
Summary and Conclusion

Soil is a highly heterogenous environment which contains a high diversity of microorganisms. These microorganisms present in soil are a valuable source of natural products providing important enzymes and bioactive compounds for industries, antibiotics for pharmaceuticals and also play a major role in the synthesis and degradation process. Screening of microorganisms from unexplored natural and man-made environments will significantly facilitate the search for enzymes. Exploring these habitats will provide access to novel bacteria and their robust enzymes that can act under multiple extreme conditions.

Microorganisms represent an excellent source of enzymes, being also the main source of production. These microbial enzymes possess a variety of biochemical, physiological and regulatory functions. Proteolytic enzymes in particular are ubiquitous in nature found in all living organisms and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community.

Due to their versatile nature, proteases represent one of the largest groups of industrial enzymes that find application in detergent, leather, food, pharmaceutical and textile industries as well as silver recovery and bioremediation processes. Proteases are present in all forms of life and are produced by microorganisms, various plants and animals. Among them bacterial proteases secure the most important place due to their enormous industrial applications.

Microbial proteases have interesting characteristics in the sense of low cost of production, good stability and specificity representing a powerful tool in the development and production of new protein hydrolysates with characteristics that can be explored industrially. Many bacteria belonging to the *Bacillus* genus are important producers of enzymes for industry and research.

The largest application of protease is in the laundry detergent and leather industries, where they remove protein-based stains from clothes and dehaire animal skin respectively. As the modern world focus on ecofriendly products and product outputs, more chemical processes are being replaced by enzymatic methods. Today there is a need for new, improved and more versatile enzymes in order to develop more novel, sustainable and economically competitive production processes.

Hence, the present study entitled ‘Characterization, Immobilization and Applications of Extracellular Protease from *Bacillus* sp. ASASBT isolated from Termite Soil’ was aimed to isolate, identify, purify, characterize, immobilize and study the applications of protease from *Bacillus* sp. ASASBT isolated from termite soil as a potential source of the enzyme protease thereby trying to reduce the production cost. The objectives of the study were to isolate bacteria from different environmental soil samples and screen them for the presence of enzymes, identify the selected protease-producing isolate and optimize the media components for the maximal production of protease, to purify, characterize and immobilize the purified protease and to study the applications of the isolated protease.

In Phase I, four different microbial sources, namely, Termite Mound Soil (TMS), Organic Waste degraded Soil (OWS), Textile Effluent degraded Soil (TES) and Marine Soil (MRS) were collected at random within the southern region of Tamil Nadu like areas around Karur District, Coimbatore District, Tirupur District and Karaikal District respectively. These samples were collected in sterile containers and stored at 4°C until use. All the samples were serially diluted and spread on nutrient agar plates which were incubated at 37°C for 48 hours. Morphologically distinct colonies were isolated from each sample and the subcultures were maintained. All the isolates were screened and assayed for the presence of the enzymes amylase, cellulase, lipase and protease.

The results showed that a total of 45 bacterial isolates (TMS1-TMS16, OWS1-OWS9, TES1-TES13, MS1-MS7) were isolated from the four different soil samples. Eventhough, all the isolates were able to produce all the four enzymes, protease showed maximum activity. Among the 45 isolates, 14 isolates showed maximum proteolytic activity on skim milk agar media. As a result, the maximum activity and zone of inhibition

of protease was exhibited by the isolate TMS1. Therefore, the isolate TMS1 from termite mound soil was subjected to identification and media optimization procedures.

In Phase II, the isolate TMS1 bacteria was identified and its media components for protease production optimized. The identification was based on the colony, morphological and biochemical characterization and scanning electron microscopy (SEM) analysis and further confirmed by 16S rRNA gene sequencing. Finally, the presence of protease in the isolated bacteria was confirmed by gene amplification using selected primer.

The protease enzyme was extracted from the nutrient broth culture after centrifugation, followed by the study of protease activity at different incubation times (12-72 hrs), pH (5.0 to 10.0), temperature (20 - 70°C), inoculum size (0.5% to 3%), agitation rate (50, 100, 150 and 200 rpm), carbon sources (dextrose, lactose, sucrose, starch and maltose), organic and inorganic nitrogen sources (gelatin, beef extract, urea, ammonium sulphate and ammonium chloride) and natural substrates (castor husk, green gram husk and black gram husk) all of which were optimized.

The findings of Phase II exhibited, the colony characteristics of the selected bacterial isolate TMS1 which showed moderate sized white, circular shiny, rough opaque and convex colonies at the end of 48 hours incubation. The morphological characteristics were found to be gram-positive, rod-shaped, motile and spore producing. The biochemical characteristics of the isolate showed positive results for Voges Proskauer, citrate utilization, urease, catalase, starch hydrolysis, nitrate reduction and gelatin liquefaction tests. However, indole production, methyl red, triple sugar iron agar and oxidase tests were negative. Thus, considering the colony, morphological and biochemical characteristics observed, it can be said that the phenotypic characteristic of the selected strain TMS1 belongs to the genus *Bacillus* sp. according to the Bergey's manual of systematic classification.

The scanning electron micrograph of the isolate TMS1 was visualized as single, rod shaped bacteria. The 16S rRNA sequence of the bacterial isolate was compared with the 16S rRNA sequences of the rRNA database of NCBI using BLAST to further identify

the species of the organism. The homologous search indicated that the isolated strain shared 94% identity with *Bacillus subtilis*. Hence, 16S rRNA gene sequence analysis and phylogenetic method were not sufficient to identify the isolated strain TMS1 at the species level. The present data thus has to be supported by more advanced genomic analysis methods for the identification at the species level. Hence the isolate TMS1 was identified as a *Bacillus* sp. and named as ***Bacillus* sp. ASASBT**. The obtained nucleotide sequence of this *Bacillus* sp. strain was submitted to the GenBank database and was assigned an accession number - MF618325. After amplification of protease gene, approximately 1.2 kb gene was amplified using the primers.

The results showed that optimization process led to maximum activity of protease at pH 7-8, temperature 40°C and the incubation time of 48 hrs. In the effect of different inoculum concentrations on protease activity, the maximum enzyme production was observed with 1% inoculum. For optimization of carbon and nitrogen sources, the results revealed that, starch was the best carbon source and gelatin the best nitrogen source with 100 rpm agitation speed. When green gram husk was used as a natural substrate, the activity of protease was maximum.

In Phase III, the protease from the selected *Bacillus* sp. ASASBT was subjected to a four step purification, that is, precipitation with ammonium sulphate at varying concentrations (0-100%), dialysis (to remove the salts present in the sample), ion exchange and gel filtration chromatographies using AKTA FPLC system. The purification profile of a protease was then determined based on its total activity, total protein, specific activity, purification fold and recovery percentage. Molecular weight determination was done by SDS-PAGE, confirmation of protease activity by zymography and identification of protein by MALDI-TOF peptide mass fingerprinting analysis.

The outcome of Phase III showed that out of the total protease activity and total protein content of *Bacillus* sp. ASASBT in all the ammonium sulphate precipitated fractions, maximum values were exhibited by the total activity (3497.00 U), total protein content (104.00 mg) and specific activity (33.62 U/mg) were observed in 40 - 60 % ammonium sulphate precipitated fraction, when compared with other fractions. Hence this

fraction was taken for further purification. The 40-60% ammonium sulphate precipitated fraction was further purified by dialysis. After dialysis, specific activity (36.52 U/mg) had increased, which indicates the purification level.

The dialysate obtained was concentrated using protein concentrator and loaded onto a Hi Trap Q FF column of ion exchange chromatography. The elution profile showed active peaks from fractions 13 to 15 which exhibited high enzyme activity and protein concentration. The active fractions from ion exchange chromatography were pooled, concentrated and run through Superdex 200 column fitted to on FPLC system. A single peak displaying protease activity and homogeneity of the purified protease was obtained. The purification profile of the isolated protease at various stages of purification is noted. It is clear that, with increase in the purification level, the specific activity (127.55 U/mg) and purification fold (11.32) of the protease also increases.

Molecular weight determination of the purified protease was done by SDS-PAGE, in combination with protein marker and stained with Coomassie Brilliant Blue and Silver stain. A single band was observed confirming the molecular weight of the purified protease to be 46 kDa, which demonstrates relatively a good purification level and it is a monomeric protein. The zymogram of the gel filtration purified protease revealed the presence of a white coloration in the area where protease had hydrolysed the substrate gelatin. The Coomassie Brilliant Blue-stained protein bands corresponding to the purified protease were cut out from the polyacrylamide gel and it was digested by trypsin. The peptides obtained were analyzed by MALDI-TOF/TOF Mass spectrometry. A mass spectrum was acquired between 800-3500 m/z and ten strongest peaks were selected for MS/MS fragmentation. Mascot results showed the presence of 'oligoendopeptidase' with 56% score, which belongs to metalloprotease family.

In Phase IV, the purified protease was characterized by studying the effect of pH, temperature, substrate concentration, metal ions, inhibitors, surfactants, oxidizing agents and organic solvents on the activity of the enzyme. The UV-visible absorption spectra of the purified protease and commercial protease were studied. The presence of various functional groups in the purified protease was confirmed by Fourier Transform Infrared Spectroscopy (FTIR) analysis.

The results showed maximum relative activity at pH 8.0 and therefore this was considered as the optimum pH. Since 40°C showed maximum relative activity it was taken to be the optimum temperature. It was also observed that, higher the temperature, lower was the enzyme activity. The findings of the effect of substrate concentration showed that there was an increase in the enzyme activity upto a certain extent beyond which the activity remained stable giving a hyperbolic curve. The isolated protease was also shown to obey the Michaelis-Menten equation with a K_m value of 0.84 μM and V_{max} of 92.36 μM .

It was also observed that divalent metal ions have positively influenced the activity of protease. Magnesium chloride (Mg^{2+}) had increased the activity of the enzyme to the maximum in 5 mM concentration followed by zinc chloride (Zn^{2+}) in 5 mM concentration. However, copper chloride (Cu^{2+}) in both concentrations were found to decrease the activity of protease to the maximum extent followed by manganese chloride (Mn^{2+}) in 5 mM concentration. The inhibitor studies revealed that PMSF, pepstatin and indoacetic acid did not affect the activity of the protease much, whereas, EDTA inhibited the enzyme at 1mM concentration and also 5 mM concentration. This indicated that the purified protease belongs to the metalloprotease family.

The effect of surfactants and oxidizing agents showed that maximum enzyme activity was obtained in the presence of Triton X-100 as neutral detergent, whereas, SDS as ionic and CTAB as cationic detergents reduced the protease activity indicating that the enzyme was very sensitive towards these detergents. Addition of hydrogen peroxide to the reaction brought about 50% conservation of enzyme activity. The studies on organic solvents showed acetone, ethanol, methanol and ethyl acetate to increase the protease activity in 25 % and 50 % concentrations, whereas the DMSO decreased the activity of protease.

The UV-visible absorption spectra of the purified protease and commercial protease showed a single peak at 285 nm. The presence of various functional groups in the purified protease was confirmed by Fourier Transform Infrared Spectroscopy (FTIR) analysis. The absorption spectrum showed strong O–H stretching vibration and presence of C=O group and C-N stretching in the protein sample which confirmed the presence of

amino acids and presence of amine group in their side chain. As there was no absorbance in the region 2220 - 2260 cm^{-1} , it indicated that there was no cyanide group present in the protein.

In Phase V, the purified protease was immobilized by using three different adsorption methods, Alginate-Chitosan, Calcium-Alginate and Agar-Agar methods. The immobilized enzymes were then characterized for optimum pH, temperature, storage stability and reusability. Finally, the surface morphology of the immobilized beads, normal beads and soluble protease was analyzed by Scanning Electron Microscopy (SEM). To determine the storage stability of the entrapped enzyme, it was analyzed at two temperatures (4°C and 27°C) and its activity noted everyday for ten days. Finally, the immobilized enzyme was checked for its reusability.

The effect of pH on the activity of proteases with all the three methods of immobilization were compared with the soluble protease. The results showed that there was no change in the optimum pH of 7.0 for protease before and after entrapment. In the case of temperature, all the entrapped protease showed an optimum of 40°C which was the same as that of the soluble enzyme.

The storage stability studies revealed that there was 100% activity in alginate-chitosan and agar-agar immobilized protease for 2 days at 27°C, after which the activity declined with increase in the number of days. At 4°C, alginate-chitosan immobilized enzyme exhibited 100 % activity for 6 days, followed by agar-agar immobilized enzyme which showed 100 % activity for 3 days.

The study on the reusability of the immobilized enzyme have shown that the three methods, alginate-chitosan method showed maximum activity in all the parameters and had higher percentage of relative activity after 6 cycles of use. Hence it was selected for application studies.

The surface morphology of entrapped beads, normal beads and soluble protease displayed significant differences in their structure - the protease without entrapment exhibited uniform even surfaces with several covered layers, whereas, entrapped protease displayed heterogenous globular aggregates on the surface.

In Phase VI, the action of commercial, purified and immobilized protease was studied for stain removal on white cotton cloth pieces stained with egg yolk and blood. Enzymatic dehairing of goat skin, anti-bacterial activity against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria, blood clot lysis, silver recovery from waste x-ray films and degumming of silk were evaluated.

The results showed that, both the purified and immobilized proteases removed the stains completely within 30 minutes like the commercial protease. Among the purified and immobilized proteases, purified protease was able to remove both the stains (egg yolk and blood) much better than immobilized protease.

The findings of dehairing study showed that both purified and immobilized proteases have maximum efficiency in hair removal as commercial protease.

The antibacterial activity using Well Diffusion Method showed maximum activity of all the three proteases against both the pathogens. As for commercial protease the purified enzyme showed the maximum ability of clot lysis.

Essentially, all the silver was removed from the enzyme treated x-ray films and separated from the solution. The solution was then qualitatively tested for the presence of silver. A black stain was obtained which confirmed the presence of silver in the solution.

The studies on degumming of silk showed that all the proteases completely and uniformly removed the sericin from silk. Degumming of silk was determined by weight loss method and quantitative estimation of sericin present in the silk was done by recording the UV spectra. The result showed a maximum yield of sericin 24.25% in silk treated with commercial protease, followed by purified protease (23.72%) with a slightly less yield obtained in silk treated with immobilized protease (20.14%). The weight loss percentage of silk fabric treated with commercial protease showed 31%, followed by 30% in purified and 18% in immobilized protease.

Thus, it can be concluded that the *Bacillus* sp. ASASBT isolated from termite soil has more potential to produce protease enzyme when compared to other isolates. Though both soluble and immobilized protease showed good activity in application studies, use of immobilized protease in the detergent, textile, leather, pharmaceutical and photographic

industries is recommended since this form of enzyme can be used several times and thereby can reduce the cost of enzyme production.