CHAPTER 3

Materials and Methods

MATERIALS AND METHODS

3.1. Chemicals and media

Nutrient broth, skim milk agar, gelatin agar, DNA isolation kit, and Luria-Bertani broth (LB) were purchased from HiMedia; agarose gel, PCR mastermix, bovine serum albumin (BSA) was obtained from ThermoFisher; trichloroacetic acid, Gentamicin and Kanamycin were purchased from Sigma-Aldrich. Primers were synthesized by Integrated DNA Technologies (IDT) and restriction endonucleases were purchased from Biolabs. All other reagents were obtained from Sigma.

3.2. Bacterial strains

Gram-positive bacteria [*Staphylococcus aureus* (MTCC 3160), *Mycobacterium smegmatis* (MTCC 14468)] and Gram-negative bacteria [*Pseudomonas diminuta* (MTCC 3361), *Escherichia coli* (MTCC40) and *Yersinia enterocolitica* (MTCC 859)] were purchased from Microbial Type Culture Collection and Gene Bank, Chandigarh India. *Escherichia coli* strains DH5α (Invitrogen) and BL21 (DE3) (Novagen) were used in cloning and protein expression analysis, respectively. Tryptone Soya broth (TSB), Luria-Bertani broth (LB), and Nutrient broth (NB) were used to culture the bacterial strains at 37°C.

3.3. Sample collection and preparation of cuchia intestinal sample

Cuchia guts were collected in a sterile container from the fish market of Tezpur, Assam, India in an aseptic condition. In the microbiology laboratory, the gut was dissected and the contents present in the canal were squeezed out and transferred to 10 mL of sterile distilled water to obtain the intestinal sample [150].

3.4. Isolation of bacteria

The serial dilution method was used for the isolation of bacteria from the gut samples, and the spread plate technique was used for the growth of bacteria using nutrient agar plates. After 24 h of incubation at 37°C different bacterial colonies showing distinctive morphological features were chosen and pure colonies were obtained by the streak plate technique. All the steps were performed in a laminar air flow chamber [151].

3.5. Screening of protease-producing bacteria

3.5.1. Primary screening

The skimmed milk agar plates were streaked using the pure culture of the isolated bacteria and incubated for 24 h at 37°C to demonstrate the proteolytic activity. The isolates that hydrolysed the media and produced clear zones by consuming skimmed milk confirmed the production of protease enzymes [152].

3.5.2. Secondary screening

The bacterial cell-free supernatant (CFS) was obtained after 72 h of incubation at 37°C. Protease production was screened on skimmed milk agar and gelatin agar media using the well-assay plate method. Then 50 µL of CFS was placed in the corresponding well made by borer. Following incubation at 37°C for 24 h, protease-producing strains were selected based on the formation of the clear zone [153]. For the detection of clear zone in the gelatin-agar media the plates were flooded with a solution of mercuric chloride at the end of incubation *Escherichia coli* and *Pseudomonas aeruginosa* served as negative and positive controls, respectively [154].

A Ninhydrin test was also performed as mentioned by Marathe et al. [155]. The colour shift to purple indicates the presence of free amino acids during the hydrolysis of casein in the presence of the protease enzyme while no colour change indicates the absence of free amino acids.

3.6. Identification of the bacteria

The isolates PRN1, PRN2, PRN3, PRN6, PRN9, and PRN10 which demonstrated efficient proteolytic activity were characterized using morphological, biochemical, and 16S rRNA gene sequencing methods.

3.6.1. Morphological Characteristics

Different colony characters like shape, surface, colour, transparency, etc. of the protease-producing bacteria were observed after incubation.

3.6.2. Gram-staining

The fresh colonies of the isolates were spread uniformly on a glass slide and were Gram stained. The stained isolates were visualized under a light microscope (Olympus CX21i) using immersion oil.

3.6.3. Biochemical characterization

Pure cultures of the protease-producing bacteria were subjected to various biochemical tests comprising of production of oxidase, catalase, citrate utilization, and IMViC by following Marathe et al. [155].

3.6.4. Molecular identification

3.6.4.1. DNA isolation and (16S rRNA) gene amplification

For molecular identification of protease-producing bacterial isolates, genomic DNA was isolated using DNA purification kit (HiMedia). Universal primers 4127F (5'-GAGTTTGATCMTGGCTCAG-3') and 421525R (5'-AAGGAGGTGATCCAGCC-3') were used to amplify 16S rRNA gene by PCR. A total reaction mixture of 25 µL comprising of Dream Taq PCR 2x master-mix (12.5 µL), nuclease-free water (9.5 µL), reverse and forward primers (1 µL), and template DNA (1 µL) was constituted. PCR amplified products were electrophorised using 0.8% (w/v) agarose gel and their size was ascertained by using 1 kb DNA ladder (ThermoFisher Scientific) [156]. Amplicons were subjected to 1st BASE DNA sequencing service (Selangor, Malaysia). Subsequently, the sequences were deposited in NCBI Data Bank.

3.6.4.2. Construction of phylogenetic tree

NCBI BLASTN suite was performed to access the sequence similarity [150]. Strains showing the highest score and number of hits corresponding to each isolate in the BLAST search results were selected. Enigmatic categories of organisms *viz.* uncultured bacteria, and enrichment culture clones were not taken into consideration.

The sequences were aligned using the ClustalW algorithm in MEGA-X and a phylogenetic tree was constructed using the maximum likelihood method with Tamura 3-parameter model in MEGA-X with 1000 bootstraps replicates [157].

3.7. Optimization of protease production

3.7.1. Protein estimation

The concentration of the total protein was measured following Chimbekujwo et al. [158]. Bovine serum albumin (BSA) was used as the standard.

3.7.2. Protease Assay

Proteolytic activity was calculated by the universal protease assay mentioned by Puri et al. [159]. Tyrosine standard curve was used for demonstrating protease activity.

One factor at-a-time protocol was used for protease production optimization at different parameters viz. time, pH, temperature, and nutritional profiles was used [160].

3.7.3. Incubation

The basal medium used for the growth and protease production optimization analysis consisted of: $Na_2HPO_4.7H_2O$, 64 g/L; NH_4Cl, 5 g/L; 1M $MgSO_4$, KH_2PO_4, 15 g/L; 1M $CaCl_2$; NaCl, 2.5 g/L; and glucose, 20 g/L. Bacterial culture of 1 mL (OD_{600nm}=1) was inoculated in the basal medium for 120 h. After every 24h, culture broth samples were taken and analysed for bacterial growth as well as protease production. UV-Vis spectrophotometer (ThermoScientific Evolution 201) was used for the determination of bacterial growth by measuring the OD at 600 nm. Subsequently, for determining the protease production, CFS was obtained by centrifugation (ThermoScientific MULTIFUGE X1R). The proteolytic activity (U/mL) of the obtained cell-free culture supernatant at different time periods was demonstrated.

3.7.4. pH and temperature

The optimum pH for protease production was demonstrated in basal media with pH ranging from pH6-10. Thereafter, 1 mL of bacterial culture (OD_{600}=1) was inoculated in fermentation media and incubated for 72 h at 37°C. After incubation, the cultures at different pHs were centrifuged, followed by the demonstration of protease activity of the cell-free supernatant as previously described. Additionally, the bacterial growth (OD_{600}) was also observed at the tested pH using UV-Vis spectrophotometer.

Similarly, to evaluate the optimum temperature for the secretion of protease, the bacteria were grown at different temperatures 30, 40, 50, 60, 70, and 80°C for 72 h at pH8. After incubation, both protease activity(U/mL) of the cell-free supernatant and bacterial growth (OD_{600}) were demonstrated at the tested temperatures.

3.7.5. Carbon Source

Different carbon sources like glucose, fructose, sucrose, lactose, and maltose were added separately to the fermentation medium to determine the best carbon source for the optimized growth and protease production by the *B. safensis* PRN1.Following incubation for 72 h at 40°C with 150 rpm shaking condition, maintaining the pH8, the enzyme activity (U/mL) and growth (OD_{600}) were monitored.

3.7.6. Nitrogen Source

Nitrogen sources like beef extract, ammonium sulphate, yeast extract, gelatin, and urea were added separately to the fermentation medium to demonstrate the best nitrogen source for the optimized growth and protease production by the *B. safensis* PRN1. After the incubation of 72 h at 40°C (pH8) the enzyme activity (U/mL) and growth (OD_{600}) were monitored as mentioned earlier.

3.8. Industrial applications

3.8.1. Effect of the protease on stain removal

The technique developed Oztas and Gormez, [161] was followed to study the stain removal property of the protease enzyme. Briefly, white cotton cloth pieces (2 x 2 cm^2) stained with blood (10 µL) were dried in a hot oven for about 10 min (95°C) and different sets were prepared: (i) Stained cloth + Water as control (ii) 20 mL Detergent solution (Surf excel) (10 mg/mL) + stained cloth (iii) 20 mL detergent solution + 400 µL of crude protease + stained cloth (iv) 20 mL tap water + 400 µL crude protease + stained cloth. Stain removal was checked qualitatively by visualization.

3.8.2. Antimicrobial activity of *B. safensis* culture supernatant

The agar well diffusion assay developed by Rather et al. [162] was employed for evaluating the antibacterial activity of the crude protease.

3.9. Purification of the crude enzyme

The bacteria were cultured in 0.5 L of nutrient broth at 37°C for 72 h followed by centrifugation at 9000 × g for 30 minutes at 4°C. The collected CFS was initially precipitated up to 70% using solid ammonium sulfate. After obtaining the desired saturation level it was allowed to settle for 18 h at 4°C followed by centrifugation at 9000 × g for 30 minutes to obtain the precipitated protein. The pellet was re-suspended and dialyzed against the continuous changes of buffer overnight and centrifuged at 10000 × g for 30 minutes to eliminate the insoluble debris. The supernatant was then fractionated by gel filtration chromatography using Shodex KW-803 column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted at 1 mL/min. The protein concentration and the protease activity of the active fractions were analyzed, concentrated with a centrifugal concentrator (10 KDa cut off membranes, ThermoFisher), and stored at -20°C.

3.9.1. Reverse phase (RP)-HPLC analysis of the purified protease

The purity of the protease was confirmed by RP-HPLC (Thermo Scientific) using HYPERSIL GOLD C18 column. Buffer systems containing acidified Milli Q (0.1% trifluoroacetic acid, mobile phase A) and acidified acetonitrile (mobile phase B) were used. The bound protein was eluted at flow rate of 1 ml/min, temperature 25°C, UV 215 nm, and 45 min run time [163].

3.9.2. SDS-PAGE

The molecular weight, subunit composition, and purity of the protease enzyme, was done in SDS-PAGE following He, [164] using 12% resolving gel and 4% stacking gel. Protein sample (15 µg) was heated at 95°C for five minutes and subsequently and stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, USA) and destaining was done with methanol: glacial acetic acid: water (40:10:50).

3.10. Characterization of the purified protease

3.10.1. Effect of pH and temperature on protease activity

The effect of pH on protease activity was determined over a wide range of pH from 4 to 10 using casein as substrate under the standard assay condition. Tris-HCl buffer (0.1 M) was used for pH 4-8 whereas glycine-NaOH buffer (0.1 M) was used with pH 9-10. The activity of the enzyme incubated in tris-HCl buffer (pH 8) was taken as 100%.

The effect of temperature on protease activity was done by incubating it at various temperatures (30-70°C) and pH 8 for 30 min using casein as a substrate. The activity of the enzyme incubated at 60°C was taken as 100%.

3.10.2. Effect of inhibitors on the protease activity

The effect of different inhibitors viz. PMSF, DTT, EDTA, and mercaptoethanol on the protease activity was determined by incubating it with 5 mM concentration of each inhibitor at 37°C for 1 h. The relative activity was then measured under standard assay conditions and the enzyme activity without inhibitor was taken as 100% [160].

3.10.3. Stability of the protease in the presence of surfactant and commercial detergent

The stability of the enzyme in the presence of various non-ionic surfactants with 5% concentration of Tween 20 and 80, Triton X-100, and SDS was studied. The enzyme was incubated at 37°C for 1 h with the surfactants and relative activity was determined under the standard assay condition. Relative activity of the enzyme incubated without any non-ionic surfactants was taken as 100% [165].

Similarly, the effect of different solid detergents like Surf Excel, Ariel, and Tide on the stability and compatibility of the enzyme was reported. The enzyme was incubated with 7 mg/mL concentration of the detergent prepared in distilled water at 37°C for 1 h. To inactivate the endogenous protease detergent solutions were incubated at 60°C for 1 h prior to mixing with the enzyme. The relative activity was determined under the standard assay condition, and the relative activity of the enzyme incubated without any detergents was taken as 100% [165].

3.11. Cloning of bacterial protease gene

3.11.1. Isolation and purification of genomic DNA from *B. safensis* PRN1

The bacterium was grown in nutrient broth media until the absorbance at 600 nm reaches 0.8 and then the genomic DNA was isolated using GeneJET Genomic DNA Purification Kit (Thermo-Scientific). Nanodrop spectrophotometer (Thermo-Scientific) was used for the determination of the DNA concentration. The purity of the DNA was determined from the UV/Vis spectrophotometric A260/A280 ratio.

3.11.2. Gene identification and amplification

The DNA sample was employed for the amplification of protease gene by PCR. A protease gene specific primer BHI-SP_F: 5'-CGCGGATCCATGAAAAAGGTGAAAATG-3' and HSP_R: 5'-CCCAAGCTTTTATTGCGCTTTCGCATAG-3' with suitable restriction enzyme sites was designed by integrated DNA technologies (IDT). The PCR amplification parameters were shown in Table 3.1:

Table 3.1 PCR amplification parameters

Steps	**Conditions**
Initial denaturation	95°C for 5 min
Denaturation	94°C for 30 s
Annealing	55.6°C for 40 s
Extension	72°C for 2 min
Final extension	72°C for 10 min
Cycle	35

3.11.3. Gel Electrophoresis

Agarose gel 0.8% electrophoresis was used for the evaluation of the quality of the isolated DNA. The DNA fragment was resolved on 0.8% agarose gel against 1 kb plus marker and extracted from the gel by using QIA quick gel extraction kit.

3.11.4. Isolation and purification of pET-28a plasmid from *E. coli*

The plasmid pET-28a was isolated from the overnight culture of *E. coli* by using Thermo-Fisher kit. The protocol is as follows:

i. The overnight bacterial culture 5 mL containing the plasmid was grown in liquid LB added with kanamycin (50 µg/mL).
ii. Bacterial cells were harvested by centrifugation at 8000 rpm for 2 min at room temperature.
iii. The cell pellet was resuspended in Resuspension solution was used to dissolve the cell pellet followed by addition of lysis solution with gentle mixing.
iv. Then neutralization solution was added followed by centrifugation.
v. The supernatant was transferred to spin column and re-centrifuged for 1 minute.
vi. The column was washed twice with 500 µL of wash solution followed by centrifugation at 13000 rpm for 1 minute.
vii. The DNA was eluted with elution buffer followed by centrifugation at 13000 rpm.
viii. The purified DNA was stored at -20°C

The plasmid was resolved on 0.8% agarose gel, and was extracted from the gel by using QIA quick gel extraction kit.

3.11.5. Restriction digestion and ligation of the gene and pET-28a

For the construction of the recombinant cloning vector, the amplified gene (24.8 ng/µl) was double digested with *Bam*HI and *Hin*dIII restriction endonucleases using the following parameters: PCR product (24.8 ng/µl)- 20 µl; Green buffer- 3 µl; *Bam*HI- 1 µl; *Hin*dIII- 1 µl. The reaction mixture was incubated at 37°C for 1h and the digested DNA fragments were resolved in 0.8 % agarose gel electrophoresis. The pET-28a plasmid (5 ng/µl) was double digested with the same restriction endonucleases. The gene was inserted into *Bam*HI and *Hin*dIII-digested pET-28a cloning vector using T_4 DNA ligase (4U/µl) by using pET-28a vector (50 ng) – 5 µl; PCR product –10 µl; Buffer – 2 µl; T4 DNA ligase – 1 µl. The reaction mixture was incubated overnight at 16°C.

3.11.6. Transformation

3.11.6.1. Preparation of competent cell

The *E. coli* DH5α/BL21 competent cells were prepared by using the calcium chloride method as described by Sambrook et al. [166]. The method is as follow

i. The glycerol stock of *E. coli* DH5α/BL21 cells were taken out from -80°C and streaked on LB media plates.
ii. Single colony was grown in LB.
iii. An aliquot of 500 µL was added to LB and incubated for 1 h at 37°C in shaking condition.
iv. The entire culture was taken in oak ridge tube and centrifuged.
v. The cell pellet was resuspended in half the initial volume of the ice cold 100 mM $CaCl_2$.
vi. The mixture was re-incubated on ice for another 45 min.
vii. The cell pellet was harvested by centrifuging at 5000 rpm for 5 min at 4°C.
viii. The resuspended pellet was collected in 2 mL of ice-cold 100 mM $CaCl_2$ solution containing 20% glycerol.
ix. Aliquots (100 µL) of the suspended cells was transferred to prechilled microcentrifuge tubes and stored at -80°C until further use.

3.11.6.2. Transformation of the competent cells by heat shock method

i. The cell suspension stored at -80°C was taken out and thawed on ice for 20 min.
ii. Added 5 µL of the ligated product, gently mixed, and incubated on ice for 30 min.
iii. Cells were subjected to heat shock for 90 second at 42°C followed by incubation on ice for 5 min.
iv. LB medium 600 µL was added to the tube followed by incubation at 37°C for 1 h
v. At the end of incubation, approximately 200 µL of the transformed cells was plated on kanamycin (50 µg/mL) supplemented LB agar plates.

vi. The cells were spread evenly on the plate and then incubated for 16 h at 37°C in static condition.

Thereafter, the recombinant plasmid pET-28a was transferred to the competent bacterial cells {*E. coli* BL21 (DE3)} as described by Singh et al. [167]. The transformed colonies were screened on LB agar plates containing 50 µg/mL kanamycin. Thereafter, to confirm the presence of the protease gene the recombinant plasmid was extracted from BL21 and digested by *Bam*HI and *Hin*dIII restriction enzymes. The recombinant plasmid was run in 0.8% agarose gel.

3.12. Protein expression and purification of the recombinant protease (r**KNBS^{SP1}**)

The expression profile of rKNBS^{SP1} protein was studied in *E. coli* cells. Primary culture was obtained by growing the *E. coli* BL21 (DE3) containing the recombinant plasmid (pPM1) overnight in LB supplemented with kanamycin at 37°C. Cells were cultured at 37°C to obtain optical density (OD_{600}) of almost 0.6 at 600 nm by transferring 1% v/v of the primary culture to 100 mL LB medium and subsequently it was induced with 0.1 mM IPTG for different incubation time (2, 4, and 6 h). After 6 h of induction at 37°C, the cells were harvested by centrifugation for 10 min at 8000 rpm in a refrigerated centrifuge (Thermo-Scientific) and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Subsequently, to release the intracellular proteins the cells were sonicated on ice for 5 mins with a 30-sec pulse. The cell free extract was centrifuged to remove cell debris at 10000×g at 4°C for 30 min and the extracellular protease activity of the culture broth was assayed using azocasein as a substrate. Ni-NTA metal affinity chromatography was used to purify the rKNBS^{SP1} protease. The supernatant containing the soluble proteins was loaded to a Ni-NTA resin (Qiagen) previously equilibrated with wash buffer (lysis buffer + 20 mM imidazole, pH 8.0). The resin was washed sufficiently with the wash buffer and the rKNBS^{SP1} was eluted by the elution buffer (lysis buffer + 300 mM imidazole, pH 8.0). The fractions collected were dialyzed and assayed for protease activity. Furthermore, the enzyme purity was investigated on 12% SDS-PAGE and stored at -20°C for further biochemical characterization.

3.13. Analysis of sequence data

The recombinant clones were subjected to 1st BASE DNA sequencing (Selangor, Malaysia). The sequence similarity searches, and the analysis of the sequenced data were performed using BLASTN program of NCBI. Amino acid sequence similarity and the multiple sequence alignment analysis were performed using the BLASTP search algorithm in NCBI and GENBANK with ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The alignment was further analyzed by ESPript 3.0 program [168].

3.14. Biochemical characterization of the alkaline serine protease

3.14.1. Electrophoresis

The molecular mass and purity of the recombinant purified trypsin like serine protease ($rKNBS^{SP1}$) was estimated by SDS-PAGE as described by He, [164].

3.14.2. Protease assay and protein measurement

The protease activity of the recombinant purified enzyme was measured by using azocasein (Sigma) as a substrate following the protocol of Sittipol et al. [169]. One unit of the enzyme activity was defined as the amount that yielded 0.01 change in absorbance at 440 nm in one minute [170]. The absorbance of the samples was taken on a UV-Vis spectrophotometer (ThermoScientific Evolution 201) against a blank control. Protein was determined with bovine serum albumin (BSA) as the standard [171].

3.14.3. Effect of pH and temperature on the activity of recombinant protease

The activity of the $rKNBS^{SP1}$ was determined over a pH range of 3-10 using azocasein as a substrate. For the estimation of pH stability, the purified enzyme was incubated in the buffers at different pH values (3-10) for 1 h at 40°C. At desirable time interval aliquots were taken out and relative enzyme activity was determined under the standard assay condition. Tris-HCl buffer and glycine-NaOH buffer were used at a concentration of 0.1 M for pH 3-8, and pH 9-10, respectively.

The protease activity was determined between 30°C to 70°C for 30 min using azocasein as the substrate under the standard conditions. Thermostability was determined

by pre-incubating the purified enzyme at 50-70°C for 1 h. At desirable time interval aliquots were taken and relative enzyme activity was determined at the optimum pH and temperature. The relative activity of the nonheated enzyme (control) was taken as 100%.

3.14.4. Effect of inhibitors and metallic ions on recombinant protease stability

To assess the influence of various inhibitors and metallic ions on the stability of the enzyme, it was pre-incubated with 5mM concentration of different inhibitors such as phenyl methane sulfonyl fluoride (PMSF), diisopropylfluorophosphates (DFP), dichlorodiphenyltrichloroethane (DTT), ethylene diamine tetra acetic acid (EDTA), β-mercaptoethanol and 2 mM concentration of various divalent metallic ions ($FeSO_4$, $CaCl_2$, $HgCl_2$, $MnSO_4$, $ZnSO_4$, $MgSO_4$, and $CuSO_4$) for 1 h at 40°C. The relative activity of the enzyme devoid of any additive was taken as 100%.

3.14.5. Effect of non-ionic surfactants and commercial detergents on enzyme compatibility

The potential of the recombinant purified enzyme as an additive in the detergent industry was investigated by assessing their stability against various surfactants (Triton X-100, Tween 20, Tween 80, and Sodium dodecyl sulfate) and an oxidizing agent hydrogen peroxide (H_2O_2). The enzyme solution was incubated with various concentration of detergent additives for 1 h at 40°C. The relative activity of the enzyme was determined at the optimum pH and temperature. The relative enzyme activity devoid of any additive was taken as 100%.

The recombinant enzyme compatibility towards the commercial detergents was investigated by preparing various detergent solutions: Surf Excel, Ariel, Tide and Ghadi in 0.1 M Tris-HCl buffer pH 8 with a final concentration of 7 mg/mL. Prior to assay the intrinsic protease available in these detergents was deactivated by heating the detergent solutions at 60°C for 1 h [172]. Initially the enzyme was incubated with each detergent at 40°C for 1 h followed by the determination of relative protease activity using azocasein as the substrate. The relative enzyme activity of the control (without detergents) was considered as 100%.

3.14.6. Determination of recombinant enzyme kinetics

For the enzyme kinetic study, different concentrations of azocasein solution (1 to 20 mg/mL) were used to determine the Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) of the protease [173]. Both the kinetic constants K_m and V_{max} were calculated from the Michaelis-Menten curve. The turnover number value (k_{cat}) was calculated from the formula of $k_{cat} = V_{max}/E$, E represented the total enzyme concentration.

3.15. *In silico* analysis of amino acid sequence

3.15.1. 3D structure of the trypsin like serine protease

I-TASSER server was used to generate the 3D structure of the protein [174, 175].

3.15.2. Docking

The structure of the ligand casein was downloaded from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) with PubChem CID: 10148509 in SDF format and converted to PDB in Open Babel server. Docking of serine protease with the ligand was done using CABS-DOCK server and the serine protease-casein complex was obtained [176].

3.15.3. Analysis of the 3D structure

3.15.3.1. Protein-protein interaction study

A region where two sets of proteins come into contact is commonly referred to as an interface area. Surface residues with significant surface areas that are exposed to the solvent are frequently characterized [177]. The interface statistics for both the complex system were obtained upon submitting the 3D structure in the PDBsum server.

We obtained the Ramachandran plot, Ligplot analysis from the PDBsum server itself [178].

3.15.3.2. Binding free energy calculation

Binding Free Energy of the complex was calculated using PPI-Affinity. It is a Web Tool for the Prediction and Optimization of Protein–Peptide and Protein–Protein Binding Affinity [179].

3.16. Industrial applications of the recombinant enzyme

3.16.1. Destaining of blood stain from cotton fabric

The blood-stained cotton cloth pieces were treated with the recombinant purified enzyme to investigate the washing performance of the protease. The cloth pieces (5 cm × 5 cm) were stained with blood and kept for 48 h at room temperature for drying. Subsequently, the stained cloth pieces were submerged in the conical flask containing 20 mL of running water, surf excel detergent (7mg/mL) and detergent supplemented with purified rKNBS^{SP1} enzyme (300 U/mL) followed by shake-incubation at 40°C for 1 h. After incubation the cloth pieces were taken out, washed with tap water and dried. The destaining potential of the enzyme was observed visually and compared to the unwashed blood-stained cloth piece that was taken as the control [180].

3.16.2. Dehairing

Fresh goat skin was obtained from a local slaughter house and cleaned properly with tap water and commercial detergent to get rid of all impurities. The skin was then cut into small pieces (5 × 5 cm^2) with a sharp knife and was soaked in two different conical flasks containing 20 mL of purified rKNBS^{SP1} enzyme (300 U/mL), and phosphate buffer pH 7.5 (control). The flasks were shake-incubated for 12 h at 37°C. After 8 h of incubation the dehairing efficiency was assessed visually by gently rubbing the skin pieces with fingers [181].

3.17. Isolation of mgDNA from the intestinal tissue of *M. cuchia*

mgDNA was isolated from the intestinal tissue of *M. cuchia* using Qiagen DNeasy Blood & Tissue Kit (Qiagen India Pvt. Ltd, India).

3.18. Assessment of cuchia blood

3.18.1. Cuchia blood sample collection

A syringe was used to draw blood from the caudal vein of the fish, transferred to a Miniplast 2 mL tube containing EDTA (3.6 mg) and haematological studies were carried out.

3.18.2. Analysis of blood haematological parameters

3.18.2.1. RBC count

Cuchia blood was diluted with appropriate diluting fluids and determined the RBC using improved Neubauer haemocytometer, and calculated [182].

3.18.2.2. Haemoglobin

The Hb concentration was determined by the Sahli's method [183]. N/10 hydrochloric acid was placed into Sahli's graduated haemoglobin tube up to the mark of 2 grams. Then the blood sample was taken in Sahli's pipette exactly up to 20 μL mark. Blood was added to the acid solution, mixed and allowed to stand for 10 min. Then, water was added till the colour of the solution matches to that of the brown glass standard.

3.18.2.3. Packed cell volume

It is the volume occupied by the red cells when a sample of anti-coagulated blood is centrifuged. It indicates relative proportion of red blood cells to plasma. It is expressed either as a percentage of original volume of blood or as a decimal fraction. PCV was estimated by macro method (Wintrobe method) [184].

3.18.2.3.1. Packed cell volume by wintrobe method

Anti-coagulated blood sample was mixed thoroughly and the blood was introduced up to the bottom of the Wintrobe tube**.** The sample was centrifuged at 2300 g for 30 min and reading of the length of column of red cells was taken.

3.18.2.4. Mean cell volume of blood

MCV is a measure of average size of red cells. It is obtained by dividing PCV by red cell count.

$$\text{MCV} = \frac{\text{PCV in \%}}{\text{Red cell count in million/cmm}} * 10$$

3.18.2.5. Mean cell haemoglobin

It is the average amount of haemoglobin in a single red cell.

$$\text{MCH} = \frac{\text{Hemoglobin in grams/dll}}{\text{Red cell count in million/cmm}} * 10$$

3.18.2.6. Mean cell haemoglobin concentration (MCHC)

It refers to the concentration of haemoglobin in 1 litre of packed red cells.

$$\text{MCHC} = \frac{\text{Hemoglobin in grams/dl}}{\text{PCV in \%}} * 100$$

3.19. Statistical Analysis

All the experiments were performed in triplicates with two independent experiments. The results obtained are expressed as the mean values and the standard deviation.