CHAPTER 4

Results

RESULTS

4.1. Isolation and screening of protease-producing bacteria from the gut of *M. cuchia*

The gut samples of *M. cuchia* were subjected to serial dilution for the isolation of bacteria through culture in media. In this study, 20 isolates were obtained. The isolates were sub-cultured and stored at -80°C for further use. Primary screening of all 20 bacterial isolates for proteolytic activity was demonstrated by streaking the pure cultures on a skimmed milk agar plate. Out of all isolates, 6 showed proteolytic activity by demonstrating a clear zone of hydrolysis as shown in Fig. 4.1A. The same was confirmed by pouring CFS of potent isolates in the wells of skim milk and gelatin agar plates (secondary screening). In skimmed milk agar plate assay zone of proteolysis around the wells confirmed the secretion of proteases with hydrolytic activity. The highest zone of hydrolysis 15±1 mm was observed in PRN1 while the least 10±1 mm in PRN2, PRN6, and PRN10 as shown in Fig. 4.1B and Table 4.1. However, the highest and lowest zone of hydrolysis in gelatin agar assay was observed in PRN1(16±1 mm), PRN6 (12±1 mm), and PRN11 (12±1 mm). No zone of hydrolysis was observed in the negative control (*E. coli*) while a prominent zone of hydrolysis could be observed in the positive control (*P. aeruginosa*) (Fig. 4.2A and Table 4.1).

Furthermore, to confirm the presence of proteases in the supernatant of the bacteria the ninhydrin test was performed. A deep purple color was observed when ninhydrin was added to the solution containing casein and enzyme indicating the presence of the free amino acids released during the hydrolysis of the casein protein by the proteases present in the supernatant. However, no color change was observed in the reaction containing only the media and ninhydrin confirming the absence of free amino acids (Fig. 4.2B).

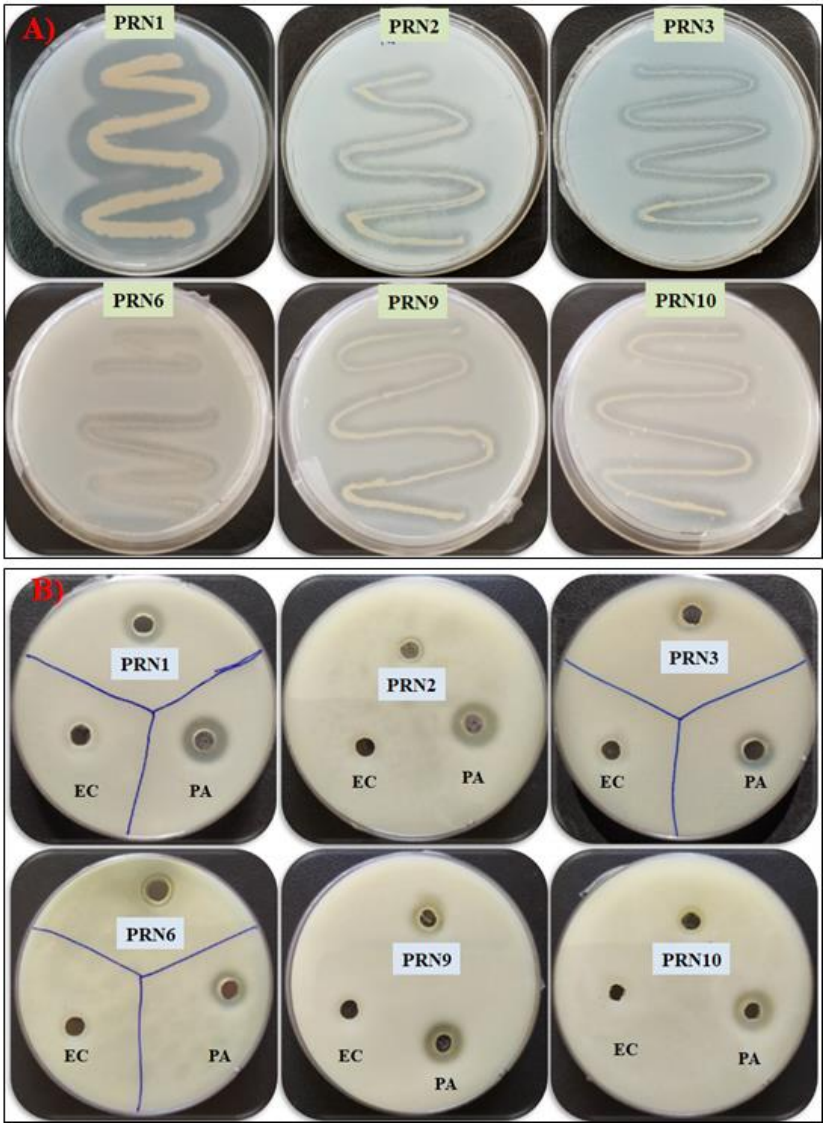

Fig. 4.1. Proteolytic activity of the isolates on skim milk agar plates. A) Streaking method **B)** Agar well diffusion method. *E. coli* (EC) and *P. aeruginosa* (PA) were used as negative and positive controls, respectively

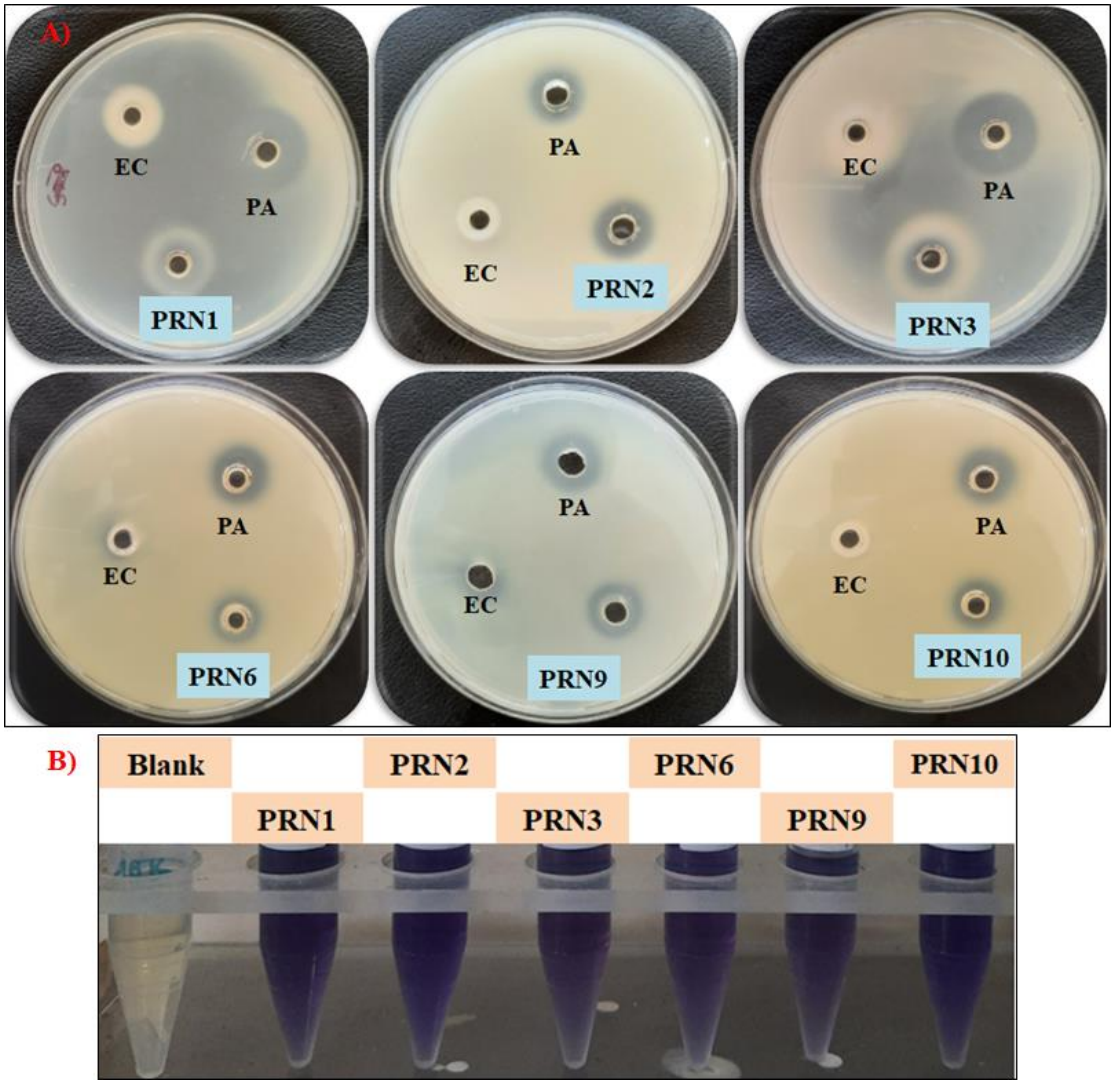

Fig. 4.2. Secondary screening of the isolates for protease production. **A)** Gelatin agar plate assay. *E. coli* (EC) and *P. aeruginosa* (PA) were used as negative and positive controls, respectively; **B)** Ninhydrin test A reaction mixture of only crude enzyme and ninhydrin represents blank

Table 4.1 Zone of hydrolysis (ZOH- mm) in skim milk and gelatin agar plates

		Skim milk agar (mm)			Gelatin agar (mm)		
Name of isolate	**Code**	**Crude protease**	***P. aeruginosa***	***E. coli***	**Crude protease**	***P. aeruginosa***	***E. coli***
Bacillus safensis	PRN1	15±1	16±1	ND	16±1	18±1	ND
Bacillus megaterium	PRN2	10±1	16±1	ND	13±1	13±1	ND
Aeromonas veronii	PRN3	12±1	14±1	ND	15±1	18±1	ND
Staphylococcus warneri	PRN6	10±1	13±1	ND	12±1	13±1	ND
Bacillus sp.	PRN9	12±1	14±1	ND	13±1	14±1	ND
Serratia oryzae	PRN10	10±1	14±1	ND	12±1	13±1	ND

ND- Not defined

4.2. Identification of bacteria

The pure culture of the bacterial isolates and Gram-staining are shown in Fig. 4.3A and Fig. 4.3B, respectively. Fig. 4.3B shows that the isolates PRN1, PRN2, PRN6, and PRN9 are Gram-positive whereas PRN3 and PRN10 are Gram-negative. Additionally, the morphological and biochemical characterization of protease-secreting bacteria are mentioned in Table 4.2 and Table 4.3.

16S rRNA PCR amplified fragment was comprised of approximately 1500 bp as presented in Fig. 4.4A. The sequencing of the DNA revealed that bacterial isolates belonged to five different genera viz. *Bacillus*, *Priestia*, *Aeromonas*, *Staphylococcus*, and *Serratia* (Table 4.4). Furthermore, the bacterial strain PRN1 showed the highest zone of hydrolysis suggesting its strong proteolytic activity as compared to the other isolated strains and was therefore, subjected to further studies. Based on 16S rRNA gene sequences of isolate PRN1 (*Bacillus safensis* accession no. OP512544) and the different related nucleotide sequences of protease-producing *Bacillus* sp. obtained from the GenBank database, a phylogenetic tree was constructed (Fig. 4.4B).

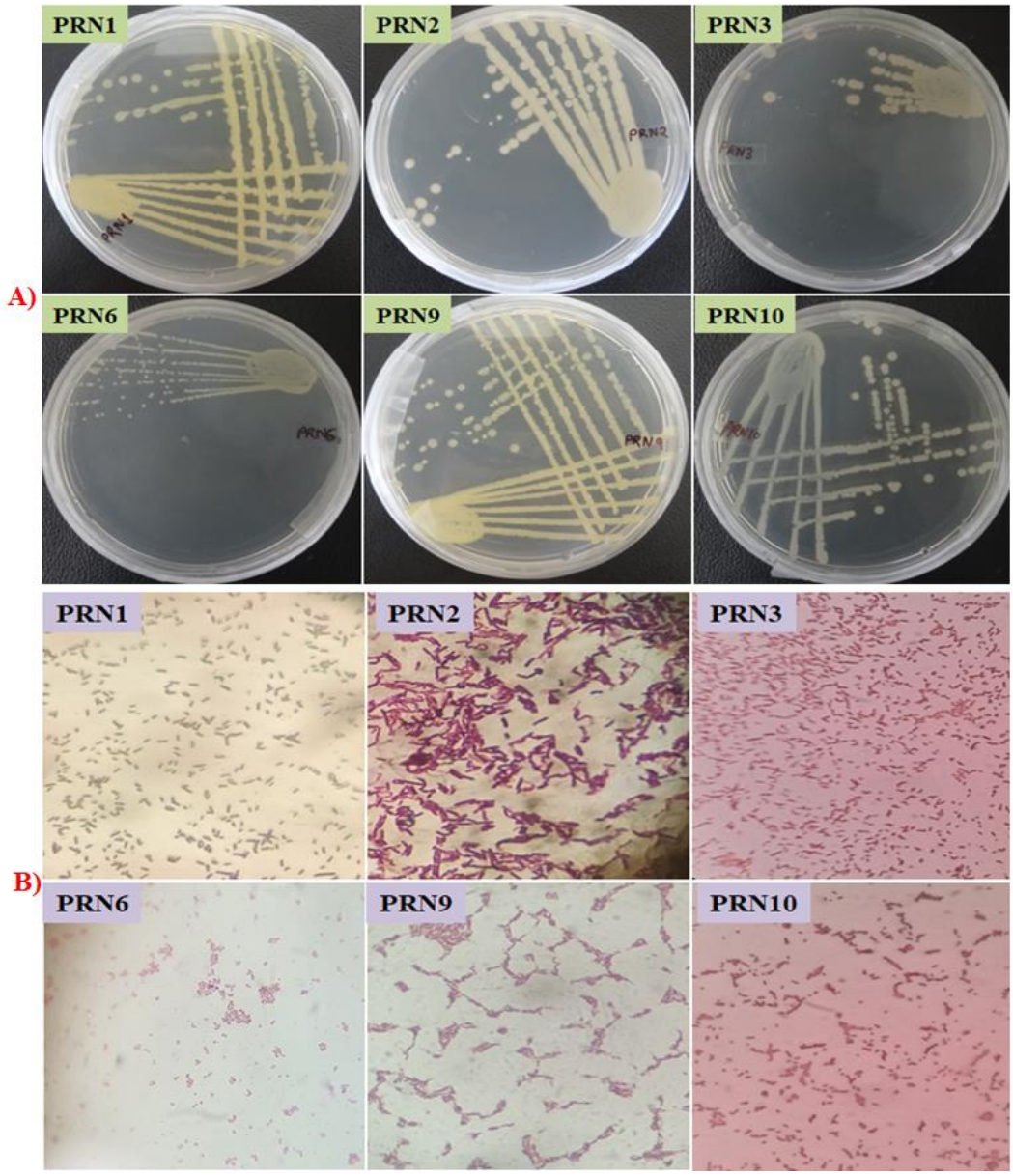

Fig. 4.3. Morphological characterization of the protease-producing isolates. A) Colony morphology; **B)** Gram-staining

Table 4.2 Morphological characterisation of protease-producing isolates

Isolate	Shape	Surface	Size	Colour	Elevation	Margin
PRN1	Circular	Smooth	Medium	Light yellow	Flat	Smooth
PRN2	Irregular	Rough	Large	Cream	Convex	Smooth
PRN3	Circular	Smooth	Large	Cream	Convex	Smooth
PRN6	Circular	Smooth	Small	White	Convex	Smooth
PRN9	Circular	Smooth	Medium	Light yellow	Flat	Smooth
PRN10	Circular	Glistening	Medium	Cream	Convex	Smooth

Table 4.3 Biochemical characterization of protease-producing isolates

Biochemical Test	PRN1	PRN2	PRN3	PRN6	PRN9	PRN10
Gram staining	+	+	-	+	+	-
Catalase	+	+	+	+	+	+
Oxidase	-	+	+	-	-	-
Simon's citrate	+	+	-	+	+	+
Voges-Proskauer	+	+	+	+	+	+
Methyl Red	+	-		+	-	-
Indole	-	-	+	-	-	-

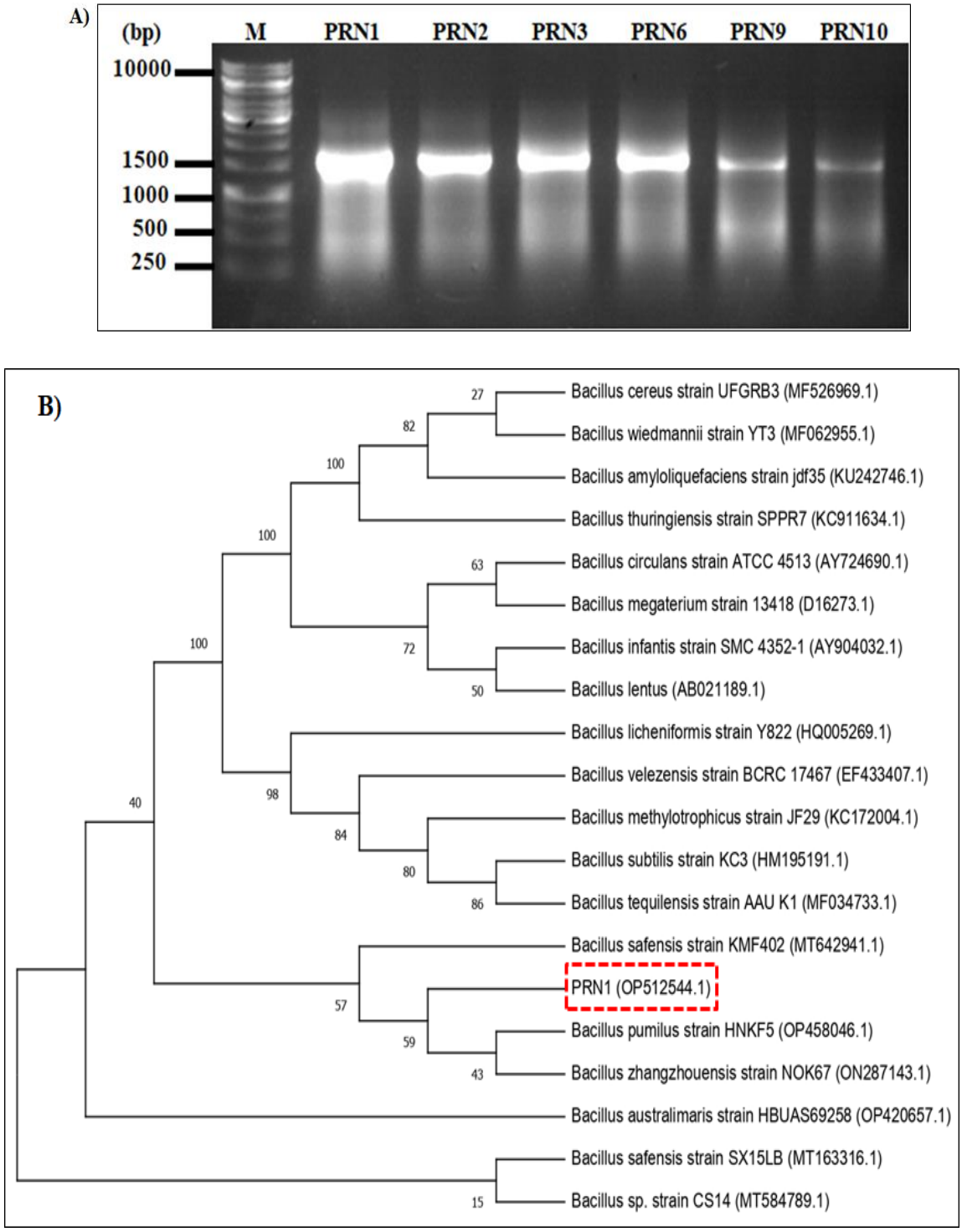

Fig. 4.4. Molecular identification of the protease-producing isolates. A) Electrophoretic separation of PCR amplification products of 16S rRNA on 0.8% agarose gel. M represents the marker (1 kb); **B)** Phylogenetic tree of PRN1 isolate

Table 4.4 % homology and Gene Bank accession no. of the isolates

Isolate	% Homology	Closs-related species	Gene Bank Accession no.
PRN1	100	*Bacillus safensis* strain KMF402 (MT642941.1)	OP512544
PRN2	98.48	*Priestia megaterium* strain 2022-XJ (OP562481.1)	OP850666
PRN3	98.83	*Aeromonas veronii* strain G12 (MK874842.1)	OP850667
PRN6	100	*Staphylococcus warneri* strain DK131 (MT642942.1)	OP850668
PRN9	100	*Bacillus* sp. Strain B46 (OP550119.1)	OP850664
PRN10	99.87	*Serratia oryzae* strain HL001 (OP020632.1)	OP850665

4.3. Optimization of protease production

4.3.1. Duration of incubation

To determine the time-dependence of protease production *B. safensis* PRN1 was incubated in the basal medium with submerged fermentation for 96 h. Bacterial growth (OD_{600}) and the proteolytic activity profile are shown in Fig. 4.5 which demonstrates an increase in the growth as well as enzyme activity up to 72 h reaching a maximum value of OD_{600}=0.47,1213 U/mL, respectively. However, a decline in enzyme activity could be observed at 96 h (1211.18 U/mL) and 120h (1115.09 U/mL) of incubation.

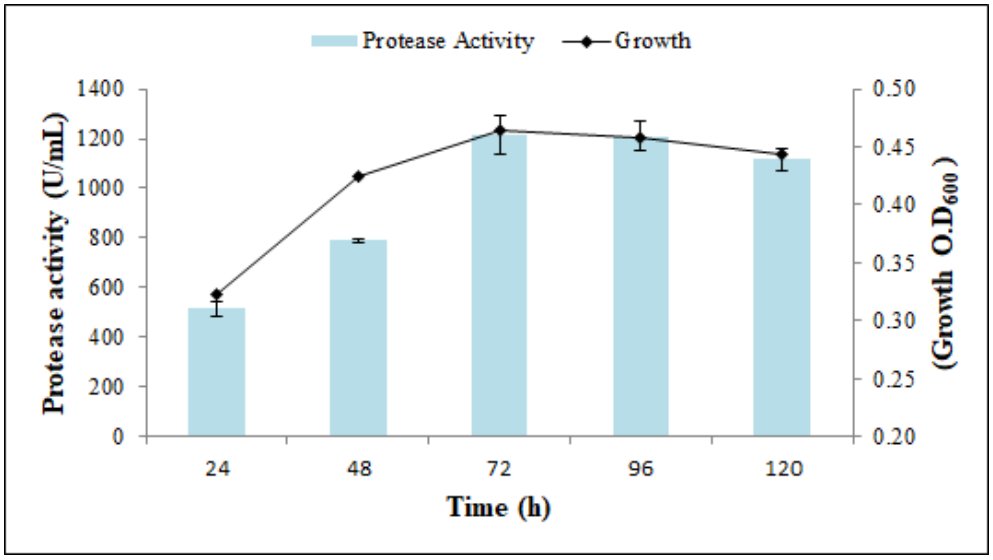

Fig. 4.5. Protease activity and growth optimization of *B. safensis* (PRN1). Effect of incubation time (24, 48, 72, and 96 h). Error bars represent the standard deviation of three measurements

4.3.2. pH and temperature

To determine the effect of pH on growth pattern and protease production, the bacteria *B. safensis* PRN1 was grown at different pH. It was found that the protease production and the growth increased as the pH of the medium increased, and the maximum protease activity (403.29 U/mL) and the growth (OD_{600}=0.83) were observed at pH8 while both decreased thereafter. The bacterial growth and the enzyme production profile in different pH were presented in Fig. 4.6A. The effect of incubation temperature on the protease production and growth of the bacteria showed that at 40°C protease activity (1021.91 U/mL) and growth (OD_{600}=1.23) were maximum. Whereas, a decrease in both growth as well as enzyme activity could be observed at 50°C (20.07 U/mL; OD_{600}=0.07) and 60°C (12.81 U/mL; OD_{600}=0.047) (Fig. 4.6B).

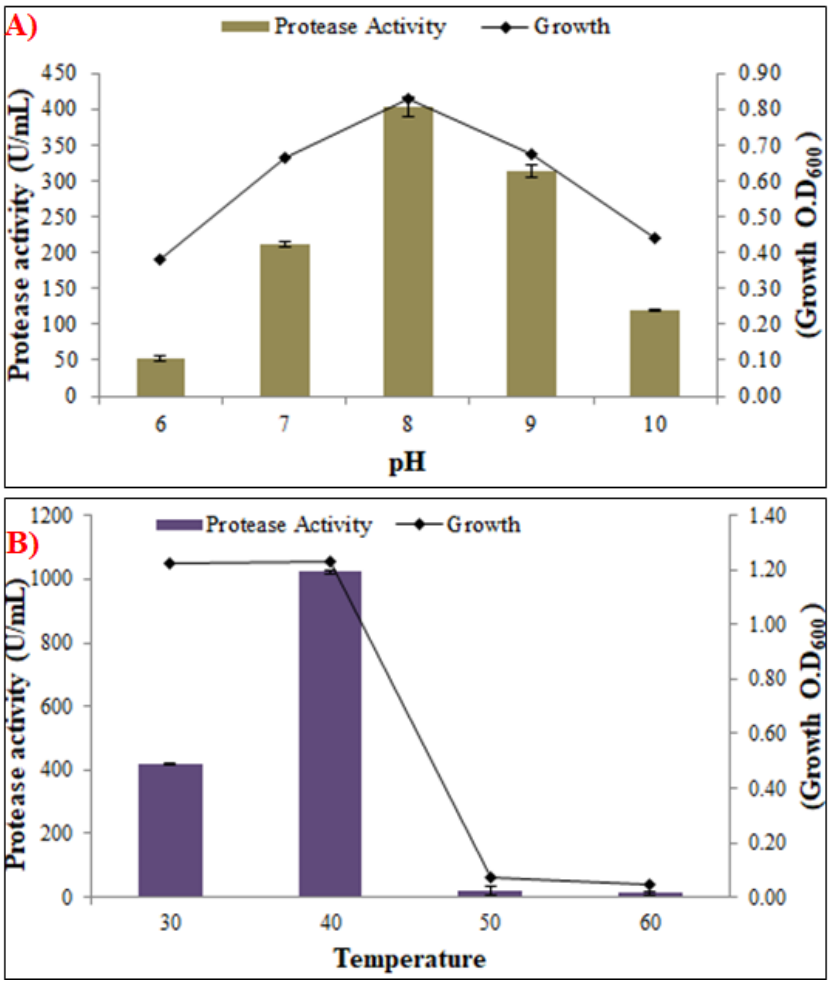

Fig. 4.6. Protease activity and growth optimization of *B. safensis* (PRN1). A) pH (6, 7, 8, 9, and 10); **B)** temperature (30, 40, 50, and 60°C).

4.3.3. Carbon and Nitrogen Source

Extracellular protease production, as well as the growth of the bacteria, varied with different carbon sources in the culture medium. The protease activity and growth of *B. safensis* PRN1 followed the order; fructose (722.00 U/mL; OD_{600}=1.14) > maltose (653.22 U/mL; OD_{600}=1.13) > sucrose (468.23U/mL; OD_{600}=0.88) > glucose (449.01U/mL; OD_{600}=0.66) > lactose (34.17U/mL; OD_{600}=0.09) (Fig. 4.7A).

Likewise, among different nitrogen sources, *B. safensis* PRN1 demonstrated maximum protease production (876.83 U/mL) and cell density (OD_{600}=1.74) in gelatin-containing medium. In the presence of beef extract, yeast extract, urea, and ammonium sulphate protease activity and bacterial growth were 724.14 U/mL, OD_{600}=1.69; 761.73

U/mL, OD_{600}=1.71; 677.78 U/mL, OD_{600}=1.08; and 648.43 U/mL, OD_{600}=0.57, respectively (Fig. 4.7B).

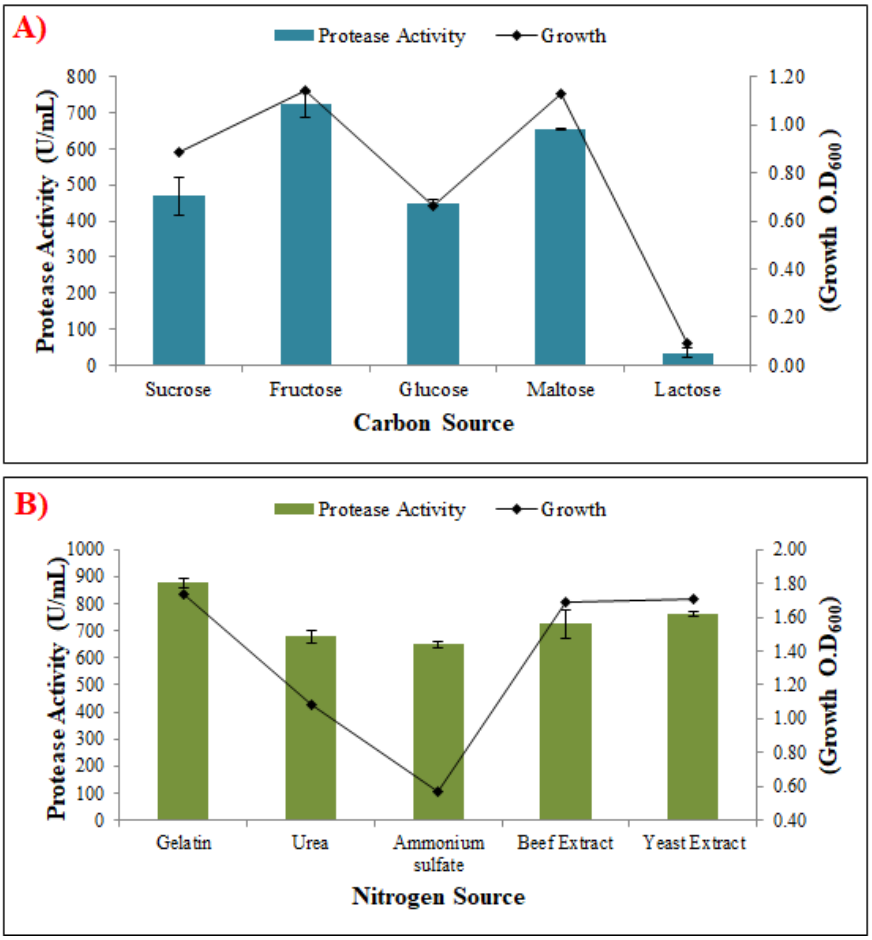

Fig. 4.7. Protease activity and growth optimization of *B. safensis* (PRN1). A) Carbon source (sucrose, fructose, glucose, maltose, and lactose); **B)** Nitrogen source (gelatin, urea, ammonium sulfate, beef extract, and yeast extract).

4.4. Industrial applications

4.4.1. Effect of protease on removing blood stain

The crude protease was found effective in removing blood stain from the cotton cloth. Insignificant stain removal was seen when the stained cloth was subjected to water, Surf Excel (10 mg/mL), and the protease alone. However, the addition of the protease with detergent demonstrated significant de-staining as compared to when the detergent was applied independently Fig. 4.8A.

4.4.2. Antibacterial activity

The crude protease demonstrated antibacterial activity against pathogenic bacteria. The maximum activity was observed against *Pseudomonas diminute* (16±1 mm) while the least was observed against *Yersinia enterocolitica* (9±1 mm). No activity was observed in negative control (media) whereas Gentamicin exhibited positive activity against all the tested strains. The same are presented in Fig. 4.8B and Table 4.5.

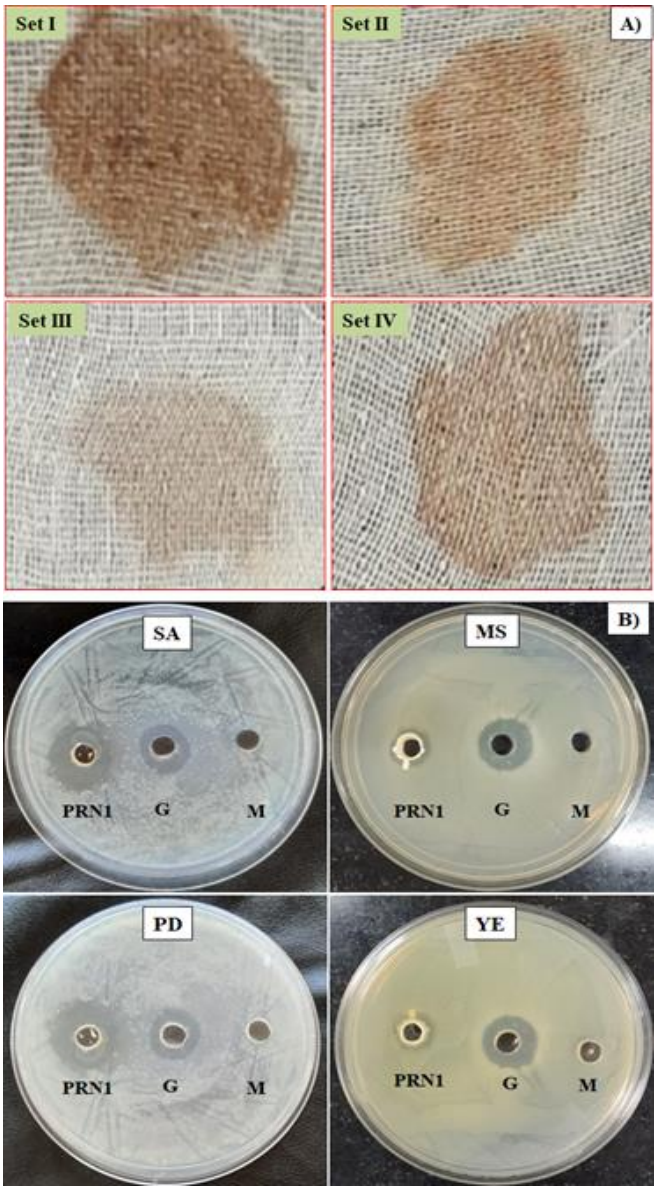

Fig. 4.8. Industrial applications of crude protease. A) Blood-stain removal property Set I: Control (Stained cloth + Water), Set II: 20 mL Detergent solution (Surf excel) (10 mg/mL) + stained cloth, Set III: 20 mL detergent solution + 400 µL of crude enzyme +

stained cloth, Set IV: 20 mL tap water + 400 µL crude enzyme + stained cloth; **B)** Antibacterial activity Agar well diffusion assay. Gentamicin (G) and blank media (M) are the positive and negative controls, respectively

Table 4.5 Zone of inhibition (mm)

	Zone of Inhibition (mm)		
Bacteria	***B. safensis* (CFS)**	**Gentamycin**	**Negative control**
Staphylococcus aureus	15±1	12±1	ND
Microbacterium smegmatis	10±1	14±1	ND
Pseudomonas diminuta	16±1	13±1	ND
Yersinia enterocolitica	9±1	14±1	ND

ND- Not defined

4.5. Purification of the crude enzyme

Purification of the crude protease was summarized in Table 4.6. Gel filtration chromatography of the protease on Shodex KW-803 column yielded three peaks as shown in Fig. 4.9A. Only peak 1 demonstrated proteolytic activity with 31.7% yield, 25.8 purification fold, and 9952.0 U/mg of protein specific activity. Furthermore, RP-HPLC was performed to confirm the purity of the protease. A single peak was eluted at a retention time of 5 min in a reverse phase C-18 column as shown in Fig. 4.9B. The SDS-PAGE of the purified protease demonstrated a single band at approximately 33 kDa indicating the homogenous and monomeric nature of the enzyme (Fig. 4.9C).

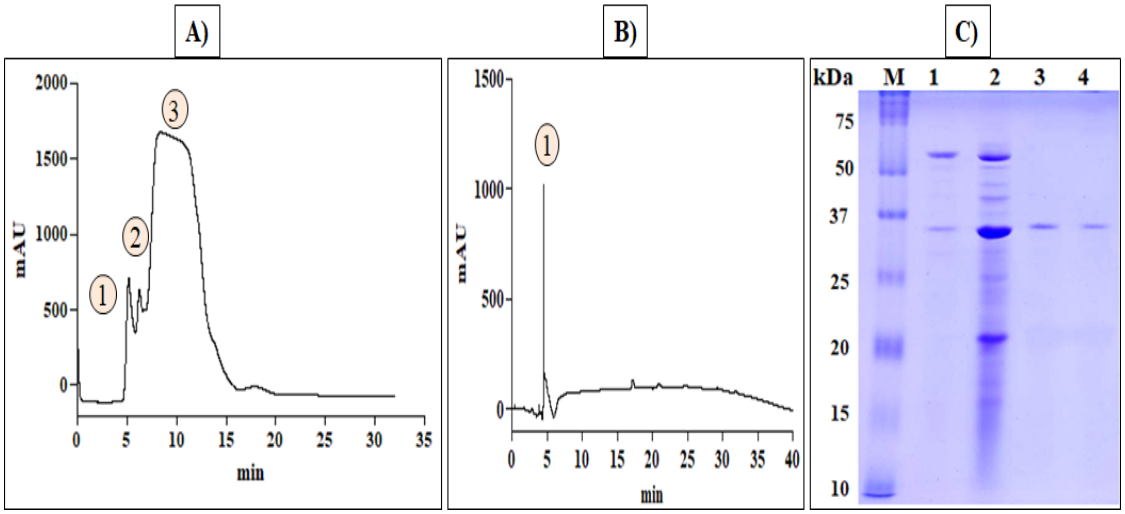

Fig. 4.9. Purification of the protease from *Bacillus safensis* PRN1. (A) Gel filtration chromatography of the protease using Shodex KW-803 column; **(B)** RP-HPLC analysis of the purified protease; **(C)** 12% SDS-PAGE of the purified protease. Lane M, protein markers. Lane 1, crude protease, Lane 2, protease after fractionation with ammonium sulfate precipitation (70%)-dialysis, Lane 3, purified protease obtained after Shodex KW-803 gel filtration chromatography (peak 1), Lane 4, HPLC analysis of the purified protease (peak 1).

Table 4.6 Purification of the protease enzyme from *Bacillus safensis* PRN1

Purification step	Total Activity (U)	Total Protein (mg)	Specific Activity (U. mg^{-1})	Yield (%)	Purification (fold)
Crude Protease	48845.74	127	384.63	100	1
$(NH_4)_2SO_4$ Precipitation (70%)- dialysis	20364.26	7.5	2715.2	41.6	7.0
Gel filtration Chromatography	15525.26	1.5	9952.0	31.7	25.8

4.6. Characterization of purified protease

4.6.1. Effect of pH and temperature on the purified protease activity

The purified enzyme demonstrated maximum activity in Tris-HCl buffer at pH 8 as shown in Fig. 4.10A. The relative enzyme activities at pH 6 and 10 were 55.2 and 35.7%, respectively. The enzyme activity was considerably reduced by decreasing pH and only 12.2% of activity was shown at pH 4. The effect of temperature on the protease activity showed that with the increase in temperature, the activity of the enzyme increases gradually and the maximum activity was observed at 60°C as shown in Fig. 4.10B. However, the protease activity decreased to 24.7 and 9.6% at 70 and 80°C, respectively.

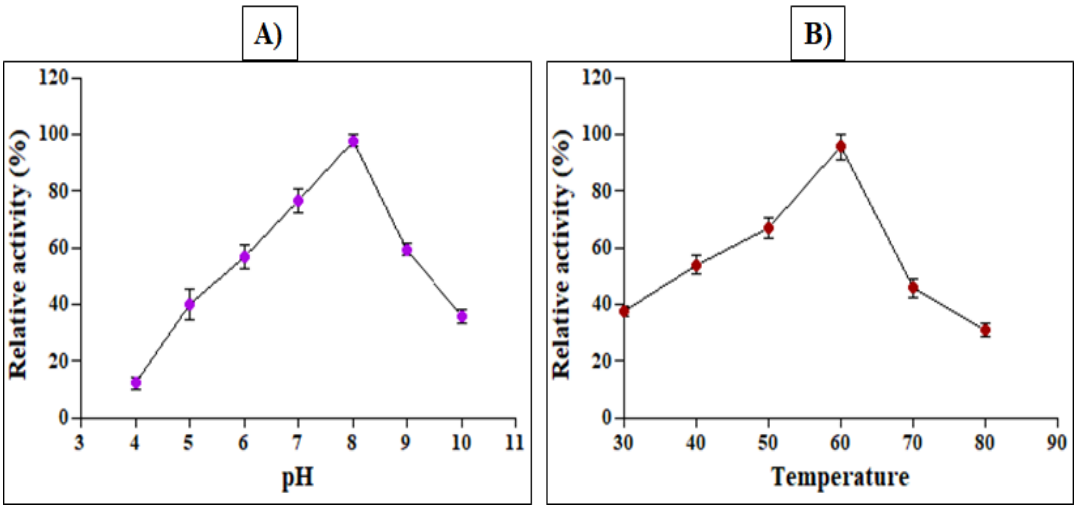

Fig. 4.10. Effect of pH and temperature on enzyme activity. A) pH; B) temperature

4.6.2. Effect of inhibitors on the protease activity

The effect of various inhibitors is shown in Table 4.7. The enzyme activity was strongly inhibited by PMSF, a well-known serine protease inhibitor. The relative activity of the enzyme was increased by 90.4%, 91.1%, and 92.5% in the presence of other inhibitors such as DTT, EDTA, and mercaptoethanol, respectively.

Table 4.7 Effect of various inhibitors and surfactants on the protease enzyme. The relative activity of the non-treated enzyme was taken as a control (100%).

Inhibitors	**Concentration**	**Relative enzyme activity (%)**
None	-	100 ± 0.7
PMSF	5 mM	7.2 ± 0.8
DTT	5 mM	90.4 ± 0.9
EDTA	5 mM	91.1 ± 0.5
Mercaptoethanol	5 mM	92.5 ± 2.0
Surfactants	**Concentration**	**Relative enzyme activity (%)**
None	-	100 ± 3.2
Tween 80	5%	82.1 ± 0.6
Tween 20	5%	97.7 ± 2.2
Triton X-100	5%	92.6 ± 2.2
SDS	1%	68.1 ± 3.6

4.6.3. Effect of surfactants and commercial detergents on protease stability

In detergent formulation stability of protease enzyme towards non-ionic surfactants is one of the criteria for selecting compatibility of enzyme in detergent industry. The enzyme demonstrated excellent stability in the presence of non-ionic surfactants at 5% final concentration. Protease retained 82.1%, 97.7%, and 92.6% of its original activity in the presence of tween 80, tween 20, and triton X-100, respectively as shown in Table 4.7. However, a decrease in relative activity (68.1%) was seen in 1% anionic surfactant SDS.

The stability of the protease towards commercial detergents was studied and shown in Fig. 4.11. The protease was highly stable retaining 80.4% to 98.2% of its original activity with all the tested detergents. The lowest relative activity of the enzyme was noticed with “tide” detergent in which 80.4% of the initial activity was retained. The relative activity of the enzyme in the presence of Surf Excel, Ariel, and Ghadi detergents

were found as. 98.2%, 84.3%, and 81%, respectively These findings indicated the potential of the protease in commercial detergents.

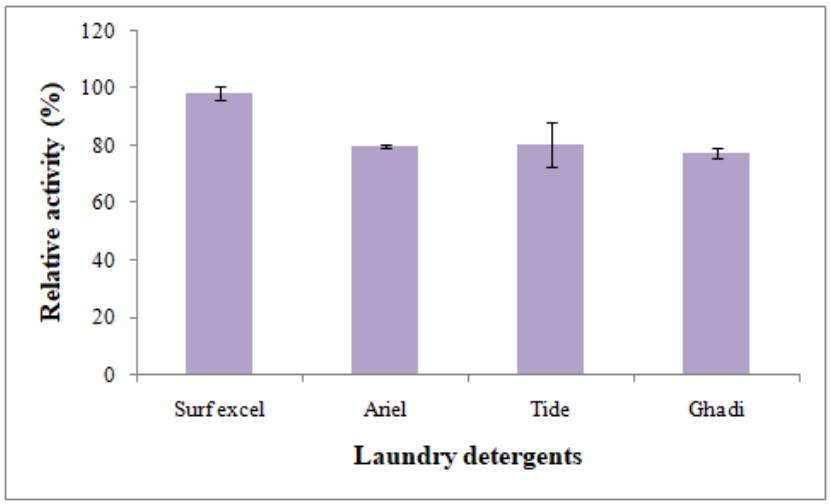

Fig. 4.11. Effect of detergents on protease activity.

4.7. Isolation and purification of genomic DNA from *B. safensis*

4.7.1. DNA yield and quality

The concentration of the isolated DNA was 24.8 ng/µL. The average value for A260/A280 was 1.759.

4.7.2. Amplification of protease gene

The DNA of *B. safensis* PRN1 was used as a template to amplify the *knbs*^{SP1} gene. A single band of the amplicon at ~909 bp was seen in agarose gel electrophoresis.

4.7.3. Electrophoresis

The genomic DNA and the amplified protease gene were examined by agarose gel electrophoresis against 1 kb plus DNA marker (Fig. 4.12A and 4.12B).

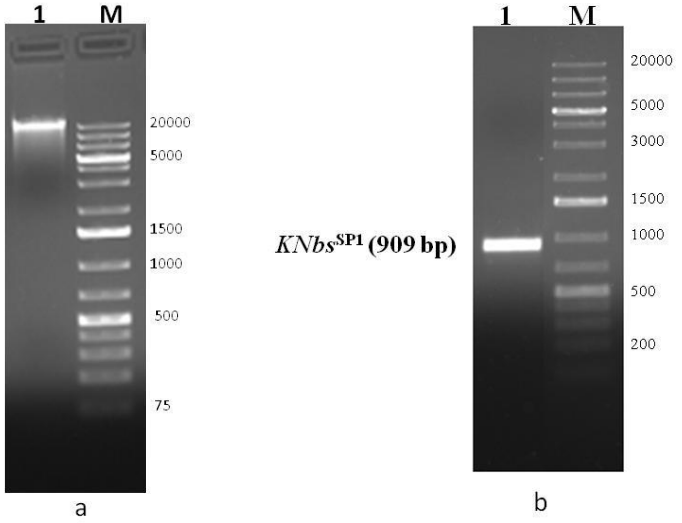

Fig. 4.12. Gel electrophoresis. A. Genomic DNA of *B. safensis* PRN1, **B.** serine protease gene (*knbs*^{SP1})

4.7.4. Serine protease gene cloning

A schematic illustration of the recombinant construct developed for this study is shown in Fig. 4.13. 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 (Fig. 4.14). The sequence data obtained were submitted in GenBank with an accession number (OR295559).

The recombinant plasmid (pPM1) containing the protease gene (*knbs*^{SP1}) was transformed into *E. coli* cells for the over-expression of the KNBS^{SP1} enzyme and was induced with 0.1 mM concentration of IPTG at a cell density (OD_{600}) of ~ 0.6. Protein expression profile was measured regularly from 2-6 h. The SDS-PAGE results demonstrated that the IPTG-induced culture produce a distinct band at ~33.0 kDa and a good level of expression was observed after 6 h of induction at 37°C (Fig. 4.15).

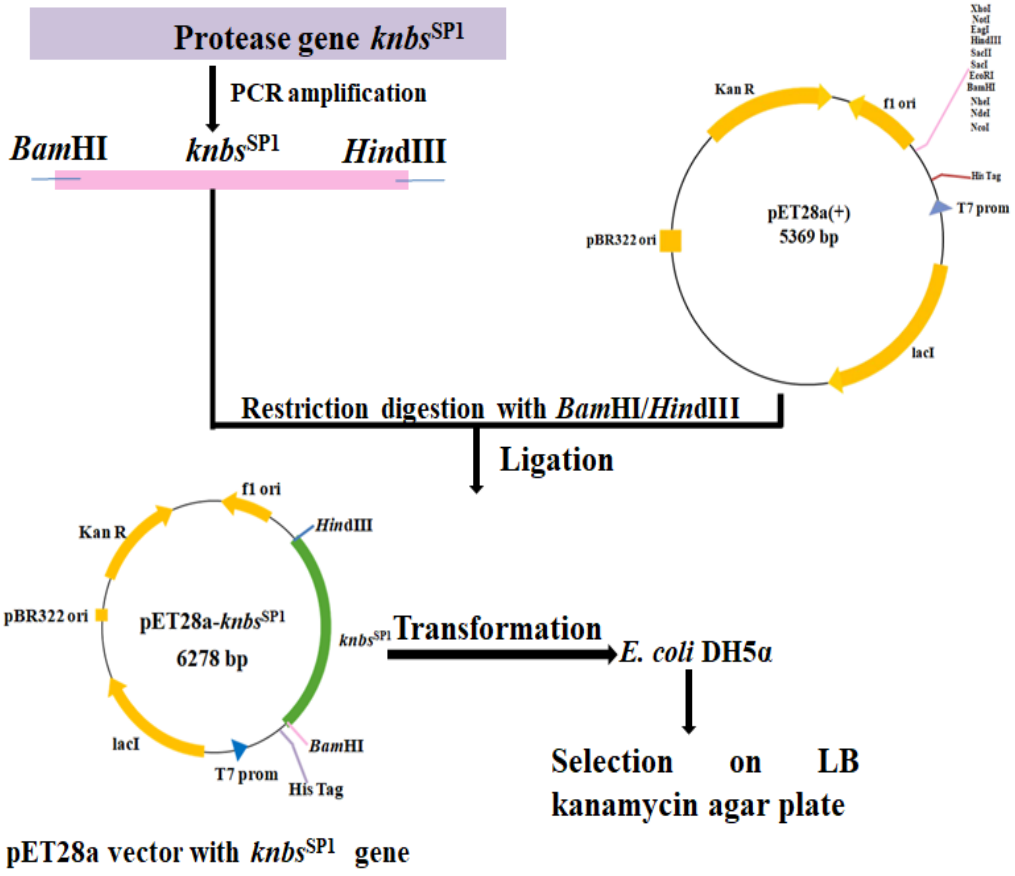

Fig. 4.13. A schematic illustration of the recombinant construct developed for this study

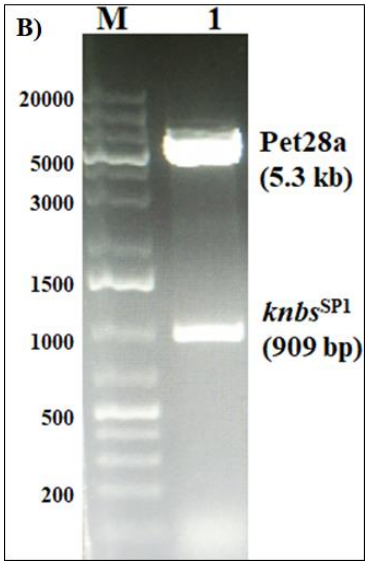

Fig. 4.14. Restriction digestion evaluation of a positive clone. M: 1 kb plus ladder; 1: PET28a-*knbs*^{SP1}. Enzyme *Bam*HI and *Hin*dIII were used for restriction digestion

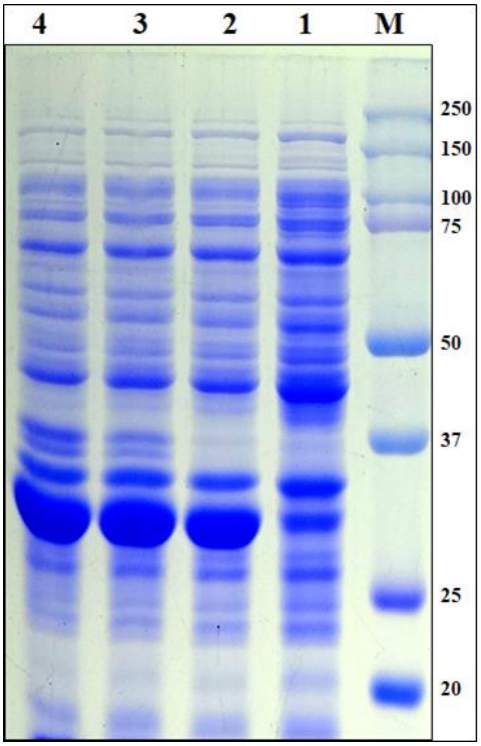

Fig. 4.15. 12% SDS-PAGE gel showing expression profiling of 6xHis-tagged KNBS^{SP1} enzyme in *E. coli* BL21 (DE3). M: Molecular weight protein marker, 1: Un-induced crude extract, 2: Crude extract after induction of 2 h with IPTG, 3: Crude extract after induction of 4 h with IPTG, 4: Crude extract after induction of 6 h with IPTG

4.8. Purification of the serine protease enzyme

For purification, Ni-NTA chromatography was used, as N-terminal region of the KNBS^{SP1}enzyme consisted of six histidine base residues that permit the enzyme purification in a single step through Ni-NTA chromatography. A single band at 33.0 kDa was observed in the purified fraction in lane 1 (Fig. 4.16).

The results of purification were shown in Table 4.8. The purity of the enzyme was assessed to be about 55.1-fold higher than the crude extract. In the optimum assay conditions, the specific activity and yield of the purified rKNBS^{SP1} was 43130 U/mg and 69.3%, respectively.

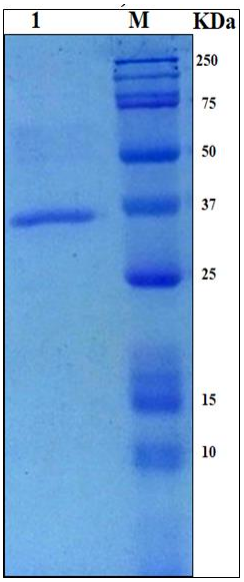

Fig. 4.16. Purification of 6xHis- KNBS^{SP1} enzyme. SDS-PAGE analysis of purified KNBS^{SP1}; M: Molecular weight protein marker, Lane 1: Purified 6xHis-KNBS^{SP1}enzyme

Table 4.8 Purifications of the recombinant protease His6-rKNBS^{SP1} of *B. safensis* strain PRN1.

Purification step	Total Activity (U)[b]	Total Protein (mg)[c]	Specific Activity (U. mg^{-1})[a]	Yield (%)	Purification (fold)
Crude extract	143490 ± 386	183.3 ± 0.04	782	100	1
Ni-NTA affinity chromatography	103512± 651	2.4 ± 0.02	43130	69.3	55.1

[a]One unit of the enzyme activity was defined as the amount that yielded 0.01 change in absorbance at 440 nm in one minute.

[b]The initial volume of crude extract solution was 100 mL.

[c]Protein concentration was determined by the Lowry method with bovine serum albumin (BSA) as a standard.

4.9. Analysis of amino acid sequence

Similarity searches between amino acid sequence inferred from the nucleotide sequence of the *knbs*^{SP1} gene in relation to other protease-producing *Bacillus* strains were represented in Fig. 4.17. The sequence reported strong similarity with those of other known extracellular trypsin like serine proteases previously obtained from *Bacillus* strains. The data displayed 99 (299/302), 97 (294/302), 95 (287/302), 97 (292/302), 97 (293/302), and 97% (294/302) similarities with the trypsin like serine proteases from *Bacillus sp.* G1, *B. australimaris*, *B. altitudinis*, *B. zhangzhouensis*, *B. pumilus* and *B. intermedius*, respectively. The nucleotide sequence alignment showed 99% sequence similarity with the other strains of *B. safensis.*

4.10. Characterization of the purified recombinant enzyme

4.10.1. Influence of pH and temperature on rKNBS^{SP1} activity and stability

The rKNBS^{SP1} demonstrated activity in a wide range of pH (3-10) and the highest activity was in Tris-HCl buffer at pH 8 (Fig. 4.18A). The relative enzyme activities at pH 7 and 10 were 83.6 and 86.0%, respectively. The enzyme activity got reduced considerably in low pH, only 39.3% of activity was observed at pH 4. The results of pH stability demonstrated that the protease was extremely stable in the range of 7-10 pH retaining 70% of activity (Fig. 4.18B).

The effect of temperature on the enzyme activity and stability demonstrated that the protease activity increased gradually with increase in temperature, and the optimum activity was reached at 60°C (Fig. 4.18C). However, the protease activity above 60°C was slightly reduced to 84.1 and 77.7% at 70 and 80°C, respectively. Thermostability of the purified rKNBS^{SP1} enzyme was observed at temperatures ranging between 50-70°C for 1 h. The thermostability profile demonstrated high stability of the protease at 60°C (98%) and retained 78.1 and 57.6% of the original activity at 70 and 80°C, respectively (Fig. 4.18D).

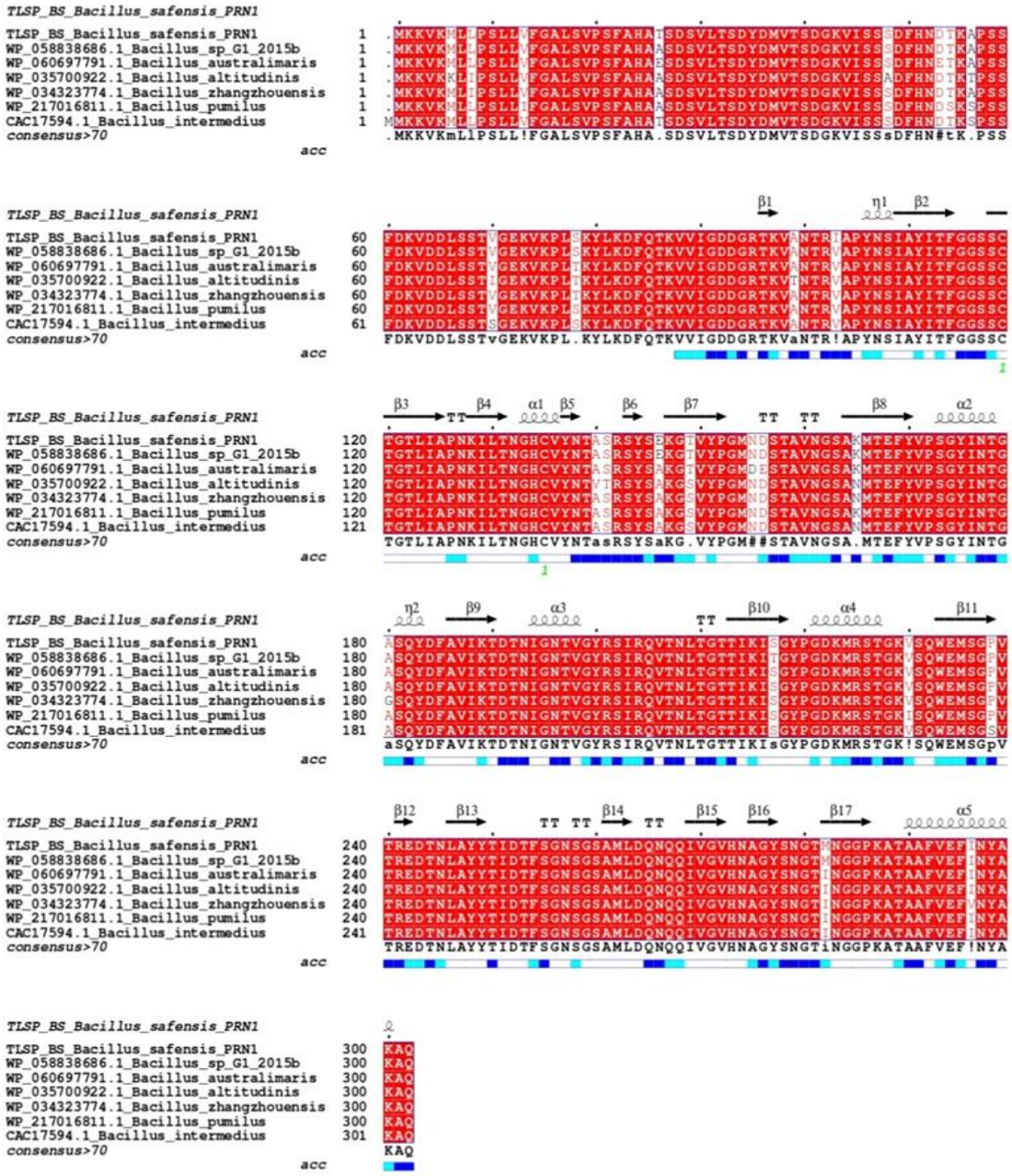

Fig. 4.17. Multiple sequence alignment of KNBS^{SP1}. Trypsin like serine protease from *B. safensis* PRN1 was aligned with other trypsin like serine proteases from *Bacillus sp.* G1, *B. australimaris*, *B. altitudinis*, *B. zhangzhouensis*, *B. pumilus* and *B. intermedius* by ClustalW2 and represented with ESPript 3.0. The top of the alignment depicts the secondary structure elements of KNBS^{SP1} enzyme. Residues conserved and identical within each group are represented by white letters in a red background. At the bottom of alignment, consensus sequences with >70% threshold are shown.

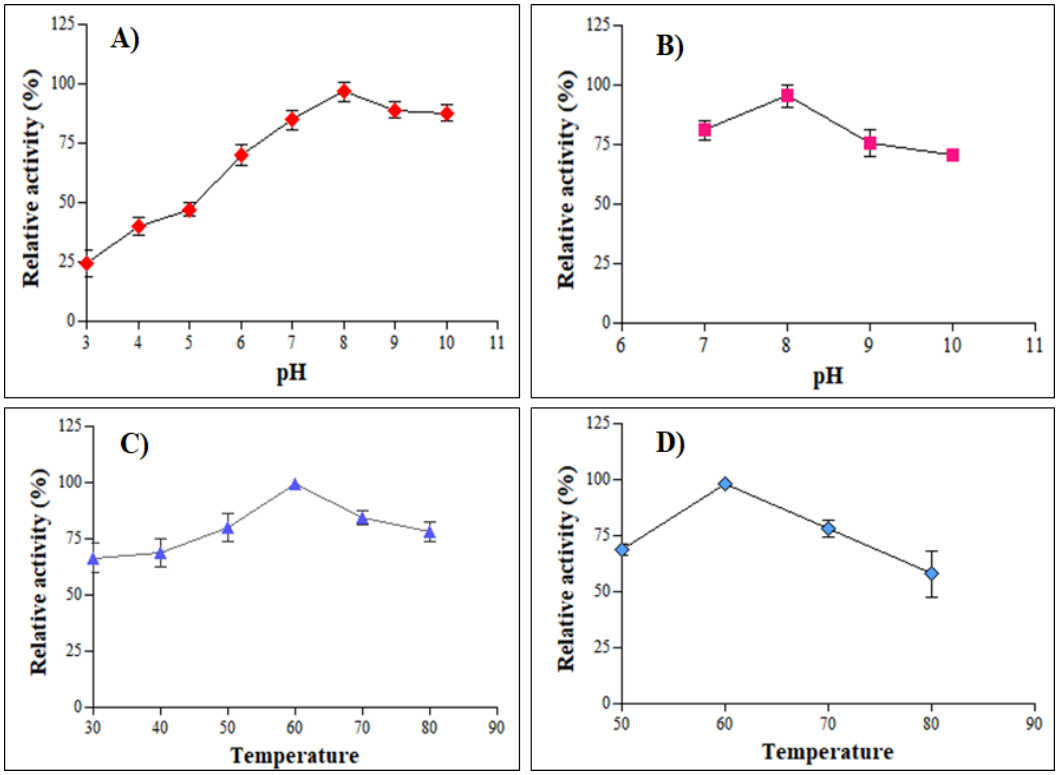

Fig. 4.18. Influence of pH and temperature on enzyme activity and stability of the rKNBS^{SP1} protease. Influence of pH on **(A)** enzyme activity and **(B)** stability. The enzyme activity at pH 8 was taken as 100%. Effect of temperature on **(C)** enzyme activity and **(D)** stability. Error bars represent the standard deviations of three measurements

4.10.2. Influence of inhibitors, oxidizing agents, and non-ionic surfactants

Exploring the influence of inhibitors on the enzyme activity demonstrated that the enzyme retained 90% to 95% of its original activity in the presence of metalloprotease (EDTA) while the protease activity enhanced in presence of thiol (β-ME, DTT) reagents (Table 4.9). However, PMSF and DFP, two popular serine protease inhibitors, strongly inhibit the enzyme activity.

The influence of various oxidizing agents, non-ionic, and anionic surfactants on the protease stability is summarized in Table 4.9. rKNBS^{SP1} retained 104.4, 89.3, and 92.0% in the presence of 5% non-ionic surfactants. Interestingly, KNBS^{SP1} was hardly affected by anionic surfactant and retained 96.9% of its relative activity in presence of 1% SDS.

Table 4.9 Effects of different inhibitors, surfactants and oxidizing agent on KNBS^{SP1} stability.

Inhibitor/surfactant /oxidizing agent	**Concentration**	**Relative protease activity (%)[a]**
None	-	100 ± 6.6
PMSF	5 mM	7.59 ± 3.2
DFP	5 mM	4.15 ± 3.7
EDTA	5 mM	99.85 ± 2.9
DTT	5 mM	94.65 ± 6.2
β-ME	5 mM	98.7 ± 1.0
Triton X-100	5% (v/v)	104.45 ± 3.5
	10% (v/v)	94.90 ± 6.5
Tween 20	5% (v/v)	89.07 ± 7.9
	10% (v/v)	85.69 ±6.1
Tween 80	5% (v/v)	92.04 ± 4.3
	10% (v/v)	89.32 ± 3.1
SDS	1% (v/v)	96.9 ± 2.0
	5% (v/v)	64.51 ± 3.8
H_2O_2	5% (v/v)	83.73 ± 2.6
	10% (v/v)	67.89 ± 4.0

[a]Values represent the means of three replicates, and ± standard deviations are reported

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4.10.3. Influence of metallic ions on the stability of rKNBS^{SP1}

The effect of some metal ions ($FeSO_4$, $CaCl_2$, $HgCl_2$, $MnSO_4$, $ZnSO_4$, $MgSO_4$, and $CuSO_4$) on the protease activity of the purified enzyme was determined (Fig. 4.19A). In comparison to the control, the protease activity was increased by 125.7%, and 105.7% after incorporation of 2 mM concentration of $CaCl_2$ and $MgSO_4$, respectively. Furthermore, the activity of the protease was decreased slightly to 98.4 and 87.1% in the presence of $MnSO_4$ and $CuSO_4$. This result suggested 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 $FeSO_4$ correspondingly inhibits the enzyme activity to 18.5, 28.4, and 32.7%, respectively.

4.10.4. Compatibility of rKNBS^{SP1} with commercial detergents

The rKNBS^{SP1} demonstrated good compatibility with commercial detergents maintaining greater than 90% of its original activity even after incubating with Surf Excel, Ariel, Tide, and Ghadi (7 mg/mL) (Fig. 4.19B).

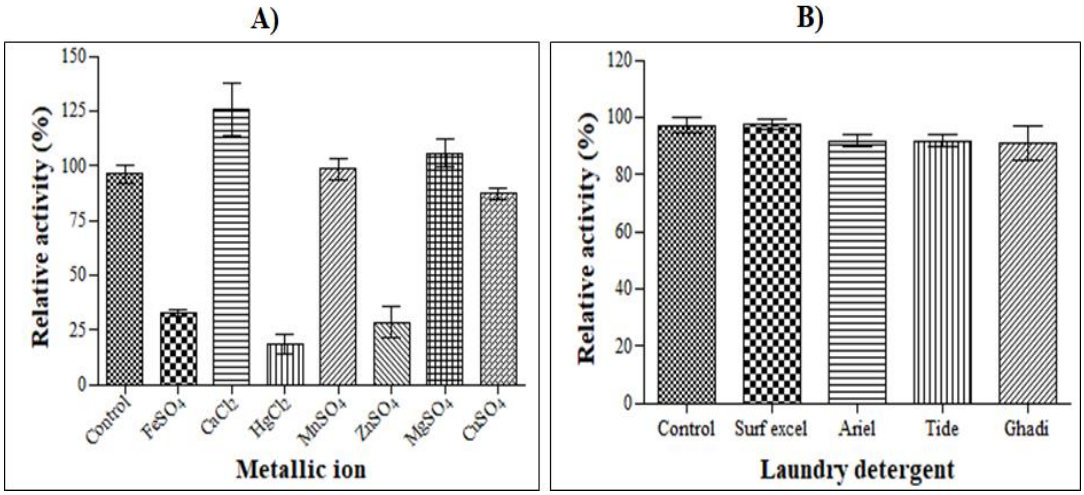

Fig. 4.19. Effect of metal ions on the rKNBS^{SP1} stability and its compatibility of with commercial detergents. (A) Influence of metal ions on the stability. **(B)** rKNBS^{SP1} compatibility with various commercial detergents (Surf Excel, Ariel, Tide and Ghadi) at 7 mg/mL. The relative enzyme activity of the control that contain no detergents was considered as 100 %. Error bars represent the standard deviations of three measurements.

4.11. K_m and V_{max} values

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 (Table 4.10). The turnover number value of 65.25 min^{-1} and catalytic efficiency of 63.34 min^{-1} mg mL^{-1} were reported in this study.

Table 4.10 Kinetic values of KNBS^{SP1} towards azocasein

Substrate	K_m (mg mL^{-1})[a]	V_{max} (U mg^{-1}) [a]	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mg mL^{-1})
Azocaesin	1.03 ± 0.14	359.7 ± 8.15	65.25	63.34

[a]Values represent the means of three replicates, and ± standard deviations are reported.

4.12. *In silico* analysis of amino acid sequence

The amino acid sequence of the KNBS^{SP1} translated from the trypsin like serine protease gene of the *B. safensis* PRN1 is shown as

MKKVKMLLPSLLVFGALSVPSFAHATSDSVLTSDYDMVTSDGKVISSSDFHNDTKAPSS
FDKVDDLSSTVGEKVKPLSKYLKDFQTKVVIGDDGRTKVANTRIAPYNSIAYITFGGSSC
TGTLIAPNKILTNGHCVYNTASRSYSEKGTVYPGMNDSTAVNGSAKMTEFYVPSGYINT
GASQYDFAVIKTDTNIGNTVGYRSIRQVTNLTGTTIKISGYPGDKMRSTGKVSQWEMSG
PVTREDTNLAYYTIDTFSGNSGSAMLDQNQQIVGVHNAGYSNGTMNGGPKATAAFVEF
INYAKAQ

4.12.1. 3D structure of the trypsin like serine protease

Several templates from the PDB library were investigated by LOMETS. For the KNBS^{SP1} protease, 1p3cA (glutamyl endopeptidase from *bacillus intermedius*) was the best template with a normalized Z-score of 1.41. Additionally, C-score, TM-score and RMSD were used to characterized the model. For the KNBS^{SP1} model C-score, TM-score and RMSD values were estimated to be -1.21, 0.56 and 12.4 respectively (Fig 4.20A). From the PDBsum server, the Ramachandran plot was constructed. The plots demonstrate the torsional angles (Φ, Ψ) in the KNBS^{SP1} protease (Fig. 4.20B) (Table 4.11).

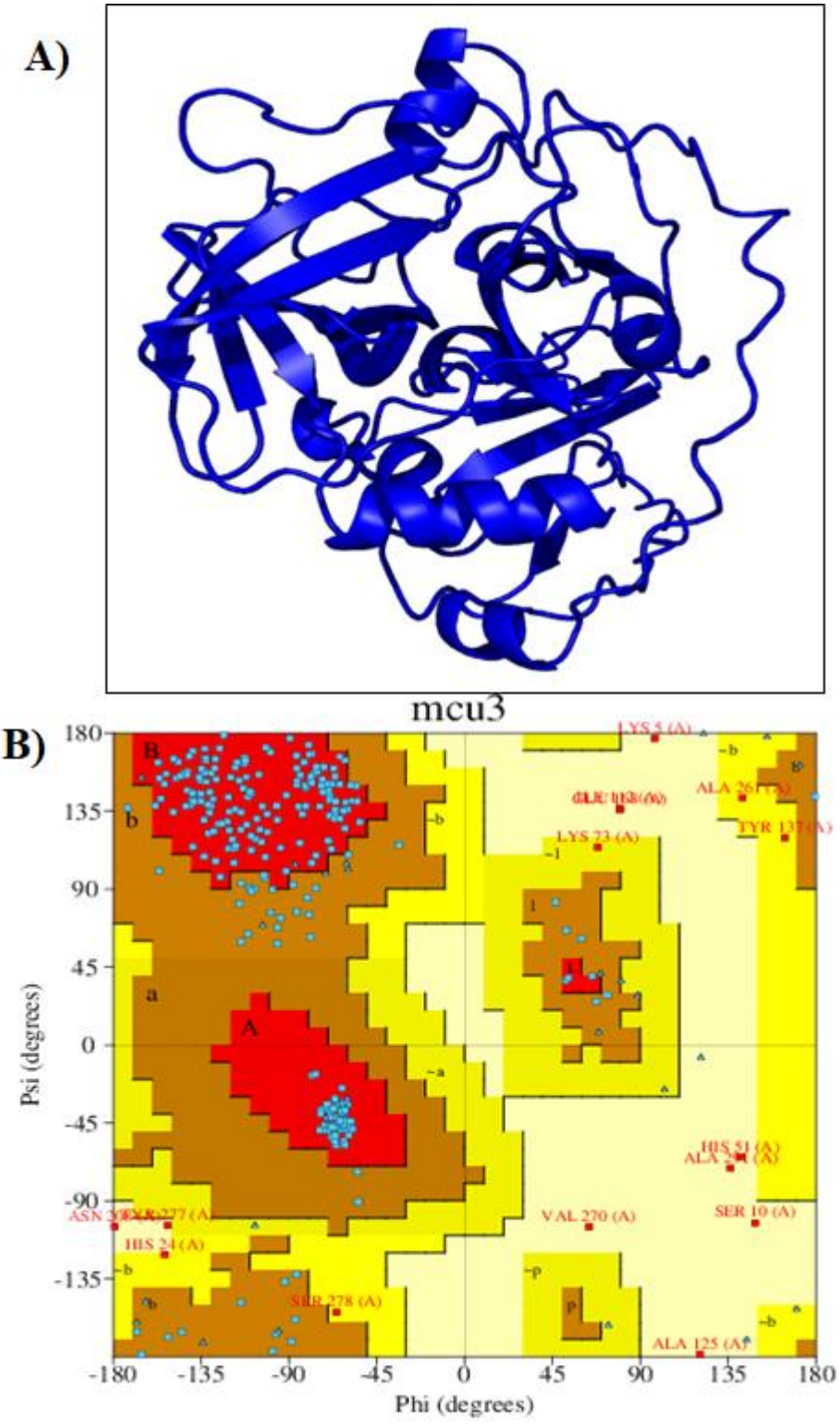

Fig. 4.20. *In silico* analysis. A) 3D Structure of the protease **B)** Ramachandran plot

Table 4.11 Ramachandran plot

Name of the model	Residues in most favoured region	Residues in additional allowed region	Residues in generously allowed region	Residues in disallowed region
1p3cA	207 (78.1%)	43 (16.2%)	5 (1.9%)	10 (3.8%)

4.12.2. Docking studies

The docking of the ligand (casein) was done using CABS-Dock server (Fig. 4.21). The interactions present between the KNBS^{SP1} with the target molecule was shown in Table 4.12 and Fig. 4.22. The binding free energy between the KNBS^{SP1} with casein was -7.8 kJ/mol.

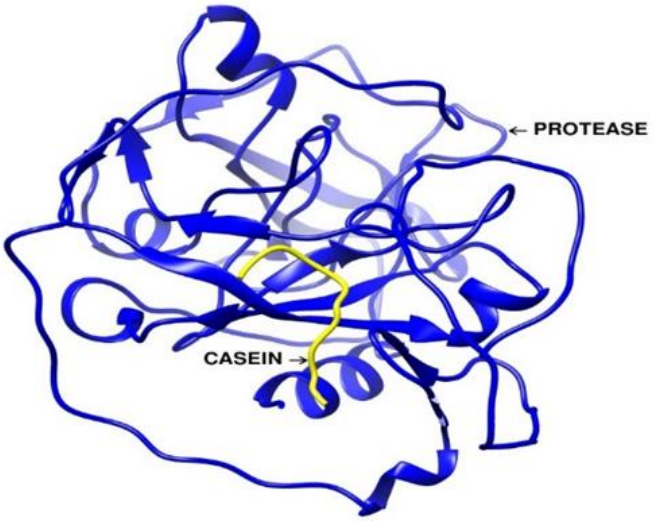

Fig. 4.21. Docking studies. KNBS^{SP1} -caesin

Table 4.12 Interactions present between the KNBS^{SP1} with the target molecule

Name of the complex	Number of H-bonds	Number of nonbonded complex
KNBS^{SP1} - caesin	5	105

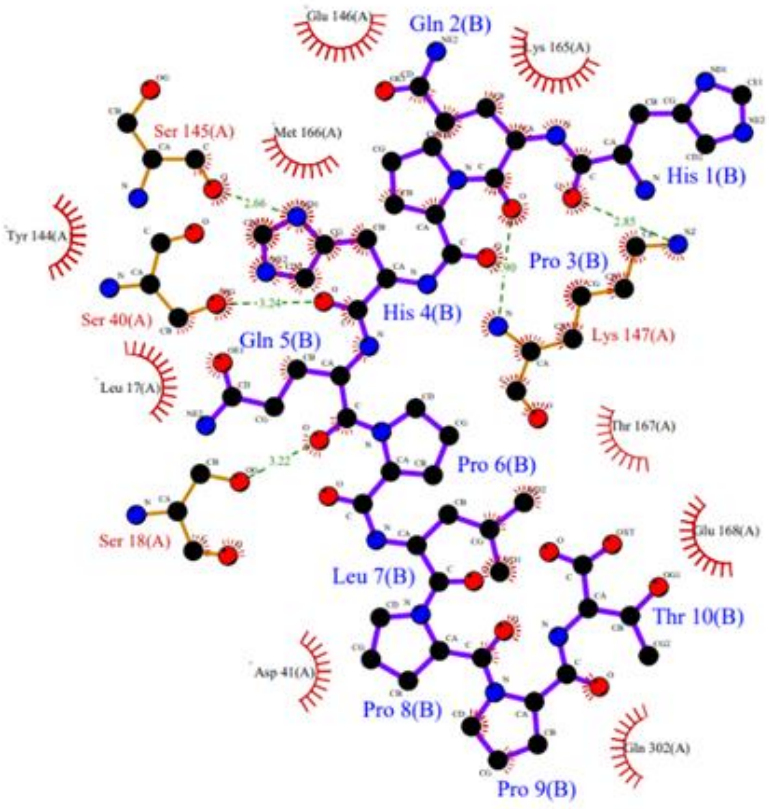

Fig. 4.22. Ligplot analysis of KNBS^{SP1} - caesin complex

4.13. Industrial applications of the recombinant enzyme

4.13.1. Blood-stain removal property

In the current study, the washing potential of rKNBS^{SP1}was evaluated by incubating the blood-stained cotton cloth at different conditions. The utilization of rKNBS^{SP1} in detergents was demonstrated in Fig. 4.23A. A limited washing performance was observed when the stained cloth was treated with detergent (Surf Excel) and purified rKNBS^{SP1} alone. The cleaning process was improved by the supplementation of rKNBS^{SP1} in detergent as confirmed by complete removal of stain from cotton cloth pieces. These findings demonstrated the potential of rKNBS^{SP1} enzyme as a bio-additive in detergent industry.

4.13.2. Hair removal potential

Dehairing potential of the rKNBS^{SP1} was assessed visually. Application of the purified rKNBS^{SP1} in the goat skin showed complete dehairing after incubation of 8 h at 37°C (Fig. 4.23B). However, hair removal was not observed in the control piece that was dipped in phosphate buffer pH8.

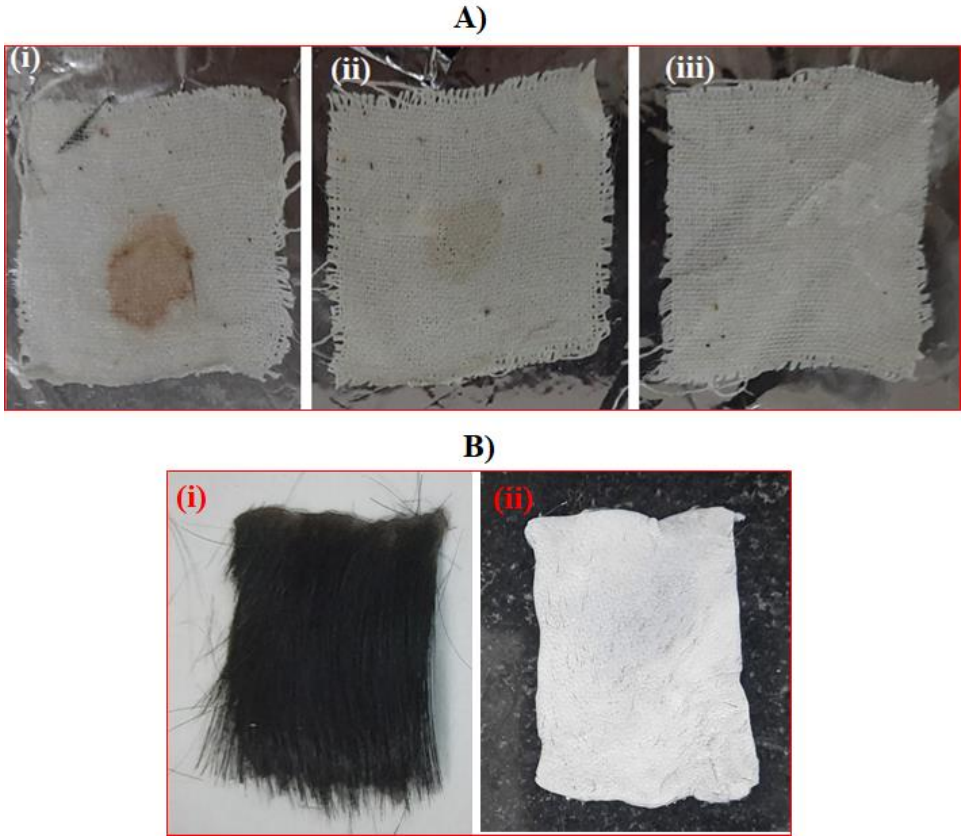

Fig. 4.23. Determination of Industrial Applications of rKNBS^{SP1}. (A) Analysis of washing performance potential of purified rKNBS^{SP1} using blood-stained cotton fabric piece (i) Blood-stained cotton cloth piece treated with water (control); (ii) Blood-stained cotton cloth piece treated with surf excel detergent (7mg/mL); (iii) Blood-stained cotton cloth piece treated with detergent supplemented with purified rKNBS^{SP1} (300 U/mL); **(B)** Analysis of dehairing potential of purified rKNBS^{SP1} using goat skin (i) Goat skin incubated in phosphate buffer (control); (ii) dehaired goat skin after incubation in purified rKNBS^{SP1} for 8 h

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

mgDNA was isolated from the intestinal tissue of *M. cuchia* was shown in Fig. 4.24. Since we have successfully isolated protease producing bacterial gene, therefore no further study was carried out using mgDNA of the cuchia intestinal tissue.

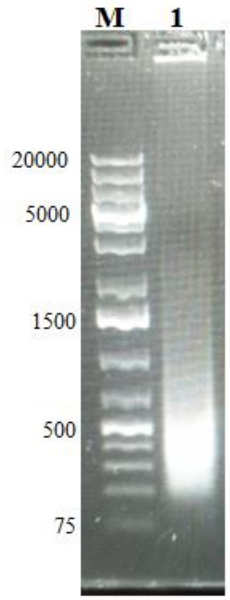

Fig. 4.24. mgDNA isolated from the intestinal tissue of *M. cuchia*. M: 1 kb plus ladder; 1: mgDNA

4.15. Analysis of blood haematological parameters

The comparative analysis of *M. cuchia* blood parameters with human blood were presented in Table 4.13. The haemoglobin value as well as other blood parameters of the *M. cuchia* blood was found to be comparatively higher than the Human blood. Therefore, the treatment of anaemic patients with the supplement of raw cuchia blood and cooked cuchia dish as prescribed by local medicineman (bej) for recovery of anaemia is found to be justified.

Table 4.13 Comparative analysis of *M. cuchia* blood with human blood

Blood Parameters	***Monopterus cuchia***	**Human**
RBC	1.9 million cells/mm^3, Nucleated	Anucleated Male-4.7-6.1 million cells/mm^3, Female- 4.2-5.4 million cells/mm^3
Haemoglobin	18.6 g/dl	Female-11.6-15 g/dl, Male-13.2-16.6 g/dl
PCV	40.7%	Male- 38.3-48.6%, Female-35.5-44.9%
MCV	214 fl	90 fl
MCH	97.9 pg	28-32 pg
MCHC	45.7 g/dl	32-36 g/dl