3

Experimental Procedure

Enzymes are used in many environmental-friendly industrial processes, as they are selective, efficient, speed up reactions by forming transition state complexes with their substrates and reduce the activation energy of the reactions. With the recent advancement in biotechnology and microbiology there has been a growing interest and demand for enzymes with novel properties. Microorganisms are the most essential sources for the production of various enzymes. Microbial enzymes are more stable and can be easily isolated (Alaria *et al.,* 2013). Their concentration can be increased by manipulating the organism's environment and genes, due to their biochemically divergent characteristics. These attempts are now being used to reduce the traditional enzyme production from complex eukaryotes (Pandey *et al*., 2000). The selection of beneficial microorganisms that producing industrial enzymes are considerable with new physiological properties and tolerance to extreme conditions used in the industrial processes such as temperature, salt concentration and pH (Umayaparvathi *et al.,* 2013).

Among the hydrolytic enzymes, proteases constitute one of the most important groups which are able to degrade proteins into small peptides and amino acids. In the total industrial enzyme market, they report for about nearly 60 percent. Proteases are widely used in detergent, leather, textile, pharmaceutical and food industries. Based on their future potential uses, there has been resumed interest in the discovery of proteases with novel properties and a constant thrust to optimize the parameters for enzyme production (Ali *et al.,* 2017). Among the different types of proteases studied, bacterial proteases are the most significant ones when compared with animal, plant and fungal proteases due to their efficient production, consistency, ease of process modification, optimization and purification. In the elevated output of desirable enzymes, selecting the correct organism plays an important role. Now-a-days, the major proportion of the commercial protease is derived from *Bacillus* strains due to their wide temperature and pH tolerance stability (Sharma *et al.,* 2017).

Purification is the most important step in studying any enzyme or protein. An enzyme purification is a collection of procedures designed to isolate a single type of enzyme from a complex mixture. Enzyme purification is essential for the characterization of the structure, functions and interaction of the enzyme of interest. The purification method also improves the specific activities of enzymes and making them more specific for industrial applications. The purification techniques used to isolate enzymes from the culture filtrate are based on various factors like solubility (ammonium sulphate precipitation), molecular weight (dialysis, gel filtration chromatography) and charge-based resins (ion exchange chromatography). FPLC is utilized to analyze or purify a mixture of proteins. The separation of complex protein samples and other biological molecules with high resolution can be done by polyacrylamide gel electrophoresis (Castillo-Yanez *et al.,* 2004).

Characterization of a purified enzyme includes determination of its optimum pH, temperature, kinetics and studying the effect of metal ions, inhibitors and oxidizing agents. By optimizing these parameters, maximum enzyme function and efficiency as desired can be ensured (Parameswaran *et al.,* 2013).

As the number of feasible applications increase, the demand of huge requirement of enzymes becomes a restricting factor. In order to solve the defects in the use of enzymes, research was initiated on a variety of reusable enzymes which could increase their applicability through improved enzyme technologies like immobilization. Furthermore, immobilization induces enzyme stiffness, prevents aggregation of enzymes and autolysis. It also provides considerable stability towards variations in temperature and organic solvents, creates a more suitable environment and high enzymatic stabilization (Obregon *et al.,* 2015).

By considering the above aspects, the present study entitled **'Characterization, Immobilization and Applications of Extracellular Protease from *Bacillus* sp. ASASBT isolated from Termite Soil'** was taken up with the view of producing industrially important enzyme using the potential source and reducing the production cost of the enzyme namely protease.

The experimental design and protocols adopted for the present study are discussed under six phases as follows:

3.1 Phase I : Isolation of bacteria from different environmental soil samples

3.1.1 Selection of microbial source

3.1.2 Collection of soil samples

3.1.3 Isolation of bacteria

3.1.4 Screening for enzymes

3.1.5 Assay of enzymes

3.2 Phase II: Identification of the selected protease-producing bacteria and optimization of media components for protease production

3.2.1 Identification of protease producing bacteria

3.2.1.1 Morphological characterization

3.2.1.2 Biochemical characterization

3.2.1.3 Scanning Electron Microscopy (SEM) analysis

3.2.1.4 16S rRNA gene sequencing analysis

3.2.1.5 Identification of protease gene

3.2.2 Optimization of media components for protease production

3.2.2.1 Basal media used for the production of protease

3.2.2.2 Extraction of enzyme

- Qualitative method
- Quantitative method

3.2.2.3 Effect of incubation time on protease production

3.2.2.4 Effect of pH on protease production

3.2.2.5 Effect of temperature on protease production

3.2.2.6 Effect of inoculum size on protease production

3.2.2.7 Effect of carbon sources on protease production

3.2.2.8 Effect of nitrogen source on protease production

3.2.2.9 Effect of agitation rate on protease production

3.2.2.10 Effect of natural substrates on protease production (Solid State Fermentation (SSF))

3.3 Phase III: Purification and molecular characterization of protease

3.3.1 Purification of protease

3.3.1.1 Precipitation with ammonium sulphate

3.3.1.2 Dialysis

3.3.1.3 Ion exchange chromatography using AKTA FPLC system

3.3.1.4 Gel filtration chromatography using AKTA FPLC system

3.3.2 Purification profile of isolated protease

3.3.2.1 Assay of protease

3.3.2.2 Estimation of protein

3.3.2.3 Specific activity of protease

3.3.2.4 Recovery percentage of protease

3.3.2.5 Purification fold of protease

3.3.3 Molecular weight of protease

3.3.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

3.3.3.2 Confirmation of protease activity by zymography

3.3.4 Identification of protein by MALDI-TOF/TOF MS analysis

3.4 Phase IV: Characterization of purified protease

3.4.1 Effect of pH on activity and stability of purified protease

3.4.2 Effect of temperature on the activity and stability of purified protease

3.4.3 Effect of substrate concentration on the activity of purified protease

3.4.4 Effect of metal ions on purified protease activity

3.4.5 Effect of inhibitors on purified protease activity

3.4.6 Effect of surfactants and oxidizing agents on purified protease activity

3.4.7 Effect of organic solvents on purified protease activity

3.4.8 UV-Visible spectroscopy of purified and commercial protease

3.4.9 Fourier Transform Infrared (FTIR) spectroscopy of purified and commercial protease

3.5 Phase V: Immobilization of purified protease

3.5.1 Activity of immobilized protease

3.5.2 Characterization of immobilized protease

3.5.2.1 Effect of pH and temperature of immobilized protease

3.5.2.2 Storage stability of immobilized protease

3.5.2.3 Reusability of immobilized protease

3.5.2.4 Surface morphology of immobilized protease

3.6 Phase VI: Applications of protease

3.6.1 Application in detergent industry

3.6.1.1 Action of protease on stains

3.6.1.2 Preparation of enzyme-based detergent cake

3.6.2 Application in leather industry

3.6.2.1 Action of protease on animal skin

3.6.3 Application in pharmaceutical industry

3.6.3.1 Antibacterial activity of protease

3.6.3.2 Clot lysis activity of protease

3.6.4 Application in photographic industry

3.6.4.1 Silver recovery from waste x-ray film

3.6.5 Application in textile industry

3.6.5.1 Activity of protease on degumming of silk

3.1 Phase I : Isolation of bacteria from different environmental soil samples

It has been observed that microorganisms grown on soil are much more efficient in the production of enzymes than plants and animals. Therefore, it was decided to select soil samples as the source of microorganisms for the present study.

3.1.1 Selection of microbial source

Proteases are essential enzymes, which are ubiquitous in nature. To satisfy the requirements of rapidly increasing sectors, it is worthwhile to screen microorganisms from new habitats for proteases with novel properties. They are isolated from different sources by spread plating on an alkaline medium and subsequent screening for the desired characteristics. A variety of proteases are produced by microorganisms depending on the

species of the producer or the strains. Based on the above, a search began for better, less expensive and more readily available sources of such enzymes and this led to the development of processes for producing microbial enzymes on a commercial scale. Hence, in the present study, four types of microbial sources were selected termite mound soil (TMS), organic waste degraded soil (OWS), textile effluent degraded soil (TES) and marine soil (MRS) for the isolation of protease and further for its purification, characterization and immobilization.

3.1.2 Collection of soil samples

The soil samples varying from natural habitats to extreme habitats were collected at random within the southern region of Tamil Nadu like area around Karur District, Coimbatore District, Tirupur District and Karaikal District respectively. They were collected with sterile spatula from 4-5 cm depth and transferred into sterile 50 ml blue capped polystyrene tubes. From each site, a minimum of two samples were collected and then immediately transferred to the laboratory and stored at 4°C. These samples were used without any pretreatment.

Table 2
Microbial sources and abbreviations

Areas/Districts	Habitats	Sample Abbreviations
Karur	Termite Mound Soil	TMS
Coimbatore	Organic Waste degraded Soil	OWS
Tirupur	Textile Effluent degraded Soil	TES
Karaikal	Marine Soil	MRS

3.1.3 Isolation of bacteria

The isolation of bacteria from samples (TMS, OWS, TES and MRS) were carried out by serial dilution technique (Lecomte *et al.*, 2011 and Dam *et al.*, 2013). The detailed procedures are explained in Appendix I.

The isolated bacterial colonies from all the samples were purified by sub culturing three times and storing as slants at 4°C. The isolated bacterial colonies were than screened for enzymes.

3.1.4 Screening for enzymes

Screening can be one of the most effective and successful ways to find new or appropriate microbial enzymes (Shimizu *et al.,* 1997). The bacterial colonies were screened for various enzymes like amylase, cellulase, protease and lipase. The detailed procedure is given in Appendix II, III, IV and V.

3.1.5 Assay of enzymes

By means of extensive screening, many novel microbial enzymes, which have excellent potential to produce biologically and chemically beneficial compounds can be identified. The main factors, which must be considered for assaying enzymes are temperature, pH, ionic strength and the proper concentrations of the essential components like substrates and enzymes (Bisswanger, 2014). The isolated bacterial colonies were also screened for various enzymes like cellulase, amylase, lipase and protease. Amylase was assayed by the method of Torrado *et al.,* (2012), cellulase by Miller (1959), protease by Pant *et al.,* (2015) and lipase by Pignede *et al.,* (2000) respectively. Appendix VI, VII, VIII and IX described the procedure for the same.

3.2 Phase II: Identification of the selected protease-producing bacteria and optimization of media components for protease production

3.2.1 Identification of protease producing bacteria

From the enzyme assays, the bacteria producing maximum protease was identified based on the morphological, cultural and biochemical characteristics by following the Bergey's Manual of Systematic Bacteriology (Sneath *et al.,* 1986).

3.2.1.1 Morphological characterization

The protease-producing colonies were morphologically identified by Gram staining, spore staining and motility study. Appendix X gives the procedure for the same.

3.2.1.2 Biochemical characterization

The maximum protease-producing strain was isolated and identified by conventional biochemical tests in accordance with Bergey's Manual of Systemic Bacteriology (Sneath *et al.,* 1986). The procedure is detailed in Appendix XI.

3.2.1.3 Scanning Electron Microscope (SEM) analysis

The morphology of the isolated bacterial strain was studied using SEM (HitachiS-3000N, Japan) at various magnifications. Appendix XII explains the detailed procedure.

3.2.1.4 16S rRNA gene sequencing analysis

When compared to morphological and biochemical characterization methods, 16S rRNA analysis is found to be the novel and accurate method for identifying unknown species. The DNA from the selected strain was isolated and the 16S rRNA was amplified and sequenced. The detailed protocol is given in Appendix XIII.

3.2.1.5 Identification of protease gene

Identification of protease gene from *Bacillus* sp. ASASBT was carried out using polymerase chain reaction (PCR). Appendix XIV describes the elaborate procedure.

3.2.2 Optimization of media components for protease production (Das and Prasad, 2010)

The enzyme production of organisms is strongly influenced by medium components. Besides the nutritional factors, the cultural parameters play a primary task in biological processes. So, the media components need to be optimized. It is essential that these organisms be provided with optimal growth conditions to increase enzyme production (Vanitha *et al.,* 2014).

3.2.2.1 Basal media used for the production of protease

Basal medium containing 1% glucose, 0.5% casein, 0.55% yeast extract, 0.2% KH_2PO4, 1% Na_2CO_3, 0.2% $MgSO_4.7H_2O$ at pH 8 was incubated at 37°C for 120 hours in an orbital shaker (Pant *et al.,* 2015).

3.2.2.2 Extraction of enzyme

In casein broth medium, the protease-producing bacterial colony was inoculated and incubated at 37°C for 48 hours. The cultured medium was filtered aseptically in a laminar air flow chamber using Whatmann No.1 filter paper. To remove unwanted particles, the filtrate obtained was subjected to centrifugation at 10,000 rpm for 10 minutes. The supernatant was used as a crude enzyme preparation for further studies.

Qualitative method

The extracted enzyme was further confirmed by zone of clearance in skim milk agar and casein agar plate. The detailed procedure is explained in Appendix XV.

Quantitative method

The quantitative estimation of enzyme was done by biomass and protease activity. Appendix XVI and VIII describes the elaborate procedure.

3.2.2.3 Effect of incubation time on protease production

The optimized protease production medium was prepared in six 100 ml conical flasks, into which 1% of 24-hour fresh culture of isolated bacteria was inoculated and incubated at 37°C for 72 hours in an orbital shaker with 150 rpm. At a regular time interval, every 12 hours each flask was filtered, followed by centrifugation at 5000 rpm for 30 minutes. The culture filtrate solution obtained was used to assay the protease activity.

3.2.2.4 Effect of pH on protease production

To study the optimum pH for protease production, a wide pH range of 5.0 to 10.0 was used. For the analysis, 100 ml of production media in 6 conical flasks were taken. The media pH was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 respectively, using 1N HCl and 1N NaOH. The 24 hour-old bacterial culture was inoculated and incubated at 37°C with agitation of 100 rpm for 48 hours. After incubation, enzyme assay was carried out (Sevinc and Demirkan, 2011).

3.2.2.5 Effect of temperature on protease production

The production medium at pH 7.0 was inoculated with 24 hour-old culture and incubated at various temperatures from 20°C, 30ºC, 40ºC, 50ºC, 60ºC and 70ºC with 100 rpm agitation for 48 hours. At the end of incubation period, the cell free culture filtrate was used for the enzyme assay (Sevinc and Demirkan, 2011).

3.2.2.6 Effect of inoculum size on protease production

The production medium at pH 7.0 was inoculated with inoculum of varying quantities - 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml per flask, from the suspension culture medium and then incubated at 37°C for 48 hours with shaking at 100 rpm. Each 1.0 ml of bacterial suspension contained 7 X 10^3 cell/ml^{-1}.

3.2.2.7 Effect of carbon sources on protease production

The effect of various carbon sources such as dextrose, lactose, sucrose, starch and maltose at the concentration of 1% was added to the production medium for their effect on enzyme production. The basal medium was used as a control. The culture was incubated at 37°C for 48 hours at 100 rpm.

3.2.2.8 Effect of nitrogen sources on protease production

Various nitrogen sources like gelatin, beef extract, urea, ammonium sulphate and ammonium chloride were supplemented individually to the culture medium to study their effect on enzyme production. The basal medium was used as the control. The culture was incubated at 37°C for 48 hours at 100 rpm.

3.2.2.9 Effect of agitation rate on protease production

The effect of agitation rate on protease production of the isolated bacterial species was studied by incubating culture flasks at four different agitation speeds of 50, 100, 150 and 200 rpm for 48 hours.

3.2.2.10 Effect of natural substrates on protease production (Solid State Fermentation (SSF))

The effect of natural substrates such as castor husk, green gram husk and black gram husk were studied. Appendix XVII gives the detailed procedure.

3.3 Phase III: Purification and molecular characterization of protease

In this phase, the extracellular protease from the isolated bacterial strain was purified and characterized. The methodology adopted for this phase is discussed below.

3.3.1 Purification of protease

Enzyme purification is very essential for developing a better knowledge of the functioning of the enzyme. Different strategies adopted for purification of enzymes are on similar lines as that of proteins. Despite the diversity in the origin of enzymes they are purified using a generalized overall approach, which involves initial recovery of protein, concentration or primary purification and ultimately high-end resolution chromatographic purification (Lakshmi *et al.*, 2018).

3.3.1.1 Precipitation with ammonium sulphate

Ammonium sulfate precipitation is a simple method and is generally used as an initial step to remove other proteins from crude extract. To part of the crude enzyme, ammonium sulphate in varying concentrations 0-20, 20-40, 40-60, 60-80 and 80-100 percent were added and left aside for 60 minutes after which the pellets were collected. The procedure for this is explained in Appendix XVIII.

3.3.1.2 Dialysis

Dialysis is a useful technique for removing salts from ammonium sulphate precipitated solution. The ammonium sulphate precipitated sample from the bacterial isolates was dialyzed against phosphate buffer. The detailed procedure is recorded in Appendix XIX.

3.3.1.3 Ion exchange chromatography using AKTA FPLC system

Fast Protein Liquid Chromatography (FPLC) is an automated liquid chromatography system intended for purification of proteins, peptides and other biomolecules. It is used to analyze or purify mixture of proteins from the crude sample. Appendix XX elaborates the detailed procedure.

3.3.1.4 Gel filtration chromatography using AKTA FPLC system

Gel filtration chromatography procedures were also performed on FPLC AKTA Purifier system at 4°C. The elaborated protocol is given in Appendix XXI.

3.3.2 Purification profile of isolated protease

3.3.2.1 Assay of protease

The protease activity was assayed for all the samples (crude, ammonium sulphate precipitated, dialyzed, ion exchange and gel filtration purified) using casein as the substrate. Appendix VIII gives the details of the protocol.

3.3.2.2 Estimation of protein

The Folin-Lowry method has been extensively used for the estimation of proteins due to its high sensitivity. The protein concentration of the mentioned samples (crude, ammonium sulphate precipitated, dialyzed, ion exchange and gel filtration purified) was determined by the method of Lowry *et al.,* (1951). The protocol for this is given in Appendix XXII.

3.3.2.3 Specific activity of protease

The specific activities of the samples (crude, ammonium sulphate precipitated, dialyzed, ion exchange and gel filtration purified) were calculated by dividing the total protease activity with the protein content. This was done according to the formula given below:

$$\text{Specific activity (U/mg of protein)} = \frac{\text{Total activity unit/ml}}{\text{Total protein mg/ml}}$$

3.3.2.4 Recovery percentage of protease

Recovery percentage of the samples (crude, ammonium sulphate precipitated, dialyzed, ion exchange and gel filtration purified) were calculated from the percentage of total enzyme activity of crude sample and total activity of the sample to be estimated. The formula for this is shown below:

$$\text{Recovery percentage (\%)} = \frac{\text{Total activity of the sample}}{\text{Total activity of crude sample}} \times 100$$

3.3.2.5 Purification fold of protease

Purification fold in each step was calculated by dividing the specific activity of the respective fraction with that of the crude sample. The calculations were done according to the formula given below:

$$\text{Purification fold} = \frac{\text{Specific activity of the sample}}{\text{Specific activity of the crude sample}}$$

3.3.3 Molecular weight of protease

3.3.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is one of the most commonly used gel electrophoretic techniques for proteins. This method provides an easy way to estimate the number of polypeptides in a sample and thus assess the complexity and purity of the sample. SDS-PAGE was carried out with the crude, ammonium sulphate precipitated, dialyzed, ion exchange and gel filtration purified samples using 10% gradient gel on a discontinuous buffer system according to the procedure of Laemmli, (1970). The protein bands obtained from the

samples were stained with Coomassie Brilliant Blue, which can only detect a 50 ng protein band. Due to its low sensitivity and narrow dynamic range, the dialyzed and gel filtration purified were stained with silver stain and viewed, since silver staining of proteins on a gel increases the sensitivity from 10 - 100 folds (Westermeier and Marouga, 2005). The procedure for this is detailed in Appendix XXIII and XXIV.

3.3.3.2 Confirmation of protease activity by zymography

Zymography is an electrophoretic technique, commonly based on sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), which contains a substrate copolymerized within the polyacrylamide gel matrix, for the detection of enzymatic activity. The protease isolated was resolved by zymography using SDS polyacrylamide gel containing substrates like gelatin and casein. The protease activity was then visualized by staining the gels with Coomassie Brilliant Blue. The procedure is detailed in Appendix XXV.

3.3.4 Identification of protein by Matrix Assisted Laser Desorption/Ionization - Time-of-Flight Mass Spectrometry (MALDI-TOF/TOF MS) analysis

MALDI is an ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation. It has been applied to the analysis of biomolecules and large organic molecules which tend to be more fragile and fragment when ionized by more conventional ionization methods. Time of Flight a mass spectrometry method that separates ions by their mass to charge ratio and determines that mass to charge ratio by the time it takes for the ions to reach a detector (Franz *et al.,* 1991). PMF is also an analytical technique for protein identification in which the unknown protein of interest is first cleaved into smaller peptides whose absolute masses can be accurately measured with a mass spectrometer such as MALDI-TOF (Clauser *et al.,* 1999). In Appendix XXVI detailed protocol is given.

3.4 Phase IV: Characterization of purified protease

The purified protease was characterized for various parameters as given below;

3.4.1 Effect of pH on activity and stability of purified protease

The pH of a solution can have several effects on the structure and activity of enzymes. The optimum pH of the purified protease was determined by measuring the activity of the purified protease in buffers of varying pH ranging from 5.0 to 10.0. The protocol for this is explained in Appendix XXVII.

3.4.2 Effect of temperature on the activity and stability of purified protease

Temperature also plays an important role in activation and inactivation of enzymes. Each enzyme has an optimum temperature for maximum activity. The optimum temperature for the purified protease was determined by incubating the enzyme-substrate mixture at various temperatures ranging from 30-80°C using casein as the substrate. Appendix XXVIII gives the protocol for this.

3.4.3 Effect of substrate concentration on the activity of purified protease

Substrate concentration also plays a significant role in enzyme velocity. As the concentration of the substrate increases, the enzyme velocity rises linearly and reaches its maximum level. This is referred to as Vmax. Further increase in substrate concentration usually has no effect on the enzyme activity. This is known as the saturation effect and is exhibited by all enzymes (Palanivelu, 2004). The procedure for this is in Appendix XXIX.

3.4.4 Effect of metal ions on purified protease activity

Metal ions are known to play a role as cofactors for enzyme activities and they often act as salt or ion bridges between two adjacent amino acid residues (Sevinc and Demirkan, 2011).

The effect of various metal ions on the activity of purified protease was determined using Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} and Na^{+} at 1mM and 5mM concentration. Appendix XXX explains the procedure for the same.

3.4.5 Effect of inhibitors on purified protease activity

Proteases can be classified according to their sensitivity to various inhibitors. The nature of the purified protease can be determined by studying the effect of different enzyme inhibitors, including chelating agents and specific group reagents on protease activity (Jellouli *et al.,* 2008).

The effect of enzyme inhibitors on the activity of purified protease was studied with serine protease inhibitor - phenylmethylsulphonyl fluride (PMSF), aspartic protease inhibitor - pepstatin, cysteine protease inhibitor - indoacetic acid and metallo protease inhibitor - ethylene diamine tetraacetic acid (EDTA). Appendix XXXI gives the procedure for this.

3.4.6 Effect of surfactants and oxidizing agents on purified protease activity

The effect of surfactants (Sodium Dodecyl Sulphate (SDS), Cetyl Trimethyl Ammonium Bromide (CTAB) and Triton X-100) and oxidizing agent (H_2O_2) on purified protease was investigated. The procedure is given in Appendix XXXII.

3.4.7 Effect of organic solvents on purified protease activity

The effect of organic solvents - acetone, ethanol, dimethyl sulfoxide (DMSO), methanol and ethyl acetate on protease activity was tested. Appendix XXXIII describes the procedure for the same.

3.4.8 UV-visible spectroscopy of purified and commercial protease

The purified and commercial protease in 100 mM phosphate buffer (pH 7.0) was subjected for the wavelength scan of 200 to 400 nm on UV-visible double beam spectrophotometer (Banik *et al.,* 2018).

3.4.9 Fourier Transform Infrared (FTIR) spectroscopy of purified and commercial protease

FTIR is useful for identification of organic molecular groups and compounds. The functional groups, cross-links and side chains involved in the compound, give rise to characteristic vibrational frequencies in the infra-red range by absorbing the light in the infra-red region of the electromagnetic spectrum. FTIR absorption spectrum of the purified protease was performed by using a Perkin Elmer FTIR spectrometer (Devakate *et al.*, 2009)

3.5 Phase V: Immobilization of purified protease

Immobilization of protease increases the active life of the enzyme and prevents the contamination of the product. The immobilization of protease in alginate-chitosan, agar-agar and calcium alginate are the various methods used. The activity of the immobilized protease was also colorimetrically assayed. The procedure for the same is given in Appendix XXXIV.

3.5.1 Activity of immobilized protease

The activity of the immobilized protease was colorimetrically assayed. Appendix XXXV gives the procedure for the same.

3.5.2 Characterization of immobilized protease

3.5.2.1 Effect of pH and Temperature of immobilized protease

The optimum pH and temperature for the immobilized protease was determined using the substrate casein prepared in buffers of varying pH ranging from 4.0 - 12.0 and temperature 20 - 80°C and assaying the activity according to the protocol in Appendix XXVII, XXVIII, XXXV and XXXVI respectively.

3.5.2.2 Storage stability of immobilized protease

In order to investigate the industrial practicability of an immobilized enzyme process, the loss of enzyme activity at low temperatures is an important parameter to take into account. The storage stability was investigated for both soluble and immobilized enzyme preparations at 4°C and 27°C. The stored immobilized enzyme was assayed everyday for a period of 10 days to determine its activity. The relative activities were calculated based on the initial activity. The protocol for this is described in Appendix XXXVI.

3.5.2.3 Reusability of immobilized protease

The reusability of the immobilized protease was determined by standard assay conditions and the activity was checked every day till there was no activity at all. The residual activity was calculated by taking the enzyme activity of the first cycles as 100%. Appendix XXXVI gives the elaborate procedure.

3.5.2.4 Surface morphology of immobilized protease

The surface morphology of alginate-chitosan, calcium-alginate and agar-agar beads with and without entrapped protease were examined through Scanning Electron Microscopy (SEM). The samples were initially dried at 40°C for 24 hours and then coated with 300°A gold particles. The surface morphology of coated beads was then observed under different resolutions (Sattar *et al.,* 2018).

3.6 Phase VI: Applications of protease

3.6.1 Application in detergent industry

3.6.1.1 Action of protease on stains

The destaining ability of protease was examined using a piece of cloth stained with egg yolk and blood, incubating it with purified, immobilized and commercial protease. The detailed procedure is recorded in Appendix XXXVII.

3.6.1.2 Preparation of enzyme-based detergent cake

Proteases in detergent industry has been used at large scale at commercial level. It is one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures (Sharma *et al.*, 2014). Appendix XXXVIII depicts the procedure for the same.

3.6.2 Application in leather industry

3.6.2.1 Action of protease on animal skin

Protease has the ability to remove hairs from skin. Enzymatic dehairing process has been gaining importance as an alternative chemical methodology in the present day scenario as this process is significant in the reduction of toxicity in addition to improvement especially in the leather industry (Rai and Mukherjee, 2010).

Goat skin was used to test the ability of the protease to dehair it by incubation with purified, immobilized and commercial protease and observing it visually. The procedure for this is given in Appendix XXXIX.

3.6.3 Application in pharmaceutical industry

3.6.3.1 Antibacterial activity of protease

The antibacterial activity of purified, immobilized and commercial protease was assayed. The detailed procedure is given in Appendix XL.

3.6.3.2 Clot lysis activity of protease

The blood clot lysis assay of the purified, immobilized and commercial protease was also determined. Appendix XLI gives the detailed procedure.

3.6.4 Application in photographic industry

3.6.4.1 Silver recovery from waste x-ray film

Removal of silver from X-ray films by decomposing the gelatin layer on the films can be achieved by proteases (Vijayalakshmi *et al.*, 2011).

The X-ray film was incubated with purified, immobilized and commercial protease and then checked for decomposition of the gelatinous coating. The presence of silver was confirmed by a silver nitrate test. The detailed procedure is recorded in Appendix XLII.

3.6.5 Application in textile industry

3.6.5.1 Activity of protease on degumming of silk

Silk fibre was treated with commercial, purified and immobilized proteases and the detailed procedure is given in Appendix XLIII.

Determination of weight loss

Weight loss of treated silk denotes the quantitative evaluation of the degumming process. Appendix XLIII gives the same.

Determination of quality and quantity of sericin

After degumming of silk the quality and quantity of sericin was determined. Appendix XLIII depicts the brief procedure.

Morphological study

Morphological characterization of silk fibre was performed by means of scanning electron microscope. The filaments of untreated and enzymatically degummed silk samples were scanned in a scanning electron microscope MIRA3 TESCAN.

The observations made at different stages of the study and the results obtained are presented and discussed in the next chapter.