Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

Mn^{2+}-Dependent Thermophilic Protease of *Thermoactinomyces Vulgaris* Tsiklinsky

Vivek Kumar Kedia[1,2] and **Ved Pal Singh**[2]
[1] Department of Botany, Govt. Degree College Nainidanda, Pauri Garhwal - 246277
[2] Department of Botany, University of Delhi, Delhi – 110007
Email – drvkkedia@rediffmail.com

Abstract Protease of *Thermoactinomyces vulgaris* was highly thermophilic, as it exhibited its optimal catalytic activity at 65°C, which was much higher than the growth temperature (50°C) of this obligate thermophile. Among the metal ions tested, Mn^{2+} was most stimulatory, enhancing the specific activity of protease by about 4.4-fold over the control, and broadened the range of its temperature optimum (i.e. 60–65°C). A heavy metal (Hg^{2+}) inhibited the enzyme activity by about 15%. The increase in slope of Arrhenius plot as well as the energy of activation (E_A) of *T. vulgaris* protease due to Mn^{2+} indicated that this divalent cation enhanced the rate of high-temperature enzyme catalysis at the expense of E_A. The increasing concentration of the substrate (casein) increased the specific activity of protease gradually in a dose-dependent manner, both in the absence as well as in the presence (10 mM) of Mn^{2+}. This divalent cation increased the V_{max} of this enzyme without affecting K_m for the substrate. The thermophilic protease of *T. vulgaris* appeared to be a metalloenzyme, in which Mn^{2+} is firmly bound to it, as even 10 mM of EDTA was not able to chelate/remove Mn^{2+} completely to inhibit its activity.

Keywords Arrhenius energy of activation · Arrhenius plots · Energy of Activation · Extracellular protease · High-temperature catalysis · Protease · *Thermoactinomyces vulgaris* · Thermophilic protease

Introduction

Thermoactinomyces vulgaris has been characterized extensively in our research laboratory with respect to its growth [36], genetics [4, 11, 34] and thermophilic hydrolases, including acid and alkaline phosphatases [35, 37, 38, 39] and membrane ATPase [5, 33].

A group of hydrolases, such as proteases are an important class of enzymes which constitute more than 59% of the total industrial enzyme market [25]. Proteases, secreted extracellularly by thermophilic bacteria, can be used in a range of commercial applications such as leather preparation, protein recovery or solubilization, meat tenderization and organic synthesis [6, 20]. Proteases are also used extensively in bread industry, as higher processing temperatures can be employed with faster reaction rates [7, 10, 40]. Thermolysin-like proteases (TLPs) are the best characterized species of proteolytic enzymes secreted by both Gram-positive and Gram-negative bacteria [43]. They hydrolyze proteins to short peptides or free amino acids and catalyze peptide synthesis in organic solvents or in solvents with low water content. Alkaline proteases, produced by thermophilic and alkaliphilic bacilli, can withstand high temperature, pH, chemical denaturing agents and non-aqueous environments. Alkaline proteases have major application in the detergent industry, as pH of laundry

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

detergents is generally in the range of 9.0–12.0. Most of the commercial alkaline proteases have been isolated from *Bacillus* species [9, 22].

Proteases have been classified according to their optimum pH such as acidic, neutral and alkaline protease. Most proteases, reported from bacteria, have been found to be alkaline proteases. Hyperthermostable proteases were characterized from five archaebacterial species (*Thermococcus celer, T. stetteri, Thermococcus* strain AN1, *T. litoralis* and *Staphylothermus marinus*) and a hyperthermophilic eubacterium, *Thermobacteroides proteolyticus* [17, 29]. A thermostable alkaline protease from *B. licheniformis* RP1 was investigated by Sellami-Kamoun et al. [31], which exhibited its optimal activity at 65-70^{o}C and at pH 10-11.

Considering diverse industrial applications of thermophilic protease, secreted by thermophilic bacteria with unique property make this group of organisms of special interest for basic and applied research. It was against this backdrop, that the present investigation was undertaken to characterize extracellular protease from an obligate thermophile – *Thermoactinomyces vulgaris* with respect to its pH and temperature optima as well as metal ion specificity and kinetic properties.

Materials and methods

Microorganism used and culture conditions

A wild-type strain (Stock no. 1227) of *Thermoactinomyces vulgaris*, which was kindly supplied by Professor D.A. Hopwood, John Innes Centre, Norwich, U.K., was used in the present investigation. The medium described by Hopwood and Wright [11] was used with certain modifications [32].

Enzyme preparation

Hopwood's medium (30 ml) was taken in a 250 ml conical flask and inoculated with 1 ml fresh spore suspension of *T. vulgaris*, containing about 10^6 spores and incubated for 12 h at 50^{o}C. The culture filtrate was collected by filtration through Whatman no. 1 filter paper. The content was centrifuged at 10,000g for 10 min at 4^{o}C and the supernatant was used as the enzyme sample.

Screening for protease production and assays for enzyme activity

The screening for protease production by *T. vulgaris* was done by using casein (1%) as a substrate and skim milk (5%) as an inducer in Hopwood's medium [11]. Sterile modified Hopwood's agar medium was poured in a sterile Petri-plate and allowed to solidify at room temperature. It was then inoculated with the wild-type (1227) strain of *T. vulgaris* and incubated for 24 h at 50^{o}C. The formation of a halo zone surrounding the colony was considered to be a positive test for protease production by this obligate thermophile.

The quantitative analysis of protease activity in the wild-type strain of *T. vulgaris* was done by the method of Folin and Ciocalteu [8]. The enzyme sample (1 ml) was mixed with 2 ml of 2% casein solution, prepared in 50 mM potassium phosphate buffer, and incubated at optimal pH and temperature for 30 min. Trichloroacetic acid (TCA) reagent (3 ml of 20% TCA) was added and kept for 10 min. It was then centrifuged at 10,000g for 15 min, and the supernatant (1 ml) was used as enzyme sample (test filtrate). Sodium carbonate solution (2.5 ml of 0.5 M)

was added to the mixture. Folin and Ciocalteu's phenol reagent (0.5 ml of 5 times diluted solution) was added for the development of colour and the absorbance read at 660 nm in a spectrophotometer (UV-Pharma Spec-1700). The enzymes assays for the specific activities of protease were done as per the method of Sellami-Kamoun et al. [31], using casein as the substrate.

Effect of pH on protease activity

In order to standardize the pH optimum of *T. vulgaris* protease, the enzyme assays were done at varying pH of the reaction mixture (i.e., at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 in 50 mM potassium phosphate buffer). The reaction mixture contained 2% casein (substrate) solution under optimal assay conditions.

Effect of metal ions on protease activity

The effect of different metal ions on protease activity was determined by the addition of the corresponding ions, each at a final concentration of 10 mM in the reaction mixture, and the assays were performed under standard conditions. The tested metal ions, included in the reaction mixtures, were the chloride salts of the following: Ba^{2+}, Ca^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}.

Effect of temperature on protease activity

To study the effect of temperature on protease activity of *T. vulgaris*, enzyme assays were done at varying temperatures (i.e., at 30, 40, 50, 55, 60, 65, 70, 75 and 80^{o}C) in the absence as well as in the presence (10 mM) of stimulatory divalent cation (Mn^{2+}).

Determination of arrhenius energies of activation of protease

The energy of activation (E_A) for the enzyme-catalyzed reaction of protease was calculated by using Arrhenius equation, as described by West et al. [41], which is given as follows:

$$E_A = 2.303(\log_{10}k_2 - \log_{10}k_1)\ R/\ (1/T_1 - 1/T_2)$$

Where T_1 and T_2 are the absolute temperatures, k_1 and k_2, the velocity constants (specific activities) of the enzymes at absolute temperatures, T_1 and T_2 respectively, R is the gas constant **(**1.987 cal/deg**)**, and E_A is expressed in terms of kilojoules per mole (1 kcal = 4.18 kJ).

E_A values for protease were determined, using the above equation. Arrhenius plots were drawn by plotting $\log_{10}$ specific activities of the enzyme, assayed in the absence as well as in the presence (10 mM) of Mn^{2+} against the reciprocals of absolute temperatures (1/T), which were equivalent to the temperatures starting from 50^{o}C of optimal growth temperature of *T. vulgaris* [i.e., 50^{o}C or (50 + 273) = 323 K] up to the temperature optimum of protease [i.e., 65^{o}C or (65 + 273) = 338 K].

Effect of substrate concentration on protease activity

To observe the effect of substrate concentration on the protease activity of *T. vulgaris*, different concentrations of casein (0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) were incubated with enzyme sample for 30 min in the absence as well as in the presence (10 mM) of Mn^{2+} at optimal pH (7.5) and temperature (65^{o}C), and their specific activities were determined.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

The values of 1/V vs 1/[S] were plotted for finding out the K_m and V_{max} of protease, where V stands for specific activity, [S] for substrate concentration, K_m for Michaelis constant and V_{max} stands for maximum velocity of the enzyme.

Effect of metal chelating compound (EDTA) on protease activity

In order to study the metal ion specificity of protease, the effect of different concentrations of a metal chelating compound, ethylene-diaminetetraacetic acid (EDTA) on its specific activity was investigated by incubating the enzyme sample without and with 5 mM and 10 mM EDTA in the absence as well as in the presence (5 mM and 10 mM) of Mn^{2+} in the reaction mixture. The enzyme activities were determined under standard assay conditions.

Results and Discussion

Screening for protease producing ability of the wild-type strain (Stock no. 1227) of *T. vulgaris* was done in the semi-solid medium, containing casein as a substrate and skim milk as an inducer, based on the zone of hydrolysis as per the method described by Rojas et al. [28] for protease production by *Eladia sacculum*. The appearance of a clear halo zone around the colony of *T. vulgaris* indicated the presence of protease activity (Fig. 1). The protease production by a thermophilic bacterium *Bacillus licheniformis* RP1 has been reported earlier by Sellami-Kamoun et al. [31].

The observations on the effect of pH on protease indicated that its specific activity increased with increasing pH up to 7.5, followed by its decrease at further higher pH values (Fig. 2). However, a couple of other bacterial species, *Bacillus cereus* KCTC3674 [16] and thermophilic *Bacillus* SMIA2 [19] showed an optimum pH of 8.0 which was slightly higher than *T. vulgaris* protease. Still higher pH optima have been observed in *Bacillus* sp. JB-99 (pH 11.0) [13], *B. licheniformis* RP1 (pH 10.0–11.0) [31] and *B. cereus* (pH 10.0–10.5) [3]. The data suggested that, similar to the proteases from *Bacillus subtilis* ITBCCB148 [42], *Bacillus* sp. HS08 [12] and *Bacillus* sp. S17110 [15], *T. vulgaris* protease is a neutral protease, and thus, it can be exploited for diverse industrial applications, as most industrialized enzymatic processes are carried out at or near neutral pH.

For studying the effect of metal ions on protease activity, enzyme assays were carried out in the absence (control) as well as in the presence (10 mM) of different metal ions in the form of their respective chlorides. Mn^{2+} enhanced the specific activity of protease of *T. vulgaris* by about 443% (i.e., 4.4-fold over the control) (Fig. 3). However, the addition of Ca^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+} and Zn^{2+} in the reaction mixture increased the specific activity by about 15%, 57%, 7%, 20% and 57%, respectively. Ba^{2+}, Co^{2+} and Hg^{2+} decreased the specific activity of protease by about 8%, 11% and 15%, respectively (Table 1). The other metal ions tested had little or no effect on protease activity. These observations suggested that *T. vulgaris* protease is a metalloenzyme, requiring Mn^{2+} for its maximal stimulation. Similarly, Mn^{2+}-dependent activation of proteases was observed by Manachini et al. [18] and Rahman et al. [24] in *Bacillus* sp. and by Johnvesly et al. [13] in *Bacillus* sp. JB-99. The present findings also showed that Cu^{2+} and Zn^{2+} increased the protease activity in *T. vulgaris* by 57%, which was similar to that observed in a hyperthermophilic bacterium, *Bacillus* strain HUTBS71 [2]. In *Streptomyces megasporus* SDP4, the presence of Cu^{2+} in the assay mixture,

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

enhanced the protease activity, which was similar to the present findings. Although, Ca^{2+}, which was found to be slightly inhibitory to *S. megasporus* protease [21], increased the specific activity of *T. vulgaris* protease by 15%. A positive regulation of the protease with addition of Ca^{2+} has already been reported in *Bacillus* sp., in which the enzyme required Ca^{2+} for its increased catalytic activity that might be attributed to involvement of this divalent cation in stabilization of its molecular structure [30].

Table 1. Effect of different metal ions on the specific activities and relative activities of protease of *T. vulgaris*.

Metal Ions (10 mM)	Specific Activity (U/mg protein)	Relative Activity (%)
Control (0 mM)	21.46 ± 1.6	100
Ba^{2+}	19.81 ± 0.4	092
Ca^{2+}	24.83 ± 1.1	115
Co^{2+}	19.20 ± 1.3	089
Cr^{3+}	21.39 ± 1.0	099
Cu^{2+}	33.83 ± 1.4	157
Hg^{2+}	18.45 ± 1.0	085
Li^{+}	21.17 ± 1.3	098
Mg^{2+}	23.05 ± 0.1	107
Mn^{2+}	95.15 ± 4.8	443
Ni^{2+}	25.85 ± 0.7	120
Zn^{2+}	33.76 ± 5.8	157

The results presented in Figure 4 indicated that, in the absence of stimulatory divalent cation (Mn^{2+}), the temperature optimum of protease was found to be 60^{o}C; whereas, in the presence of this divalent cation, its activity was almost the same both at 60^{o}C and 65^{o}C. These observations suggested that, in native state, the extracellular protease of *T. vulgaris* was highly thermophilic, exhibiting its temperature optimum of 60^{o}C, which was much higher than the growth temperature (50^{o}C) of this obligate thermophile. However, Mn^{2+} broadened the temperature optimum to 60-65^{o}C, suggesting that this divalent cation provides an added adaptive advantage to the enzyme to function in a broad range of temperatures, required for enzyme-catalyzed reactions under diverse industrial operations. Similarly, the protease of *Bacillus* sp. JB-99 was also thermophilic, exhibiting its temperature optimum of 70^{o}C in the absence of its stimulatory divalent cation (Ca^{2+}) and 80^{o}C in the presence (10 mM) of Ca^{2+} [14]. The extracellular thermophilic proteases have also been reported in other thermophilic bacteria, such as *B. mojavensis* [1, 23] and *Bacillus* HS08 [12], which exhibited their temperature optima of 60^{o}C and 65^{o}C, respectively. However, the temperature optima of other thermophilic proteases were still higher, ranging from 70^{o}C, as in case of *B. circulans* [26] to 85^{o}C, as in case of *B. stearothermophilus* sp. [27] and *Geobacillus* sp. YMTC1049 [44].

The Arrhenius plot analysis of the data indicated that the plots were of a linear nature, and Mn^{2+} increased the slope of the plot of *T. vulgaris* protease. Mn^{2+} increased the Arrhenius

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

energy of activation (E_A) from 2.16 kJ/mol (for native enzyme) to 3.22 kJ/mol in presence (10 mM) of this divalent cation (Fig. 5). These observations suggested that Mn^{2+} enhanced the protease activity at the expense of E_A for its high-temperature catalysis.

In order to study the kinetic properties of *T. vulgaris* protease, the effect of varying concentrations (0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) of the substrate (casein) on the specific activity of protease was studied in the absence as well as in the presence (10 mM) of Mn^{2+}. It was found that, as the concentration of the substrate increased up to 1.5%, the specific activity increased gradually in a dose-dependent manner, followed by uniform activity up to 2.0% and then increase again up to 3.0% substrate concentration (Fig. 6). The K_m and V_{max} of the enzyme, in its native form, were found to be 0.27% and 25.3 U/mg protein, respectively (Fig. 7). In the presence of Mn^{2+}, the specific activity of protease increased with increasing concentration of the substrate from 0.5% to 3.0%, in a dose-dependent manner, with maximum activity (94.95 U/mg protein) achieved at 3.0% substrate concentration (Fig. 6), and the K_m and V_{max} of the enzyme were found to be 0.28% and 104.16 U/mg protein, respectively (Fig. 8). The data, indicated that the Mn^{2+} increased the catalytic activity (V_{max}) of *T. vulgaris* protease drastically from 25.3 to 104.16 with almost no change in the K_m for the substrate (i.e., from 0.27% to 0.28% only). Thus, the stimulatory divalent cation (Mn^{2+}) enhanced the rate of high-temperature catalysis (V_{max}) of protease in *T. vulgaris* without affecting K_m for the substrate.

The specific activity of protease increased with increasing concentrations of Mn^{2+}, the activity being 23.97 in its absence (as control), and 89.57 U/mg protein and 99.71 U/mg protein in the presence of 5 mM and 10 mM of this divalent cation, respectively (Table 2). A metal chelating compound (EDTA) decreased protease activity from 23.97 (in native enzyme preparation) to 16.23 U/mg protein and 8.6 U/mg protein at its 5 mM and 10 mM concentrations, respectively (Table 2).

Table 2. Effect of varying concentrations of the metal chelating compound (EDTA) on the specific activity of protease of *T. vulgaris* in the absence as well as in the presence (5 mM and 10 mM) of the activatory divalent cation (Mn^{2+}).

Mn^{2+}	Without EDTA	With EDTA	
		5 mM EDTA	10 mM EDTA
0 mM	23.97 ± 0.3	16.33 ± 0.1	08.60 ± 0.8
5 mM	89.57 ± 1.8	49.70 ± 0.2	31.38 ± 1.0
10 mM	99.71 ± 3.8	80.95 ± 1.1	67.08 ± 0.5

Thus, *T. vulgaris* protease exhibited a high degree of metal ion specificity, as Mn^{2+} recovered the activity of this enzyme from its EDTA-dependent inhibition. A concentration of 10 mM of Mn^{2+} was able to recover about 81% protease activity at 5 mM EDTA and 67% activity at 10 mM EDTA (Table 2). These observations indicated that *T. vulgaris* thermophilic protease appears to be a metalloenzyme (a metalloprotease), in which Mn^{2+} is firmly bound to the enzyme molecule, so that even 10 mM EDTA (a metal chelating compound) was not able to chelate or remove it and to inactivate it completely, suggesting

thereby that the cation-dependant thermo-catalysis of enzyme could be an added advantage to the adaptive evolution of *T. vulgaris* so as to enable it to thrive best at high temperature.

Acknowledgments We are thankful to Professor Sir D.A. Hopwood, FRS, John Innes Centre, Norwich, U.K. for providing the wild-type and mutant strains of *Thermoactinomyces vulgaris*. The financial support by the Council of Scientific and Industrial Research, New Delhi and University of Delhi under Doctoral Research Programme is gratefully acknowledged.

References

1. Adinarayana K, Ellaiah P and Prasad DS (2003) Purification and characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. Pharm Sci Technol 4:1–9
2. Akel H, Al-Quadan F and Yousef TK (2009) Characterization of a purified thermostable protease from hyperthermophilic *Bacillus* strain HUTBS71. Eur J Sci Res 31:280–288
3. Banik RM and Prakash M (2004) Laundary detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol Res 159:135–140
4. Bhatnagar K and Singh VP (2003) Ca^{2+}-dependence and inhibition of transformation by trifluoperazine and chlorpromazine in *Thermoactinomyces vulgaris*. Curr Microbiol 46:265–269
5. Bhatnagar K and Singh VP (2004) Ca^{2+}-dependence and inhibitory effects of trifluoperazine on plasma membrane ATPase of *Thermoactinomyces vulgaris*. Curr Microbiol 49:28–31
6. Cowan D (1996) Industrial enzyme technology. Trends Biotechnol 14:177–178
7. Cowan D, Danial R and Morgan H (1985) Thermophilic protease: properties and potential applications. Trends Biotechnol 3:68–72
8. Folin O and Ciocalteu V (1929) On tyrosine and tryptophane determinations in proteins. J Biol Chem 73:627–627
9. Gupta R, Beg QK and Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:13–32
10. Gusek T and Kinsella E (1988) Properties and potential applications of a unique heat-stable protease. Food Technol 49:102–107
11. Hopwood DA and Wright HM (1972) Transformation in *Thermoactinomyces vulgaris*. J Gen Microbiol 71:383–393
12. Huang G, Ying T, Huo P and Jiang YZ (2006) Purification and characterization of a protease from thermophilic *Bacillus* strain HS08. Afr J Biotechnol 5:2433–2438
13. Johnvesly B and Naik GR (2001) Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. Process Biochem 37:139–144
14. Johnvesly B, Manjunath BR and Naik GR (2002) Pigeon pea waste as a novel, inexpensive, substrate for production of a thermostable alkaline protesase from thermoalkalophilic *Bacillus* sp. JB-99. Biores Technol 82:61–64

15. Jung SC, Paik H, Kim MS, Baik KS, Lee W, Seong CN and Choi SK (2007) InhA-like protease secreted by *Bacillus* sp. S17110 inhibited in turban shell. J Microbiol 45:402–408
16. Kim SS, Kim YJ and Rhee I (2001) Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC3674. Arch Microbiol 175:458–461
17. Klingeberg M, Hashwa F and Antranikian G (1991) Properties of extremely thermostable proteases from anaerobic hyperthermophilic bacteria. Appl Microbiol Biotechnol 34:715–719
18. Manachini PL, Fortina MS and Parini C (1998) Thermostable alkaline protease produced by *Bacillus thermorubber*: a new species of *Bacillus*. Appl Microbiol Biotechnol 28: 409–413
19. Nascimento WC and Martins ML (2004) Production and properties of an extracellular protease from thermophilic *Bacillus* sp. SMIA2. Braz J Microbiol 35:91–96
20. Olga LT (2013) Protein hydrolysis using proteases: An important tool for food biotechnology. Journal Mol Cata B: Enzymatic 90:1-11
21. Patke D and Dey S (1988) Proteolytic activity from a thermophilic *Streptomyces megasporus* strain SDP4. Lett Appl Microbiol 26**:**171–174
22. Priest FG (1977) Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol Rev 41:711–753
23. Qasim KB and Rani G (2003) Purification and characterization of an oxidation stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enzyme Microbiol Technol 32:294–304
24. Rahman RNZA, Razak CN, Ampon K, Basri M, Yunus WMZW and Salleh AB (1994) Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1. Appl Microbiol Biotechnol 40:822–827
25. Rao MB, Tanksale AM, Ghatge MS and Deshpande VV (1998) Molecular and Biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635
26. Rao S, Sathish T, Ravichandra P and Prakasham RS (2009) Characterization of thermo and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. Process Biochem 44:262–268
27. Razak C, Rahman R, Salleh A, Yunus W, Ampon K and Basri M (1995) Production of a thermostable protease from a new high pH isolate of *Bacillus stearothermophilus*. J Biosci 6:94–100
28. Rojas JA, Cruz C, Mikan JF, Villalba LS, Caridad M, Garcia C and Restrepo S (2009) Isoenzyme characterization of proteases and amylases and partial purification of proteases from filamentous fungi causing biodeterioration of industrial paper. Int Biod Biodegrad 63:169–175
29. Saggu SK and Mishra PC (2017) Characterization of thermostable alkaline proteases from *Bacillus infantis* SKS1 isolated from garden soil. PLOS ONE 1-18
30. Sana B, Ghosh D, Saha M and Mukherjee J (2006) Purification and characterization of a salt, solvent, detergent and bleach tolerance protease from a new gamma-Proteobacterium isolated from the marine environment of the Sundarbans. Process Biochem 41:208–215

31. Sellami-Kamoun A, Haddar A, Ali NE, Ghorbel-Frikha B, Kanoun S and Nasri M (2008) Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. Microbiol Res 163:299–306
32. Singh VP (1980) Investigations on the phosphatases of *Thermoactinomyces vulgaris*. Ph.D. Thesis, University of Delhi, India
33. Singh VP (1985) *In vivo* effects of Ca^{2+} on ATPase of an obligate thermophile – *Thermoactinomyces vulgaris* under shake culture conditions. Symp Biologica Hungarica 32:399–401
34. Singh VP (1987) Effects of -SH blocking and -SH protecting agents on genetic transformation in *Thermoactinomyces vulgaris*. Acta Bot Ind 15:62–64
35. Singh VP (2007) Mg^{2+} decreases arrhenius energies of activation for high temperature catalysis of phosphatases in *Thermoactinomyces vulgaris*. Curr Microbiol 55:179–184
36. Singh VP and Sinha U (1982) Ca^{2+} dependence and metabolic status of an obligate thermophile - *Thermoactinomyces vulgaris* under shake culture conditions. Experientia (Cellular and Molecular Life Sciences) 38:670–671
37. Singh VP and Sinha U (1982) Thermostability and turnover of phosphatases in an obligate thermophile - *Thermoactinomyces vulgaris*. Ind J Exp Biol 20:26–30
38. Sinha U and Singh VP (1980) Phosphate utilisation and constitutive synthesis of phosphatases in *Thermoactinomyces vulgaris* Tsiklinsky. Biochem J 190:457–460
39. Sinha U, Singh VP and Srivastava S (1981) Cation dependent activity and stability of phosphatases of *Thermoactinomyces vulgaris*. Ind J Exp Biol 19:453–457
40. Snajder M et al., (2019) Extracellular production of the engineered thermostable protease pernisine from Aeropyrum pernix K1 in Streptomyces rimosus Microbial Cell Factories 18:196
41. West ES, Todd WR, Mason HS and Bruggen JTV (1966) Textbook of biochemistry, Arnold, New Delhi
42. Yandri TS, Dian H and Sutopo H (2008) The chemical modification of protease enzyme isolated from local bacteria isolate, *Bacillus subtilis* ITBCCB148 with cyanuric chloride polyethylenglycol. Eur J Sci Res 23:177–186
43. Zabolotskaya MV, Demidyuk IV, Akimkina TV and Kostrov SV (2004) A novel neutral protease from *Thermoactinomyces* species 27a: sequencing of the gene, purification and characterization of the enzyme. Protein J 23:483–492
44. Zhu W, Cha D, Cheng G, Peng Q and Shen P (2007) Purification and characterization of a thermostable protease from a newly isolated *Geobacillus* sp. YMTC1049. Enzyme Microb Technol 40:1592–1597

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

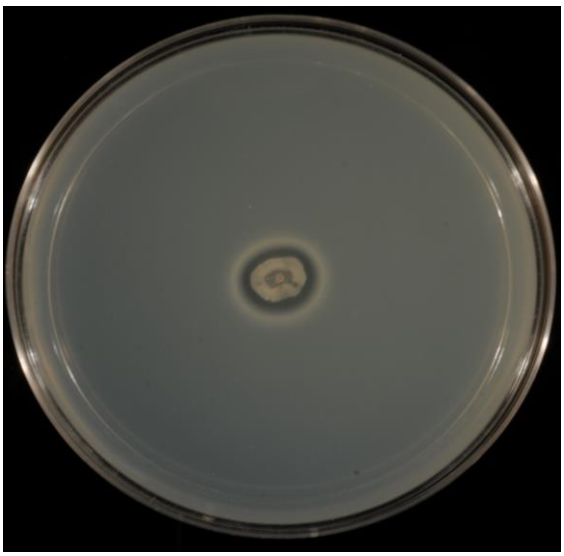

Fig. 1 Culture plates showing zone of hydrolysis of protease produced by the wild-type strain 1227 of *T. vulgaris* after 24 h of growth at 50°C, using casein as the substrate and skimmed milk as inducer.

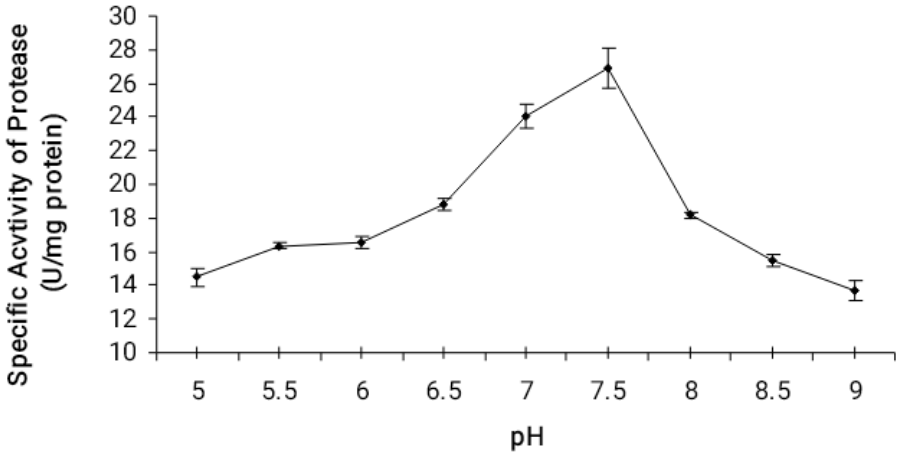

Fig. 2 Effect of pH on protease activity of wild-type strain (1227) of *T. vulgaris*.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

Fig. 3 Effect of metal ions on protease activity of wild-type strain (1227) of *T. vulgaris*.

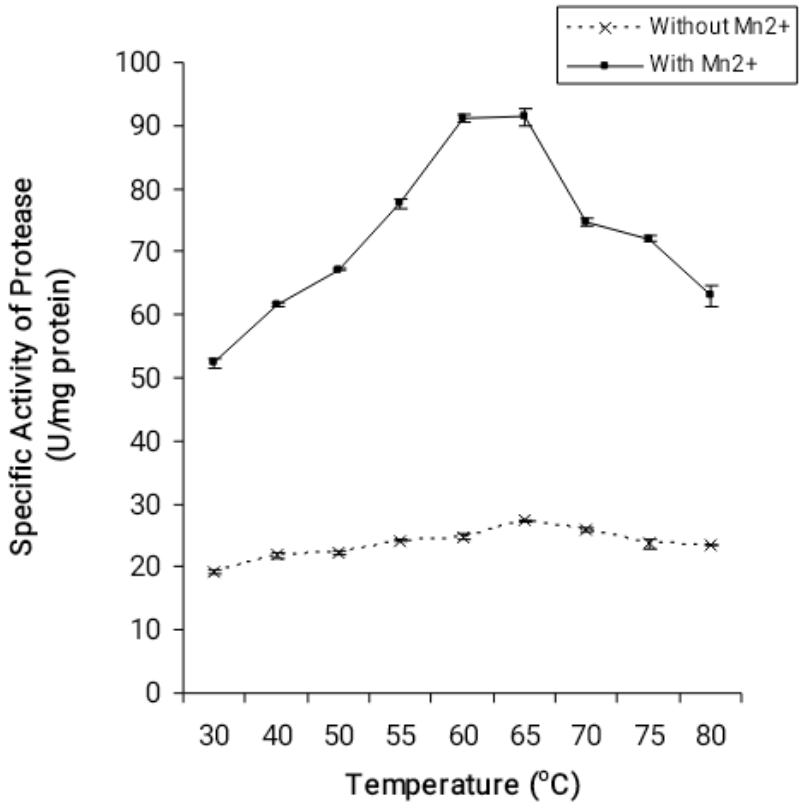

Fig. 4 Effect of temperature on specific activity of protease of wild-type strain (1227) of *T. vulgaris* in the absence and presence (10 mM) of Mn^{2+}.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

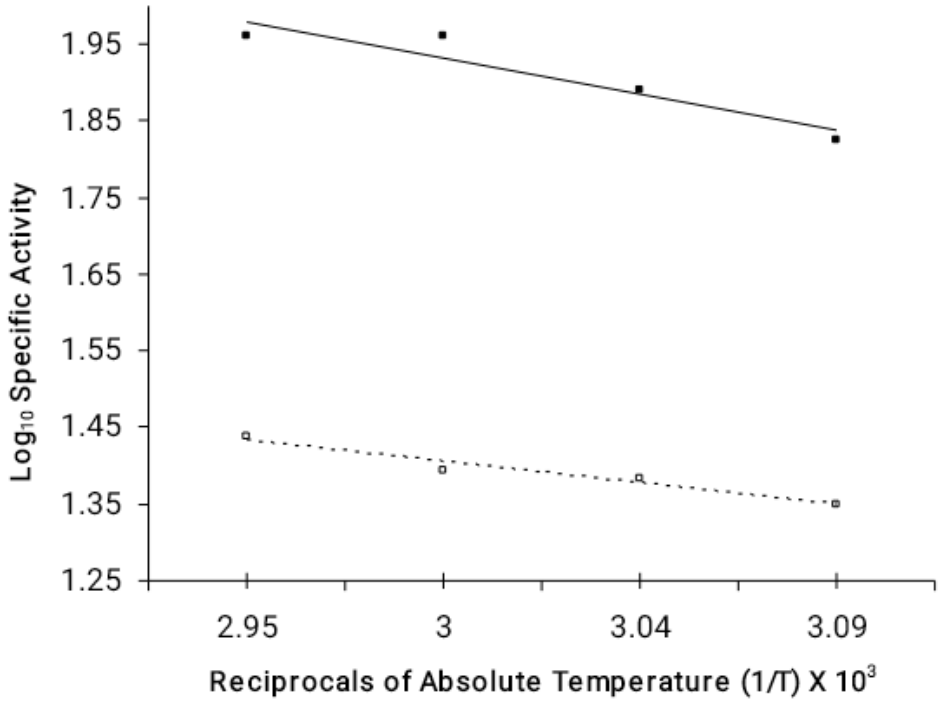

Fig. 5 Arrhenius plots drawn for log10 specific activities of protease of wild-type strain (1227) of *T. vulgaris* in the absence and presence (10 mM) of Mn^{2+} against reciprocals of absolute temperature.

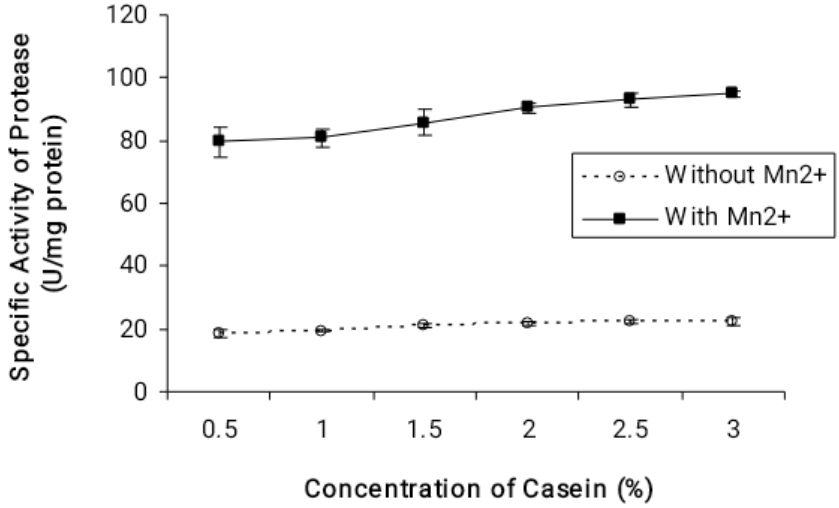

Fig. 6 Effect of substrate concentration on specific activity of protease of wild-type strain (1227) of *T. vulgaris* in the absence and presence (10 mM) of Mn^{2+}.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 2101 - 2113
Received 20 October 2020; Accepted 04 December 2020

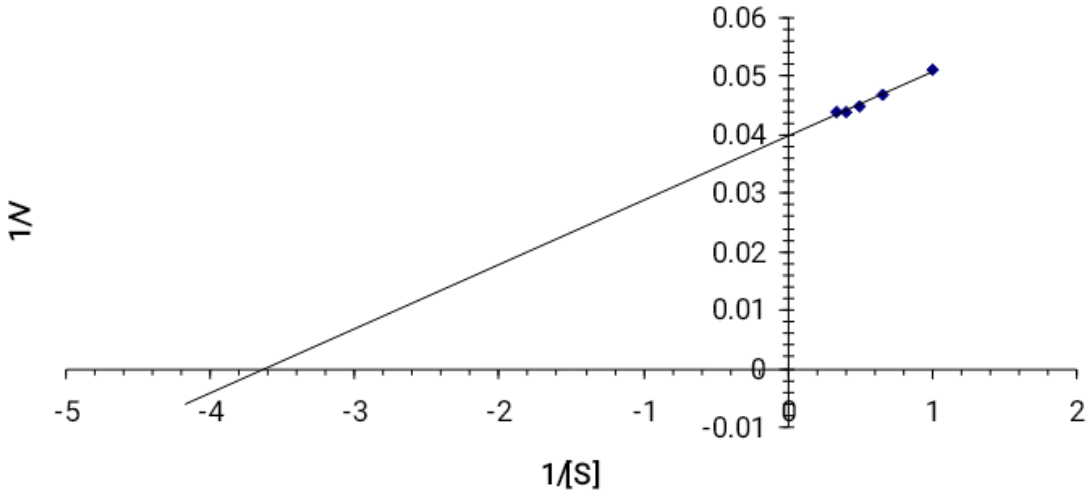

Fig. 7 Plot of 1/V (reciprocal of specific activities of protease) against 1/[S] (reciprocal of substrate concentration) in the absence of Mn^{2+}.

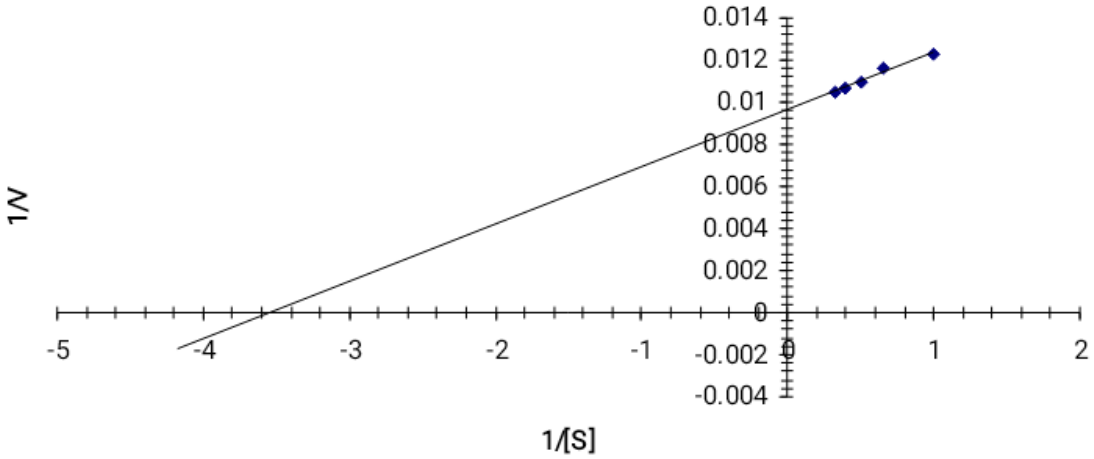

Fig. 8 Plot of 1/V (reciprocal of specific activities of protease) against 1/[S] (reciprocal of substrate concentration) in the presence (10 mM) of Mn^{2+}.