

## Purification and Characterization of Alkaline Protease from a Mutant *Bacillus licheniformis* B18

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**Abstract:** Alkaline protease from a mutant of *Bacillus licheniformis* B18 was purified from the culture supernatant by employing the methods such as ammonium sulphate precipitation, DEAE cellulose chromatography followed by Gel filtration using Sephadex G-100. The yield of the enzyme after purification was found to be 10%. Protease was found to be homogenous when examined by SDS-PAGE and the enzyme showed that it has a molecular weight of 28 KDa. Characterization studies were carried out using the purified enzyme. While the optimum pH and temperature for the activity of alkaline protease was found to be 10 and 50°C and stable in the pH range 5.0 - 12.0. The thermo stability exhibited by protease ranged from 30-70°C. Among various protease inhibitors PMSF strongly inhibited the enzyme activity revealing that the enzyme in the present study is serine alkaline protease.  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  had a slight enhancing effect on the activity of the enzyme. High level of hydrolytic activity was shown by casein and also found that purified alkaline protease digested the human blood clot, coagulated white egg to soluble form and also digested chicken skin upon the prolonged incubation of enzyme with chicken skin. The protease showed good compatibility and stability in the presence of  $\text{CaCl}_2$  and glycine with Nirma, Surf excel and Wheel. The enzyme retained 20-40% activity with most of the detergents tested even after 3hrs. The supplementation of the enzyme preparation in detergent completely removed the blood stain of the cloth. The enzyme followed a typical Michaelis-Menten kinetics. Apparent  $K_m$  value was found to be  $3.2\text{mgml}^{-1}$ .

**Key words:** Alkaline Protease • Mutant *Bacillus licheniformis* B18 • Purification • Characterization

### INTRODUCTION

Proteases are the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields. The detergent industry is the largest user of industrial enzymes, accounting for more than one third of the global market which is considered to total 1.6 billion US dollars. The genus *Bacillus* is among the most important organisms for commercial protease production [1] due to their high thermo stability and pH stability. In our earlier studies, we obtained a mutant strain of *Bacillus licheniformis* B18 which produced 63.64% higher titers of alkaline protease over the wild strain which is a significant increase of yield and found to be stable for the high

yielding nature [2]. The present investigation dealt with purification and characterization of alkaline protease of this mutant and its suitability for various commercial application.

### MATERIALS AND METHODS

**Microorganism and Cultural Conditions:** The bacterial strain of *Bacillus licheniformis* B18 isolated from alkaline soil of the milk processing unit and was mutated by UV irradiation [2]. Alkaline protease production was carried out in a 2L fermentor (Lark Bioreactor) containing 1 L modified production medium. The composition of modified production medium (2) was (g/l) maltose 10, yeast extract 5, peptone 5,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 and  $\text{K}_2\text{HPO}_4$

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1 (pH 10), 10% level of inoculum was added and the fermentor was run at 40°C for 72 h. After the completion of fermentation the whole fermentation broth was centrifuged using Eitek centrifuge at 10,000 rpm at 40°C and the clear supernatant was separated. The supernatant (Crude enzyme) was subjected to recovery and purification process.

### **Enzyme Purification**

**Ammonium Sulphate Precipitation:** The supernatant obtained was fractionated by precipitation with ammonium sulphate at 70% saturation [2]. The precipitate obtained was separated by centrifugation at 8000g for 20 min. The pellet obtained was re suspended in 0.01M potassium phosphate buffer pH (7.5) and dialyzed against the same buffer for 20h by changing buffer for every 6 h. After dialysis, the solution was centrifuged and the pellet obtained was re dissolved in the same buffer.

**DEAE – Cellulose Chromatography:** The dialyzed material was applied to DEAE cellulose column (Sigma) (2.6 x 25 cm) pre equilibrated with buffer 1. Elution was done with 1:1 linear gradient from 0.1M-1M NaCl in buffer 1 at a flow rate of 35 mlh<sup>-1</sup>. The fractions with alkaline protease activity were pooled and concentrated.

**Gel Filtration Chromatography:** The pooled active fractions obtained were applied on to a sephadex G-100 column (Sigma) (1.6x67cm) equilibrated with 0.01M potassium phosphate buffer. The column was eluted with the same buffer at a flow rate of 12mlh<sup>-1</sup>. Filtrate fractions showing the enzyme activity were pooled, dialyzed against distilled water, lyophilized and stored at 20°C.

**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS - PAGE):** The electrophoresis was performed according to Laemmli [3] using 10% acrylamide. The gel was stained for 1h with 0.25% Coomassie blue R-250 in methanol/ water/acetic acid (50:40:10) and the gel was finally de stained in a de staining solution containing water/acetic acid/methanol (87.5:7.5:5)

**Enzyme Assay:** According to Boominadhan and Rajakumar [4], the enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M carbonate –bicarbonate buffer pH 9.5 and 1ml enzyme

solution in a total volume of 3.0ml. Reaction mixture was incubated for 5 min at 40°C. The reaction was terminated by adding 3ml of 10% ice-cold trichloroacetic acid. The tubes were incubated for one hour at room temperature. Precipitate was filtered thorough whatman no.1 filter paper and the filtrate was collected. For the color development for the assay of tyrosine in the filtrate, 5ml of 0.4 M sodium carbonate and 0.5 ml of Folin phenol reagent were added to 1ml of filtrate, vortexed immediately and incubated for 20 min at room temperature and optical density was taken at 660 nm. Concentration of tyrosine in the filtrate was read from a standard curve for tyrosine already prepared. One unit enzyme activity was taken as the amount of enzyme producing 1µg of tyrosine under standard assay conditions and expressed as units ml<sup>-1</sup> enzyme.

**Protein Assay:** The concentration of protein during purification studies was calculated from the absorbance at 280nm [3].

### **Characterization of Purified Alkaline Protease**

#### **Effect of pH on Purified Enzyme Activity and Stability:**

The influence of pH on the alkaline protease activity was determined by measuring the enzyme activity at varying pH values ranging from 7 to 12 at 40°C for 15 min using different buffers viz., 0.05 M Potassium phosphate-Sodium hydroxide buffer (pH 7.0, 8.0) and Glycine –Sodium hydroxide buffer (pH 9.0, 10, 11, 12). The purified enzyme was incubated in relevant buffers with different pH for 24 h at 40°C. The relative activity was determined before and after incubation. The percentage of activity remaining was calculated.

#### **Effect of Temperature on Enzyme Activity and Stability:**

The influence of temperature on the activity of alkaline protease was determined by measuring the enzyme activity at various temperatures ranging from 30 to 70°C under the standard assay conditions, using Glycine Sodium hydroxide buffer 0.2M (pH 10.0) and using casein as substrate. The stability of the enzyme was determined both in the absence and presence of calcium chloride. The enzyme was incubated at different temperatures for 30 min in 0.2M glycine Sodium hydroxide buffer with and without calcium chloride (5mM). Enzyme activities were determined under the standard assay conditions. The percentage of activity remaining after the heat treatments was calculated.

#### **Effect of Inhibitors on the Activity of Alkaline Protease:**

The enzyme was incubated with various inhibitors at 5 Milli Molar concentrations for 15min at 40°C and the residual activities were determined. Inhibitors used were PMSF, P-CMB, Idoacetic acid and EDTA.

#### **Effect of Metal Ions on the Activity of Alkaline Protease:**

The enzyme was incubated with various metal ion sources. (10mM) viz, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Co<sup>+2</sup>, Cd<sup>+2</sup>, Fe<sup>+3</sup>, Na<sup>+</sup>, Zn<sup>2+</sup> and Cu<sup>2+</sup> for 30min at 40°C and relative protease activities were measured.

#### **Effect of Different Substrates on the Alkaline Protease**

**Activity:** Protease activity was measured with various substrates including bovine serum albumin, casein, egg albumin and gelatin. 1ml enzyme was incubated with different protein substrates as described earlier and the activities were measured.

#### **Effect of Substrate Concentration on Activity of the**

**Enzyme:** Substrate concentration on the activity of purified alkaline protease was determined by incubating 1ml of purified protease with different concentrations of casein viz, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mg respectively and the activity of enzyme was measured (Km value of the enzyme was calculated).

**Digestion of Natural Proteins:** 2ml of the enzyme was incubated with human blood clot, coagulated egg white and chicken skin in glycine-NaOH buffer (pH 9.5) at 37°C for 10h [10].

**Detergent Stability of the Enzyme:** The compatibility of purified alkaline protease with local laundry detergents was studied in the presence of 10mM CaCl<sub>2</sub> and 1M glycine. The detergents used were Ariel, (Procter and Gamble, India), Surf excel, Rin, Wheel (Hindustan Lever Ltd, India) and Nirma (Nirma chemicals, India). The detergents were diluted in distilled water (0.5% w/v), incubated with protease for 3 h at 40°C and the residual activity was determined. The enzyme activity of a control sample was taken as 100%.

**Destaining Property of Purified Alkaline Protease:** The destaining property was studied by dipping two pieces of cloth artificially stained with blood either in detergent solution or detergent solution supplemented with enzyme followed by incubation for 10 min at 40°C.

## **RESULTS AND DISCUSSION**

#### **Purification of Alkaline Protease of *Bacillus licheniformis* B18:**

Table 1 summarizes the extent of purification of alkaline protease produced by the mutant of *Bacillus licheniformis* B18. The enzyme was purified 3.15 folds with a specific activity 2.55 Umg<sup>-1</sup> protein after ammonium sulfate fractionation. The enzyme was then purified with DEAE cellulose chromatography and showed 8.59 folds enzyme purification with a specific activity of 6.96 Umg<sup>-1</sup> protein. The final purification step with sephadex G-100 column chromatography showed 26.33 folds enzyme purification with a specific activity of 21.33 Umg<sup>-1</sup> protein. These results indicated that the effectiveness of purification method applied in this research. However, the yield of the enzyme after purification was found to be low (10.6%). This might be due to the result of autolysis of the enzyme in each purification step [10].

The purified protease along with standard molecular weight markers were run on SDS –PAGE. The protein in the sample migrated as a single band (Plate 1) which indicated its homogeneity. The molecular mass standards used were bovine serum albumin (67 KDa), ovalbumin (45 KDa) carbonic anhydrase (30 KDa), trypsinogen (24KDa) and  $\alpha$  –lactalbumin (14KDa). Depending on the relative mobility the molecular weight of the protein band was calculated to be around 28KDa (Plate 1). Varieties of molecular mass for protease from different *Bacillus* species have been reported viz., 30.9 KDa from thermophilic *Bacillus* strain HSO8 [5]; 27.0 KDa from *Bacillus megaterium* [6]; 75.0 KDa from *Bacillus* sp. S17110 [7]; 34.0 KDa from *Bacillus thuringiensis* [8]; 38.0 KDa from *Bacillus cereus* KCTC 3674[9]; 15.0 KDa from *Bacillus subtilis* PE-11 [10]; 34.0 Kda from *Bacillus cereus* BG1 [11]; 66.2, 31.0 KDa and 20.1 KDa from *Bacillus licheniformis* strains BLP1, BLP2 and BLP3 respectively [12].

**Characterization of Purified Alkaline Protease:** The highest protease activity was found to be at pH10 using glycine NaOH buffer (Fig. 1). The maximum activity shown has been taken as 100%. Alkaline proteases generally have broad pH optima in the range of pH 8 – 12 [13] and pH optima was 10.0 – 10.5 for protease from *Bacillus* spp, *Thermos aquaticus*, *Xanthomonas maltophila* and *Vibrio metschnikovii* [14, 15]. The enzyme was stable over a broad range of pH 7 to 10 (Fig. 2) and retained 70% of its original

Table 1: Summary of Purification steps of alkaline protease from mutant *Bacillus licheniformis* (B18)

Purification Steps	Total Protein (mg)	Enzyme activity Uml <sup>-1</sup>	Total enzyme activity (U)	Specific activity Umg <sup>-1</sup>	Purification (fold)	Yield
Crude enzyme	370	300	150,000	0.81	1	100
70%(NH <sub>4</sub> ) SO <sub>4</sub>	55	140	70,000	2.55	3.15	46.6
DEAE-cellulose Chromatography	13.5	94	47,000	6.96	8.59	31.3
Sephadex G-100 Chromatography	1.5	32	16,000	21.33	26.33	10.6

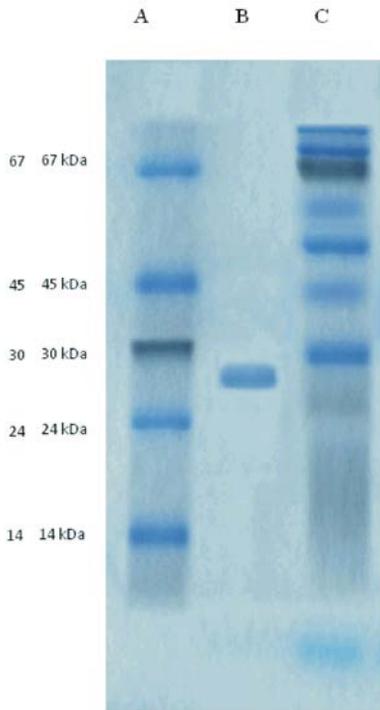


Plate 1: SDS-Polyacrylamide gel electrophoresis of protease from mutant *Bacillus licheniformis* B18; where, lane A, protein markers (67, 45, 30, 24, 14 kDa), lane B, purified protease, lane C, crude enzyme.

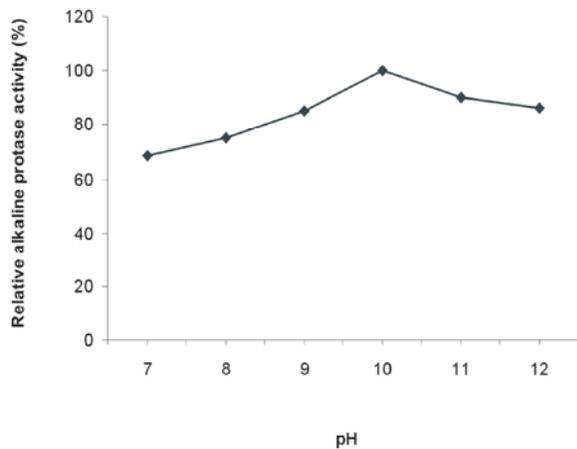


Fig. 1: Effect of pH on the activity of purified alkaline protease

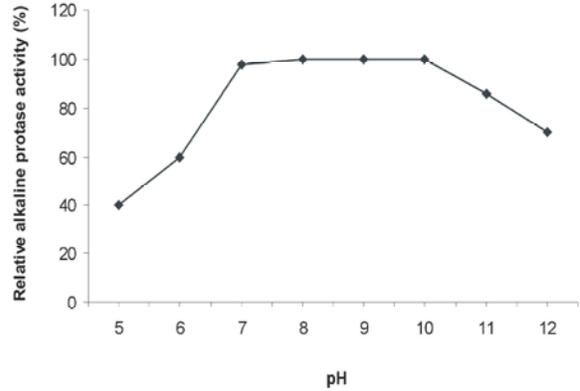


Fig. 2: Effect of pH on the stability of purified alkaline protease Temperature °C

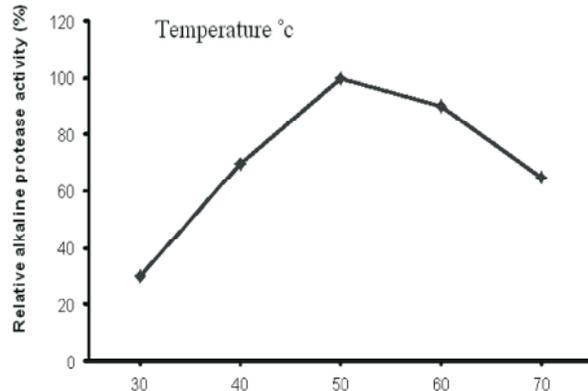


Fig. 3: Effect of temperature on the activity of purified alkaline protease

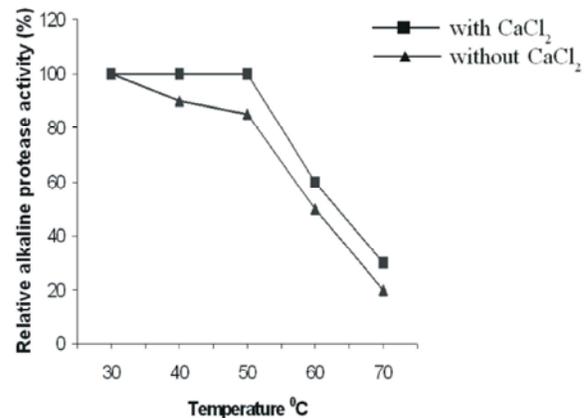


Fig. 4: Effect of temperature on the stability of purified alkaline protease

Table 2: Effect of protease inhibitors on the alkaline protease activity

Inhibitor (5mM)	(%) Relative enzyme activity
Control	100
PMSF (Phenyl methyl sulphonyl fluoride)	00
PCMB (Para chloro mercuric benzoate)	95
Iodoacetic acid	90
EDTA (Ethelene Diamine Tetra Acetic Acid)	93

activity after incubation in Glycine NaOH buffer with pH 12.0 at 40°C for 24h. This was comparable with the stability shown by the highly alkali stable proteases such as those obtained from *Bacillus* sp. GX 6638 [13], *Bacillus subtilis* RM 615 [16] and *Vibrio metschnikovii* RH530 [15].

The optimum temperature for caseinolysis was recorded was at 50°C (Fig. 3). Alkaline proteases of *Bacillus* with similar temperature optima have been reported [13, 17-20]. The enzyme was also stable with about 100% activity at 30, 40 & 50°C for 30 min (Fig. 4). Reduction in activity could be observed after incubation at 60°C and above. The presence of calcium chloride was found to improve the thermo stability of the enzyme. These results suggested that concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperatures. The thermo stability shown by this enzyme was comparable with or better than that reported for the alkaline proteases from different *Bacillus* species [13, 17, 18, 21].

The enzyme was strongly inhibited by PMSF and slightly by EDTA (Table 2). A complete loss of enzyme activity in the presence of PMSF [22] and slight inhibition of enzyme activity by EDTA has been reported in alkaline serine protease from bacterial sources such as *Bacillus licheniformis* [20, 23], *Bacillus thermorube* [24], *Bacillus* sp. [21], *Halobacterium halobium* [25], *Myxococcus viriscens* [26] and *Streptomyces* sp. [27]. In the present study also the enzyme purified from mutant *Bacillus licheniformis* (B18) showed similar inhibition by PMSF and EDTA as reported by various researchers. This indicated that it is a serine alkaline protease. Among the other inhibitors studied, slight inhibition was observed with iodoacetate and PCMB.

None of the metal ions showed considerable enhancing effect on the activity of the protease (Table 3). Ca<sup>2+</sup> & Mn<sup>2+</sup> had a slight enhancing effect on the activity of the enzyme. Of the different metal ions tested Zn<sup>+2</sup>

Table 3: Effect of metal ions on the activity of alkaline protease

Metal ion Source	Relative activity (%) (10mM)
Control	100
CaCl <sub>2</sub> (Ca <sup>2+</sup> )	102.1
MgCl <sub>2</sub> (Mg <sup>2+</sup> )	101
CoCl <sub>2</sub> (Co <sup>2+</sup> )	90
CdCl <sub>2</sub> (Cd <sup>2+</sup> )	90
FeCl <sub>3</sub> (Fe <sup>3+</sup> )	80
NaCl (Na <sup>+</sup> )	98
ZnCl <sub>2</sub> (Zn <sup>+2</sup> )	82
CuCl <sub>2</sub> (Cu <sup>+2</sup> )	90

Table 4: Effect of different substrates on the alkaline protease activity

Substrate (1 ml)	Relative enzyme activity (%)
Casein	100
Bovine serum albumin	54
Egg albumin	32
Gelatin	12

showed the maximum inhibition. Mn<sup>+2</sup> has been reported as enhancing the activity of alkaline serine proteases from some bacterial sources such as *Bacillus sterothermophilus* F1 [28] and *Nocardiopsis derssonvillei* [29].

High level of the hydrolytic activity was shown by casein and with poor to moderated hydrolysis of BSA and egg albumin (Table 4). However the hydrolysis was hardly observed with gelatin.

The ability of purified protease to digest some natural proteins was tested. The results were shown in Plate 2. These results showed that enzyme can convert the insoluble forms of human clot and coagulated white egg to soluble form. The enzyme was also able to digest chicken skin after incubation for a long time with it. The results suggest usefulness of this enzyme for different application such as, extraction of collagen from skin for collagen replacement therapy, waste treatment and others.

Enzyme activity and stability in the presence of some available commercial detergents was studied with a view to exploit the enzyme in detergent industry. A good protease is expected to be stable in the presence of commercial detergents. The proteases showed good

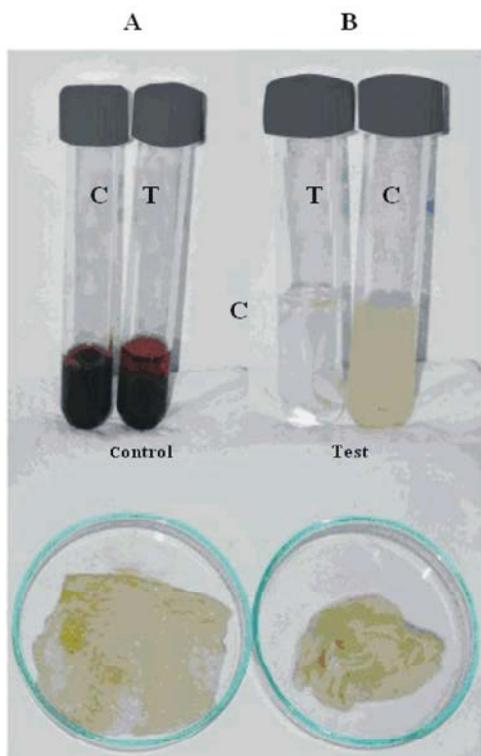


Plate 2: Digestive ability of purified protease

- A. Blood clot
- B. White egg
- C. Chicken skin

Table 5: Detergent stability of the enzyme

Detergent	(%) Retention of enzyme activity							
	Time	0 hr	0.5 hr	1	1.5	2	2.5	3.0
Control	100	100	100	100	100	100	100	100
Wheel	100	90	80	70	60	50	46	
Ariel	100	74	62	51	43	36	20	
Surf excel	100	84	82	64	62	48	35	
Rin	100	65	59	47	36	30	22	
Nirma	100	90	86	74	50	48	40	

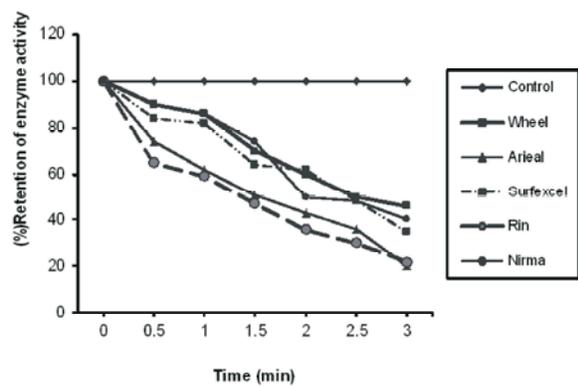


Fig. 5: Effect of detergents on the stability of purified alkaline protease

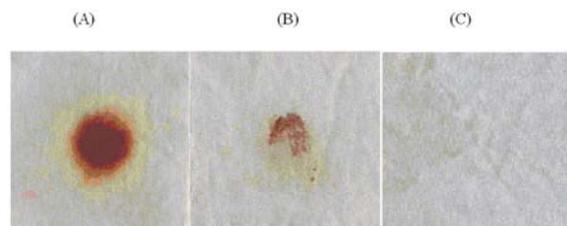


Plate 3: Washing performance of alkaline protease from mutant *Bacillus licheniformis* (B18)

- (A): Cloth stained with Blood
- (B): Blood stained cloth washed with detergent only
- (C): Blood stained cloth washed with detergent and enzyme

stability and compatibility in the presence of Nirma, Surf excel and wheel. The results were shown in Table 5 and Fig. 5. Enzyme activity and stability in the presence of detergents was reported [30]. They observed that enzyme retains 84.5% of activity in the presence of Vim and more than 40% in the presence of Nirma super, Wheel and Nirma. Interestingly, in the present study, the enzyme retained around 20-40% activity with most of the detergents tested even after 3 hours incubation. Compatibility of alkaline protease of *Condiobolus cornatus* (Ncl 86.8.20) with commercial detergents was reported [31] and observed that the enzyme protease retains more than 80 percent of its activity in the presence of detergents viz, snow white, Nirma, Revel and more than 56 percent of its activity in the presence of wheel and surf when incubated for 1h. Similarly among the three proteases isolated from *Tritirachium album limber*, proteinase R and T were reported to retain 90 and 89 percent activity respectively up to 1h in the presence of detergents like ERA plus and Dyname. BPN was highly unstable in all the detergents and retained just 4 percent activity even after 10 min [32].

The results of distaining experiment showed the complete removal of stain in detergent solution supplemented with enzyme whereas blood stain was not completely removed from cloth dipped in detergent solution alone (Plate 3). This suggested usefulness of this enzyme in detergent industry. From this study the protease could be recommended as an additive in detergents to improve their washing performance of the detergent.

The results showed that the enzyme followed a typical Michaelis- Menten kinetics from a double reciprocal plot (Fig. 6). The apparent  $K_m$  of the enzyme for casein was found to be 3.2 mg/ml. The  $K_m$  values of 0.4 and 1.3 towards casein ( $\text{mg ml}^{-1}$ ) have been reported for alkaline

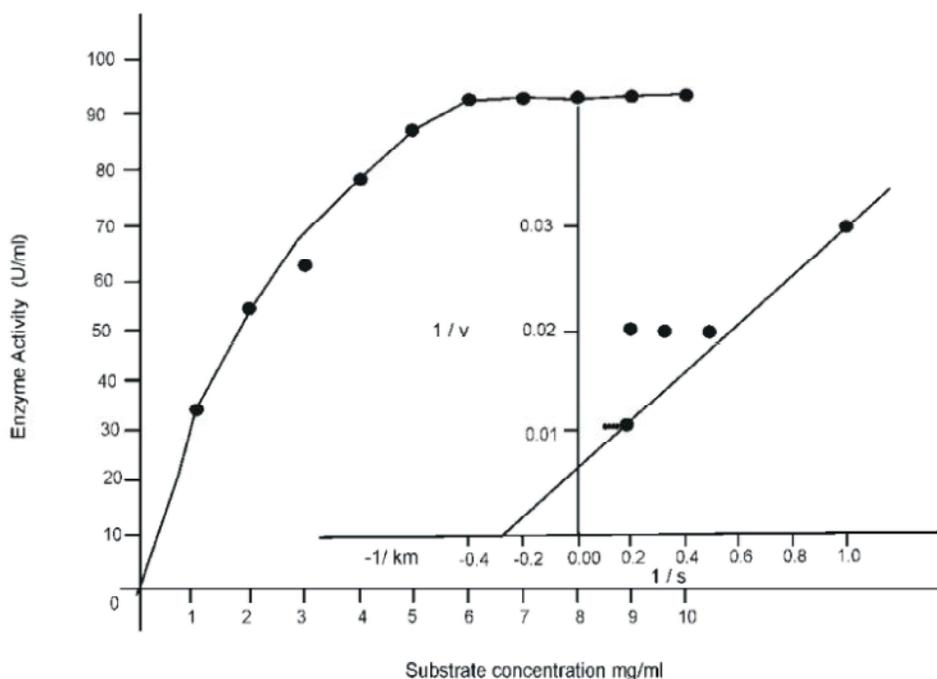


Fig. 6: Effect of substrate concentration on purified alkaline protease (Inset Line weaver-Burk plot)

proteases of *Bacillus alcalophilus* var. *halodurans* [17] and *Brevibacterium linens* [33] and the higher values 3.7, 7.4 and 9.4 mg ml<sup>-1</sup> have been reported for the proteases of *Bacillus polymyxa* [34], *Halomonas* sp. Es10 [34] and *Bacillus licheniformis* N3 [35], respectively. The lower km value of 3.2 mg ml<sup>-1</sup> in the present study indicated lower affinity of the enzyme from mutant *Bacillus licheniformis* (B18). Apparent km of the enzyme for casein was found to be 2.9 mg ml<sup>-1</sup> for mutant *Bacillus polymyxa*. It has lower km value than the wild *Bacillus polymyxa* protease [30].

### CONCLUSIONS

The purified alkaline serine protease exhibited desirable properties such as high pH and temperature optima, detergent stability and good washing performance. So this enzyme can be suggested for application in detergent industry, after proper evaluation of the performance in field trails. The results also suggested that the usefulness of this enzyme for different application such as, extraction of collagen from skin for collagen replacement therapy, waste treatment and others. The suitability of this protease for other commercial applications was not investigated. However it is reasonable to assume the promising nature of this enzyme for other commercial applications.

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