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Production, purification and characterization of a thermostable alkaline serine protease from *Bacillus lichniformis NMS-1*

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Alkaline proteases are used in food industry, leather tanning and processing industry, preparation of pharmaceuticals and also in the fiber industry. An alkaline serine protease producing strain was isolated using soil sample from a natural hot water spring in Sri Lanka. It was identified based on morphological, biochemical and 16s rRNA identifications as *Bacillus licheniformis* NMS-1. The extacellular protease enzyme was purified by two steps procedure involving ammonium sulfate precipitation followed by DEAE-Sephadex A-25 gel chromatography. The purification gave a 56 fold increase of the specific activity with a yield of 16%. The optimal pH and optimal temperature of the protease were pH 9 and 60°C, respectively. The protease was relatively stable between 20– 80°C. The enzyme was stable within the pH values of 8 – 12. The K_m and V_{max} values calculated from Lineweaver – Burk plot were $2.7x10^{-3}$ mg/ml and 263 mU/mg. Among the protease inhibitors that were tested, PMSF completely inhibited the enzyme activity indicating that the protease is a serine protease. The enzyme retained more than 50% of its activity after 60 min incubation at 60°C. The major protease types used commercially are heat stable alkaline proteases. Alkaline serine proteases are enzymes that cleave peptide bonds in protein in which serine serves as the nucleophilic amino acid at the enzyme active site. Properties of this protease have shown it's suitability for industrial applications such as detergent industry.

Key words: Alkaline protease, purification, characterization, *Bacillus licheniformis* NMS-1, thermophilic, serine protease.

INTRODUCTION

Proteases count for nearly 65% of the world enzyme market (Rao et al., 1998). Commercial proteases are mostly produced from the bacteria and it is reported that 35% of total microbial enzymes used for detergent industry

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Abbreviations: PAGE, Polyacrylamide gel electrophoresis; **PMSF,** phenylmethylsulfonyl fluoride.

are the proteases from bacterial sources (Ferrero et al., 1996). Proteases are commonly classified according to their optimum pH as acidic protease, neutral protease and alkaline protease. There have been extensive researches on functions of acidic and alkaline proteases. Bacterial alkaline proteases generally have an optimum pH of 10 and optimum temperature at 60°C. The genus *Bacillus* has been studied in considerable depth and the ability of *Bacillus* strains to produce and secrete large quantities (20-25 g/l) of extracellular enzyme has placed them among the most importance industrial enzyme producers (Schallmey et al., 2004). These properties make them suitable for use in the enzyme industry. Alkaline proteases are used in food industry, leather tanning and processing industry, preparation of pharmaceuticals and also in the fiber industry (Van-kessel et al., 1991; Ming chu et al., 1992). Therefore research is done on large scale production of alkaline proteases. Thermophilic bacteria from hot water springs produce thermostable enzymes. Proteases produced from thermophilic bacteria are used in a range of commercial applications (Sonnleitner and Fiechter, 1983; Rehman et al., 1994; Rao et al., 1998; Adams and Kelly, 1998; Zeikus et al., 1998; Singh et al., 2001). Enzymes isolated from these organisms are not only thermostable and active at high temperature but are also often resistant to and active in presence of organic solvents and detergents. Thermophiles such as *Bacillus licheniformis* (Ferrero et al., 1996) and *Bacillus clausii* (Kumar et al., 2004), have been studied for their ability in producing thermostable proteases. In recent years, there has been a great amount of research and development effort focusing with the aim of obtaining high yields of alkaline protease in the fermentation medium (Varela et al., 1996; De Coninck et al., 2000; Puri et al., 2002). Serine proteases are characterized by the presence of a serine group in their active site, found in virus, bacteria, and eukaryotes; this class comprises two distinct families, the chymotrypsin family which include the mammalian enzyme such as chymotrypsin, trypsin, elastase, and the sublilisin family include the bacterial enzymes such as subtilisin although the three dimensional structure is different in the two families, they possess similar active site geometry and catalytic mechanism. Serine proteases exhibit different substrate specificities. Serine alkaline proteases are produced by several bacteria, molds, yeast, and fungi; optimum pH of serine alkaline protease is around 10 (Ibrahim et al., 2011; Jayakumar et al., 2012). They are inhibited by diisopropyl-fluorophosphate (DFP) or a potato protease inhibitor but not by tosyl L-lysine chloromethyl ketone (TLCK). Their molecular masses are in the range of 15 to 30 kDa (Rao et al., 1998).

In this paper we report the purification and characterization of a serine protease from *B. licheniformis* NMS 1.

MATERIALS AND METHODS

Isolation of bacterial strain using enrichment media

B. licheniformis strain NMS 1 was isolated from a soil sample collected from Nelumwewa hot water spring, in Polonnaruwa, Sri Lanka. The bacterial strain was grown in an enrichment medium containing 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 0.5 g/l glucose, 0.4 g/l Na_2HPO_4, 0.085 g/l Na_2CO_3, 0.0 2g/l $ZnSO_4$, 0.02 g/l $MgSO_4$, 0.02 g/l $CaCl_2$ and pH 7.2. Cultures were incubated in conical flask at 37, 55 and 60°C for 48 h in an orbital shaker at 150 rpm and then centrifuged at 15000 *g* for 30 min at of 4°C (Kubota, 6500). To determine the growth of the microorganism, absorbance was measured at 600 nm. Streak plate isolation procedure and the dilution plate technique were used in isolation of bacterial colonies. The supernatant was assayed for proteolytic activity. Screening test was performed for all bacterial strains isolated using a plate technique to detect the production of protease using skimmed milk as the substrate.

Study of the colony morphology of bacterial species

The bacterial colonies isolated were streaked on MacConky and Nutrient agar. Plates and slants of MacConky agar and nutrient agar then were incubated for 24 hat 37°C. Morphological characteristics of colonies appeared on MacConky agar and nutrient agar of the bacteria were studied. Isolated bacterial species NMS1 was the only bacterial species which produced clear zone in skim milk media at 50°C. It was selected for further studies. This bacterial species was identified by morphological characteristics, biochemical test and 16s r RNA analysis.

PCR amplification and sequencing of 16S rRNA identification

Total DNA was isolated from small colony of the NMS1 bacterial sample and 5 µl of extracted DNA was subjected to the polymerase chain reaction (PCR) using 27F/800R and 518F/1492R. Amplified DNA was subjected to DNA sequencing using 518F and 800R primers and the obtained DNA sequences were compared with already existing DNA sequences in Gene bank. Bacterial universal 16s rRNA primers were 27_forward 5'-AGAGTTTGATCCTGGCTCAG-3' 1492_reverse 5'-TACGGTTACCTTGTTACGACTT-3' PCR products were sequenced in Genetech laboratory, Sri Lanka, forward and reverse primers were aligned using ABI Auto assembler software, and the over lapping consensus sequence was compared with sequence in the NCBI data base using FASTA3 sequence homology searches.

Selection of the optimum temperature for protease production

To determine optimum temperature for protease production, enrichment culture medium was used with casein in place of peptone.10 ml of initial cultures were inoculated into 150 ml of culture media at different temperatures separately. The cultures were incubated at 37, 50, 55, and 60°C in an orbital shaker at 150 rpm for 48 h. Optical densities at 600 nm, pH, protease activity, and cell number were estimated in culture media at different temperatures at 4 hour intervals.

Purification

Purification was done at 4°C. The bacterial strain NMS1 grown in a

fresh 300 ml culture medium for 32 h at 50°C was harvested by centrifugation at 15000 *g* for 20 min. The supernatant was precipitated in ammonium sulphate solution of 90% saturation. Saturated solution was centrifuged at 15'000 *g* for 30 min. The pellet obtained was dissolved in 50 mM phosphate buffer at 7.5. The solution was dialysed in the same buffer for 16 h before applying to a DEAE-Sephadex A-25 column (2.6×30 cm). The protease was eluted with a 1:1 gradient of NaCl (0.1 to 0.7 N NaCl in 50 mM pH 7.5 phosphate buffer) at a flow rate of 0.8 ml/min. The fractions were assayed for protease activity. The protein content was determined using Lowry's method (Lowry et al., 1951) using bovine serum albumin as the standard.

Protease assay

The protease activity was determined using the method described by Cupp – Enyard (2008) with slight modifications. The enzyme activity was calculated using a tyrosine standard graph. Activity is defined as the amount of enzyme that hydrolyzed one micromole (µmol) of casein per minute.1 ml of each culture supernatant was mixed with equal amount of Tris-HCl buffer and was mixed thoroughly.1 ml of this mixture was incubated with 5 ml of 0.65% casein for 10 min at 37°C. The reaction was terminated by addition of 5 ml of 110 mM TCA to each tube. The blanks were prepared by incubating the buffer without supernatant. After 30 minutes of incubation each test solution was centrifuged and filtered. Then 2 ml of each filtrate and blank was added to separate vials and 5 ml of 0. 5 M Na_2CO_3 added to each tube and immediately added 1 ml Folin- Ciocalteus reagent was added. After incubating at 37°C for 30 min, absorbance was measured at 660 nm.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out according to the method described by Davis (1964) using Shandon apparatus.

Characterization of protease

All assays were done in triplicate.

Effect of temperature on protease activity

To determine the optimum temperature, protease activity was measured using 5 ml of 0.65% casein solution in 50 mM pH 7.2 phosphate buffer solutions incubated at 10 to 60^{0}C, respectively. 1 ml of partially purified enzyme was added to each tube and assayed for protease activity

Stability of enzyme at different temperature

5 ml of the purified protease was incubated at temperatures ranging from 10 to 98°C for 1 h. 1 ml of each enzyme solution was added to tubes containing 5 ml of 0.65% casein in 50 mM phosphate buffer (pH 7.5). The protease assay was done under standard assay conditions.

Effect of pH on protease activity

Protease activity was measured at 29°C in the following buffer systems: HCl-KCl buffer solution of pH 2, citrate buffer solution of pH 3, 4, 5, 6 and phosphate buffer of pH-6.5, 7, 7.5, 8, 9 bicarbonate buffer of pH 10, 11, 12 to measure the optimum pH. The protease assay was performed as described under standard assay conditions.

Stability of enzyme at different pH

To test the pH stability of protease HCl-KCl buffer solution of pH- 2, citrate buffer solution of pH -3, 4, 5, 6 and phosphate buffer of pH-6.5, 7, 7.5, 8, 9 bicarbonate buffer of pH 10, 11 were prepared. 5 ml of each buffer solution and 1 ml of partially purified enzyme were incubated at 29°C over a period of 1 h. The protease assay was performed as described under standard assay conditions.

Effect of substrate concentration on enzyme activity

The protease assay was performed varying the substrate concentration and the K_m and V_{max} values were calculated using Lineweaver-Burk plot.

Determination of protease type

To determine the type of protease, the purified protease was pretreated for 30 min at 37°C and 5 mM PMSF residual enzyme activity was measured under standard assay conditions.

RESULTS AND DISCUSSION

Three bacterial strains were isolated based on their morphology. The extracellular protease producing ability was screened by streaking bacterial strains on skimmed milk agar and incubation for 24 h at 55°C. It has been reported that *B. licheniformis* produces very narrow zone of hydrolysis on casein agar despite being a very good producer of proteases in sub merged cultures (Mao et al., 1992) but *B. licheniformis* NMS 1 produces a very prominent and distinct zone of hydrolysis. *B. licheniformis* NMS 1 grows in the temperature range of 30 to 60°C. After 28 h incubation at 50°C *B. licheniformis* NMS 1 strain produced a maximum protease activity of 200 mU/ml but at lower temperatures like 30°C a lower production was observed. These results are compatible with findings of Atalo and Gashe (1993) and Johnvesly et al. (2002).

Razik et al. (1994) isolated a *Bacillus stearothermophilus* AP-4 with a optimum protease production at 55^{0}C. Mabrouk et al. (1999) found optimum temperature as 37°C for *B. lichniformis* 21415 after 5 days of incubation. Rehman et al. (2005) also observed optimum temperature for enzyme production in *P. aeruginosa* strain K as 37°C. NMS1 Colonies were convex, large, and sticky, off white/yellow in colour and had rough edges, and were extremely mucoid and had a characteristic smell. This bacterial strain was used for the further studies. The bacterium was Gram positive, rod shaped, with a bulge

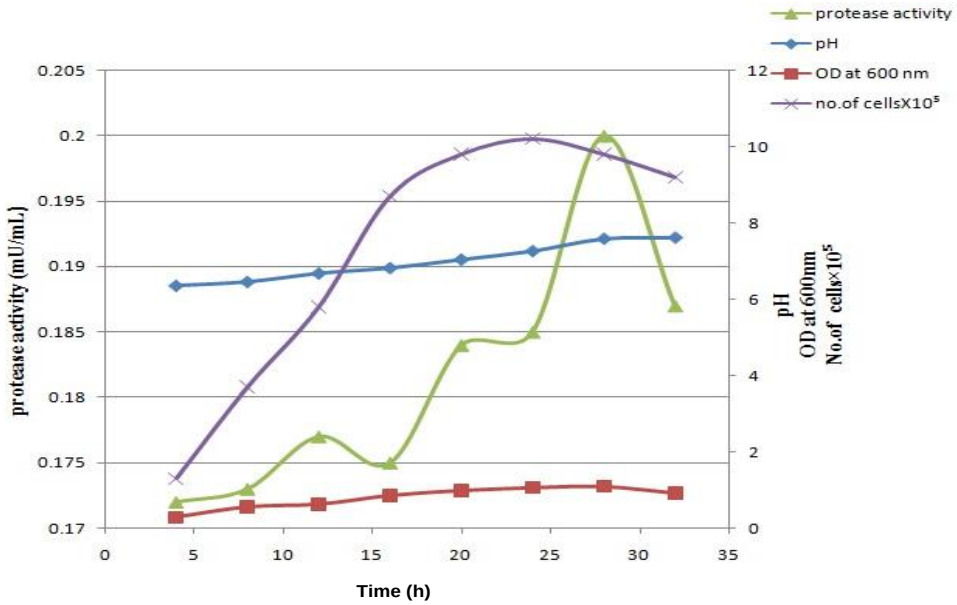

Figure 1. Extracellular protease production by *Bacillus licheniformis* NMS-1 grown at 50°C.

Table 1. Purification of extracellular proteases from *Bacillus licheniformis NMS1*

Purification stage	Total volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Recovery (%)	Purification fold
Initial crude Supernatant	500	45110	829	54	100	1
0-90% ammonium sulphate fractionated pellet	100	22240	49	453	49	8
DEAE ion exchange chromatography	30	7380	2.4	3049	16	56

central spore. These features were observed by light microscopy. The bacteria were highly motile as observed by the hanging drop method. The isolate could ferment various types of sugars as shown in biochemical test results. At the late stationary phase the organism produced spores abundantly and this showed a relationship with protease production. This isolated organism grows at temperatures ranging 30 to 60°C and pH of 4 to 11 and up to 10% of NaCl.

PCR amplification and sequencing of 16S rRNA identification

99% homology sequence was shown with *B. licheniformis.* NCBI BLAST result shows a significant relation with isolates NMS1 with other *Bacillus* in NCBI with a 99% identity and query recovery. Based on biochemical, morphological tests and 16S Rrna, identification of isolated bacteria was found to be *B. licheniformis*- NMS1.

Optimization of the temperature for production of extra cellular protease

The maximum protease production was observed at 50°C as 200 mU/ml. After 48 h of incubation at 50°C (Figure 1).

Purification of the protease enzyme

The purification of enzyme by ammonium sulphate precipitation and ion exchange chromatography gave a specific activity increase of 54 mU/mg (Table 1). From DEAE sephadex A-25 gel chromatography, two enzymes peak were obtained indicating the presence of isoenzymes (Figure 2). Only one peak was pooled and

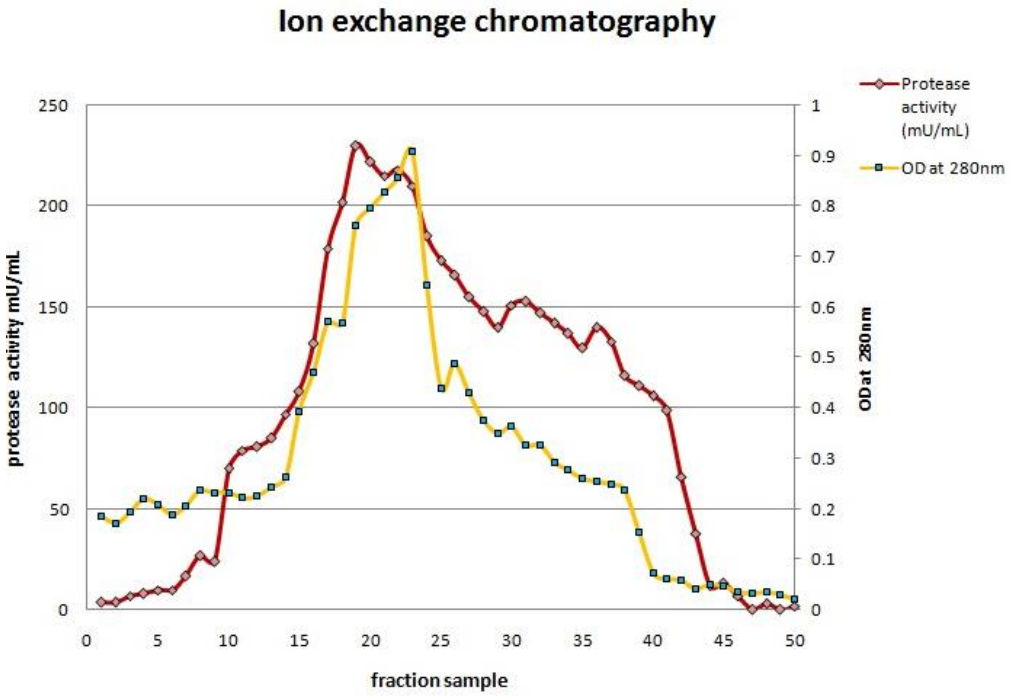

Figure 2. Ion exchange chromatography on DEAE sephadex A-25 of the crude extract of protease from *Bacillus licheniformis* NMS 1.

used for further studies. The number of folds purification was 56 folds with yield of 16%. The specific activity was 3049 mU/mg. Two protein bands were observed in polyacrylamide gel electrophoresis indicating that the enzyme has been partially purified. Rai and Mukharjee (2009) reported that a bacterium may produce arrays of extracellular protease isoenzymes for its survival and growth in particular habitat. Only a limited number of studies have been done on isoenzymes of alkaline proteases produced by Bacillus genus. Among these studies, Mala and Srividya (2010) reported isolating two different isoenzymes with molecular weights of 66 and 18 KDa from a Bacillus species. Tekin et al. (2012) reported similar results, isolating alkaline proteases from *Bacillus cohnii* APT5.

Characterization

Effect of temperature on enzyme activity and stability

Enzyme activity increases from 20 to 60°C (Figure 3). The results show that it has stability between 20 to 80°C after 1 hof incubation (Figure 4). Therefore this enzyme can be classified as a thermostable enzyme. Gupta et al. (2008) found optimum temperature for protease from *Virgibacillus pantpthenticus* as 50°C with a retained activity of 82%, when incubated at this temperature for 1 h. Comparison of these results of El-Hawary and Ibrahim (1992) and Nilegaokare et al. (2002) concluded that optimum temperature of proteolytic activity frequently exceed the optimum temperature for enzyme production. It was also suggested that the stability of protease enzyme could be due to their genetic adaptability to carry out their biological activities at a higher temperature (Gaure et al., 1989; Whittle and Bloomfield, 1999; Kanekar et al., 2002)

Effect of pH on enzyme activity and stability

The maximum activity was observed at pH 9 (Figure 5) and enzyme was stable in the pH range 4-12 (Figure 6). These types of protease are very unique and are widely used in detergent industry. Prescott et al. (1995) observed Wai 21a protease stable over the pH range 2-5. Kumar (2005) observed that *Bacillus pumilus* protease had pH optimum for hydrolysis of casein from 10.5-12 with optimum activity at 11.5. He reported that the enzyme was stable over a broad pH range of 6-12 for 4 hand a pH range of 6-11 for 24 h at 30°C. Related results were also given by Johnvesly and Naik (2001) who reported a pH optimum of 11 for protease enzyme from *Bacillus* species JB-99 and a high activity in the range of pH 8-12.

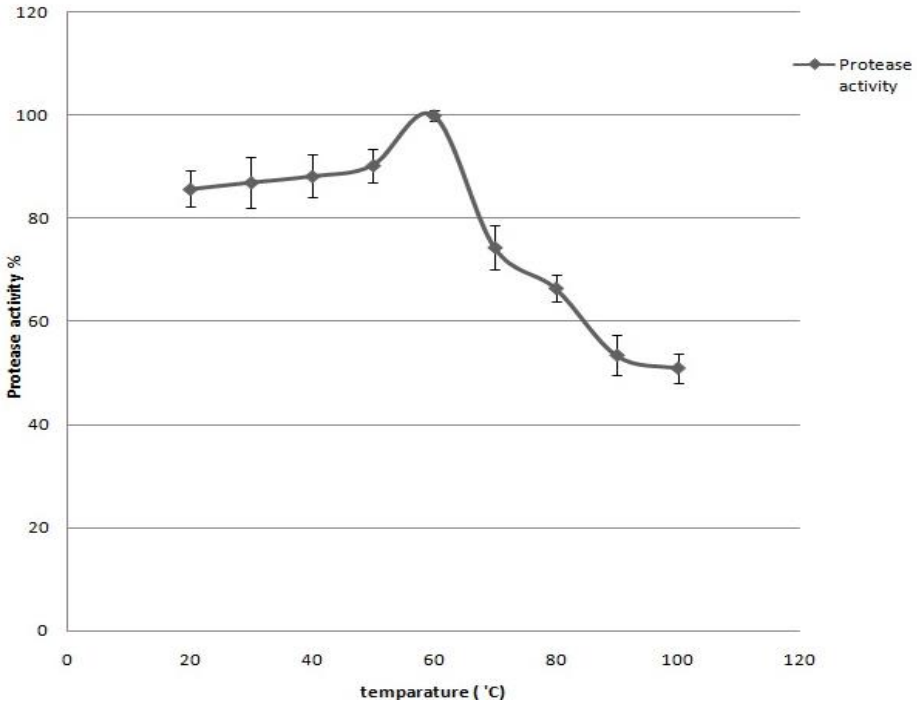

Figure 3. Effect of temperature on activity of purified extracellular thermostable protease.

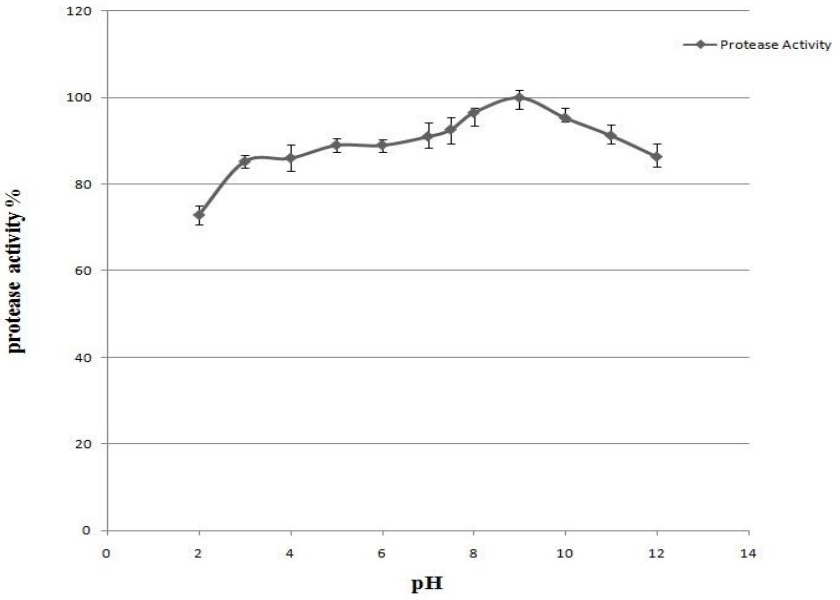

Figure 4. Effect of pH on activity of purified extracellular thermostable protease.

Calculation of K_m and V_{max}

The results show K_m value as 2.7×10^{-3} mg/ml and V_{max} as 263 mU/mg (Figure 7). K_m value of *B. licheniformis* NMS 1 protease was lower than those of Bacillus species TKU004 metallo-protease (2.98 mg/ml) (Wang et al., 2006) and TKU2007 protease (0.13 mg/ml) (Wang and Yah, 2006), *P aeruginosa* Psa A protease (2.69 mg/ml)

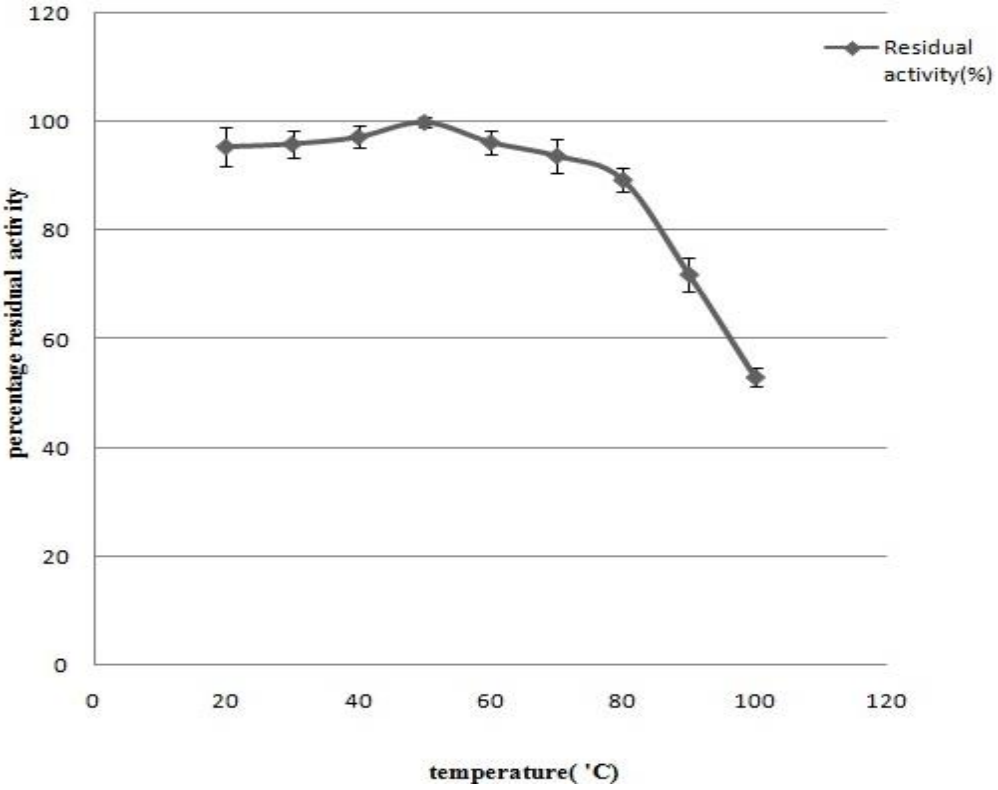

Figure 5. Effect of temperature on the stability of purified extracellular thermostable enzyme.

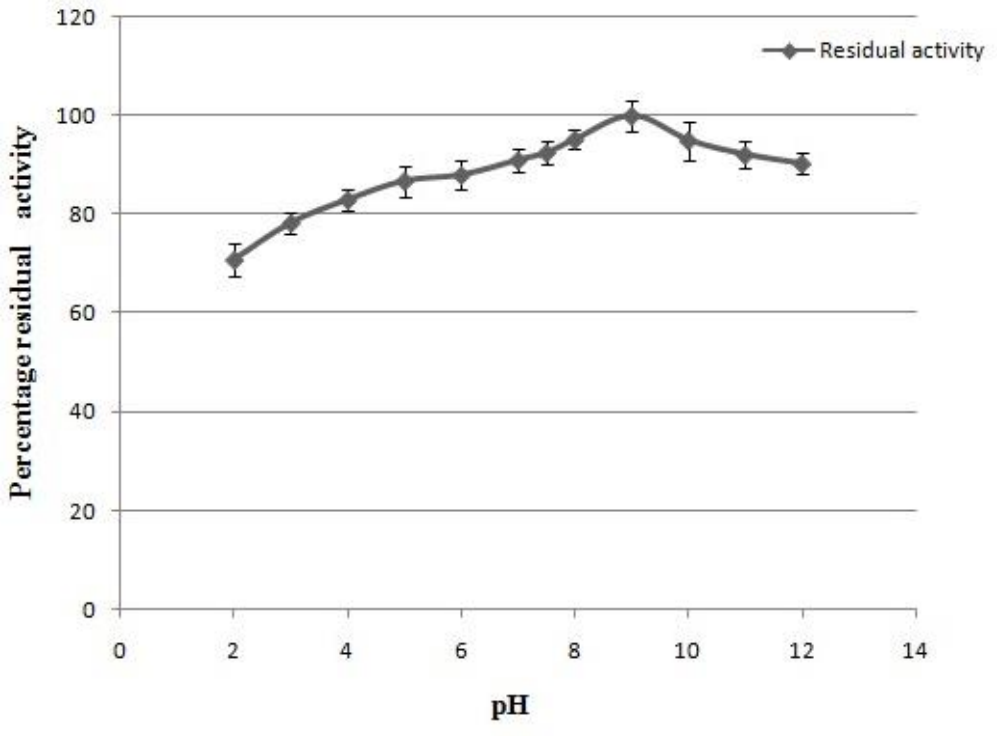

Figure 6. Effect of pH on the stability of purified extracellular thermostable enzyme.

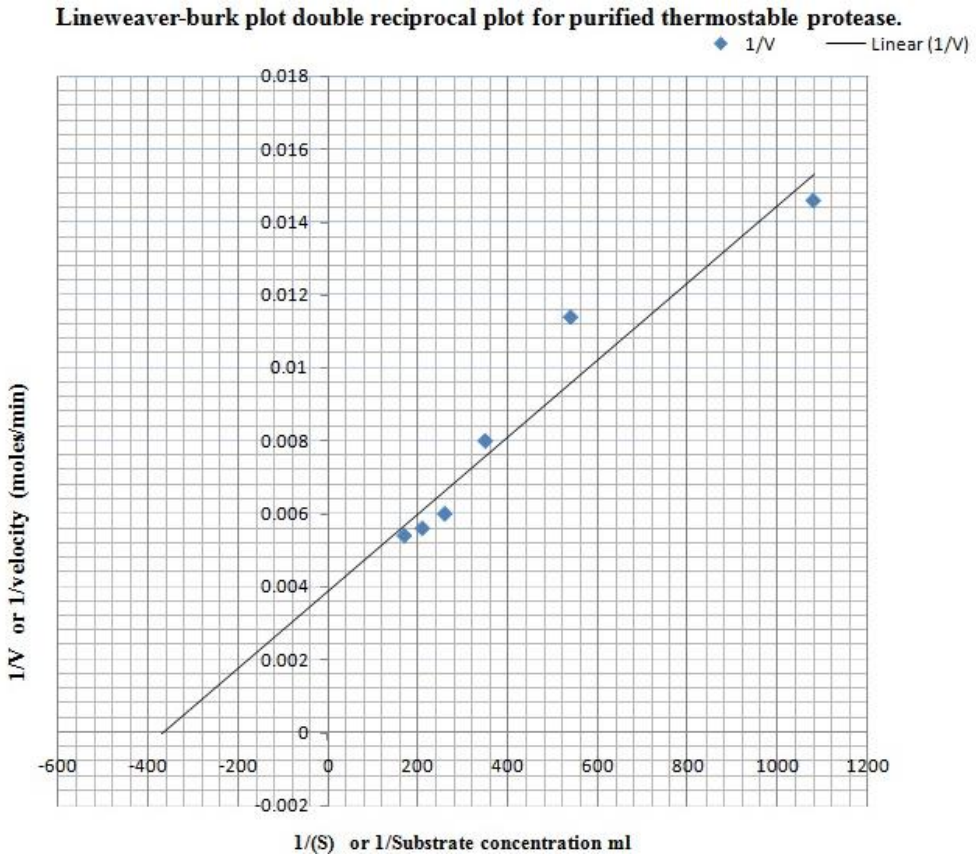

Figure 7. Lineweaver- Burk double reciprocal plot for purified protease enzyme.

(Gupta et al., 2005a). The V_{max} of *B. licheniformis* NMS 1 was higher than that of TKU007 (0.86 U/ml) (Wang and Yeh, 2006), 0.14 U/ml of *Bacillus* species TKU004 metallo-protease (Wang et al., 2006) 1.26 U/ml of *Bacillus cereus* (Sierecka, 1998) and 3.03 U/ml of *P. aeruginosa* Psa A (Gupta et al., 2005a).

Determination of protease type

Inhibition by phenylmethylsulfonyl fluoride (PMSF) indicated NMS 1 protease to be a serine protease. Such reports have been shown earlier for bacillus species (Dhandapani, 1994) PMSF causes Sulphonation of the serine residues residing in the active site of the protease and has been reported to result in the complete loss of enzyme activity (Beynon and Bond, 2001).

Conclusion

Optimum temperature for production of *B. licheniformis* NMS 1 extracellular protease was at 50°C. The enzyme was stable at 20 to 60°C with maximum protease activity at 60°C. The enzyme showed a high activity between pH values 8 - 12 with optimum pH at 9. Enzyme was stable between pH values 4 - 12. K_m and V_{max} values were 2.7×10^{-3} and 263 mU/mg. Characteristics of enzymes show that it is an alkaline serine protease suitable for use in the detergent industry.

Conflict of interests

The authors did not declare any conflict of interest.

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