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Review Article

Biochemical Characterization of Alkaline Protease from Bacillus Circulans Mtcc 7906

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Abstract

In the present study, an alkaline protease of Bacillus circulans MTCC 7906 was purified 16.2 folds with specific activity of 102815 U/mg in comparison to crude extract using ammonium sulphate precipitation (30-60%), dialysis and DEAE-Cellulose anion exchange chromatography. The molecular weight of the purified enzyme was found to be 46 kDa on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The characterization of partially purified enzyme revealed 9.0 and 60°C as an optimum pH and temperature, respectively, with Km and Vmax of 4.5 mg/ml and 5555 U/ml using casein as substrate. The enzyme was activated by Ba^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} and inhibited by EDTA and ammonium hydroxide. The results thus indicated that the alkaline protease of B. circulans is a metalloprotease with serine at its active centre. Therefore it was concluded that this alkaline protease may be suitable candidate for commercial applications.

***Key words:** Alkaline protease; Bacillus circulans; Purification; Characterization; Inhibition; SDS-PAGE.*

Alkaline proteases (EC 3.4.4.16) accounting for 25% of the total enzyme market are being largely used in detergent and leather industries [1-2]. They have wide range of commercial usage in food, feed, pharmaceutical based industries, recovery of silk from cocoons and that of silver from used X-ray films [3-4]. In addition, alkaline proteases may also find wide applications in clearing of ultra-filtration and reverse osmosis membrane systems, by playing a fundamental role in breaking down proteinaceous foulants that block membranes during concentration of liquid foods like milk, whey and beverages [5]. Among microorganisms, the genus "*Bacillus*" is probably the only genus being commercialized for alkaline protease production with a number of *Bacillus* sp. such as *B. licheniformis*, *B. lentus*, *B. alcalophilus*, *B. subtilis*, *B. amyloliquefaciens* and *B. mojavensis* documented in different research reports [2, 6-8]. The first alkaline protease Carlsberg (BIOTEX) from *B. licheniformis* was commercialized as an additive in detergents in the 1960s [9]. The reason for this monoply of *Bacillus* sp. is due

to their wide temperature, pH tolerance and thermal stability [10], rapid growth, feasibility of mass culture, limited space for cultivation, broad biochemical diversity, simplicity for generation of new recombinant enzymes with desired properties (genetic manipulation) and the ease of separation of the extracellular enzyme [11-12]. The biochemical characterization of alkaline proteases has revealed them to be mostly serine centered or metallo-proteases with a pH between pH 9 and 11 [13-14], temperature range from 40 to 80°C [15-16] and molecular weight of nearly 30 kDa [17].

Although strains like *B. licheniformis* and *B. lentus* have been commercialized [18], efforts are being carried out in different laboratories to isolate and characterize more *Bacillus* species and strains with high alkaline protease activities [19-20]. In this context, a potential alkaline protease producing bacterial strain was isolated and identified as *Bacillus circulans* MTCC 7906 in our laboratory [21]. The latter has been characterized for optimum fermentation parameters for enzyme production with respect to development of low cost and easy available medium ingredients to fit for commercial use [22-23] and also molecularly characterized [24]. The strain was found to be compatible with local detergent powders [21] and had dehairing property [22] too thus making it a potential isolate for alkaline protease production.

In order to make *B. circulans* MTCC 7906 strain commercially viable, this strain needs to be characterized at biochemical levels with reference to enzyme purification and study of kinetic parameters so that it may be physiologically manipulated for higher alkaline protease production.

Materials and Methods

Inoculum preparation and production of alkaline protease

The inoculum of *B. circulans* MTCC 7906 was prepared by transferring a loopful culture of *B. circulans* into 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculum medium composed (g/l) of Glucose-10.0, Casein-5.0, Yeast Extract-5.0, K_2HPO_4-1.0, $MgSO_4$-0.2 and Na_2CO_3-10.0 with a pH of 9.5 [25]. The inoculated medium was incubated on an orbital shaker at 150 rpm, 28°C for 48 h. Flasks (500 ml capacity) in triplicate containing 200 ml of production medium were inoculated with 2% inoculum (10^6 cells/ml) of 48 hours old inoculum culture. The flasks were incubated on an orbital shaker incubator (150 rpm) at 28°C. The samples were drawn (aseptically) periodically after every 24 hours, spun at 10,000 rpm for 10 min at 4°C and supernatant so obtained was used for estimation of enzyme activity and total soluble proteins [26].

Assay of Proteolytic Activity

Alkaline Protease activity was determined in a reaction mixture (3 ml) containing 0.1 ml of enzyme, 2 ml of 0.5% casein (in carbonate-bicarbonate buffer, 0.1 M, pH 9.5) and 0.9 ml of distilled water and was incubated at 60°C for 15 minutes. The reaction was stopped by adding 3 ml of 5% trichloroacetic acid (TCA) and free amino acids released by crude protease from casein hydrolysis were estimated by Lowry method [26]. An enzyme blank was also run along with the sample. The alkaline protease activity was defined as nano moles (nM) of tyrosine released per minute per ml of crude enzyme.

All experiments were conducted in triplicate and mean values were reported.

Purification of alkaline protease

Ammonium Sulphate Precipitation

The 2500 ml cell free supernatant (obtained by centrifugation at 10,000 rpm for 20 min at 4°C) was concentrated by adding solid ammonium sulphate to get 0-30%, 30-60% and 60-90% saturations sequentially as per protocol [27], stirred for 60 min and left overnight at 4°C. The precipitates were harvested by centrifugation at 10,000 rpm for 20 min, dissolved in minimum volume of 0.1 M Tris-HCl buffer (pH 9.5) and dialyzed against the same buffer for 48 h at 4°C. The dialyzed samples were assayed for protease activity, protein content and purified further by DEAE-cellulose column chromatography.

DEAE cellulose column chromatography

Dialyzed enzyme was loaded on to a 0.1 M Tris-HCl buffer (pH 9.5) preequilibrated DEAE-cellulose column (3.0 cm × 45 cm). The same buffer containing sodium chloride gradient (0.1-1.2 M) was used for elution of protein with a flow rate of 1 ml min^{-1}. Thirty ml buffer of each molarity was used for elution. Fractions of 5 ml each were collected and analyzed for alkaline protease activity and protein content as described earlier. Alkaline Protease active fractions were pooled for further characterization.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to [28] using a 11% crosslinked polyacrylamide gel. Commassie blue (0.25%) staining was used to detect the protein bands.

***Characterization of partially purified alkaline protease of B. circulans* MTCC 7906**

Determination of optimum pH

The experiment was carried out to investigate the effect of different pH values on the partially purified alkaline protease enzyme. The purified enzyme extract was incubated at different pH values ranging from 7 to 11 using casein as a substrate and preparing buffers of different pH values (7-8 in phosphate buffer and 9-11 in carbonate-bicarbonate buffer). The enzyme activities for each case were determined under standard assay conditions as described earlier.

Determination of optimum temperature

Alkaline protease activity was determined at different temperature (40-80°C) levels by incubating the reaction mixture at appropriate temperatures for 15 minutes and analyzed for amino acids released under standard assay conditions.

***Effect of Substrate concentration*: Determination of Km and Vmax**

The concentration of casein was varied (1 to 15 mg/ml) to study the effect of substrate on partially purified protease activity under optimized conditions of pH, temperature and enzyme concentration. The kinetic parameters, Km and Vmax, of purified protease were determined by plotting the values of 1/V vs 1/[S] (Lineweaver Burk plot). From this plot, the apparent Michaelis constant (Km) and the maximum velocity (Vmax) of alkaline protease were determined.

Effect of various activators and inhibitors

Impact of various compounds $BaCl_2$, $CuCl_2$, $CaCl_2$, $MnCl_2$, $HgCl_2$, $CoCl_2$, $ZnCl_2$, EDTA and ammonium hydroxide as activators and inhibitors on purified alkaline protease was studied with 10 mM of concentration by incubating them in reaction mixtures under conditions optimized for alkaline protease

[29-30].

Statistical Analysis

The experiments were carried out in triplicates and results were analyzed statistically by CPCS 1 software.

Results And Discussion

Enzyme purification

The supernatant with alkaline protease activity of 9970250 U/2.5 L and specific activity of 6330 U/mg was used as crude enzyme and subjected to partial purification by ammonium sulphate precipitation in three sequential fractions of 0-30%, 30-60% and 60-90%. A purification of 2.9 folds was achieved with a specific activity of 18641 U/mg of protein in 0-30% fraction. The process when repeated by increasing ammonium sulphate concentration to 30-60% and 60-90% sequentially, revealed fold purification level of 11.0 and 5.2 folds and specific activity of 69677 and 32947 U/mg with a yield of 40.7% and 33.6%, respectively (Table 1). This showed that maximum purification could be achieved in 30-60% ammonium sulphate concentration. Hence, this fraction was subjected to DEAE cellulose anion exchange chromatography for further purification.

Different workers have reported variable fold purification and yield (%) of purified alkaline proteases. The yield and fold enzyme purification was 62% and 1.5 times having specific activity of 45.8 U/mg of protein in *B. pseudofirmus* [31]. In another report, [32] observed 2.2 fold increase of specific activity (1679 U/mg) with 62.2% recovery in *B. circulans*. Similarly, [33] precipitated the crude enzyme of *B. subtilis* with 1.11 fold purification and specific activity of 55.71 U/mg with 13.54% yield. Hence, our results present a better alkaline protease activity than earlier reports of [32] in case of *B. circulans.*

DEAE-cellulose anion exchange chromatography

The dialyzed protein fraction (30-60%) was eluted in the form of 78 fractions of 5 ml each (collected at a flow rate of 1 ml min^{-1}) and analyzed for alkaline protease activity and protein concentration. Alkaline protease eluted at a NaCl concentration gradient of 0.6-0.7 M and elution patterns of proteins and alkaline protease activity showed overlapping single peaks suggesting that fraction in the peak had alkaline protease as the major protein (Fig. 1). This also revealed the purity obtained with column chromatography. Hence, the protein fractions corresponding to the peak (fractions 40 to 45) were pooled that had a combined specific activity of 102815 U/mg with 16.2 fold purification (Table 1).

These findings are in accordance with several earlier reports showing 11.9% fold purification using Sephadex G-100 with 9000 U/mg specific activity in *B. circulans* [32]. Among other reports, fold purifications of 40.38, 50 and 1.49 with specific activities of 34171.46 U/mg, 143550 APU/mg and 74.66 U/mg have been reported in *B. polymyxa* B-17, *Bacillus* sp. 2-5 and *B. subtilis*, respectively [33-35].

SDS-PAGE of alkaline protease

The partially purified alkaline protease resolved on a SDS-PAGE (5% stacking and 11% running gel) was found to be a homogenous monomeric protein as evident by a single band corresponding to 46 kDa (Fig. 2). In the available literature on *B. circulans,* it has been reported to be a 30 and 39.5 kDa [32, 36]. In other species of *Bacillus*, the alkaline protease is reported as a single

band with a molecular weight ranging from 15 to 35 kDa [37-38] with few reports of higher molecular weights of 42 kDa from *Bacillus* sp. PS719 [39] and a very high (90 kDa) from *B. subtilis* [40]. Halophilic alkaline proteases with molecular weight in range from 40 to 130 kDa have also been reported [41-42]. Hence, a wide variation in the molecular weight of alkaline protease from different *Bacillus* sp. is observed.

Effect of pH on the activity of alkaline protease

The enzyme produced by *Bacillus circulans* MTCC 7906 was found to be active in alkaline pH range with maximum activity of 3164.4 U/ml at pH 9.0 (Fig. 3a). However the activity declined sharply near neutral pH. However, the enzyme activity decreased rather marginally at alkaline pH levels. Any further variation of the pH of the reaction mixture caused reduction in catalytic activity.

In literature, pH optimum for alkaline proteases has been reported to be species specific in a range of 7 to 11 [4, 14, 36 and 43] with a few exceptions of higher pH optima of 11-12 [32] and 12-13 [3, 5]. As stated above, our results presented an alkalophilic alkaline protease of *B. circulans* with a pH range of 8.5-11.0.

Effect of temperature on the activity of alkaline protease

Analysis of the temperature dependent alkaline protease activity revealed it as a broad range (50-65°C) enzyme with maximum activity at 60°C (Fig. 3b). However, further increase in the incubation temperature drastically reduced the enzyme activity. This inactivation of enzyme shows the destruction of enzyme at higher temperature incubation. The results showed that the alkaline protease appeared to be heat stable at temperature between 50-65°C. For a variety of industrial applications relatively high thermostability is an attractive and desirable characteristic of an enzyme [8, 44]. Elsewhere, reports from literature also suggest the alkaline proteases display maximum activity between 40°C to 85°C [3, 15 and 45]. Earlier, the optimum temperature of 40 and 70°C for alkaline protease of *B. circulans* has also been reported [32, 36].

Determination of kinetics of alkaline protease

Enzyme activities were measured under standard assay conditions as described earlier and results obtained were plotted as a graph of enzyme activity (U/ml) against concentration of substrate (mg/ml), which yielded a typical hyperbolic curve showing 12 mg/ml casein as the optimum concentration with an enzyme activity of 4051 U/ml (Fig. 4a). The enzyme followed the expected kinetics on which a double reciprocal plot was prepared that revealed an apparent Km of the enzyme as 4.5 mg casein/ml and a Vmax of 5555 U/ml (Fig. 4b).

Our results were in contrast to [32] for *B. circulans* as *B. circulans* MTCC 7906 had a lower affinity and catalytic rate. Literature reports a wide variation in the optimum substrate concentration for alkaline protease activity in different alkalophiles which is species as well substrate specific. Among other reports, Km values of alkaline protease of *Bacillus* sp. have been reported to be between 2-2.5 mg/ml [43, 46]. Jaouadi [7] exhibited substrate specificity in case of *B. pumilus* CBS as when casein was used as substrate, it gave Km value of 0.4 mM with Vmax of 27,160 U/mg. On the other hand,

with synthetic substrate, N-succinyl-L-Ala-Ala-Pro-Phe-p-nitroanilide the value of Km and Kcat was found to be 0.3 mM and 44,100 min^{-1}, respectively.

Effect of various activators and inhibitors on the activity of alkaline protease

Among different activators and inhibitors studied for their influence on alkaline protease activity, the results presented in Table 2 revealed that the enzyme activity is increased by Ca^{2+} (54.1%), Mn^{2+} (43.6%), Zn^{2+} (38.8%), Co^{2+} (35.9%) and Ba^{2+} (34.7%), indicating that enzyme may be a metalloprotease. This fact was further strengthened by the use of a metal chelator EDTA that inhibited the enzyme activity by 65.6%. The two metal ions Hg^{2+} and Cu^{2+} inhibited the enzyme activity by 87.4% and 25.7%, respectively. The liquor ammonia used in the incubated reaction mixture resulted in 28.0% reduction in alkaline protease activity, which suggests that the enzyme may be carrying serine residue at its active site also.

The results thus suggest that alkaline protease of *B. circulans* is a protease with metalloprotease being the major component or a metal dependent serine protease. Earlier, [32] also reported alkaline protease of *B. circulans* as a serine protease. Elsewhere, alkaline proteases in the form of metalloproteases and/or serine proteases have been reported in literature. Whereas the former are normally activated in the presence of divalent ions like Cu^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} etc. [32, 47-49], heavy metal ions like Hg^{2+}, Ag^{+} and compounds like PMSF and DFP etc. have been reported to be inhibitory to alkaline proteases [16, 37 and 50].

Conclusion

An alkaline protease of *Bacillus circulans* MTCC 7906 was purified and characterized. The purification to homogeneity of the enzyme was achieved by ammonium sulphate precipitation and anion exchange chromatography using DEAE cellulose. The enzyme was purified 16.2 fold with a specific activity, on casein as a substrate, of 102815 U/mg. The purified enzyme was homogenous on SDS-PAGE, and its molecular weight was estimated to be 46 kDa. The optimum pH and temperature for proteolytic activity were 9.0 and 60°C, respectively, with Km and Vmax of 4.5 mg/ml and 5555 U/ml using casein as substrate. The enzyme was found to be inhibited by EDTA and ammonium hydroxide and activated by Ba^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} confirmed it as a metalloprotease with serine at its active centre. Considering the high activity at high alkaline pH and temperature, *B. circulans* alkaline protease may find potential application as a laundry detergents additive or can be suitable for various commercial applications. Further work is also needed to improve the stability of this alkaline protease to make it commercially viable strain.

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Table 1 Purification Profile of Alkaline protease produced by *B. circulans* MTCC 7906

Purification Step	Volume (ml)	Total Enzyme Units (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	% Yield
Crude enzyme extract	2500	9970250	1575	6330	1	100
Ammonium Sulphate Saturation (%)						
0-30	12.5	281481	15.1	18641	2.9	2.8
30-60	15.0	4062217	58.3	69677	11.0	40.7
60-90	20.0	3354070	101.8	32947	5.2	33.6
DEAE-Cellulose anion exchange column chromatography	30.0	123378	1.2	102815	16.2	1.2

Table 2 Relative alkaline protease activity of partially purified alkaline protease in the presence of various activators and inhibitors

Activators and Inhibitors	Relative alkaline protease activity (%)
Control	100 ± 8.8
Ba^{2+}	134.7 ± 9.5
Cu^{2+}	74.3 ± 7.2
Ca^{2+}	154.1 ± 3.5
Mn^{2+}	143.6 ± 4.3
Hg^{2+}	12.6 ± 2.9
Co^{2+}	135.9 ± 7.7
Zn^{2+}	138.8 ± 5.7
EDTA	34.4 ± 6.6
NH_4OH	72.0 ± 4.8

- The activity in the absence of activators and inhibitors (control), referred to 100%

relative protease activity.

- The values shown are means of three separate determinations ± SE.

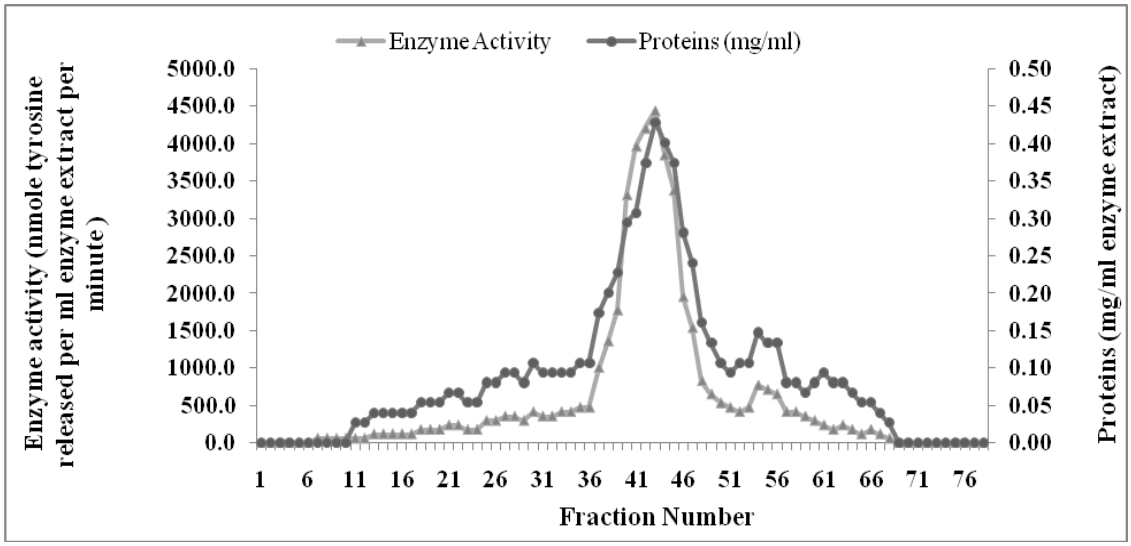

Fig. 1 DEAE cellulose anion exchange chromatographic profile of saturated ammonium sulphate (30-60%) concentrated alkaline protease of *B. circulans* MTCC 7906.

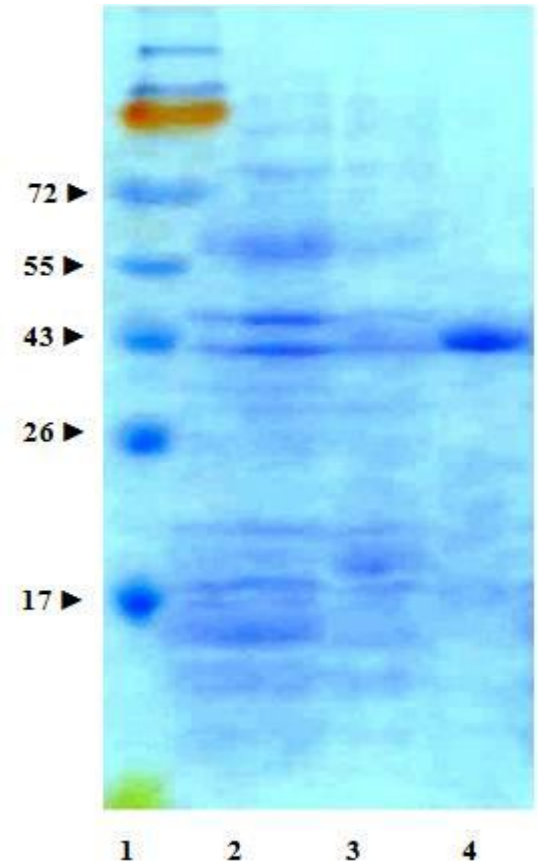

Fig. 2 SDS-PAGE profile of alkaline protease in *B. circulans* MTCC 7906. Lane 1: Protein ladder (10-170 kDa), Lane 2: Crude alkaline protease, Lane 3: Ammonium sulphate precipitated alkaline protease, Lane 4: Purified alkaline protease

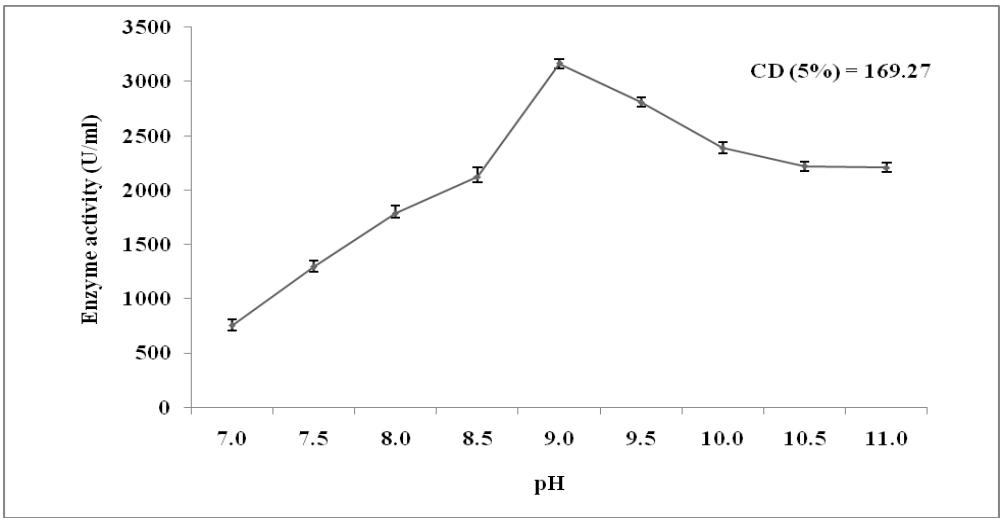

Fig. 3a

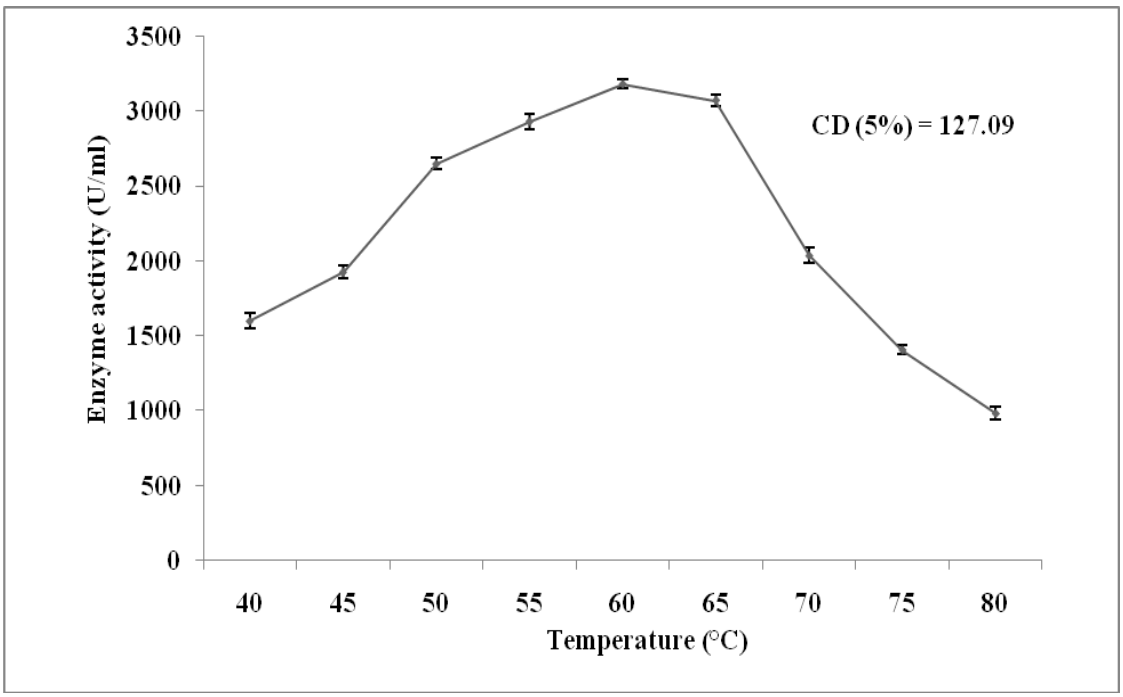

Fig. 3b

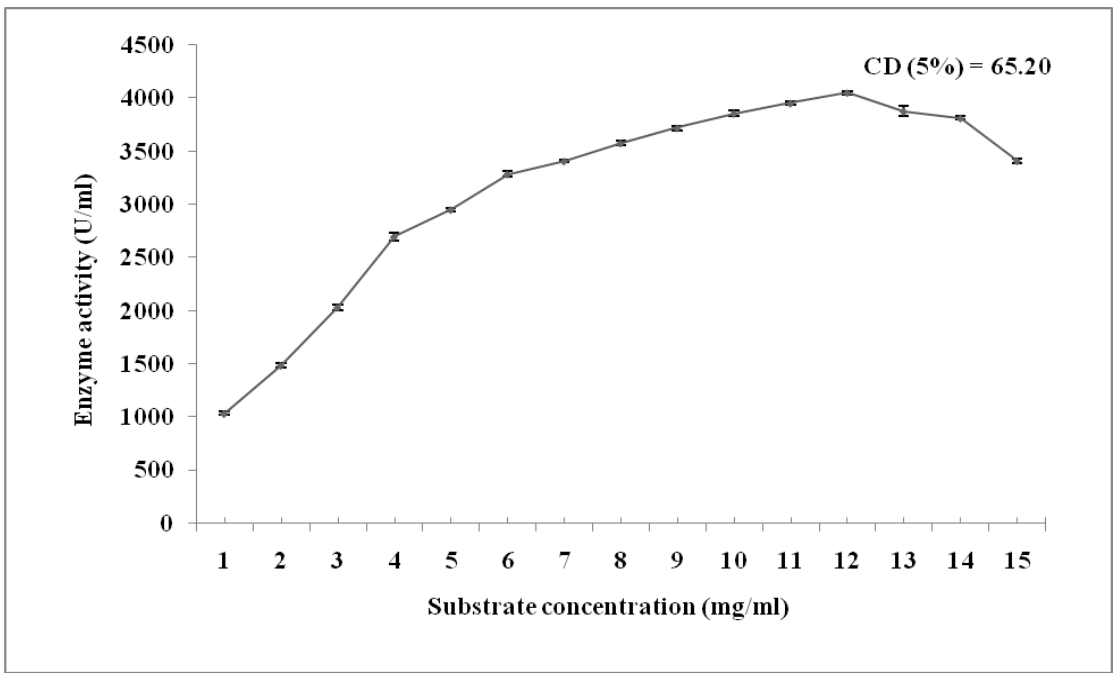

Fig. 4a

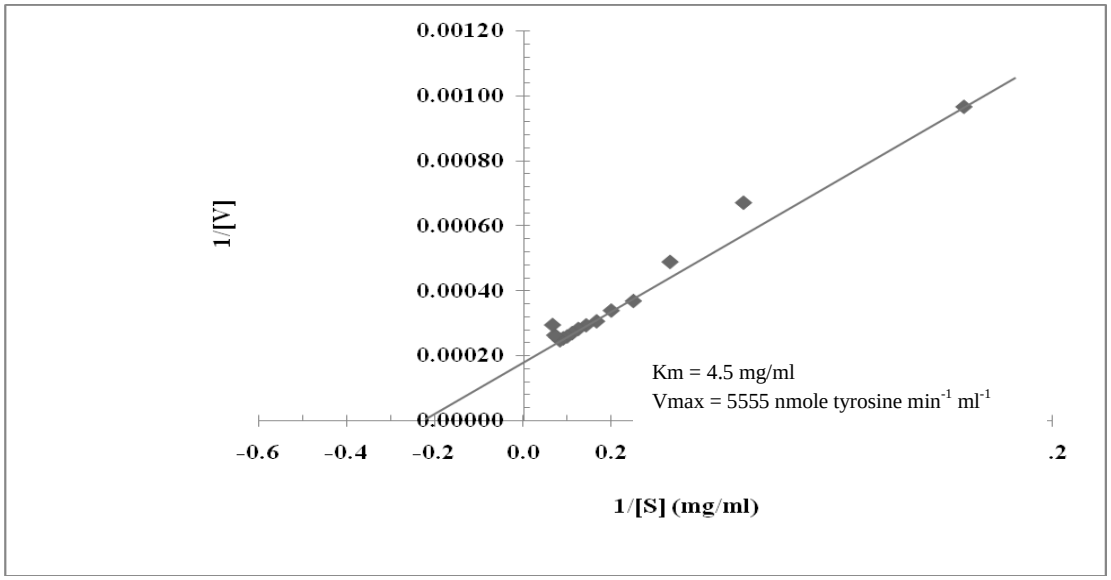

Fig. 4b

Fig. 3 and 4 Effect of pH **(3a)**, Temperature **(3b)** and substrate concentration **(4a and 4b)** on alkaline protease activity of partially purified enzyme for *B. circulans*