CHAPTER 5

Discussion

DISCUSSION

5.1. Asiatic mud eel (cuchia)

The Asiatic fresh water mud eel (*Monopterus cuchia*) is carnivorous in feeding habit, therefore, prefers animal-based food like small fish, echinoderms, amphibians, crustaceans, etc. [6]. The fish needs the production of protein degrading protease enzyme in the gut for the digestion of the feed. Animal gut harbours various enzyme producing microorganisms (bacteria and fungi) for the digestion of the feed. In this regard, the GI tract of *M. cuchia* was dissected out to isolate the protease-producing bacteria.

5.2. Protease producing bacteria

Owing to the better catalytic efficiency, specificity, stability, and bioavailability enzymes are preferred over chemical catalysts in various industries like pharmaceutical, leather, food, etc. The enzymes obtained from microorganisms (bacteria and fungi) have been studied thoroughly and preferred over the plant or animal derived enzymes due to the presence of all desired characteristics applicable to industrial utilities [185]. Proteases constitute the major sector (60%) of the industrially essential enzymes with an annual global production amounting to $1.5–1.8 billion. Bacteria belonging to genera *Staphylococcus, Bacillus, Pseudomonas,* and *Aeromonas* are the predominant protease producers. However, *Bacillus* species is the most dynamic and active producer of proteases, therefore, they are the most studied bacteria for the secretion of proteases at the commercial level [186]. Various sources have been explored for the isolation of protease-producing bacteria, including water, soil, industrial effluents, slaughterhouses, etc. The gastrointestinal (GI) tract of carnivorous fish could be the potential source of protease-producing bacteria since it has been reported that gut microbiota helps in the digestion of food ingredients [187].

In this study, twenty (20) bacterial isolates were obtained from the GI tract of *M. cuchia* in nutrient broth medium through serial dilution and the pure culture of the bacterial isolates were done. The isolates were screened for proteolytic activity on skimmed milk and gelatin agar plates. Six (6) isolates could hydrolyse the substrate revealed by a clear zone of hydrolysis. The Ninhydrin test also confirmed the existence

of protease in the bacterial supernatant. Characterization of bacteria revealed that these protease producers belong to different genera viz. *Bacillus, Priestia, Aeromonas, Staphylococcus,* and *Serratia* with sequence homology ranging between 98 and 100%. PRN1 strain showing 100% homology to *Bacillus safensis* demonstrated the highest zone of clearance in both skimmed milk and gelatin agar plates. Hence, was selected for further studies including optimization and application experiments.

5.3. Protease production by bacteria

In order to assess the potential of the bacteria in the production of protease enzyme, the culture parameters were optimized for the best protease production and the optimum bacterial growth. The culture conditions tend to strongly affect the secretion of bacterial proteases, so it is pertinent to determine the nature of protease [188]. Protease production is affected by temperature, incubation time, type of C and N source, and pH of the fermentation media.

5.4. Media optimization for protease production

The present investigation showed that the maximum protease production (1213 U/ml) of *B. safensis* PRN1 was at 72 h. The result is in agreement with Lakshmi et al. [188] who demonstrated the maximum production of protease by *Bacillus licheniformis* at 72h of incubation. Kate, [189] studied the production potential of keratinase enzyme by *Bacillus cereus* using feather meal agar as a low-cost substrate and further reported maximum proteolytic activity at 72 h.

The pH of media greatly effects enzyme production as the production rate of protease could be greatly influenced by pH, temperature, and species of the bacteria [190]. It was observed that the protease activity of *B. safensis* PRN1 gradually increased and reached the maximum of 403 U/ml at pH8. This result is in accordance with the outcome of Govarthanan et al. [191] reported the highest yield of protease by *Bacillus* sp. at pH8. Sayem and Hoq, [192] reported an optimum pH of 8.5 for the maximum protease production from *Bacillus licheniformis*. For the optimum secretion of protease, most of the *Bacillus* sp. reported have optimum pH ranging from 7-11 [193, 194].

Besides pH, temperature is also an essential parameter for the optimum proteolytic activity [62]. The rate of cellular production of microorganisms reduces below or above the optimum temperature resulting in slower growth [195]. The optimum temperature for the protease secretion by the *B. safensis* PRN1 was demonstrated at 40°C (1021 U/ml). This is supported by the findings of Naidu, [196] who characterized and purified protease enzyme from *B. subtilis* which potentially active at 40°C. Ray et al. [197] demonstrated that the maximum protease production of *B. licheniformis* BF2 and *B. subtilis* BH4 was at 40°C.

Microorganisms tend to respond differently to the type of carbon source used in the culture medium for protease production as reported by Rao et al. [70]. The study recorded the maximum protease activity (722 U/ml) in fructose containing medium. The findings of Ibrahim et al. [198] reported the maximum production of protease in fructose containing medium by *Bacillus* sp. NPST-AK15.

Nitrogen source plays an important role in microbial fermentation. For effective protease production, different bacterial strains use different substrates as nitrogen source [199]. The current study reports gelatin as the best nitrogen source since the highest proteolytic activity (876.83 U/mL) was obtained. In the presence of gelatin, the enhanced growth of *B. safensis* PRN1 with an increase in the turbidity of the culture medium could be due to the proteinaceous nature [200]. Similar observation was reported by Patel et al. [201] in haloalkaliphilic *Bacillus* sp.

The cost-effective production of microbial proteases from growth media has gained a great deal of attention in fermentation biotechnology [202]. Proteases have a wide range of applications in various industries *viz.* detergent, aquaculture, pharmaceuticals, leather, food, meat tenderization etc. In the present study, the role of protease from *B. safensis* PRN1 in blood stain removal was investigated to observe the potential of the enzyme solution in the detergent industry. Bloodstain of cotton cloth treated with the mixture of crude protease and the detergent was removed rapidly as compared to that of detergent and water-alone. The previous reports have shown the use of bacterial proteases against protein stains [70, 202]. Therefore, it is proposed to isolate

the protease from this isolate (*B. safensis*) which following purification could be used as a cleaning additive in detergents for releasing the proteinaceous stains.

5.5. Purification and characterization of protease

In the present study, the crude protease from *B. safensis* PRN1 was purified and characterized. After dialysis, the purification fold was found to be 7.0 with the enzyme yield of 41.6%. After gel filtration chromatography, the purification fold increased to 25.8 with 31.7% enzyme yield. The purified protease showed a single band at approximately 33 kDa in the SDS-PAGE. Anbu, [203] isolated alkaline protease from *Bacillus koreensis*, and was purified by gel filtration chromatography having the fold of 5.0 and yield of 23%. Sun et al. [204] purified alkaline protease from *Pediococcus pentosaceus* with a purification fold of 25.6 and a yield of 13.1%. Similarly, Haddar et al. [205] purified alkaline serine protease with a purification fold and yield of 14.5% and 5.78 respectively. Proteases from *Bacillus* species in general tend to have the molecular weight of 28 to 38 kDa [203].

The purified protease demonstrated the highest activity at pH8 and temperature 60°C. The imbalance in the optimum conditions leads to denaturing of enzymes resulting in loss of catalytic activity. At the optimum pH and temperature, each enzyme performs best and the activity declines with the increase or decrease in its optimum condition. Changes in temperature and pH may also alter the conformation of an enzyme, substrate binding, and its catalytic activity. Initially, the rate of the reaction increases with the increase in temperature because of the increase in kinetic energy of the reacting molecules, and with a further increase in temperature the kinetic energy of the enzyme surpasses the activation energy which results in the breakage of intramolecular bonds that maintain the structure of the enzyme. In a study carried out by Shah et al. [206], the detergent stable protease purified from *Bacillus cereus* demonstrated highest activity at pH8 and temperature 60°C. In another study, the protease enzyme purified from *Bacillus cereus* WQ9-2 showed the maximum activity at pH8 and temperature 50°C [207]. Ferrareze et al. [208] reported 30 kDa keratinolytic protease enzyme that has the optimal activity at 60°C and under a wide pH range 6 to 11.

The protease activity was inhibited to a greater extent at a concentration of 5 mM of PMSF indicating it to be serine in nature [209]. The other inhibitors like DTT, EDTA, and β-mercaptoethanol have little effect on the relative activity of the enzyme, that further confirms that the enzyme is unlikely to be cysteine or metalloprotease. This is in accordance with the findings of Suberu et al. [210] who reported the relative activity of serine protease which was increased by 129.36, 103.52, and 119.09% in the presence of DTT, EDTA, and β-mercaptoethanol, respectively. For the potential application in the detergent industry as a bio-additive chelator, insensitivity would be an important feature of the enzyme, as detergent formulation contains a significant portion of chelating agent that functions as water softener and help in stain removal [211].

Following incubation with various surfactants and commercial detergents for 60 min at 37°C, protease enzyme retained more than 80% of its activity. However, a decrease in the protease activity was found in the presence of SDS. This inhibition could be due to a decrease in the protein-protein connection and hydrophobic interactions that play an important role in stabilizing the tertiary structure of protein [212]. These characteristics of the enzyme from *B. safensis* support its potential application in the detergent industry. Accordingly, the blood stain removal ability of the enzyme was investigated affirming that the addition of the enzyme to the detergent formulation improves its washing performance.

5.6. Antibacterial activity of protease

Pathogenic microorganisms continue to be a threat to human health, food industry etc. due to the emergence of antibiotic resistance to conventional drugs. Therefore, efforts are being made to explore antimicrobial agents from natural sources [213, 214]. Proteases of microbial origin are being utilized in therapeutics due to their antibacterial properties. The present study reports the antibacterial potential of proteases, therefore could be developed as a source of antimicrobial agents. A similar study was carried out by Bhaskar et al. [215] and reported the antibacterial activity of proteases isolated from *Bacillus proteolyticus* CFR3001 against *E. coli, Y. enterocolytica, L. monocytogenes, and B. cereus.*

5.7. Recombinant protease

The trypsin like serine protease gene (*knbs*^{SP1}) from *Bacillus safensis* PRN1 was isolated and cloned using a pET-28a (+) vector. The size of the amplified *knbs*^{SP1} gene was in accordance with the expected gene size of 909 bp. The recombinant plasmid was subjected to colony PCR and restriction enzyme digestion. Furthermore, DNA sequencing was done for confirmation of the in-frame cloning of the *knbs*^{SP1} gene. The sequence data obtained were deposited in the NCBI GenBank database with the accession number (OR295559). The *knbs*^{SP1} gene was successfully expressed in competent *E. coli* BL21 cell. The SDS-PAGE demonstrated the IPTG-induced culture produce a distinct band at ~33.0 kDa which was consistent with the theoretically predicted molecular weight of the KNBS^{SP1}enzyme. The enzyme was purified through Ni-NTA affinity chromatography. A single band of 33.0 kDa was found in the purified fraction and the size was consistent with that of the recombinant KNBS^{SP1} enzyme, having a 6xHis-tag.

There have been several reports on the cloning of protease gene from *Bacillus* sp. Ariyaei et al. [216] cloned, expressed and characterized the serine protease gene from native Iranian *Bacillus* sp. An intracellular serine protease of 35 kDa from *Bacillus* sp. LCB10 was cloned, expressed and characterized by Hou et al. [217]. Suberu et al. [210] cloned 1203 bp serine protease gene from *Bacillus subtilis* RD7.

In the optimum condition, the specific activity and yield of the purified rKNBS^{SP1} was 43130 U/mg and 69.3%, respectively. The purified recombinant protease enzyme showed remarkably high specific activity, better purification fold and yield in comparison to the alkaline proteases reported previously from *Bacillus altitudinis* [218], *Bacillus lehensis* [219], and *Shewanellaarctica* [220].

Before determining the prospects of an enzyme, it is essential to characterize biochemically for industrial application. The rKNBS^{SP1} enzyme demonstrated the maximum proteolytic activity of 17407.1 ± 621 U/mL with azocasein as the substrate. Shakilanishi and Shanthi [221], reported azocasein as a good substrate for protease enzyme isolated from *B. cereus*.

5.8. Characterization of recombinant protease

The purified recombinant enzyme demonstrated activity in a wide range of pH 3-10 and the highest activity was seen in Tris-HCl buffer at pH 8. The result of pH stability demonstrated that the protease was extremely stable in the range of pH 7-10. Results suggested that the fresh protease is active in the alkaline pH. Mechri et al. [222], Suberu et al. [210] and Akram et al. [223] reported similar observation in the case of proteases isolated from *Bacillus* sp. NKSP-7, *Bacillus subtilis* RD7 and *Anoxybacillus kamchatkensis* M1V at the optimum pH value ranging from 7-11.

The result of the influence of temperature on the enzyme activity and stability demonstrated that the protease activity increases with the increase in temperature and the optimum activity was reached at 60°C. The thermostability profile demonstrated that the protease was highly stable at 60°C. The thermostability of the enzyme is better than previously reported proteases from *Bacillus* strains [224]. The stability of the enzyme in alkaline pH and at higher temperature is one of the salient properties considering the potential acceptance of enzymes in industrial procedures operating under high pH and temperature. Furthermore, the thermostable enzymes can lower the chance of contaminants at high temperature and also the cost of external cooling in industries [225]. Moreover, the recombinant protease is found to be more stable than native protein secreted in the culture broth in harsh conditions (high temp. and pH) due to the presence of N-terminal extension. This sequence might have interacted with the amino acid sequence of the enzyme due to which stability of the enzyme was increased [226].

PMSF and DFP were found to be strong inhibitor of the serine protease family. Proteases are generally grouped based on their sensitivity towards the different inhibitors [227]. According to these findings, it was affirmed that the enzyme belonged to the thiol-dependent serine protease group. In detergent and laundry industries, alkaline serine proteases are widely applied. Recently, a novel alkaline serine protease isolated from *Nocardiopsis alba* strain OM-5 was inhibited by PMSF. However, the enzyme activity remained unaffected by EDTA and DTT, indicating it to be a serine protease [228].

According to Daroit et al. [230] the effect of some metal ions on the protease activity of the purified enzyme demonstrated that the alkaline protease needed Mg^{+2}, Ca^{+2}, and Mn^{+2} for its optimum activity. However, heavy metals such as $HgCl_2$, $ZnSO_4$, and $FeSO_4$ inhibit the enzyme activity. Mercury ions are supposed to cause enzyme inactivation by interacting with the protein's thiol groups. In a similar type of study, Jagadeesan et al. [230] reported Ca^{+2}, Mg^{+2} and Mn^{+2} increased the keratinase activity indicating the role of metallic ions in stabilizing the enzyme at high temperatures by forming salt bridges.

The protease enzyme was found to be oxidant and surfactant stable. The protease from *Bacillus licheniformis* and *Micromonospora chaiyaphumensis* supported these results [231, 232]. Interestingly, the purified enzyme was hardly affected by the anionic surfactant. The stability of the PRN1 protease was similar to the serine alkaline protease obtained from *Bacillus mojavensis* which is highly stable against 1% SDS after incubation of 1h at room temperature [233]. These characteristics support the promising application of $rKNBS^{SP1}$ protease in detergent formulations.

5.9. Recombinant protease as a bio-additive agent

The alkaline protease plays a vital role in the digestion of protein-based stains demonstrating a potential bio-additive in laundry detergents [234]. The $rKNBS^{SP1}$ protease demonstrated good stability with the available commercial detergents like Surf Excel, Ariel, Tide, and Ghadi. These findings are in accordance with the previously reported alkaline proteases isolated from *Bacillus sp.* NKSP-7 and *Bacillus subtilis* PTTC 1023 [235, 223]. Interestingly, the stability of $rKNBS^{SP1}$ protease towards detergents was higher than the commercially available detergent protease Alcalase Ultra 2.5 L that retained less than 80% of its initial activity [222]. The high compatibility of $rKNBS^{SP1}$ protease towards commercial detergents is attributed to its ability to withstand harsh industrial condition of higher temperature and pH.

5.10. Kinetics of recombinant protease

The kinetic constant values of $rKNBS^{SP1}$ enzyme were 1.03 ± 0.14 mg mL^{-1} and 359.7 ± 8.15 U mg^{-1} with the substrate azocasein. The k_{cat} and k_{cat}/K_m were 65.25 min^{-1}

and 63.34 min^{-1} mg mL^{-1}, respectively. The alkaline serine protease from *Mucor subtilissimus* URM 4133 showed K_m and V_{max} values of 2.35 mg mL^{-1} and 333.33 U mg^{-1}, with the substrate azocaesin [235]. The fibrinolytic protease purified from *Streptomyces sp.* CS684 displayed K_m, V_{max} and k_{cat} values of 4.2 mg mL^{-1}, 305.8 8 µg min^{-1} mg^{-1} and 188.7 s^{-1} for azocasein [236]. Similarly, the kinetic study performed by Suberu et al. [210] found K_m and V_{max} values of 2.80 and 4.248 for azocasein substrate.

5.11. Bioinformatics and structural biology of recombinant protease

I-TASSER server was used to generate the high-quality model for the $rKNBS^{SP1}$ protease [237]. The best model 1p3cA was selected on the basis of parameters like Z score, C score, TM score and RMSD [238]. The Z-score value measured with the standard deviation, is the variation between the raw and average scores. The quality of the template is specified by I-TASSER using a normalized Z-score. Consequently, a normalized Z-score greater than one denotes an alignment with high confidence. A C-score is a score of confidence that determine the quality of the estimated model by I-TASSER [237]. Generally, the C-score has the value between -5 to 2 where higher the C-score, higher is the confidence of the model [239]. The TM-score and RMSD is a scale for evaluating the structural closeness of protein structures. The value of TM-score ranges between 0 to 1 where 1 denote a good match between two structures [240]. Furthermore, the 3D structure was validated by Ramachandran plot. The plots demonstrate the torsional angles (Φ, Ψ) in the $KNBS^{SP1}$ protease [241]. This predicted model is of excellent quality as most of the residues are in the favoured region (>95%). Analysis of the serine protease-substrate complex helps to understand the connection modes of the substrate placed at the active site [242]. The docking of the modelled trypsin like serine protease showed good affinity towards casein substrate.

The bio-additives are on high demand in the detergent industry to remove various stains from fabrics [243]. Enzyme mediated elimination of proteinaceous stains from cotton cloth is a safe and green process [222]. In the present study, the cleaning process was improved on supplementing the $rKNBS^{SP1}$ protease with the detergent as confirmed by the complete elimination of blood stain from the cotton cloths. These findings demonstrated the potential of $rKNBS^{SP1}$ enzyme as a bio-additive in the detergent industry.

The application of several proteases such as SAPLF, SAPTEX, and SAPB isolated from *Lysinibacillus fusiformis* C250R, *Penicillium chrysogenium*X5, and *Bacillus pumilus* CBS in the removal of blood stain from the cotton fabrics have been reported by Jaouadi et al. [224], Mechri et al. [222], and Benmrad et al. [244].

5.12. Industrial application of recombinant enzyme

In the present-day scenario enzyme-based dehairing process are gaining popularity over the chemical method as this process is crucial in reducing toxicity as well as improving the leather quality [245]. Majority of the dehairing proteases reported are stable in alkaline pH lacking keratinase and collagenase activities [221]. The non-keratinolytic protease remove the intact hairs by acting on proteins surrounding the hair roots [221]. The purified rKNBS^{SP1} demonstrated dehairing property on the goat skin. Eco-friendly dehairing by the alkaline protease from *Bacillus* strains [246, 223] have also been reported. However, these enzymes were directly produced from the strain cultures resulting in low activity. On the contrary, in the current study, the protease was expressed in *E. coli* cells resulting in excellent dehairing property. Moreover, both the crude and purified protease has taken less time for dehairing as compared to the previous reports that needed 12 h, 16 h and 24 h, for the dehairing of the skin, respectively [246-248].

5.13. Comparative analysis of cuchia and human blood parameters

Additionally, comparative analysis of the blood parameters of *M. cuchia* with Human blood exhibited the haemoglobin value as well as other blood parameters of *M. cuchia* blood are comparatively higher. Therefore, the intake of raw cuchia blood by acute anaemic patients as prescribed by traditional medicinemen (bej) has some scientific basis that needs further investigation.