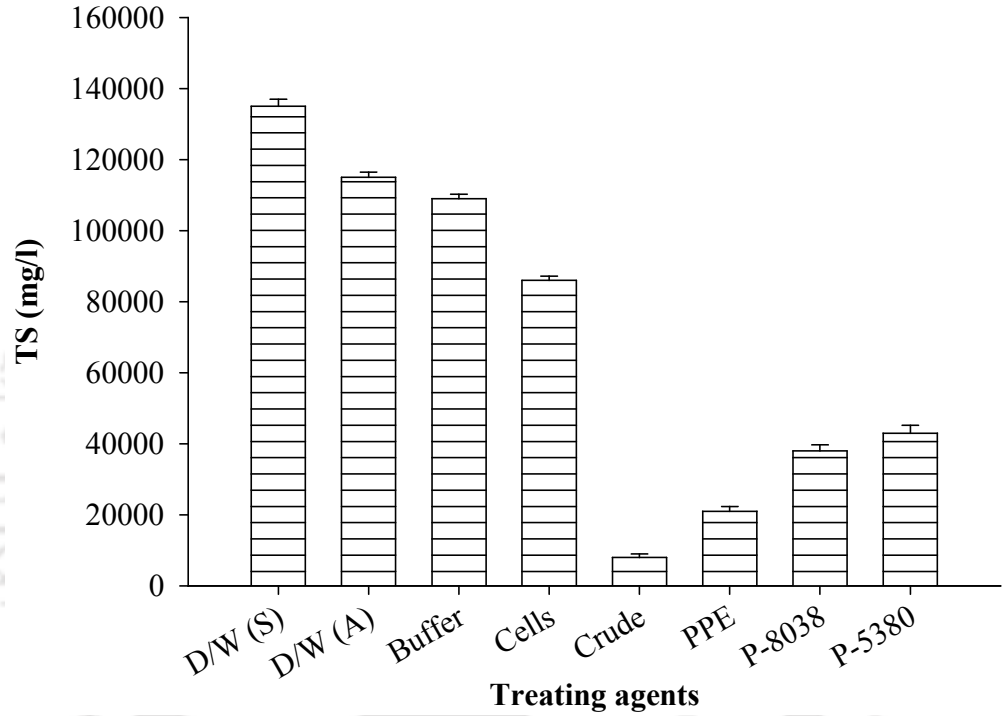


Figure 7.1 (a) Reduction in TS of the tannery effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

The maximum reduction in TS was achieved with crude alkaline protease from SVB1 and then with the partially purified alkaline protease from SVB1. Commercial enzymes P-5380 and P-8038 were also showed a good efficiency by reducing TS to 31% and 28%, respectively (Fig. 7.1 a).

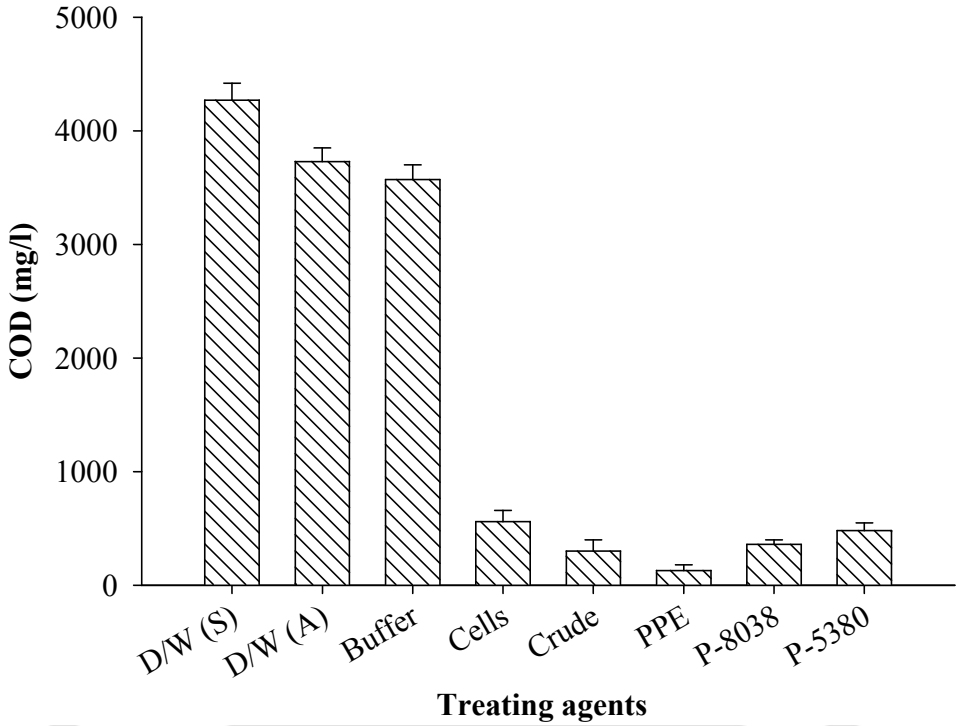


Figure 7.1 (b) Reduction in COD of the tannery effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

The live cells of SVB1 gave very poor performance as TS was decreased only to 64%. This was very obvious as the biomass was increased, TS would certainly increase. The performance of the crude alkaline protease from SVB1 was found to reduce TS by 94%. COD was decreased by 86% in all the experimental flasks (Fig. 7.1 b). The best efficiency was achieved with partially purified alkaline protease from SVB1 that resulted in a reduction of 97%. But, if the cost of purification is taken into consideration, crude alkaline protease from SVB1 was the most economic with 93% reduction. BOD was also reduced to more than 11% in all the experimental flasks, and the best with crude alkaline protease (Fig. 7.1 c). The pH was reduced to near to neutral (7.4) by the partially purified alkaline protease from SVB1 and to 7.8 by the crude alkaline protease from SVB1 (Fig.

7.1 d). The reduction in pH may be due to production of some acidic peptides during the proteolysis of the wastewater.

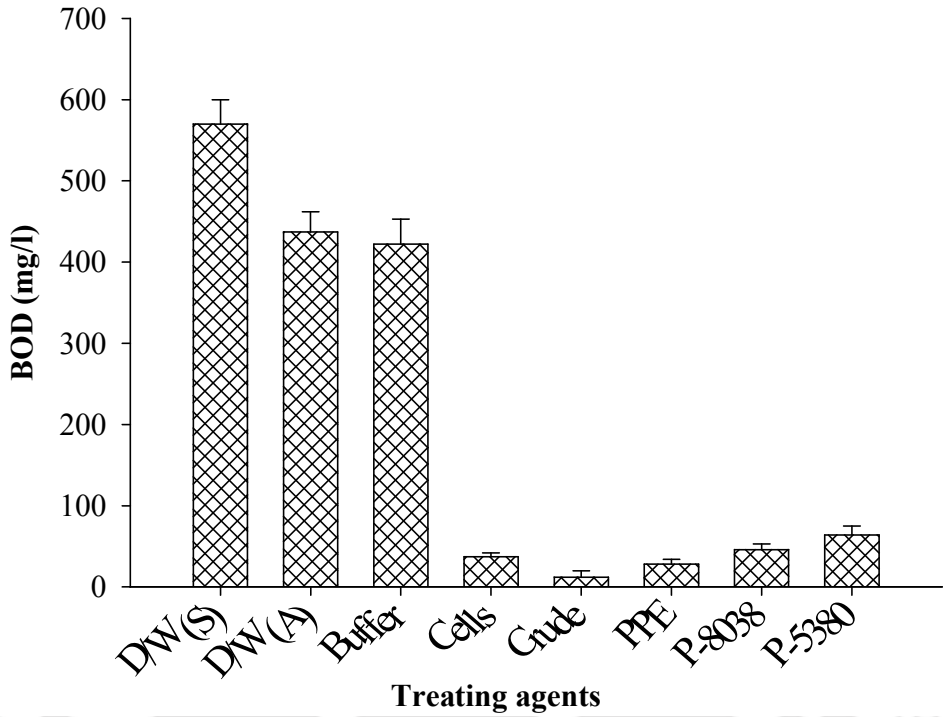


Figure 7.1 (c) Reduction in BOD of the tannery effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

Tannery wastewater, a primary effluent stream in leather processing industry is produced by soaking the hides and skins in fresh water to remove excess salt and alkalinity. The highly saline (1–10% w/v) nature of this waste stream hinders biological treatment (Dhaneshwar, 1990). In recent years there have been a few studies on biological treatment of tannery soak liquor. Lefebvre *et al*. (2006) made an attempt to treat tannery saline wastewater biologically. They studied anaerobic digestion of tannery soak liquor in an up-flow anaerobic sludge blanket (UASB).

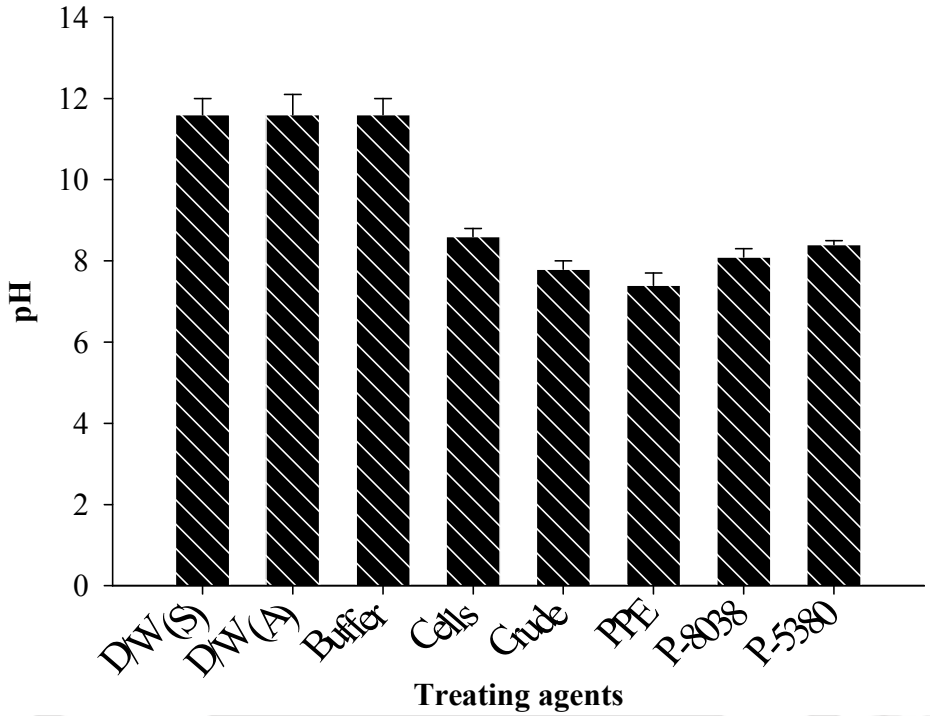


Figure 7.1 (d) Reduction in pH of the tannery effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

A 78% COD removal was achieved by an unidentified microbial consortia and halophillic/moderate halophillic cultures. A reduction of COD from 2020 to 175 mg/l was obtained using activated sludge process (Rajagopalu and Kammani, 2008). In our study, much higher reduction in COD (from 4270 to 130 mg/l) was achieved using the crude/partially purified alkaline protease from SVB1. Similar order of reduction in TS, COD and BOD was reported in few previous studies using membrane filtration or reverse osmosis (Purkait *et al*., 2009; Das *et al*., 2006; Purkait *et al*., 2005).

7.3.3 TS, COD and BOD Reduction in Dairy Effluent

The reduction in TS, COD and BOD in dairy effluent with different treating agents (live cells, partially purified enzyme and commercially available purified enzymes) is represented in Fig. 7.2.

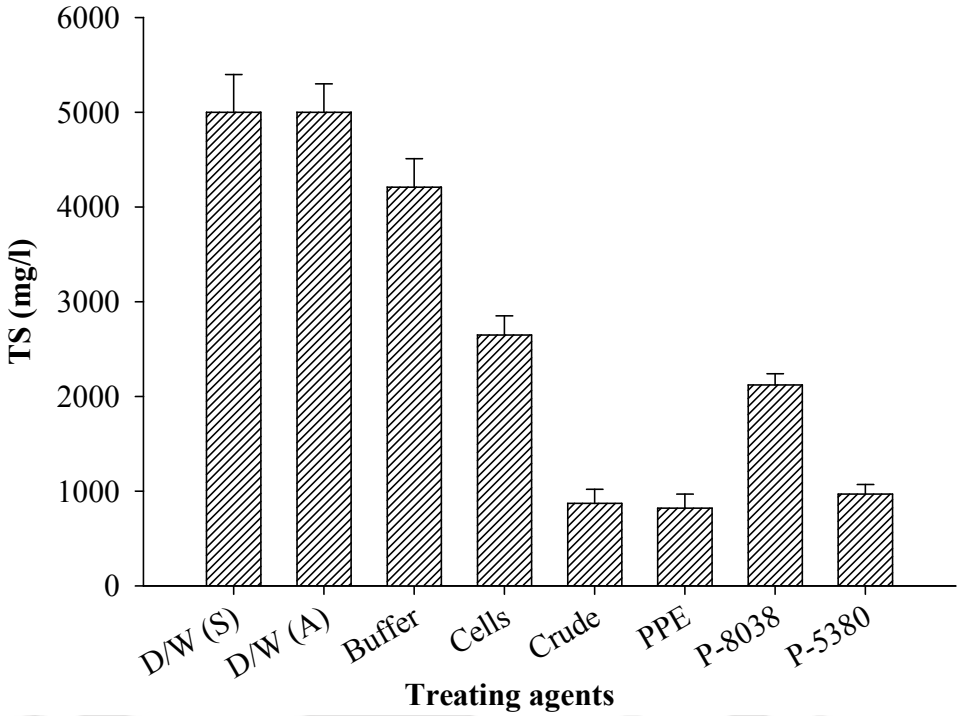


Figure 7.2 (a) Reduction in TS of the dairy effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

The maximum reduction in TS (from 5000 to 1000 mg/l) was achieved with partially purified alkaline protease from SVB1. The reduction in TS by the crude alkaline protease from SVB1 closely followed that of the partially purified enzyme. P-5380 was also efficient in reduction of TS (TS was decreased by 19%). However, in presence of the viable cells of SVB1 and P-8038, TS decreased only to 53% and 42%, respectively, where the poor efficiency of the first could be attributed to increase in biomass. The

maximum reduction in COD was achieved with crude alkaline protease from SVB1 giving a reduction to 0.6% of the COD before treatment (Fig. 7.2 b).

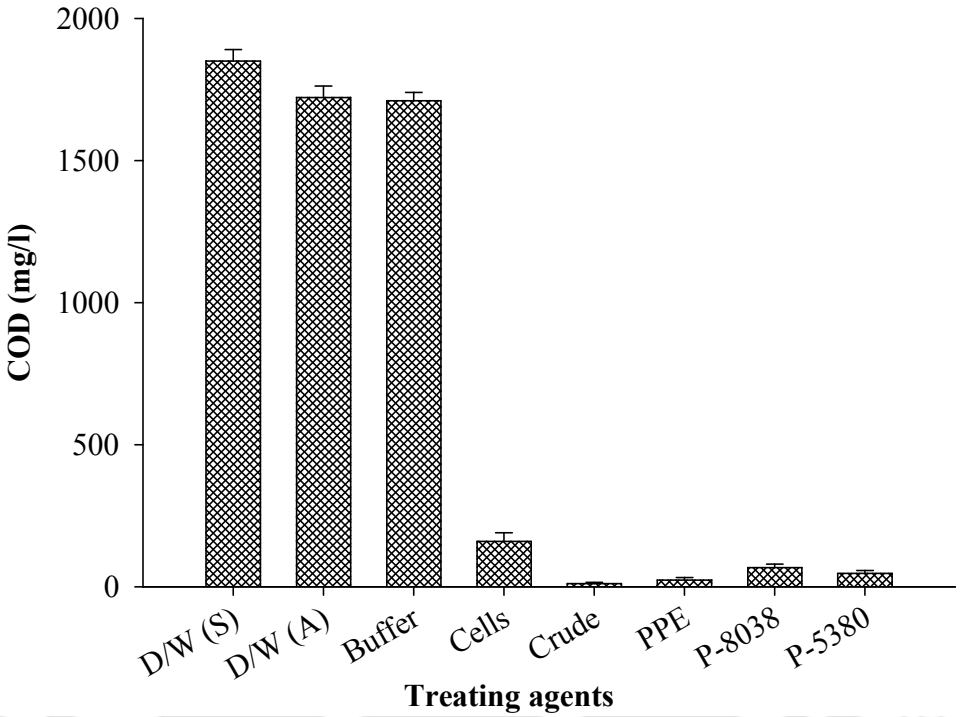


Figure 7.2 (b) Reduction in COD of the dairy effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

The other alkaline proteases (partially purified from SVB1, P-8038 and P-5380) were also showed very good results for COD reduction (to 1.3, 3.6 and 2.5% of the COD before treatment, respectively). BOD was reduced to negligible value (less than 4% of the original BOD) in all the experimental flasks, but the most efficient reduction was obtained using crude alkaline protease (Fig. 7.2 c).

Dairy wastewater generally does not contain conventional toxic chemicals like those listed under EPA's Toxic Release Inventory. However, it has high concentration of dissolved organic components like whey proteins, lactose, fat and minerals

(Mukhopadhyay *et al*., 2003), which upon decomposition generates foul smell that causes discomfort to the surrounding population.

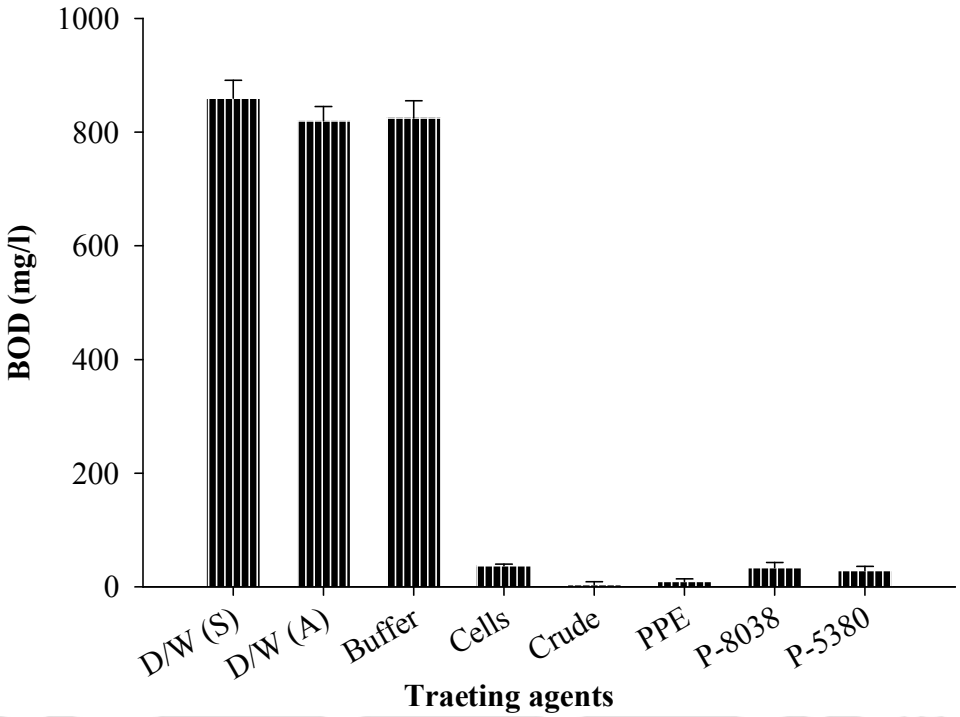


Figure 7.2 (c) Reduction in BOD of the dairy effluent: treatment with viable cells of SVB1, crude and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water, D/W (A); or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition (D/W (S)).

The dairy waste consists mainly of raw materials lost during handling, processing and cleaning materials mixed with the processing water. Great care has to be exercised while discharging the dairy wastewater into the general pool as they impose relatively high oxygen demand. Anaerobic hybrid reactor was used for the primary treatment of dairy wastewater (Rajesh Banu *et al*., 2008) that resulted in 95% removal of COD. In a previous study COD removal efficiency was achieved more than 90% in a series of batch reactors (Mohseni-Bandpi and Bazari-Iranian, 2004). Lipase and protease have been used successfully in dairy waste water treatment for removal of lipid/fat or hydrolysis of whey

proteins in earlier studies (Leal *et al*., 2006; Lamas *et al*., 2001). However, treatment of dairy waste water using alkaline protease has not been reported.

7.3.4 TS, COD and BOD Reduction and Sludge Solubilisation in Domestic Sewage

The extent of sewage sludge solubilization with different treating agents (viable cells, partially purified enzyme and commercially available purified enzymes) was determined from the reduction in TS, COD and BOD in domestic sewage (Fig. 7.3).

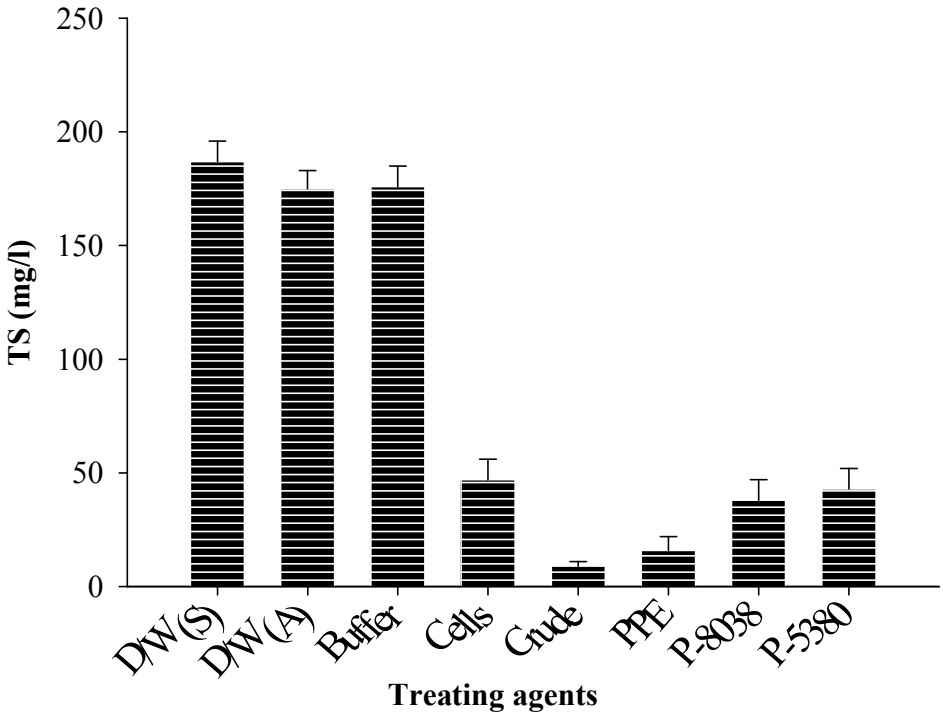


Figure 7.3 (a) Reduction of TS of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

The maximum reduction in TS was achieved with the crude alkaline protease from SVB1 This was closely followed by the partially purified alkaline protease from SVB1. P-8038, P-5380 and the viable cells of SVB1 also showed a good efficiency in

reducing TS to 20, 23 and 25%, respectively. On the other hand, the maximum reduction in COD was achieved with crude alkaline protease from SVB1 giving a reduction of 9.7% (Fig. 7.3 b). Even the partially purified alkaline protease from SVB1 also showed appreciable efficiency in COD reduction (12%) while the other alkaline protease *viz*., P-8038 and P-5380 was not that efficient (37 and 27%, respectively). Though, BOD was reduced to negligible value (less than 15%) in all the experimental flasks, but the crude alkaline protease reduced it most efficiently *viz*., 3.4% (Fig. 7.3 c).

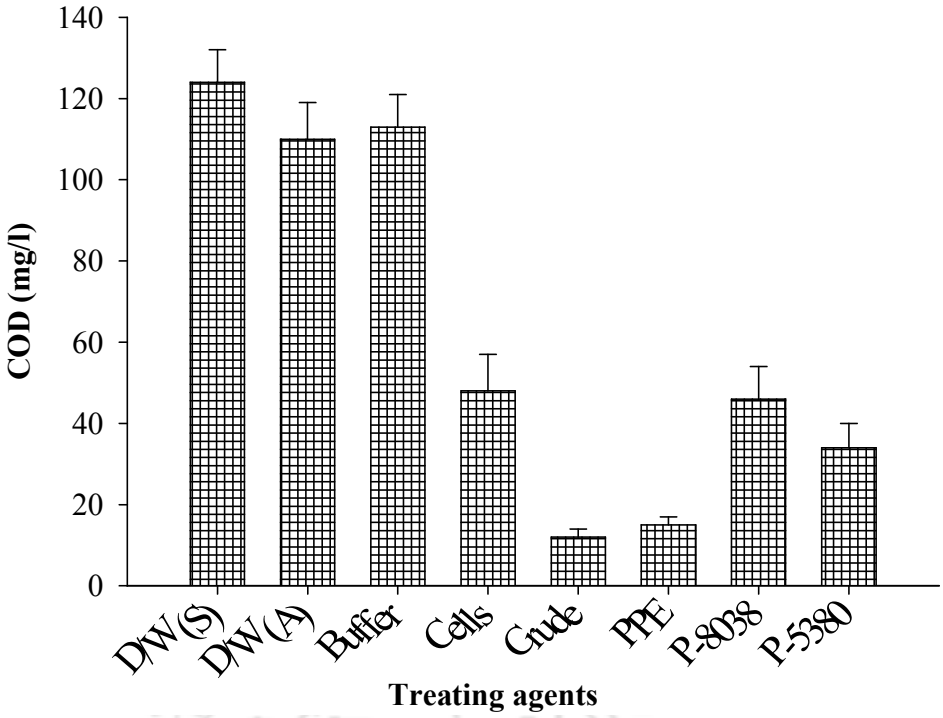


Figure 7.3 (b) Reduction of COD of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

These results illustrate the potential to use the alkaline protease from SVB1 to reduce sludge volume considerably without any primary purification step, which reduces the treatment cost. The dominance of protein in sewage sludge and its ability to bind flocs

and form different matrices is emphasized previously (Frolund et. al., 1995). The removal of protein with protease is thought to weaken the floc, which explains the role of proteases in solid settling (Higgins and Novak, 1997).

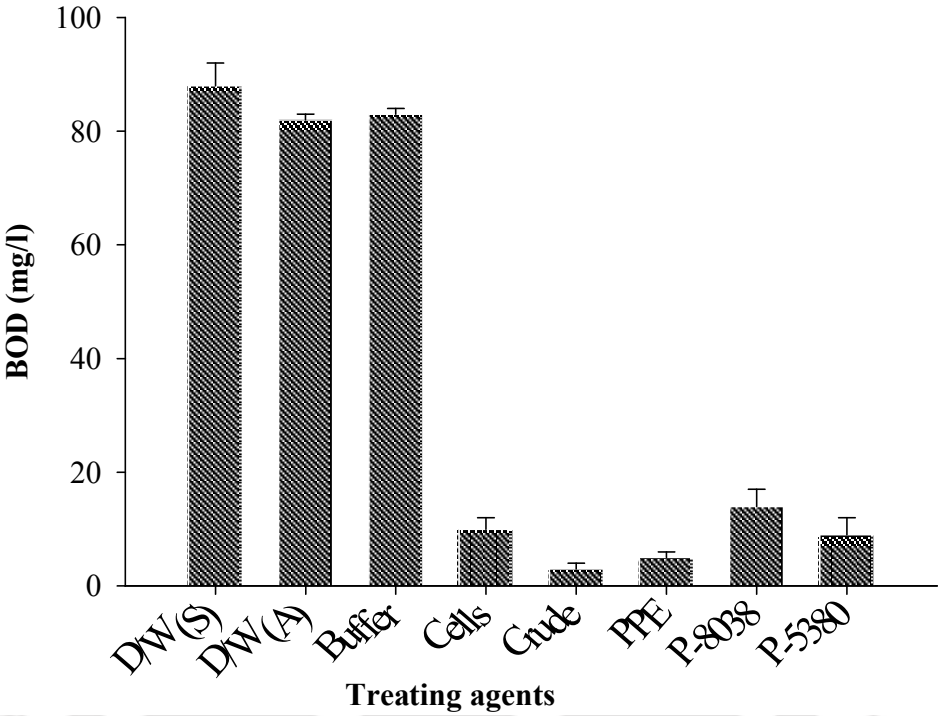


Figure 7.3 (c) Reduction of BOD of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

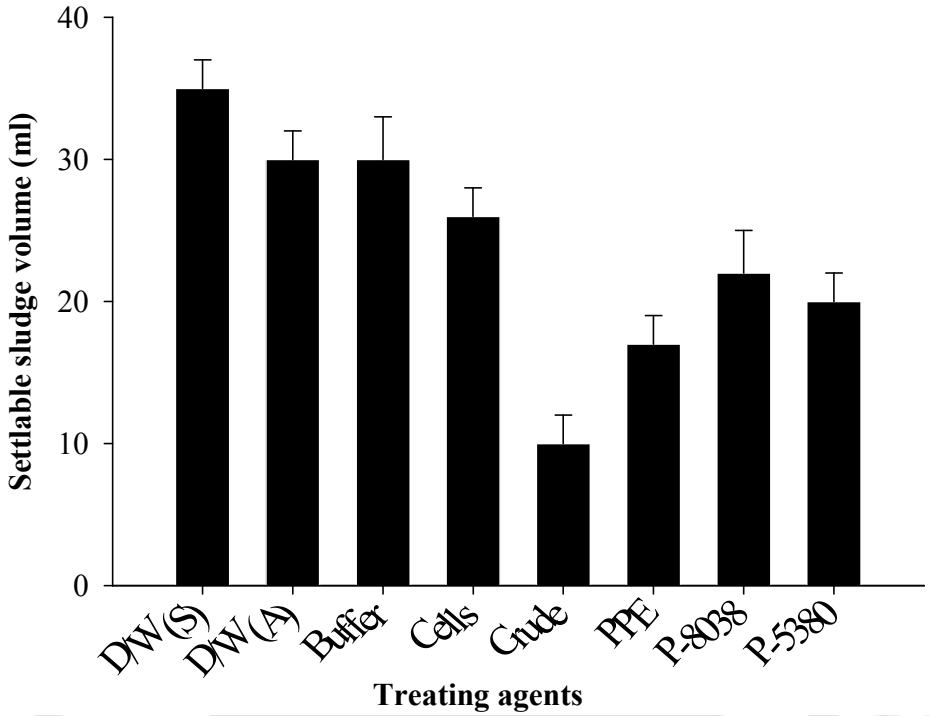
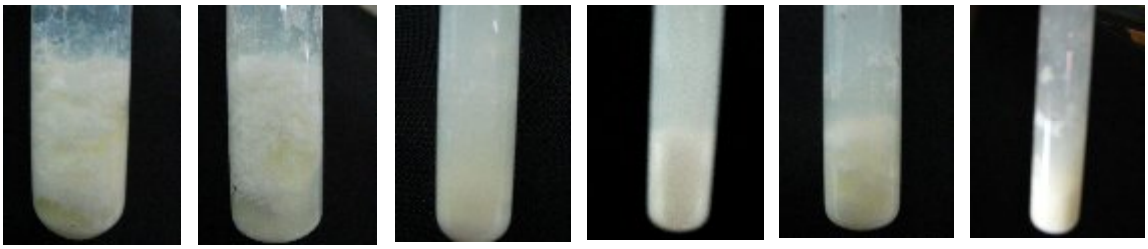


Figure 7.3 (d) Reduction of settlable sludge volume (SSV) of the domestic sewage: treatment with viable cells of SVB1 (cells), crude (crude) and partially purified (PPE) alkaline protease from SVB1, P-8038 and P-5380. Negative control: sterile distilled water or borax NaOH buffer (pH 10.0) incubated at 30°C and 180 RPM. Alternative control flask contained distilled water incubated at static condition D/W (S)).

7.3.5 Slaughter House Waste Solubilization

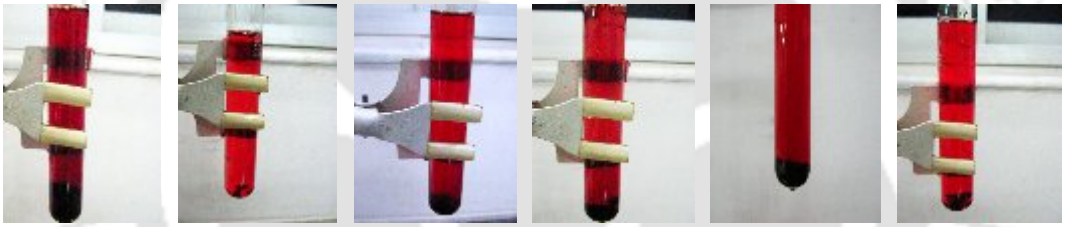
Insoluble coagulated egg white (Fig.7.4) and coagulated blood (Fig. 7.5) were converted to soluble form after incubation with the alkaline protease (crude as well as partially purified forms). Results were compared with the procured enzymes (P-5380 or P- 8038). It was found that the egg white was completely solubilized by the partially purified alkaline protease. On the other hand, though the egg white was partly solubilized using the crude enzyme and P-8038, however, the solubilization with viable cells of SVB1 or P-5380 was not significant.



(a) (b) (c) (d) (e) (f)

Figure 7.4 Coagulated egg white solubilization experiment: (a) control, (b) crude alkaline protease from SVB1, (c) partially purified crude alkaline protease from SVB1, (d) P-5380, (e) P-8038 and (f) Viable cells of SVB1.

From the blood clot solubilization experiments, it was observed that the crude alkaline protease was more efficient than its partially purified counterpart (Fig. 7.5). Viable cells of SVB1 were also found to solubilize blood clot very efficiently and P-5380 was found to be more efficient than P-8038.



(a) (b) (c) (d) (e) (f)

Figure 7.5 Coagulated blood solubilization experiment: (a) control, (b) crude alkaline protease from SVB1, (c) partially purified crude alkaline protease from SVB1, (d) P-5380, (e) P-8038 and (f) Viable cells of SVB1.

Slaughterhouse wastewater contains high levels of fats and proteins that present low biodegradability. Since, the use of high-cost commercial enzymatic preparations would make the pre-treatment procedure economically infeasible. The use of low-cost enzymatic preparations represents a vital development in the treatment of effluents. In this context, the higher efficiency of the alkaline protease from SVB1 (both crude and partially purified) in solubilising the coagulated egg and blood as compared to the other alkaline proteases used in this study envisages the potential of alkaline protease from SVB1 for effluent treatment.

7.3.6 Antibacterial Activity

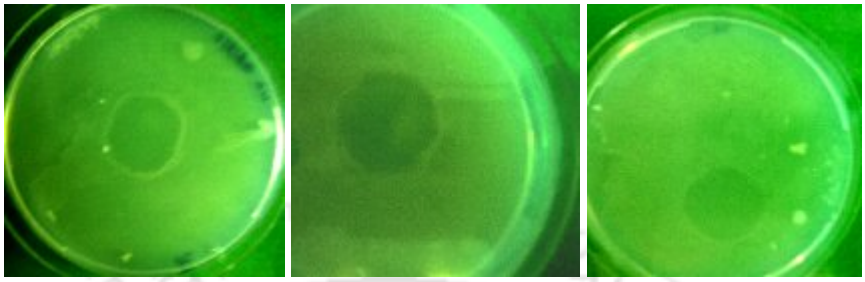
Agricultural utilization of sewage sludge requires successful implementation of pathogen reduction techniques (Dumontet *et al*., 1999), so that after treatment it could safely be used without the risks of disease transmission. However, anaerobic and aerobic sludge digestion processes in sewage treatment plants are inadequate for destruction of pathogens (Parmar *et al*., 2001). Alkaline proteases used for treating tannery, dairy and domestic sewage reflect its efficiency in terms of reductions of cfu count in treated effluents. Table 7.2 gives the microbial count in terms of colony forming unit (cfu/ml) after treatment with the treating agents as mentioned in section 7.2.4.

Table 7.2 Microbial count (cfu/ml) of the domestic sewage effluent after treatment with different treating agents

Sl. No.	Treating agents	Microbial count (cfu/ml)
1	D/W (S)	$15 \times 10^4 \pm 0.02 \times 10^4$
2	D/W (A)	$18 \times 10^4 \pm 0.03 \times 10^4$
3	Buffer	$17 \times 10^4 \pm 0.01 \times 10^4$
4	Cells	$12 \times 10^3 \pm 0.03 \times 10^3$
5	Crude	$4 \times 10^2 \pm 0.05 \times 10^2$
6	PPE	$6 \times 10^2 \pm 0.04 \times 10^2$
7	P-5380	$10 \times 10^2 \pm 0.09 \times 10^2$
8	P-8038	$9 \times 10^2 \pm 0.06 \times 10^2$

Count of effluent before treatment and that of control were found to be the same. Since the alkaline protease from SVB1 was observed to bring down the cfu/ml of the domestic sewage, its possible effect on different potential human and plant pathogens were also studied and results are represented in Fig. 7.6. The zone of clearance is clearly visible in all the three cases. This can be inferred from Fig. 7.6 that the alkaline protease

from SVB1 had shown strong inhibitory effect on growth of the pathogenic microorganisms, *P. carotovorum*, *S. marcescens* and *W. eutropha*.



(a) (b) (c)

Figure 7.6 Antibacterial activity of the alkaline protease from SVB1 against (a) *P. carotovorum*, (b) *S. marcescens* and (c) *W. eutropha*.

Lysozyme and other enzymes, which lyse gram-positive bacteria by hydrolytic attack of the peptidoglycan cell wall, have minimal effect on gram-negative bacteria because their bacterial envelopes are protected by outer cell membranes (Slein and Logan, 1967). Pavlova *et al*. (1987) reported that enzyme complexes from thermophilic soil organisms were capable of lysing vegetative cells of various gram-negative organisms. Alkaline protease from *Bacillus* sp. has previously been shown to lyse cells of *E. coli* (Dean and Ward, 1991). It is likely that the lytic action of protease toward pathogens is responsible for its observed role in lysis of pathogens. The morphological change in the protease treated cells of target organisms (*B. coagulans*, *P. carotovorum*, *S. marcescens* and *W. eutropha*), as revealed by SEM is presented in Fig. 7.7.

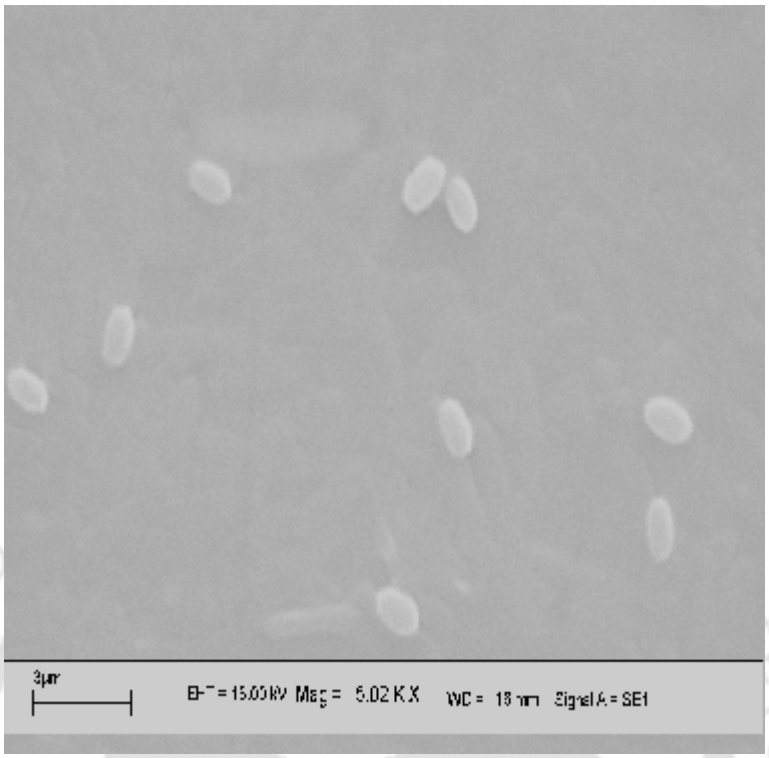


Figure 7.7 (a) Untreated *P. carotovorum*.

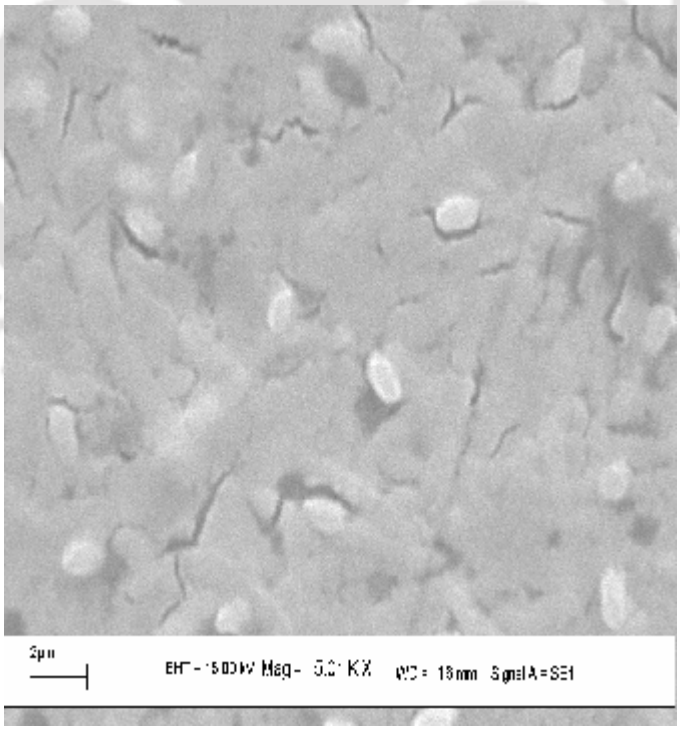


Figure 7.7 (b) *P. carotovorum* treated with the alkaline protease from SVB1.

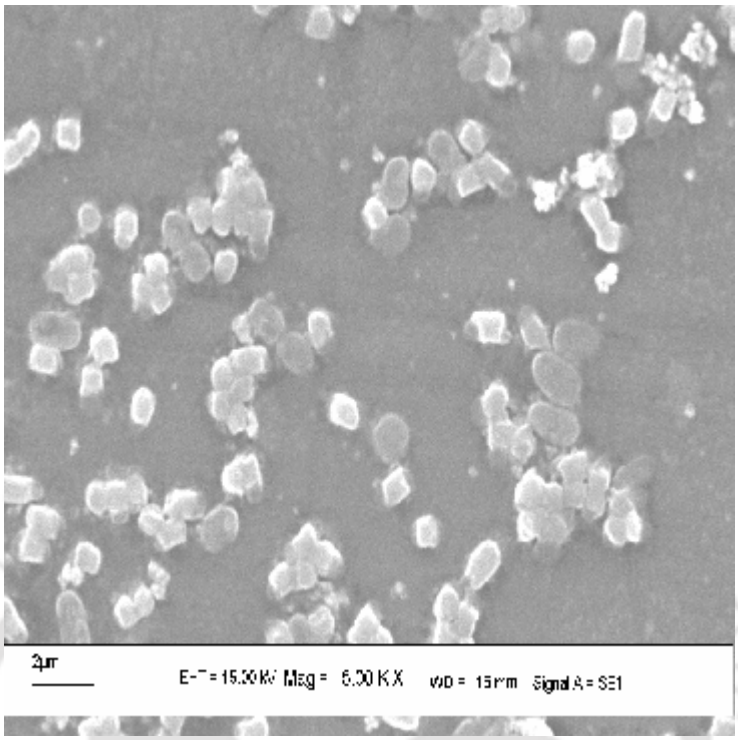


Figure 7.7 (c) Untreated *S. marcescens*.

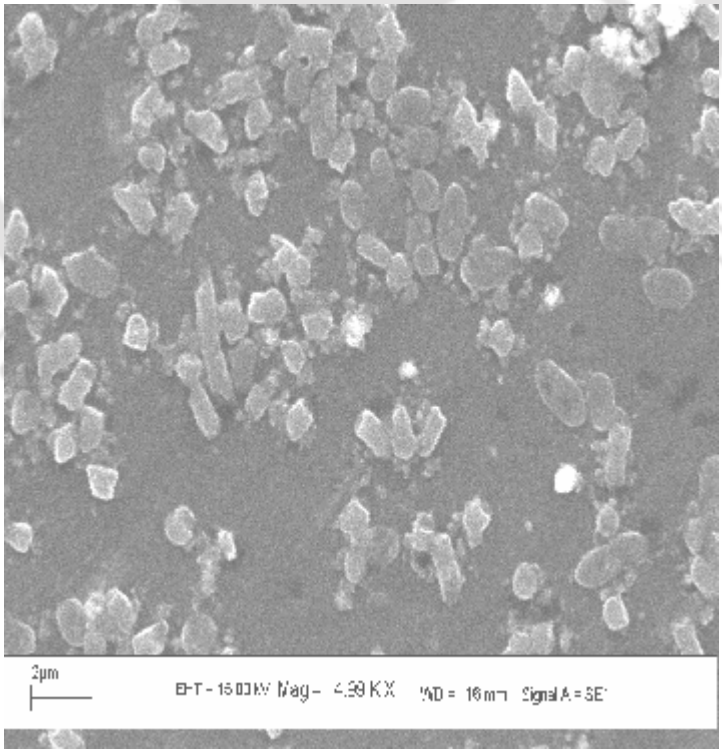


Figure 7.7 (d) *S. marcescens* treated with the alkaline protease from SVB1.

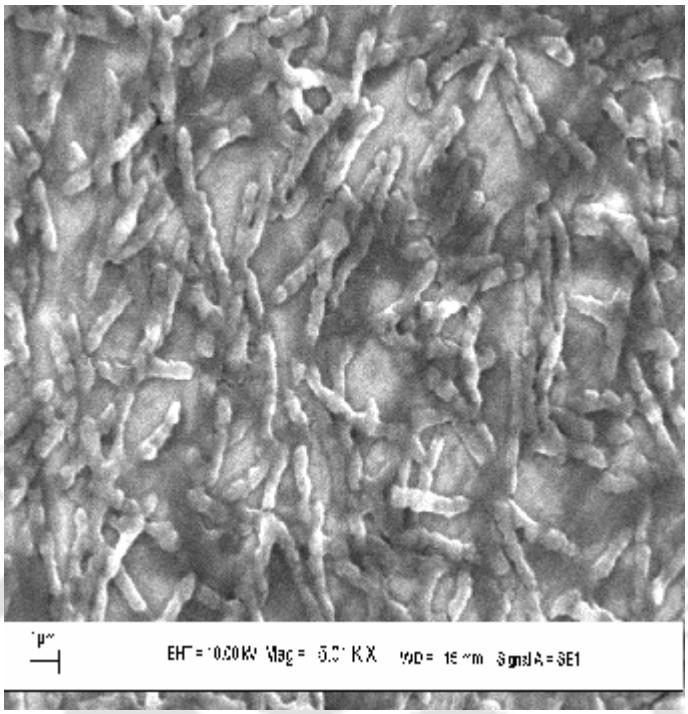


Figure 7.7 (e) Untreated *W. eutropha*.

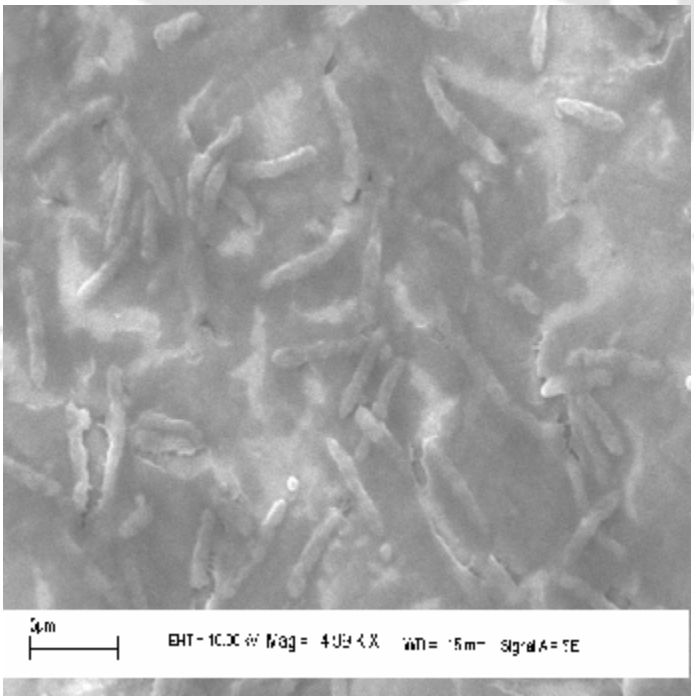


Figure 7.7 (f) *W. eutropha* treated with the alkaline protease from SVB1.

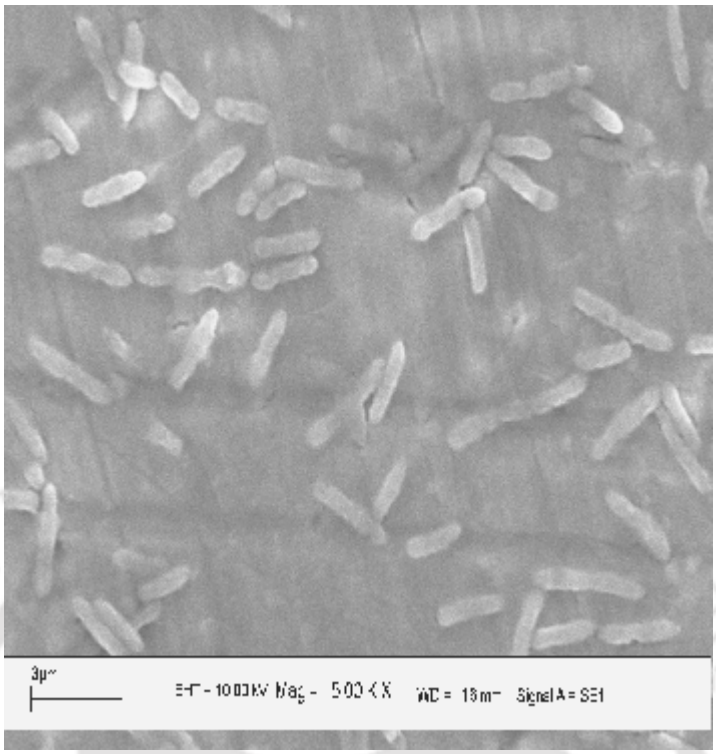


Figure 7.7 (g) Untreated *B. coagulans*.

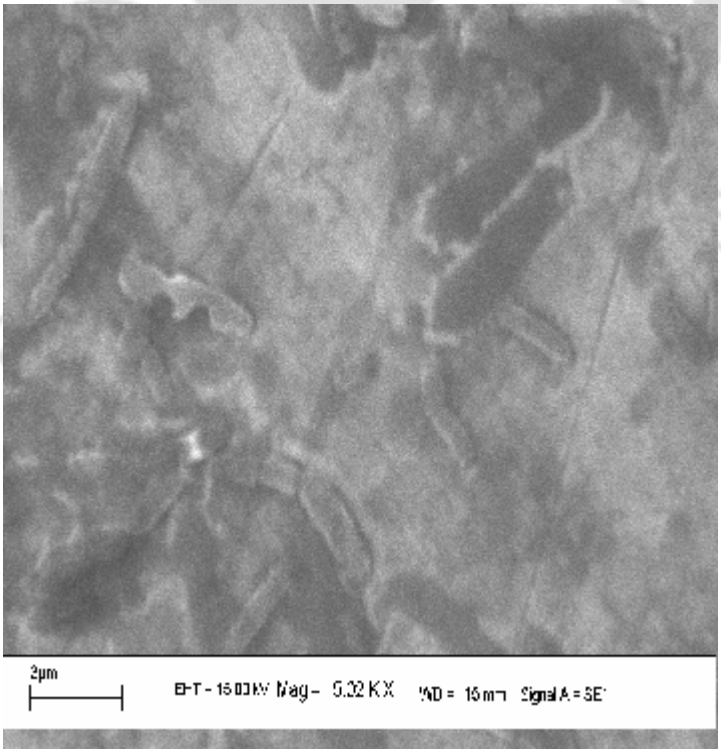


Figure 7.7 (h) *B. coagulans* treated with the alkaline protease from SVB1.

7.4 Conclusions

The following conclusions were drawn from the research work presented in this chapter. Both crude and the partially purified alkaline proteases performed significantly for the treatment of effluents from tannery, dairy and domestic sewage. The reduction of TS, COD and BOD in all the effluents was achieved within 48 h using all the enzymes, and 72 h with the viable cells of SVB1. In the control flask, even after 90 h, the reduction in either of the parameter (TS, COD and BOD) was not at all significant. Disintegration of sludge structure using crude alkaline protease from *Bacillus pseudofirmus* SVB1 was studied to improve the degradation of organic matter in the digestion step, leading to better dewaterability, better compactation and less volume resulting in reduced transport costs and easier handling of the sludge after dewatering step. Both the crude as well as the partially purified enzymes was found to reduce the microbial count in the domestic sewage. The enzyme was found to lyse and inhibit growth of the cells of both Gram positive and Gram negative species (*B. coagulans*, *P. carotovorum*, *S. marcescens* and *W. eutropha*). The application of the alkaline protease has potential for sewage sludge processing through its combined beneficial effects in pathogen reduction, solids reduction and improved dewatering (solids settling). Alkaline protease from *Bacillus pseudofirmus* SVB1 was found to solubilize abattoir solid wastes efficiently.

References

Batstone, D.J., Keller, J., Newel, R.B and Newland, M. (1997) Model development and full scale validation for anaerobic treatment of protein and fat based wastewater. *Wat. Sci. Tech.*, **36**: 423-431.

Bhaskar, N., Sudeepa, E.S., Rashmi, H.N. and Selvi, A. T. (2007) Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresour. Technol.*, **98**: 2758-2764.

Caixeta, C.E.T., Cammarota, M.C. and Xavier, A.M.F. (2002) Slaughterhouse wastewater treatment: evaluation of a new three-phase separation system in a UASB reactor. *Biores. Technol.*, **81**: 61-69.

Cammarota, M.C., Teixeira, G.A. and Freire, D.M.G. (2001) Enzymatic pre-hydrolysis and anaerobic degradation of wastewaters with high fat content. *Biotechnol. Lett.*, **23**: 1591-1595.

Dalev, P.G. (1994) Utilisation of waste feathers from poultry slaughter for production of a protein concentrate. *Biores. Technol.*, **48**: 265-267.

Danalewich, J.R., Papagiannis, T.G., Belyea, R.L., Tumbleson, M.E. and Raskin, L. (1998) Characterization of dairy waste streams, current treatment practices and potential for biological nutrient removal. *Water Res., 32*: 3555-3568.

Das, C., Patel, P., De, S. and Das Gupta, S. (2006) Treatment of tanning effluent using nanofiltration followed by reverse osmosis. *Sep. Purif. Technol.*, **50**: 291-299.

Dean, C.R. and Ward, O.P. (1991) Nature of Escherichia coli cell lysis by culture supernatants of Bacillus species. *Appl. Environ. Microbiol.*, **57**(7): 1893-1898.

Dhaneshwar, R.S. (1990) Tannery Wastewater Treatment and Disposal - State of Art. *Indian J. Environ. Protection*, **10**:131-141.

Dumontet, S., Dinel, H. and Baloda, S.B. (1999) Pathogen reduction in sewage sludge by composting and other biological treatments: A review. *Biol. Agric. Horticulture*, **16**: 409–430.

Frølund, B., Griebe, T. and Nielsen, P.H. (1995) Enzymatic activity in the activated-sludge floc matrix. *Appl. Microbiol. Biotechnol.* **43**: 755-761.

Gajju, H., Bhalla, T.C. and Agrawal, H.O. (1996) Thermostable alkaline protease from *Bacillus coagulans* PB-77. *Indian J. Microbiol.*, **36**:153-155.

Higgins, M.J. and Novak, J.T. (1997) Characterization of exocellular protein and its role in bioflocculation. *J. Environ. Eng.*, **123**: 479-485.

Jung, J., Jinho Jung, X.K., Xing, X. and Matsumoto, K. (2001) Kinetic analysis of disruption of excess activated sludge by DynoMill and characteristics of protein release for recovery of useful materials. *Biochem. Eng. J.*, **8**: 1-7.

Lamas, E.M., Barros, R.M., Balcão, V.M. and Malcat, F.X. (2001) Hydrolysis of whey proteins by proteases extracted from *Cynara cardunculus* and immobilized onto highly activated supports. *Enzyme Microb. Technol.*, **28**: 642-652.

Leal, M.C.M.R., Freire, D.M.G., Cammarota, M.C. and Sant'Ann, Jr.G.L. (2006) Effect of enzymatic hydrolysis on anaerobic treatment of dairy wastewater. *Process Biochem.*, **41**: 1173-1178.

Lefebvre, O., Vasudevan, N., Torrijos, M., Thanasekaran, K. and Moletta, R. (2006) Anaerobic digestion of tannery soak liquor with an aerobic post-treatment. *Water Res.*, **40**: 1492-1500.

Ludzack, F.J. and Noran, P.K. (1965) Tolerance of high salinities by conventional wastewater treatment process. *J. Wat. Pollut. Control Fed.*, **37**: 1404-1416.

Marshall, K.R. and Harper, W.J. (1988) Whey protein concentrates. *Bull. Int. Dairy Fed.*, **233**: 22-32.

Martínez, J., Borzacconi, L., Mallo, M., Galisteo, M. and Viñas, M. (1995) Treatment of slaughterhouse wastewater. *Water Sci. Technol.*, **32**(12): 99-104.

Massé, D.I. and Masse, L. (2001) The effect of temperature on slaughterhouse wastewater treatment in anaerobic sequencing batch reactors. *Bioresour. Technol.*, **76**: 91-98.

Masse, L., Massé, D.I. and Kennedy, K.J. (2003) Effect of hydrolysis pretreatment on fat degradation during anaerobic digestion of slaughterhouse wastewater. *Process Biochem.*, **38**: 1365-1372.

Mohseni-Bandpi, A. and BazariIranian, H. (2004) Biological treatment of dairy wastewater by sequencing batch reactor. *J. Env. Health Sci. Eng.*, **1**: 65-69.

Mukhopadhyay, R., Talukdar, D., Chatterjee, B.P. and Guha, A.K. (2003) Whey processing with chitosan and isolation of lactose. *Process Biochem.*, **39**: 381-385.

Omil, F., Garrido, J., Arrojo, B. and Méndez, R. (2003) Anaerobic filter performance for the treatment of complex dairy wastewater at industrial scale. *Water Res.*, **37**(17): 4099-4108.

Onyeche, T.I., Schläfer, O., Bormann, H., Schröder, C. and Sievers, M. (2002) Ultrasonic cell disruption of stabilised sludge with subsequent anaerobic digestion. *Ultrasonics*, **40**: 31-35.

Parmar, N., Singh, A. and Ward, O.P. (2001) Enzyme treatment to reduce solids and improve settling of sewage sludge. *J. Ind. Microbiol. Biotechnol.*, **26**: 383-386.

Pavlova, I.N., Zholner, L.G. and Tinyanova, N.Z. (1987) Lytic characteristics of extracellular enzymes from thermophilic strain spore rod. *In Proceedings of Fourth Europiam congress on Biotechnology*, vol **2**, eds. Neijssel O.M., van der Meer R.R. and Luyben K.C.A.M. PP. 201, Weinheim, Verlag Chemie. ISBN 0444428283.

Purkait, M.K., Bhattacharya, P.K., De, S. (2005) Membrane filtration of leather plant effluent: Flux decline mechanism. *J. Memb. Sci.*, **258**: 85-96.

Purkait, M.K., Dinesh Kumar, V. and Maity, D. (2009) Treatment of leather plant effluent using NF followed by RO and permeate flux prediction using artificial neural network. *Chem. Eng. J.*, **151**: 275-285.

Rajagopalu, V. and Kammani, S. (2008) Activated sludge process treatability studies on combined tannery and domestic waste water. J. Indian Water Works Assoc., **40**:143-149.

Rajesh Banu, J., Anandan, S., Kaliappan, S. and Yeom, I-T. (2008) Treatment of dairy wastewater using anaerobic and solar photocatalytic methods. *Solar Energy*, **82**: 812-819.

Rao, J.R., Chandrababu, N.K., Muralidharan, C., Nair, B.U., Rao, P.G. and Ramasamy, T. (2003) Recouping the wastewater: a way forward for cleaner leather processing., *J. Clean. Prod.*, **11**: 591-599.

Roman, H.J., Burges, J.E. and Pletschke, B.I. (2006) Enzyme treatment to decrease solids and improve digestion or primary sludge. *Afri. J. Biotechnol.*, **5**(10): 963-967.

Ruggaber, T.P. and Talley, J.W. (2006) Enhancing Bioremediation with Enzymatic Processes: A Review. *Practice Period. Haz. Toxic Radioactive Waste Manage.*, **10**: 73-85.

Slein, M.W. and Logan, G.F. Jr. (1967) Lysis of Escherichia coli by ethylenediaminetetraacetate and phospholipases as measured by ,B-galactosidase activity. *J. Bacteriol.*, **94**: 934-941.

Tiehm, A., Nickel, K., Zellhorn, M. and Neis, U. (2001) Ultrasonic waste activated sludge disintegration for improving anaerobic stabilization, *Water Res.*, **35**(8): 2003-2009.

Watson, S.D., Akhurst, T., Whiteley, C.G., Rose, P.D. and Pletschke, B.I. (2004) Primary sludge floc degradation is accelerated under biosulphidogenic conditions. Enzymological aspects. *Enzyme Microb. Technol.*, **34**: 595-602.

CHAPTER 8
CONCLUSIONS AND FUTURE ASPECTS

8.1 Significant Findings of the Present Research Work

A new strain growing in a wide range of pH was isolated from the premises of tannery industry. Based on 16S rRNA sequence analysis, the strain was identified to be *Bacillus pseudofirmus* having 98% similarity with the nearest neighbor *Bacillus pseudofirmus* (GenBank accession no. AB029256.1) in the phylogenetic tree and was designated as *Bacillus pseudofirmus* SVB1 (GenBank accession no. EU533950). It was observed to be capable of dehairing goat skin and hydrolyzing different poultry slaughter house waste, clotted blood, egg albumin, poultry feather. A new screening media containing gelatin as sole source of carbon and nitrogen was designed for alkaline protease producers.

Statistical design of experiments including Plakett-Burman design and central composite designs were employed successfully for optimization of alkaline protease production. Multi-response optimization was compared with the single response optimization for the optimization of physical process parameters. It was observed that there was not much compromise in the optimal levels of the responses when multi-response optimization was performed as compared to the single response optimization study. The optimal levels of pH, temperature and rpm of the shaking incubator were found to be 9.6, 28°C and 191, respectively, and an enhancements of 30%, 18% and 32% were achieved in cell growth, alkaline protease activity and specific activity respectively. A low cost medium was formulated for alkaline protease production, in which low cost

C/N source, casein that replaced the high cost media components *viz*., yeast extract and beef extract. Under the optimal levels of medium components, a significant improvement (4.9 fold) in the production of alkaline protease by SVB1 was accomplished using casein as sole source of carbon and nitrogen.

An unusually large (85 kDa) serine alkaline protease was purified in a two step purification process. The enzyme was found to be stable in a wide range of pH, 7.0-12.0. The optimum activity was exhibited by the enzyme at pH of 10.0. The enzyme had shown activity in the mesophilic range of temperature from 20-50°C. The optimum activity was achieved at 40°C. The low K_m value (1.83 mg/ml) and high V_{max} value (533.83 U) of the alkaline protease inferred that the high substrate affinity and catalytic efficiency, respectively. Its activity (under harsh conditions) in the presence of bleaching agent, surfactants, organic solvents and high metal ion concentrations was very interesting from industrial viewpoint.

The introduction of an enzyme-based cleaning step is likely to reduce the need for subsequent acid and/or alkali treatment in food industry for bioprocess equipments. The performance of alkaline protease from SVB1 was found to be better than the other commercial alkaline proteases used in this study.

High efficiency in dehairing and maintaining good quality of the leather was achieved with application of the alkaline protease obtained in this study, which was even better than few other commercial alkaline protease preparations available in the market. The effluent from the dehairing process carried out with the alkaline protease from SVB1 was having less pollution as compared to the other commercial alkaline proteases used in this study as well from other process reported in the literature.

The efficiency of the alkaline protease from SVB1 for the treatment of tannery, dairy and domestic sewage effluents was explored and evaluated in this investigation. TS, BOD and COD was reduced reasonably in the case of all the effluents from tannery, dairy and domestic sewage used in this study after treatment with the alkaline protease from SVB1. The pH of tannery effluent was reduced to 7.4 from 11.6. The settable sludge was reduced by 67% in domestic sewage. The microbial count of domestic sewage was reduced from 15×10^4 to 4×10^2 after treatment with the alkaline protease from SVB1.

The antibacterial activity of this enzyme against the domestic sewage borne microbes and few potential human and plant pathogens draws special attention for its use in aquaculture bioremediation.

8.2 Future Aspects

The present research work is carried out with the wild strain. Strain improvement may be of particular interest to improve the production further. Protein engineering studies may be carried out to improve the functions of the alkaline protease further.

Biochemical characterization of the recombinant alkaline protease obtained from protein engineering to evaluate the industrial value of the recombinant enzyme.

The toxicity or allergenic response induced by the protein, if any, is to be tested before applying it to food processing sector.

Scaling up of production is to be acquired to meet the required demands of the industries. Scaling up of the applications (dehairing and effluent treatment) may find potential use in the large scale industries.

APPENDIX I

BLAST ALGORITHM

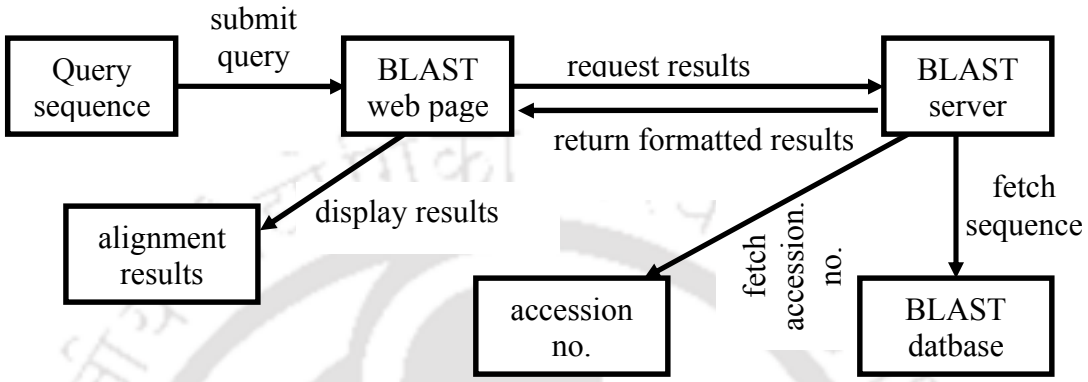


Figure A.I.1 Algorithm for bacterial identification by analyzing its DNA sequence using BLAST

APPENDIX II

SCHEMATIC PRESENTATION OF STATISTICAL EXPERIMENTAL DESIGN

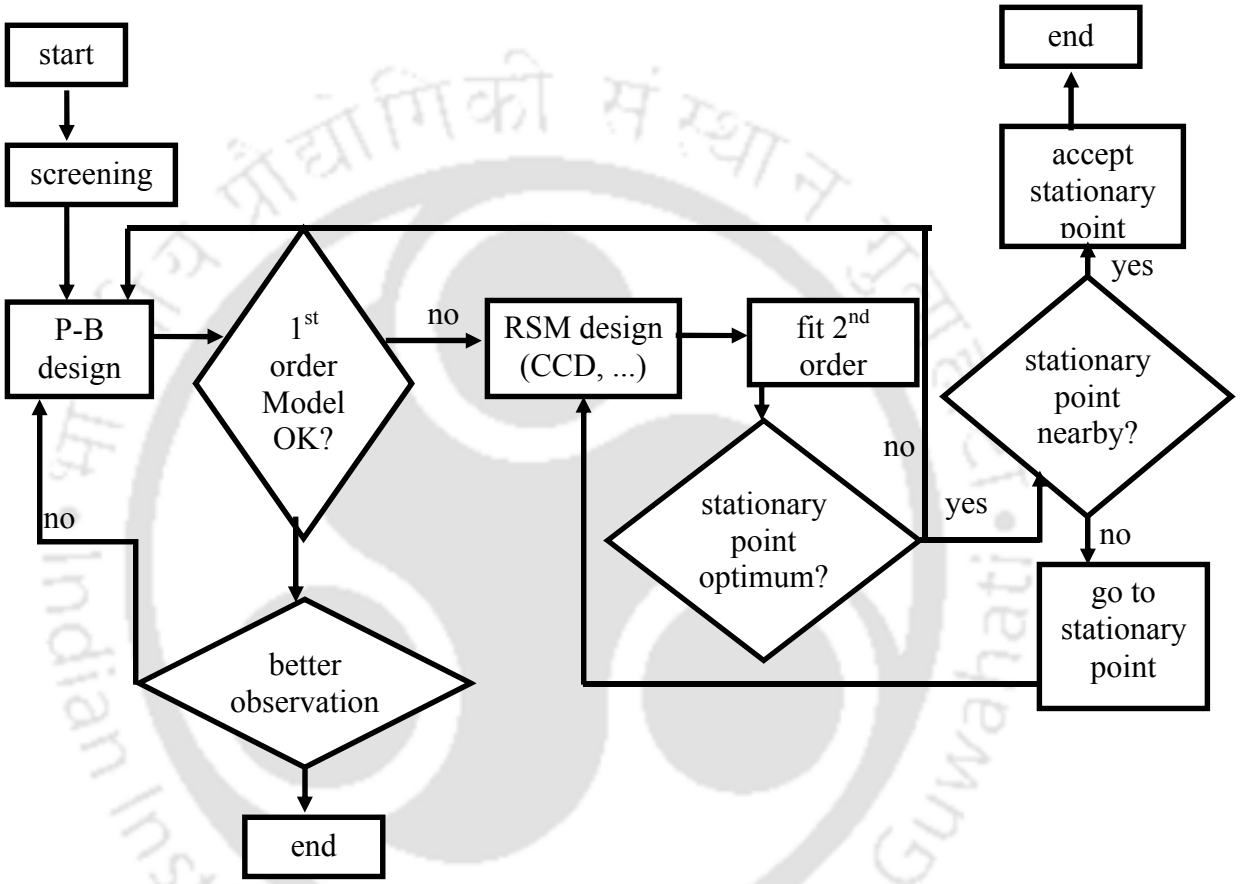


Figure A.II.1 Algorithm of optimization starting from screening of the statistically significant variable to find out the optimal value.

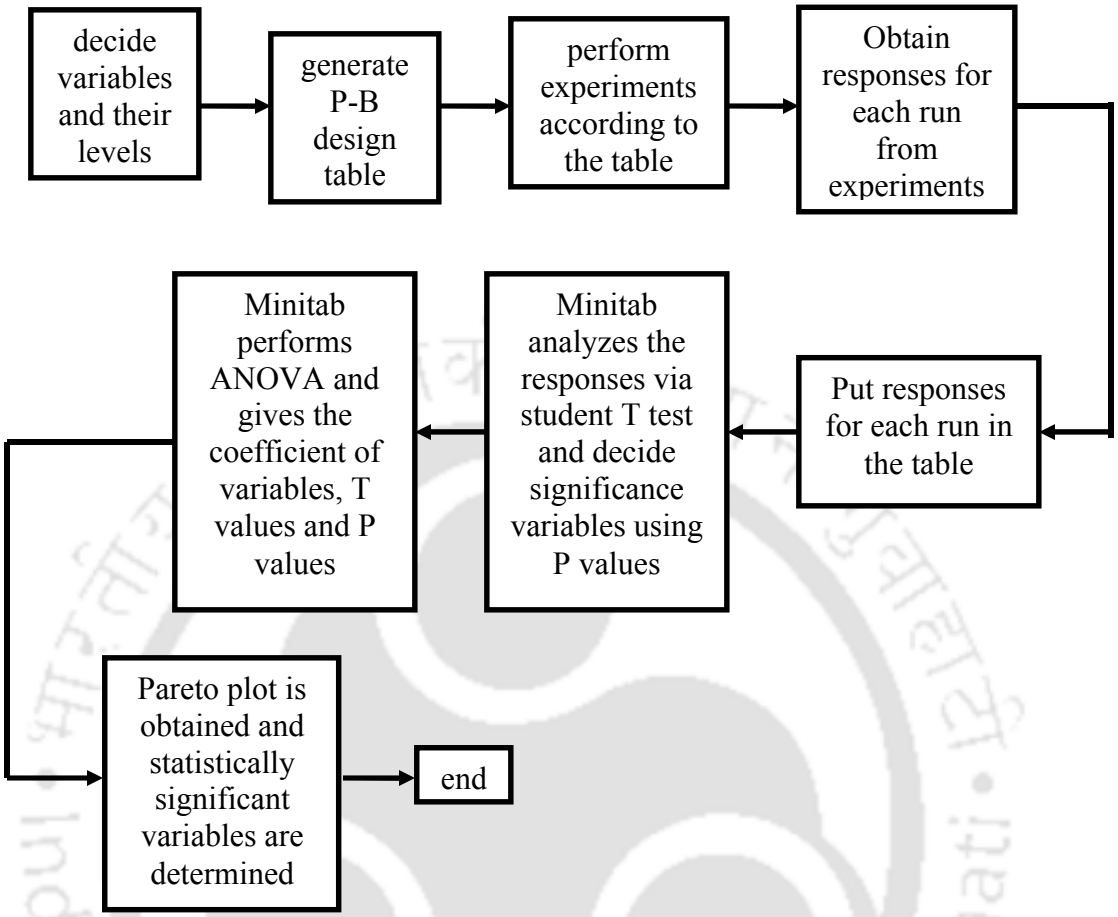


Figure A.II.2 Algorithm of Placket-Burman experimental design for screening of the statistically significant variable.

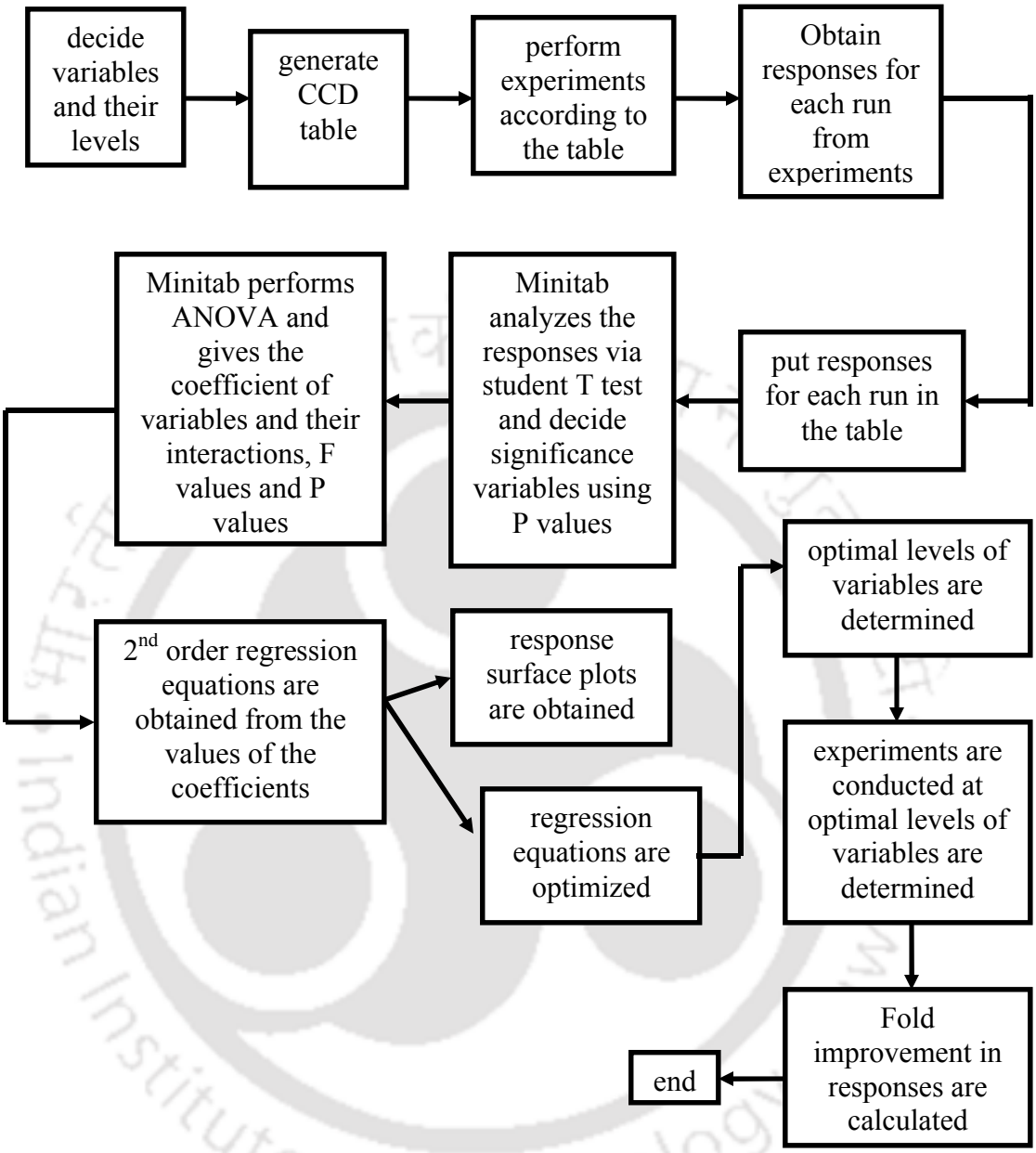


Figure A.II.3 Algorithm of central composite design for optimizing the responses.

APPENDIX III

PLOTS FOR DETERMINATION OF $t_{1/2}$ AND k_d

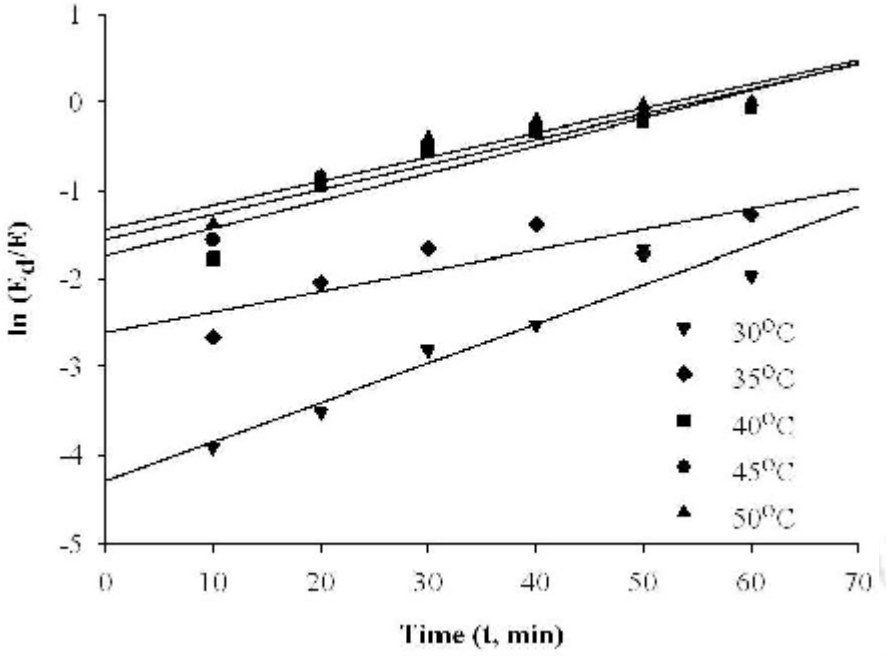


Figure A.III.1 Plot of ln (E_d/E) Vs. time at pH 7.0.

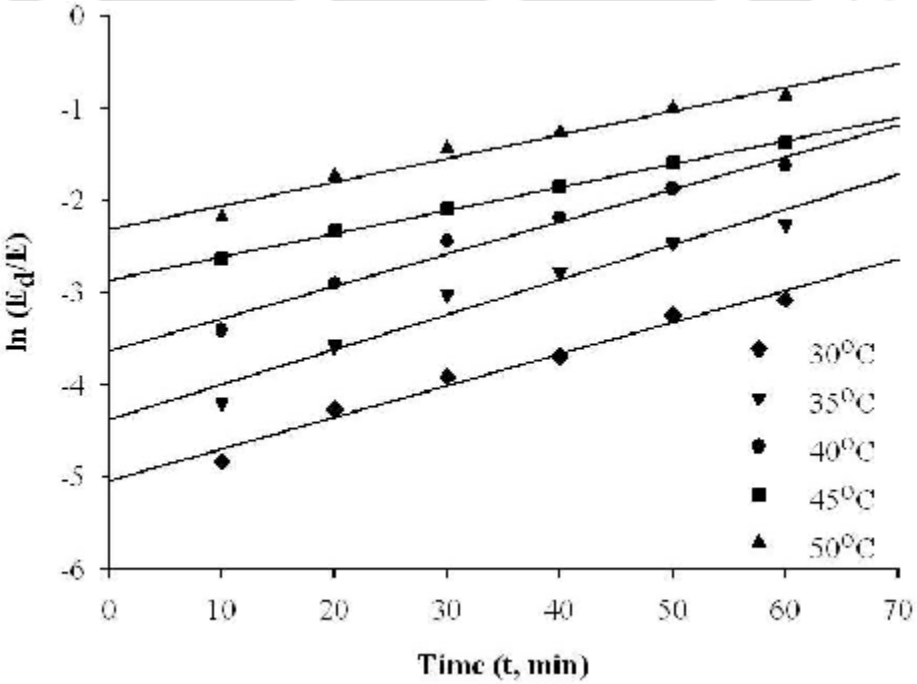


Figure A.III.2 Plot of ln (E_d/E) Vs. time at pH 9.0.

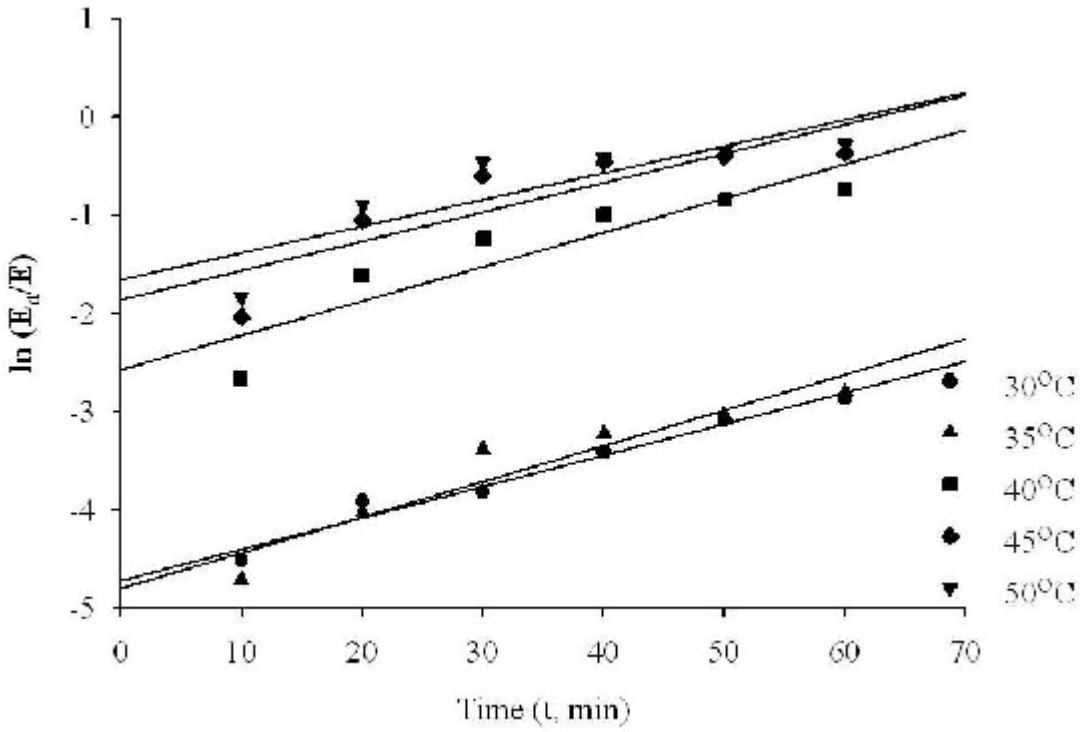


Figure A.III.3 Plot of ln (E_d/E) Vs. time at pH 10.0.

APPENDIX IV

PLOTS FOR DETERMINATION OF ΔH*, ΔS*, E_A, AND k_0

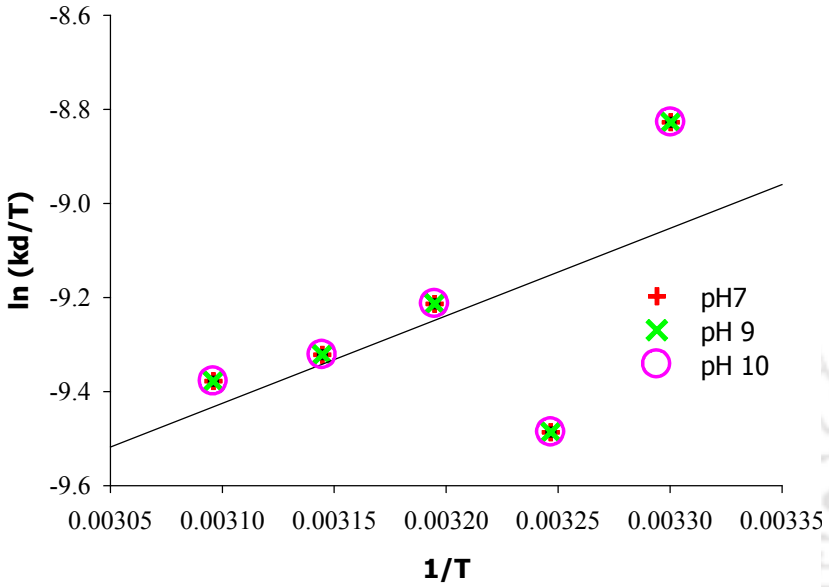


Figure A.IV.1 Plot of ln (k_d/T) Vs. 1/T.

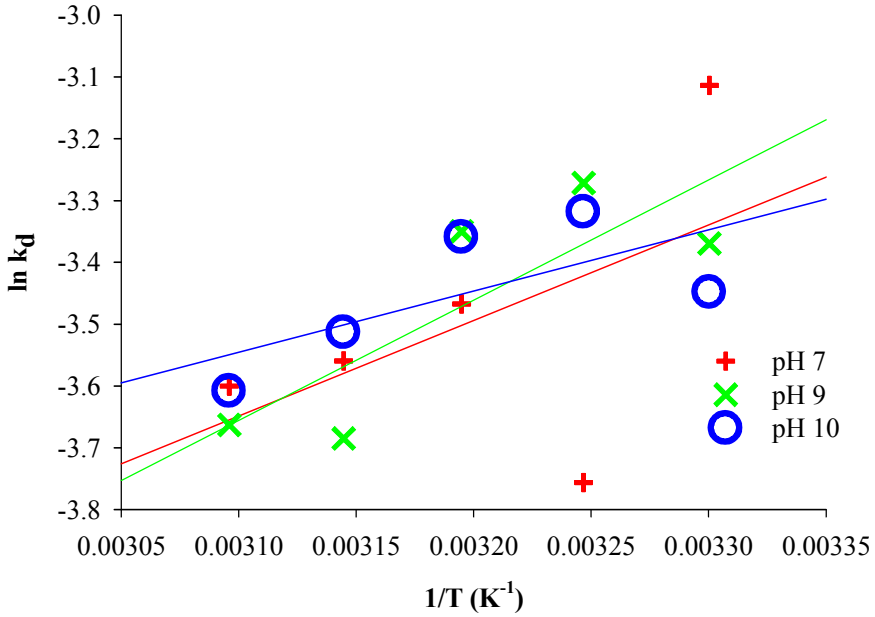


Figure A.IV.2 Plot of ln (k_d) Vs. 1/T.

LIST OF PUBLICATIONS

Journals

1. **Sen S.,** Venkata Dasu V. and Mandal B. (2007) Developments in Directed Evolution for Improving Enzyme Functions. *Appl*. *Biochem*. *Biotechnol*., **143**: 212-223.
2. **Sen S.,** Venkata Dasu V. and Mandal B. (2009) Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1. *Ann*. *Microbiol*., **59**(3): 1-8.
3. **Sen S.,** Venkata Dasu V. and Mandal B. (2009) Medium Development for Enhanced Production of Alkaline Protease from a Newly Isolated *Bacillus pseudofirmus* SVB1. *Asia Pac*. *J*. *Chem*. *Eng*. (*In Press,* DOI: **10.1002/apj.417**).

International Conference Proceedings

1. **Sen S.**, Venkata Dasu V. and Mandal B. Screening of medium components for alkaline protease production from a newly isolated microorganism using Plackett-Burman experimental design. International Conference on New Horizons in Biotechnology **'NHBT 2007'** November 26-29, 2007, Trivandrum, India
2. **Sen S.**, Venkata Dasu V. and Mandal B. The efficiency of crude alkaline protease from newly isolated *Bacillus pseudofirmus* SVB1 in removing protein residues from model solid (mica) surface. International congress on Bioprocesses in Food Industries & 5th BRSI Convention of the Biotech Research Society **'ICBF 2008'** November 6-8, 2008, Hyderabad, India

3. **Sen S.**, Venkata Dasu V. and Mandal B. Potential application of alkaline protease from *Bacillus pseudofirmus* SVB1 for removal of pathogens from sewage sludge and its solubilisation. International Congress of Environmental Research **'ICER 2008'** December 18-20, 2008, Goa, India.
4. **Sen S.**, Venkata Dasu V. and Mandal B. Applications of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1 in slaughter house waste biodegradation. 1st International Society BioTechnology Conference **'ISBT2008'** December 28-30, 2008, Gangtok, Sikkim, India.
5. **Sen S.**, Mandal B. and Venkata Dasu V. Alkaline protease: a tool to clean environment. **AIChE** Spring National Meeting and 5th Global Congress on Process Safety, Apr 26-30, **2009**, Tampa Convention Center, Tampa.

National Conference Proceedings

1. **Sen S.,** Venkata Dasu V. and Mandal B. Studies on effect of physical process parameters on production of alkaline protease from tannery waste soil isolate. 59th Annual session of Chemical Engineering Congress **'CHEMCON 2006'** December 27-30, 2006, Gujarat, India.
2. **Sen S.,** Venkata Dasu V. and Mandal B. Studies on effect of fermentation medium components on production of alkaline protease from tannery waste soil isolate. 60th Annual session of Chemical Engineering Congress **'CHEMCON 2007'** December 27-30, 2007, Kolkata, India.
3. **Sen S.**, Venkata Dasu V. and Mandal B. Ecofriendly applications of alkaline protease from newly isolated *Bacillus pseudofirmus* SVB1. **ChEmference-2008** July 5-6, 2008, Kanpur, India.

VITAE

The author was born on June 6, 1973 in Rourkela, Orissa, India. She passed the High School Certificate Examination conducted by Board of Secondary Education, Orissa (10th Class), India in 1988, and Higher Secondary Examination conducted by Council of Higher Secondary Education (12th Class) Orissa, India in 1990. She completed B.E. in Chemical Engineering from Indira Gandhi Institute of Technology, Sarang, Orissa, India, in 1998. She did M.Tech. in Biotechnology from Jadavpur University, Kolkata, India in 2003. Her M.Tech. project was on site directed mutagenesis that included cloning of Cholera Toxin (CT) gene from a pathogenic strain of Vibrio cholerae, transformation, conjugation and expression of the toxin in a non-pathogenic strain of Vibrio cholerae.

Ms. Shampa Sen worked for nearly four years in different engineering colleges teaching various Chemical Engineering courses viz., Mass Transfer, Heat Transfer etc., and Biotechnology courses viz., Molecular Biology, Biochemistry, Genetic Engineering, and Biochemical engineering to both under graduate and post graduate students.

Ms. Sen joined the Ph.D. program in January 2006 in the Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati 781 039, Assam, India. She had qualified the National Eligibility Test for lecturership conducted jointly by the Council of Scientific and Industrial Research and University Grants Comission. She received Senior Research Fellowship during her Ph.D. under the scheme run by the Ministry of Human Resource and Development (MHRD), India. She successfully completed the course work with 9.25/10 CPI. She gave the Open (Ph.D. Synopsis) Seminar on November 23, 2009 and presented her thesis work before the Doctoral Committee and her performance was satisfactory. She submitted the Ph.D thesis in January 2010. Presently, she is continuing as Associate Professor in the School of Biotechnology, Chemical and Biomedical Engineering of Vellore Institute of Technology University, India.