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INFLUENCE OF DIFFERENT FACTORS ON PRODUCTION OF PURIFIED PROTEASE BY *BACILLUS SUBTILIS* DKMNR

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ABSTRACT

The extracellular protease produced by *Bacillus subtilis* DKMNR was purified using ammonium sulphate precipitation and Sephadex G-100 column chromatography. SDS-PAGE analysis revealed that this protease is monomeric in nature and has molecular weight of 43 kDa. The protease belongs to serine-type with more specificity for casein compared to BSA, egg albumin and gelatin. The enzyme activity was positively influenced by the presence of Ca^{2+}, Mg^{2+} and Mn^{2+} ions and negatively affected by Fe^{2+} and Ba^{+2} ions. The produced enzyme has showed highest activity at 70°C and also possesses good thermostablity at above 70°C. The kinetic data revealed that the obtained enzyme has K_m of 0.7614 mg mL^{-1} and V_{max} of 2582 U min^{-1}.The lower activation energy of 41.082 kJ/mol/k shows that the enzyme is more stable at higher temperature and also highly reactive.

KEY WORDS

Protease production, *Bacillus subtilis*, purification, activation energy, thermostability of enzyme and enzyme kinetics.

INTRODUCTION

Proteases constitute one of the most important groups of enzymes both industrially and therapeutically. The annual sales accounts 60% of the total world enzyme market[1] . Of these, bacterial alkaline proteases are most useful in laundry, food, leather and silk industries[26,2,3] as compared to animal and fungal proteases. Recent developments indicated that proteases with high activity profile at alkaline pH and high temperatures have potential applications in variety of fields including enzymatic debridement and the natural healing process in the skin ulcerations [4,5,6]. They have also gained importance in view of their cleaving of peptide bonds in aqueous environments and synthesize them in non-aqueous conditions [25].

Physical, biochemical, thermal, molecular and catalytic properties of proteases vary with the producing organism[7,8]. In general, most of the industrial proteases face some limitations [9] and their use highly depends on their stability during isolation, purification and storage in addition to their robustness against solvents, surfactants and oxidants[10,11] Hence, in depth knowledge on protease kinetics of fermentation and catalytic level of protease production by newly isolated strain is one of the pre-requisites for evaluation of its potential for biotechnological application[12,13]. In the present studies a strain of *B. subtilis* DKMNR was assayed for its ability to digest casein.

MATERIALS AND METHODS

1. ***Bacterial strain, media and growth conditions:***
   Bacillus subtilis culture used in this study was isolated from garden soil sample around the department of Chemical Engineering, Andhra University, Visakhapatnam, A.P, India. The production medium, yeast extract-peptone-dextrose (YPD) medium, consisting ($g.l^{-1}$) of glucose 10.0; peptone, 7.5; yeast extract, 7.5; K_2HPO_4, 0.50, $MgSo_4$ 0.05 and $CaCl_2$ 0.02 and pH 8.0 was inoculated with 1% (v/v) of culture medium and incubated at 30^0C on rotary shaker at 120 Rpm for 48 hrs. At the end of incubation period, bacterial cell mass was separated by centrifugation and the supernatant was dialyzed against distilled water overnight and served as enzyme for assaying enzyme activity.

2. ***Measurement of protease activity:***
   Protease activity was determined by the modified Auson–Hagihara method [14]. In this 1 ml of the enzyme solution was added to 1 ml casein solution (1%, w/v casein solution prepared in 50 mM glycine–NaOH buffer, pH 11.0) and incubated at 70°C for 20 min. The reaction was terminated by adding 4 ml of 10% trichloroacetic acid and the contents were filtered through a Whatman No. 1 filter paper. The filtrate absorbance was read at 280 nm using UV–Visible spectrophotometer and the protease activity was calculated using tyrosine standard curve. One unit of alkaline protease activity was defined as 1 μg of tyrosine liberated ml^{-1} under the assay conditions. All experiments were repeated twice and run in triplicate. Since the variations in values were insignificant, average of values having 3% experimental error are presented.

3. *Protein estimation:*

The protein content of enzyme was determined by the Lowry method [15] using BSA as standard.

4. *Purification and molecular weight determination of protease:*

(i) Ammonium sulphate precipitation:

The crude broth obtained after fermentation was centrifuged at 5000 X g for 10 min to remove the cell biomass. Solid ammonium sulphate was added slowly to the culture supernatant to get 60% saturation, stirred for 60 min and left for overnight at 4^{o}C. The precipitate was harvested by centrifugation at 10,000 X g for 10 min, dissolved in 50 mM glycine-NaOH buffer (pH 11.0) and dialyzed against same buffer overnight (4^{o}C). The dialyzed sample was then assayed for protease activity and protein content.

(ii) Sephadex G-100 Chromatography:

Dialyzed enzyme was loaded on to a column of sephadex G-100 (1.5 x 90 cm) previously equilibrated with 50mM glycine-NaOH buffer (pH 11.0) and then eluted at a flow rate of 10 ml/h with the same buffer containing sodium chloride gradient from 0.1 to 1.0M. The absorbance of fractions was checked at 280 nm. Fractions were assayed for protease activity with casein as substratum. Protease active fractions were pooled and concentrated for further characterization.

(iii) Polyacrylamide gel electrophoresis:

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [16] using a 10% crosslinked polyacrylamide gel on a Tarson gel electrophoresis unit (Tarson, India). Silver staining was performed to visualize protein bands on the gel. Native PAGE was performed according to the method of Davis[13] with Tris/Glycine buffer, (pH 8.3). Coomassie Brilliliant Blue (0.1%) staining was used to detect the protein bands on the gel.

(iv) Determination of the pH optimum and pH stability:

The pH optimum for purified protease was assayed by analyzing its activity in the pH range of 4.5 to 13.0 using casein as a substratum and buffer systems of 0.05 mol l^{-1} phosphate buffer for pH 5.0 to 7.5, Tris-HCl for pH 8.0 to 9.0, glycine-NaOH for pH 9.5 to 11.0, sodium phosphate for 11.5 to 12.0 and sodium carbonate for 12.5 to 13.0. pH stability study of the protein was analyzed by pre-incubating 5ml of purified enzyme in 3.5 ml of selected pH buffer at 37° C for 0-120 min and subsequent analysis of residual activities under standard assay conditions.

(v) Determination of optimum temperature and thermal stability:

To study the temperature optima of enzyme, the enzyme reaction mixture was incubated at different temperatures ranging from 35°C to 90°C in glycine-NaOH buffer (pH 11.0) and measured protease activity using casein (1%) as substratum. For determining thermal stability, the enzyme was pre-incubated for 1.0 h at different temperatures ranging from 35 to 90 °C and the residual activity was measured under standard assay conditions after incubating with casein.

Thermo-inactivation assays were carried out by preheating 950 μl of standard buffer at the corresponding temperature, then adding 1 μg protein in 50 μl of the same buffer and pre-incubating the mixture at the same temperature. Samples were collected every 1.0 h at 75, 80, 85 and 90 °C and cooled to 50°C before assaying the protease activity.

5. *Effect of various metal ions:*

Effect of different metal ions like Ca^{2+},Co^{2+},Cu^{2+}, Ba^{2+}, Mg^{2+},Mn^{2+},Fe^{2+},Hg^{2+},Na^{+} and Zn^{2+}, K^{2+}, Ag^{2+}, Pb^{2+} and EDTA, on the enzyme catalytic behavior was studied by pre-incubating purified enzyme in a specified ion (10mM final concentration) containing buffer

solution. After 1 hr of incubation, casein was added and residual activity of the enzyme was measured as described above.

6. Effect of protease inhibitors:

Effect of different protease inhibitors such as phenyl methyl sulphonyl fluoride (PMSF), diisopropylflurophosphate (DFP), serine protease (SP) inhibitor, EDTA (metalloprotease inhibitor), *p*-chloro mercuric benzoate (*p*CMB), Ido acetic acid (cysteine protease inhibitor) and β-mercaptoethanol on protease activity was investigated by incubating the enzyme for 30 min at 30 °C in the selected protease inhibitor containing reaction mixture in a final concentration of 1.0 and 5.0 mM the proteolytic activity was assayed by Auson-Hagihara method.

7. Effect of surfactants and oxidatives on protease activity:

The impact of 1.0% final concentration of different surfactants and oxidatives (SDS in w/v, Tween-80, Triton X-100 in v/v and H_2O_2 in v/v) on proteolytic activity of the purified alkaline protease was studied by pre-incubating the enzyme in above surfactant solutions at 37°C before being tested for protease activity. After 4 hr incubation, casein was added and residual activity of the enzyme was measured as described above. A parallel control was maintained with enzyme, buffer along with substrate and the control activity value was considered as 100%.

8. Hydrolysis of protein substrates:

Protease activity using other than casein as substrate material was assayed by mixing 100 µl of enzyme and 1.9 ml of assay buffer containing 1 mg/ml BSA, egg albumin and gelatin in a separate reaction mixtures and measured the protease activity. The casein dependent protease activity was considered as control for calculating the percent activity.

9. Estimation of kinetic parameters:

(i) Determination of activation energy:

The activation energy of the isolated protease was determined by measuring the enzyme activity at different temperatures. Arrhenius plot constructed between the reciprocal of the temperature and enzyme activity, was used to determine the activation energy [2].

(ii) Determination of V_{max} and K_m values:

The kinetic parameters, V_{max} and K_m, of the purified alkaline protease were determined by measuring of enzyme activity at different concentrations of the substratum (casein, 0.4 -1.6%). The kinetic parameters (K_m and V_{max}) values were determined using Michaelis-menton equation through non-linear regression analysis using the program enzyme kinetics module 1.3 in Sigmaplot - 10.0.

RESULTS AND DISCUSSION

1. Enzyme purification and molecular weight:

Table 1 summarizes the results of the alkaline protease purification. This enzyme was purified 2.45-fold starting from the culture filtrate and achieved near homogeneity by ammonium sulfate precipitation (60%), ion exchange chromatography using Sephadex G-100 and gel filtration. The specific activity of the finally purified enzyme was 486.18 $U.mg^{-1}$of protein indicating 30% recovery. Purified alkaline protease was homogeneous as evidenced from by a single protein band in SDS-PAGE. The apparent molecular mass of the purified enzyme was estimated to be 43 kDa (Figure 1). The above results suggest that this is a monomeric enzyme. These results are in accordance with the literature reports where molecular masses of *Bacillus* species proteases were rarely more than 50 kDa[17,2]. The protease from *Bacillus circulans* has 39.5 kDa molecular weight.

Table 1
Purification of alkaline protease of B. subtilis DKMNR.

	Total activity	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification fold (%)
Crude	171548	864	198.55	100	1.00
$(NH_4)_2SO_4$	85051	234	363.47	60.24	1.83
Sephadex-G100	18961	39	486.18	30	2.45

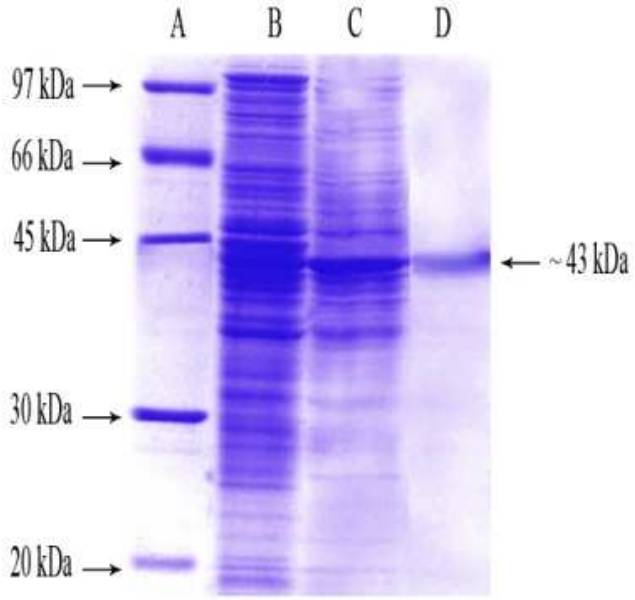

Figure 1
SDS-PAGE analysis of the purified protease. Lane A Marker; B crude enzyme; C, ammonium sulphate precipitated and dialyzed sample; D, Purified protease by Sephadex G-100.

2. Effect of substrate concentration on protease activity:

The substrate concentration also critically affected the enzyme activity. The constant amount of purified protease from *B. subtilis* DKMNR was added to the different concentrations of casein solution. The protease has shown the highest activity of 1697 U mg^{-1} min^{-1} at the highest concentration of substratum (1.6%). The nature of the curve indicates that the increase in substrate concentration increased the protease activity at certain point after that it reaches the saturation. From Figure 2 it is clear that the isolated protease followed the pseudo first order mechanism.

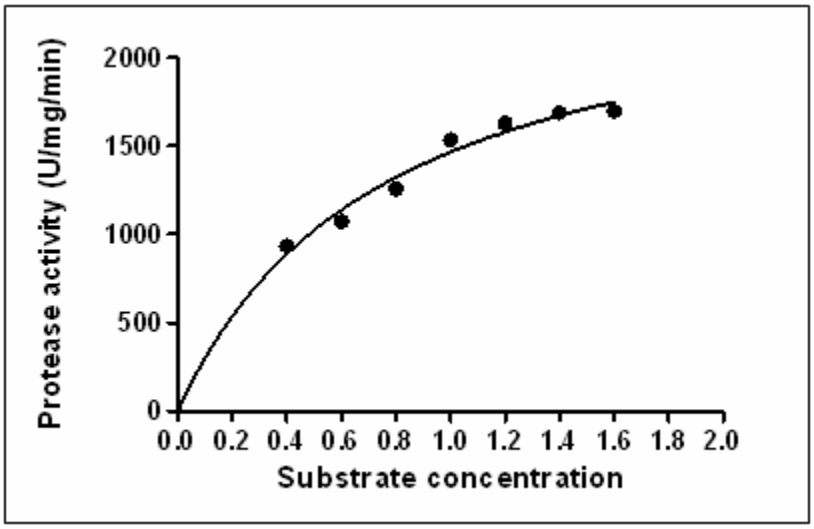

Figure 2
Effect of substratum concentration (0.4 to 1.6%) on the protease activity.

3. Effect of pH on the protease activity and stability:

In general, species of *Bacillus* are reported to secrete mostly two types of extracellular proteases, a neutral or metallo-protease, exhibit optimum activity at pH 7.0 and an alkaline protease having pH optima between 9.0 and 11.0[8]. The enzyme produced by *B. subtilis* DKMNR exhibited its optimum activity at pH 11.0 indicating the enzyme to be alkaline protease group. Any further variation in p^H of reaction mixture resulted reduced catalytic activity Figure 3. This activity variation was more and drastic with increase of reaction mixture pH towards alkalinity. More than 50% reduction was noticed with the change of 0.5 pH unit from 11.0 to 11.5 and further increase of 0.5 pH unit resulted in 90% inhibition Figure 3.

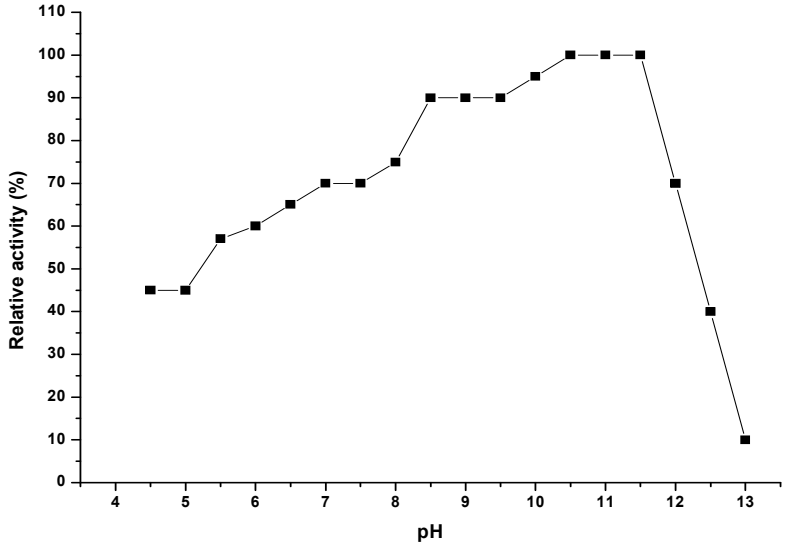

Figure 3
Effect of pH on the activity of protease of B. subtilis DKMNR

A progressive reduction of enzyme activity was observed with change of reaction mixture pH towards acidic side indicating its robust nature in pH range of 5.0 to 11.0. The protease was active more than 70 % in the range of pH 5.5-7.0 (Figure 3). Even though its activity at pH 5.0 was only 50-70 % of its activity below pH 7.0 with respect to its activity at pH 11.0 indicating its alkaline nature [18] have reported protease with broad pH range from 6.0 to 12.0 having optimum activity at pH 10.5 to 11.5. Protease of *B. subtilis* DKMNR was radically reduced with the change of pH in either side of pH optima indicating the more activity enzyme.

4. pH stability studies:

pH dependent enzyme stability studies were performed by incubating it at a range of pH solutions from 10.5 to 12.0 for 0 – 120 min at room temperature and measuring the proteolytic activity at pH 11.0. The catalytic activity profile denoted that the enzyme activity varied with incubation time and storage pH Figure 4.

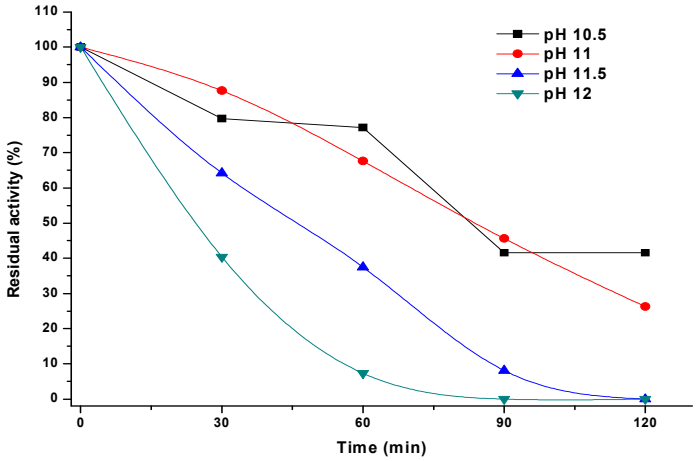

Figure 4
Influence of pH and incubation time on the activity of B. subtilis DKMNR

Incubation of protease for first min in 10.5 to 11.5 pH solution did not show any reduction in activity profile, whereas approximately 30 percent reduction in activity was noticed with the enzyme incubated in the pH range of 10.5 to 11.5 indicating its stable nature at this pH range Figure 4. The increase in incubation time to 90 min revealed that enzyme activity was adversely affected and showed 40 % and 50% of its activity at pH 10.5 and 11.5 respectively, with a maximum stability in the pH range of 10.5 to 11.0. Similar kind of pH stability for protease produced by *Bacillus* species has been observed[10] .

5. Effect of temperature on protease activity and stability:

The influence of incubation temperature on protease activity was studied by incubating the enzyme for 20 min in specified temperature prior to determination of proteolytic activity. Protease activity was not affected by pre incubation of the enzyme at 35 to 70°C but decreased markedly at higher temperatures more than 70°C Figure 5. Analysis of the data revealed a phase activity profile with initial constant activity phase in the temperature range of 35 to 45 °C followed by rapid accelerated phase in the range of 45 to 60 °C and slow accelerated phase in the range of 60 to 70 °C Figure 5. This type of three phase activity has not been reported earlier.

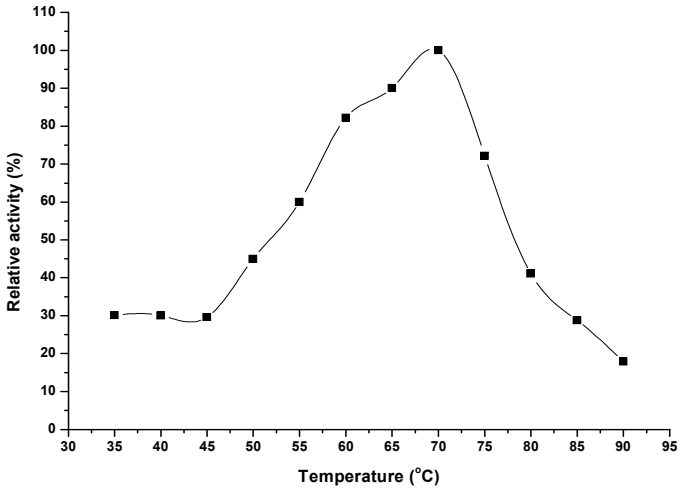

Figure 5

***Effect of temperature (35℃ to 90 ℃) on the activity of protease of B. Subtilis* DKMNR.**

6. Thermostability studies:

The thermostability of protease was studied by incubating reaction mixture at different temperatures ranging from 75 to 90 °C for 0 to 210 min at optimum pH (11.0). The catalytic activity profile denoted that the enzyme activity varied with incubation time and temperature Figure 6. Incubation of protease at 75°C for more than 60 min did not show any effect on the enzyme but at temperatures from 80 to 90°C caused a severe decline in protease activity when the incubation time was less than 60 min. While, increase of incubation time more than 120 min adversely affected the enzyme activity and showed only 53 % and 41% of its activity at incubation time of 150 and 210 min, respectively. The enzyme activity decreased abruptly with further increase of incubation time.

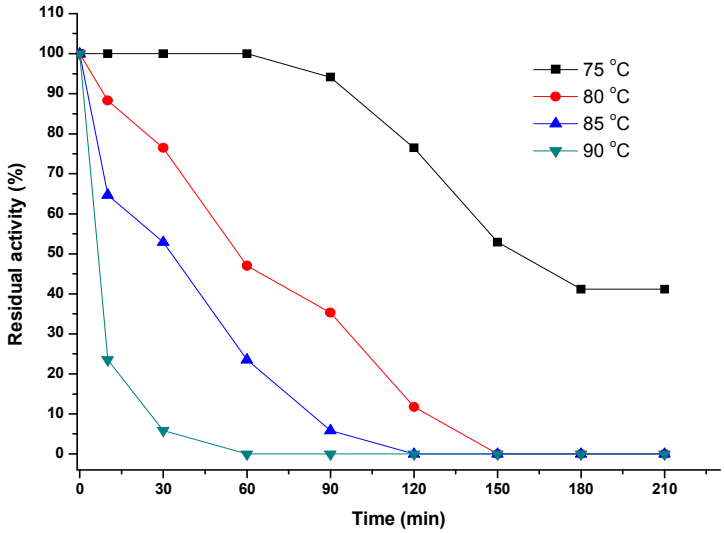

Figure 6

***Effect of temperature on protease from 0 to 210 min, stability of B. Subtilis DKMNR*.**

7. Effect of metal ions on purified alkaline protease activity:

To understand the role of different metal ions on the regulation of protease activity, the enzyme activity was monitored in the presence of 10 mM concentration of selected metal in a reaction mixture. The effect of different metal ions on the activity of protease is shown in Figure 7.

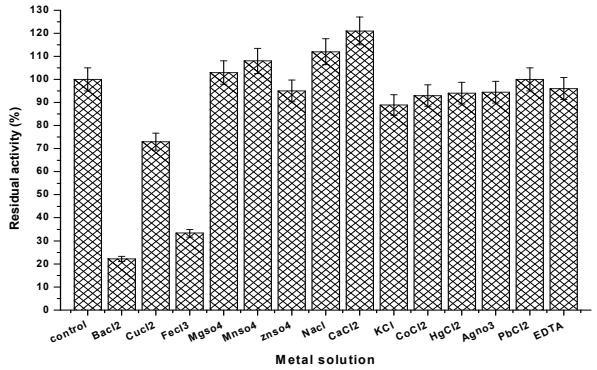

Figure 7

Effect of different metal ions on activity of purified protease of B. Subtilis DKMNR.

Presence of $PbCl_2$ did not show any inhibition of alkaline protease activity indicating enzyme from *B. subtilis* DKMNR is not a metalloprotease. Na^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} ions positively regulated the enzyme activity, while other tested metal ions did not show much influence, except Ba^{2+} and Fe^{2+}, compared to control. The activity regulation was depended on the nature of ion. This was evident from the observations that 21, 12, 8 and 3 % enzyme activity improvement was noticed with the supplementation of Ca^{2+}, Na^{2+}, Mn^{2+} and Mg^{2+} ions, respectively. On the other hand, > 60 % inhibition was observed in the presence of Ba^{2+} and Fe^{2+} ions. The Ca^{2+} ion stimulated the enzyme activity indicating calcium ion involvement in stabilization of the molecular structure of enzyme as reported earlier[19,20,21]. In fact, calcium ions are known to be inducer and stabilizer of many enzymes and protect them from conformational changes[17]. Such type of metal dependent variation in proteases activity has also been reported with serine proteases [22].

8. Effect of Inhibitors on protease activity:

North[23] has classified proteases based on their sensitivity to various inhibitors. To identify the class to which the alkaline protease produced by *B. subtilis* DKMNR belongs to, the enzyme activity was assayed in the presence of different inhibitors and results are presented in Table 2.

Table 2

Residual activity of purified alkaline protease of B. Subtilis DKMNR at two concentrations (1 and 5 mM) of inhibitors.

Inhibitor	Residual activity (%)	
	1 mM	5 mM
Control	100	100
β-mercaptoethanol	94	85
PMSF	9	0
Ido acetic acid	98	95
EDTA	96	96
PCMB	93	91
DFP	24	5

Presence of 1mM and 5mM of EDTA (Metallo protease inhibitor), Idoactetic acid (IAA,) and *p*-chloromercuribenzoate (pCMB) which are inhibitors of cysteine protease showed little or no effect on the alkaline protease of *B. subtilis* DKMNR suggesting this protease does not belongs to the metallo protease and cysteine protease Table 2. The aspartic protease inhibitor, ß-mercaptoethnol, was also ineffective as it showed only 6 and 15% inhibition of proteolytic activity. In presence of 1 and 5mM concentration of ß-mercaptoethnol. Serine protease inhibitor PMSF, completely inhibited the enzyme activity even at very low concentration and the inhibition pattern was typical of serine proteases.

9. Effect of surfactants and reducing agents on the activity of *B. subtilis* DKMNR alkaline protease:

Table 3 depicts the influence of different surfactants and reducing agents on the activity of alkaline protease of *B. subtilis* DKMNR. The purified enzyme has shown stability in the presence of all the surfactants studied Table 3. Infact the non-ionic detergents, Triton X-100 and Tween-20, enhanced its residual activity to 21 and 6 % respectively. In the presence of strong anionic surfactant (SDS 1%) the enzyme retained 84% of its residual activity. In general the proteases of *Bacillus* are unstable against the oxidants and bleaching agents[5]. However, the enzyme under investigation did not show any inhibition in the presence of 1% hydrogen peroxide Table 3 suggesting the purified enzyme is stable for tested cationic, anionic, nonionic and even for different commercially available detergents Hence, protease of *B. subtilis* DKMNR is most suitable in the formulations of the commercial detergents as most of the commercial detergents contain cationic and anionic components in its formulations [5].

Table 3

Residual activity of purified alkaline protease of B. Subtilis DKMNR in the presence of different surfactants:

Surfactants	Residual activity (%)
Control	100
Trirton X-100	121
Tween-20	106
SDS	84
H_2O_2	97

10. Hydrolysis of proteinaceous substrates:

The substrate specificity of *B. subtilis* DKMNR protease was tested with natural proteins like casein, BSA, egg albumin, and gelatin. Present protease exhibited the highest activity with casein. Considering the casein dependent activity as control, BSA and egg albumin and gelatin hydrolysis activity was assayed. The data revealed that enzyme showed only 75, 56 and 26 % respectively of activity with BSA, egg albumin and gelatin as substrates. The present observations are in agreement with the report on *Bacillus subtilis* PE-11 [24] Figure 8.

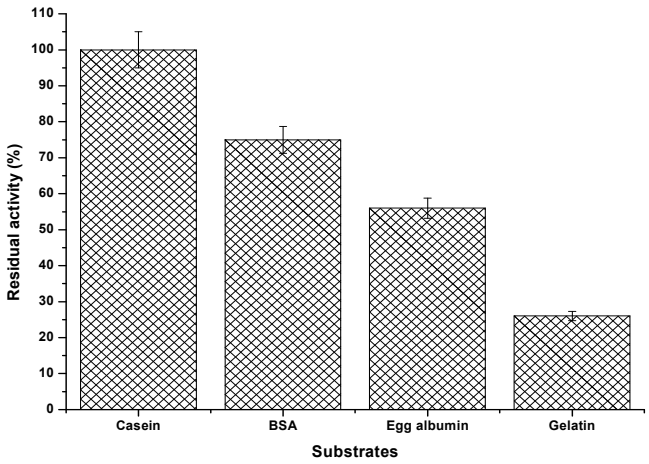

Figure 8
Influence of substrates on protease activity of B. Subtilis DKMNR.

Kinetic parameters of pure protease:

1. Activation energy:

The activation energy of purified protease of *B. subtilis* DKMNR was investigated using the Arrhenius plot by plotting the residual activity in natural logarithm (ln*v*) versus inverse of the temperature (T^{-1} K) after fitting the data in linear regression (Figure 9). The observed correlation coefficient was higher than 0.95 which indicate the deactivation follows the first order kinetics. From figure 8 activation energy was calculated as 41.082 kJ/mol/k such variation of enzyme activation energy indicates the conformational changes especially at the catalytic site which improves affinity towards substrate binding. This could be further confirmed based on the observation that the noticed change of rate of enzyme activity (62 and 31 U per increase of one degree centigrade in the temperature range of 45 to 60°C and 60 to 70°C, respectively).

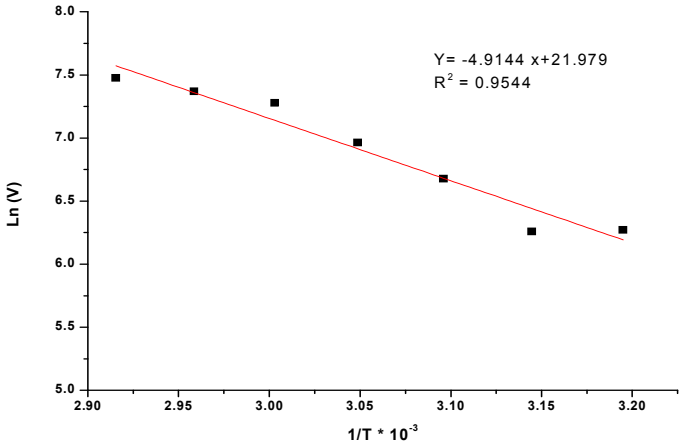

Figure 9
Arrhenius plot for protease of B. subtilis DKMNR

2. K_m and V_{max} values:
The protease of *B. subtilis* DKMNR was characterized further for its K_m and V_{max} towards casein as a substratum at 70 °C. The K_m and V_{max} values were determined by using Michaelis-menton equation through nonlinear regression analysis. The results were recorded as K_m of 0.7614 mg mL^{-1} and V_{max} of 2582 U min^{-1}. Similar results were reported for protease obtained from *B. circulans*[2].

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