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Purification, Characterization and Thermodynamic Assessment of an Alkaline Protease by *Geotrichum Candidum* of Dairy Origin

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Abstract

Background: Alkaline proteases is the important group of enzymes having numerous industrial applications including dairy food formulations.

Objectives: The current study deals with the purification and characterization of an alkaline serine protease produced by *Geotrichum candidum* QAUGC01, isolated from indigenous fermented milk product, Dahi.

Material and Methods: In total twelve *G. candidum* strains were screened for their proteolytic activity by using standard protease assay. The protease production from *G. candidum* QAUGC01 was optimized by varying physio-chemical conditions. The protease was purified by using two-step method: ammonium sulfate precipitation and gel filtration chromatography. Protease was further characterized by studying various parameter like temperature, pH, modulators, metal ions and organic solvent. A thermodynamic study was also carried out to explore the half-life of protease.

Results: The *G. candidum* grew profusely at 25 °C and at an initial pH of 4.0 for 72 h of incubation producing 26.21 U/ml maximum extracellular protease. Protease revealed that V_{max} and K_m was 26.25 U.ml⁻¹.min⁻¹ and 0.05 mg.mL⁻¹, respectively using casein as substrate. The enzyme was stable at a temperature range (25-45 °C) and pH (8-9). Residual enzyme activity was strongly inhibited in the presence of PMSF (7.5%). The protease could hydrolyze proteinaceous substrates, casein (98%) and BSA (95%). The thermodynamic studies explored that the half-life of the enzyme that was 106.62 min, 38.72 min and 15.71 min at 50, 60 and 70 °C, respectively.

Conclusions: Purified protease from *G. candidum* GCQAU01 is an ideal candidate for industrial application.

Keywords: *Geotrichum candidum*; Alkaline Serine Protease, Kinetics; Thermodynamics

1. Background

Nearly two-third of commercial proteases are produced by fungal species, yeasts and bacteria (1). Proteases (EC 3.4.21) are the significant class of industrially produced enzymes, which constitute around 60% of the total world enzyme production (2). Proteases are exploited in the food, dairy, leather, detergents and brewing industries (3). Proteases also have health and medical applications for instance: tissue inflammation reduction and burns treatment (4).

Thousands of proteases have been isolated from plants, animal tissues and microorganisms. Among these sources, microorganisms especially, bacteria and fungi are preferred for their high enzyme production (5).

Alkaline proteases constitute 60–65% of the global industrial market. Alkaline serine proteases of microbial origin possess considerable industrial potential (6). Bacterial protease production requires cost intensive processes for the separation of enzymes from cells. To satisfy the escalating industrial need for potent enzymes, it is mandatory to search for novel enzymes with desired properties. This was the aim of the present work to use the well-known fungus *Geotrichum Candidum*, which is a dominant yeast in fermented dairy products to meet the industrial demands (7).

The *G. candidum* is widely distributed in dairy environment and naturally appears in raw milk, brines

and equipment surface, and it was recently found in fermented milk product Dahi (8, 9). The *G. candidum* ranks high amongst the rapidly growing fungus and it is on the borderline between mold and yeasts. It has been classified as a filamentous yeast-like fungi. Microscopic examination of *G. candidum* showed arthroconidia formed by hyphal fragmentation and with cream-colored, yeast-like colonies. This uniqueness suggests that it belongs to imperfect fungi group (10). Safety assessment of *G. candidum* was reported (11) and technological beneficial use was recognized in "2002 IDF inventory" (12). Moreover, its genomic data was recently reported (13).

The industrial production of protease from *G. candidum* has not been exploited to a large extent (14). Presence of *G. candidum* extracellular proteases have been reported four decades ago (15) but no other study has followed since then.

2. Objective

The objective of the present study is the extracellular production of proteases by a proteolytic strain of *G. candidum* isolated from Dahi. Furthermore, characterization and assessment of thermodynamic properties of protease was also evaluated in laboratory conditions.

3. Materials and Methods

3.1. Materials

All the chemicals and reagents were commercial products of analytical or higher grade unless mentioned otherwise.

3.2. Yeast strains and Culture Conditions

G. candidum strains were isolated from indigenous fermented milk product (Dahi) by plating at 25 °C on Oxytetracycline glucose agar (OGA) prepared by using 20g/L glucose and yeast extract 5 g.L⁻¹, oxytetracycline 0.10 g.L⁻¹ and 15g.L⁻¹ agar pH 6.6 ± 0.2. The culture was stored in the form of slant at 4°C. Pre-culturing of microbial isolates was done by transferring one colony from 5 days old OG agar slant of *G. candidum* having rich growth to 5 ml of OG broth medium. After incubation at 25 °C for 48 h under 200 rpm shaking, culturing was done by transferring 1 ml of pre-culture (5 ± 1x10⁸ cells per mL) to 100 mL of the same broth

All isolated strains were selected for proteolytic activity based on appearance of clear hydrolysis zones on the casein agar plate (Nutrient agar with 1% casein) around the colonies. Microbial colonies that displayed the largest clearance zones, were purified and protease activity was assessed.

Three reference strains *G. candidum* UCMA 91 (ATCC 204307), *G. candidum* UCMA 103, *G. candidum* UCMA 322 were also used in the present study and which were obtained from Université de Caen, Normandy, France.

3.3. Protease Assay

The standard protease assay was used with casein as a substrate with minor modification to determine proteolytic activity (16). One International Unit (IU) of enzyme activity was expressed as the fraction of enzyme which produces one μmol of tyrosine/ml/min. The protein concentration was measured by using BSA as a standard at 650 nm.

3.4. Media for Protease Production

Effect of media composition on the production of protease was studied by cultivating the organism in five different growth broth media:

Maltose based medium (MPY; adapted from (17)) (g.L⁻¹): Maltose 10.0; Peptone 2.5; yeast extract, 1.0, Na₂CO₃ 7.5, K₂HPO₄ 1.0.

Gelatin based medium (GCG; adapted from (18)) (g.L⁻¹): gelatin 15 g, casein hydrolysate 5.0, and 3ml of glycerol (20% in water);

Beef extract based medium (BPN; adapted from (19)) (g.L⁻¹): beef extract 1.0; peptone 5.0, Yeast Extract 2.0, NaCl 5.0;

Peptone based medium (PYG; adapted from (20)) (g.L⁻¹): peptone 10.0, yeast extract 0.2, glucose 1.0; K₂HPO₄ 0.5, calcium chloride 0.1, and magnesium sulphate 0.1;

Casein based medium (CGY; adapted from (21)) (g.L⁻¹): glucose 10.0; casein 5.0, yeast extract 5.0; K₂HPO₄ 2.0, KH₂PO₄ 2.0, MgSO₄ 7H₂O 0.1.

The initial pH of these media was adjusted at 5.0 with 1N HCL/NaOH, since it has been reported to be in the range of optimal pH for *G. candidum* growth and proteolysis (22). Each experiment was conducted at 25 °C in a one-liter conical flask, containing 250mL of broth culture media inoculated with standardized inoculum, left under gentle agitation of 200rpm for 96 h. The standard protease assay for the *G. candidum* QAUGC01 was performed while *G. candidum* growth was simultaneously assessed. The growth index of the microorganisms was determined by measuring absorbance of a diluted solution at 600 nm.

3.5. Purification of Protease

All procedures were performed at 4 °C. After 72 h, the culture medium (250ml) was centrifuged at 4,000 rpm for 30min at 4°C (Kokusan Model H-251 centrifuge) and was filtered on filter paper (Whatman No. 1, Sigma Aldrich) to remove biomass. The filtrate was then precipitated with ammonium sulfate at 60% saturation. The precipitates was collected by centrifugation (8000 rpm for 10 min at 4 °C), and mixed in 10ml Tris-HCL buffer (pH 8.5). The crude protein was dialyzed and then subjected to Sephadex G-100 for gel filtration chromatography. In total five mL of protein precipitate suspension was carefully loaded and eluted by using 50 mM Tris-HCL buffer pH 8.5 at a flow rate of 0.5 mL/min. Each 3mL collected fractions were examined both for protease activity and total protein content estimation. The fractions with highest protease activity

were pooled and then lyophilized. The molecular mass was estimated by SDS-PAGE.

3.6. Characterization of Purified Protease

3.6.1 Effect of Substrate Concentration

To assess the effect of different concentrations of substrate (casein) on enzyme activity, the purified enzyme was assayed by standard protein assay with varying substrate concentration from 0.3 to 0.025% (w/v). The Data obtained was plotted in the form of Lineweaver-Burk plot in order to calculate K_m and V_{max} (23).

3.6.2. Effect of Temperature, pH and Inhibitors on Protease Activity

To determine the effect of reaction temperature and pH on protease activity, the protease activity was assayed at temperature ranges from 5 to 65°C and pH 4 to 13. Stability of protease was determined by pre-incubation of the enzyme with a pH range of 4 to 13. The effect of selected inhibitors; PMSF, EDTA, β -mercaptoethanol, ascorbic acid and cysteine, at 10mM concentration was examined by incubating purified enzyme solution and inhibitor for 1 h at 25°C. The relative protease activities were determined under protease assay standard conditions.

3.6.3. Effect of Metal Ions and Organic Solvents on Protease Activity

The effect of various chloride salts of divalent ions (Co^{2+} , Ca^{2+} , Mn^{2+} , Cu^{2+} , Mg^{2+} , Hg^{2+} , Zn^{2+} and Cd^{2+}) of concentration 10mM were added separately in a reaction mixture for 1 h at 25°C. The effect of different selected solvents was checked in separate conical flasks by adding each solvent at 10 mM concentration for 1 h at 25 °C and protease stability was determined by using the standard protease assay.

3.6.4. Effect of Substrate Specificity

The purified protease activity was examined at 1% (w/v) of varied proteinaceous substrates such as BSA, casein, skim milk, azo-casein and gelatin under the standard protease assay conditions.

3.7. Thermodynamics of Enzyme

Thermodynamic parameters related to irreversible denaturation of protease were calculated by incubating the protease in Tris-HCL buffer (pH 8.5) at four different temperatures i.e. 45, 50, 55 and 60 °C. Aliquots were taken out and assayed at different times for enzyme activity. The data were plotted to the first order plots and analysed as described (23). The reaction rate constant for thermal inactivation (k_d) was determined from a logarithmic plot of percent of activity remaining versus time, according to the equation.

$$\ln\% \text{ residual activity} = -k_d t \text{ (I)}$$

To determine the activation energy for thermal inactivation (E_a), an Arrhenius plot was obtained from a plot of $\ln(k_d)$ versus $1/T$ (temperature of incubation).

The line slope was calculated from the following equation:

$$\ln(k_d) = -E_a/RT \text{ (II)}$$

The half-life ($t_{1/2}$) was calculated from the following equations:

$$t_{1/2} = 0.693/k_d \text{ (III)}$$

Thermodynamic parameters like enthalpy (ΔH) free energy (ΔG) and entropy (ΔS) were calculated as follows:

$$\Delta H = E_a - RT \text{ (IV)}$$

$$\Delta G = -RT \ln(k_{cat} h / k_B T) \text{ (V)}$$

$$\Delta S = (\Delta H - \Delta G) / T \text{ (VI)}$$

4. Results

4.1. Screening and Characterization of Proteolytic Activity

Twelve strains of *G. candidum* were isolated from indigenously fermented milk product Dahi, were screened for protease activity along with three reference strains and results are given in (Fig. S1). All strains except QAUGC12 were identified as protease producers at different levels with an effective hydrolysis zone reaching 1 to 15 mm increasing of diameter around the microbial spot loaded.

4.2. Effect of Fermentation Media

Keeping in view the economic concerns involved in the fermentation process, the protease production was experimented with five different media. Beef extract based medium (BPN) gave the maximum yield with a specific activity of 26.21 Umg^{-1} (Fig. S2A). Thus, medium BPN was used for further studies.

4.3. Optimization of Various Cultural Conditions

Four different incubation time periods 24, 48, 72 and 96 h were examined for the production of protease in BPN broth. Maximum protease production (26.21 Umg^{-1}) was recorded after 72 h of incubation (Fig. S2B). A set of experiments were carried out at temperature range 15 °C - 65 °C to optimize the incubation temperature for protease production. A gradual increase in the incubation temperature the protease activity increased and reached to maximum at 25 °C (27.30 Umg^{-1}). Finally, it reduced to minimum at 65 °C (10.0 Umg^{-1}) (Fig. S2C). The effect of pH (3-11), on the production of protease was examined. Protease production was higher in the pH range 3 to 5 with a maximum at pH 4 (that is also maximum growth pH) with specific activity 28.9 Umg^{-1} but it was consistently decreased with an increase in pH which parallels growth behavior evaluated by growth index (Fig. S2D).

4.4. Purification of Protease

Summary of purification procedure of extracellular protease is summarized in Table 1. Protease was purified around 46 fold with specific activity of 34.60 IUmg^{-1} (Table 1). The SDS-PAGE analysis revealed a single band with a molecular weight of 81 kDa (Fig. 1).

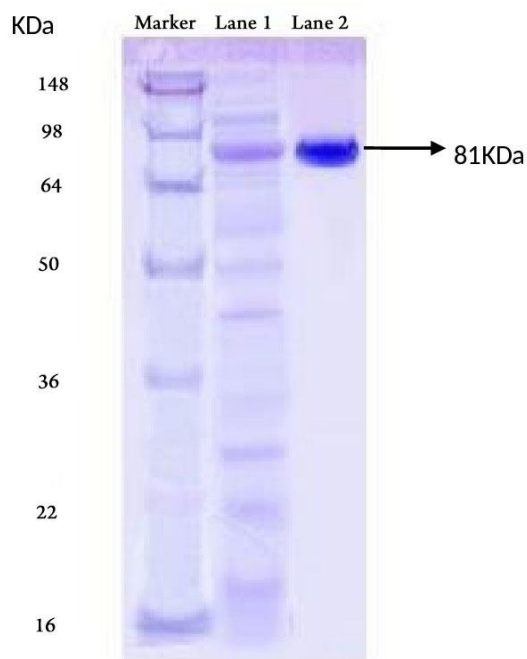


Figure 1. SDS-PAGE analysis of purified protease by *G. candidum* (QAUGC01) Lane 1, Crude enzyme, Lane 2 Purified Protease.

4.5. Characterization of Purified Protease

4.5.1. The effect of substrate concentration, temperature and pH on protease activity

The data on substrate effect on enzyme activity were plotted and kinetic constant were calculated. V_{max} was calculated as 26.25 U/ml/min while K_m was calculated 0.05 mg/ml (Fig. 2). Thermophilicity can accelerate the exploitation of enzyme in biotechnological processes to avoid contamination by common microorganism. Hence, the thermostability of purified enzyme was tested by imposing various temperature regimes (5-75 °C). The relative activity of purified protease at 55 °C was 141.54% (Fig. 3A). Purified protease showed maximum relative activity of 144.33% at pH 12 (Fig. 3B).

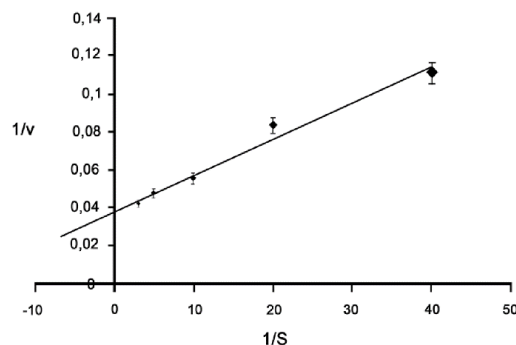


Figure 2. Lineweaver-Burk Plot showing effect of substrate (casein) concentration on protease activity. "V" denotes initial velocity of enzymatic reaction (units are $\mu\text{Mole}/\text{mg}/\text{ml}$) while "S" denotes substrate concentration in percentage

4.5.2. Effect of Inhibitors on Protease Activity

The complete loss of protease activity happened in the presence of inhibitor 10mM PMSF with relative activity of 7.5% (Fig. 3C).

Thus, *G. candidum* protease is categorized as a serine protease. This inhibitor causes covalent and irreversible modifications at the active site residues. β -mercaptoethanol and cysteine did not affect the protease activity.

4.5.3. Effect of Metal Ions and Organic Solvent on Protease Activity

The *G. candidum* QAUGC01 protease is not metal dependent. The effect of various metal ion salts such as Co^{+2} , Ca^{+2} , Mn^{+2} , Cu^{+2} , Mg^{+2} , Hg^{+2} , Cd^{+2} and Zn^{+2} were checked at (10 mM) concentration on purified protease. Calcium (107%), magnesium (96.10%) and manganese (93%) at 10 mM concentration did not affect the protease activity to much significant extent (Fig. 3D).

Protease showed maximum stability in the presence of polar solvents such as methanol, ethanol and butanol with an increasing activity up to 158.7%, 164.64% and 173.10%, respectively (Fig. 3E).

Table 1. Summary of Purification of Protease from *G. candidum* QAUGC01

Purification step	Volum e (mL)	Total Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Culture supernatant	250	431.0	6510	15.10	1.0	100
Ammonium sulfate precipitation	15	38.5	4673	121.30	8.03	71.74
Sephadex G-100	3	4.35	3012	692.0	45.83	46.27

Table 2. Thermodynamics Parameters for Denaturation of Protease

Temperature (°C)	Temperature (K)	K_d (min^{-1})	Half-life (min)	Enthalpy (kJ mol^{-1})	Free energy (kJ mol^{-1})	Entropy ($\text{Jmol}^{-1} \text{K}^{-1}$)
45	318.15	0.0049	141.43	5.21	89.87	-266.10
50	323.15	0.0065	106.62	5.16	91.29	-266.53
55	328.15	0.0122	56.80	5.12	92.65	-266.73
60	333.15	0.0179	38.72	5.08	94.11	-267.24
65	338.15	0.0258	26.86	5.04	95.47	-267.42
70	343.15	0.0441	15.71	5.00	96.88	-267.75

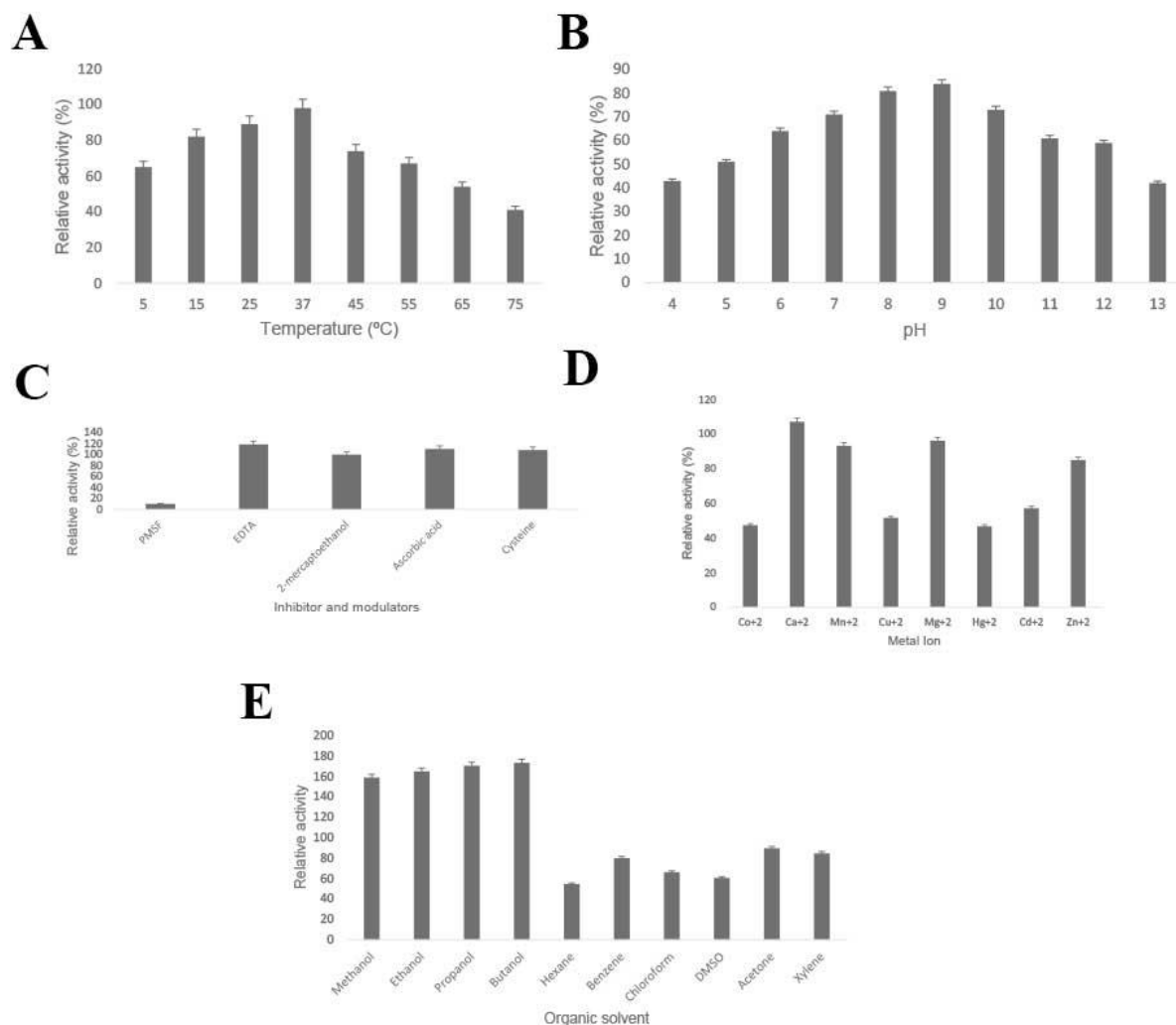


Figure 3. (A) Effect of temperature (B) pH (C) inhibitors (D) metal ion (E) organic solvent (F) substrate specificity, on the stability and activity of protease without any treatment was taken as 100%. Y error bars indicates the standard deviation among the three replicates which differ significantly at < 0.05

4.5.4. Effect of Substrate SPECIFICITY

Broad range of substrate specificity of the protease was observed in the current study. Among different substrates tested protease showed a better hydrolyzing ability against casein and skim milk (98% and 95% of relative activity) followed by gelatin, BSA and casein with 89%, 62% and 35% relative activity, respectively (Fig. 3F).

4.6. Thermodynamics of Protease Denaturation

The resistance of enzymes against temperature is an important criterion for its commercial significance. Purified protease was incubated at 50, 55, 60, 65 and 70°C. The data obtained from the experiments were plotted as shown in Figure 4.

The values of K_d were 0.0122, 0.0258, 0.0441 min^{-1} while the half-life calculated were 56.8 min, 26.8 min and 15.7 min at 55, 65 and 70°C respectively (Table 2). The values of enthalpy, free energy and enthalpy for denaturation of protease are presented in (Table 2).

Moreover less values of entropy shows that the denaturation of protease is a favorable process.

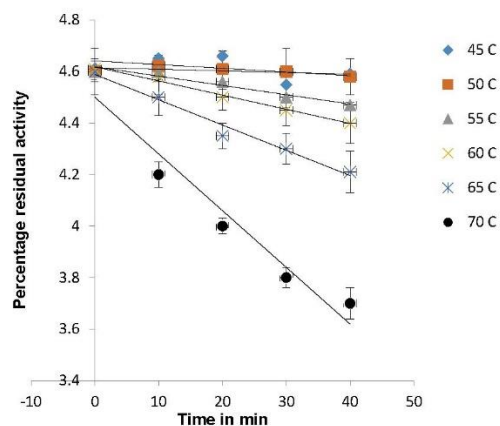


Figure 4. Pseudo-first-order plots for irreversible thermal denaturation of protease at 45-70 °C. The slope of each graph corresponds to the k_d (thermal inactivation constant) for the respective temperature

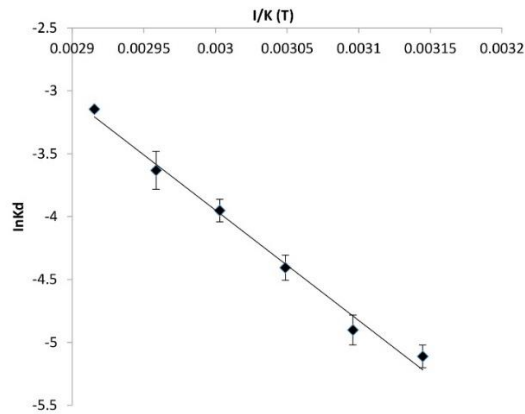


Figure 5. Arrhenius plot to calculate activation energy E_a for irreversible thermal inactivation of protease. k_d denotes denaturation constant while T represents temperature in Kelvin. $E_a = -\text{slope} \times R$, while R is Gas Constant (8.314 J/K/mol).

5. Discussion

The present study deals with the evaluation of proteolytic activity of *G. candidum* based on the clear hydrolysis zone formation. The protease activity was found to be strain dependent and this was confirmed by quantifying by standard protease assay. The *G. candidum* QAUGC01 (NCBI accession No. KT280407) showed highest activity and it was used for further studies. Various conditions were considered and maximum specific activity was obtained at 25 °C at an initial pH of 4.0 for 72 h of incubation producing 26.21 U.mL⁻¹ maximum extracellular protease. Prolonged incubation and static condition showed reduction in the growth and protease production. The longer period of incubation results in the formation of inhibitory substances and/or non-availability of nutrients and this causes a decline in the formation of enzymes. Our results were also corroborated with an alkaline protease from *Bacillus* sp (24).

Temperature affects the protease synthesis non-specifically by altering the biochemical reaction rate specifically through inducing or repressing enzyme production. Temperature is an important parameter that controls biochemical pathways (25). In the case of extracellular enzymes, the temperature allows altering of structural properties of plasma membrane. The *G. candidum* can be cultivated in the temperature range from 5 °C to 38 °C, with an optimal production around 25 °C (26). Temperature not only affect protease enzyme production but also growth rate of *G. candidum*. Medium pH is also important for cellular transport of protein through the membrane bilayer (27, 28). *G. candidum* can grow in broad pH range (3 to 11), best at pH below 4.4 and however, few strains can grow even at pH 3.4 which makes this organism suitable for industrial use (22).

The present study indicated that protease was stable at wide range of elevated temperature and pH with maximum range (25-45 °C) and pH (8-9). The results

were in accordance with the earlier report for *Bacillus* sp (29). It was reported earlier that an alkaline protease from marine yeast *Aureobasidium pullulans* was stable in the range of pH 4.0 and 12.0 (30). The alkalophilic *Bacillus pumilus* produced another thermo-stable protease in alkaline pH 4.0-12.0. The results obtained in present study were in agreement with past studies (31, 32).

Proteases have also been categorized according to their sensitivity to various inhibitors. Inhibition studies suggest understanding regarding the nature of an enzyme, its active site nature and cofactor requirements (33). Thus, *G. candidum* protease is categorized as a serine protease, because the EDTA had no effect on activity, which suggests that the enzyme may not be a metalloprotease and only depends on the mono and divalent cations as stabilizers. However, the EDTA-independent nature of the enzyme suggests that it may be a useful laundry additive (34). It was observed that Ca⁺² ions have the ability to stabilize the enzyme structure when exposed to extreme temperature and it depicts its role in stabilizing ternary complex of enzymes (35). However, Ca⁺² dependent proteases are less studied and information about them is scarce. Inhibition of proteolytic activity in the presence of cadmium ion (57.11 ± 7.5%) is due to the interaction with disulfide and thiol groups (36).

Protease stability with polar solvents is a very valuable characteristic for fermentation industry (37). The maximum inhibitory effect was observed with non-polar organic solvents such as hexane, benzene, chloroform and DMSO. It has now been realized that the catalytic performance of the enzymes could be correlated with their dynamic properties and interactions with the solvent system. Though, enzymes are generally inactivated or denatured in the presence of non-polar organic solvents. Maximum hydrolytic activity of protease was observed against casein, it is similar to the earlier report from alkaline protease from *Bacillus mojavensis* (38, 39). Extracellular enzyme activity of *G. candidum* is higher in soft cheese than hard cheeses and this also confirms the proteolytic role of *G. candidum* in semi-solid indigenous fermented milk product Dahi (7). Half-life and K_d , clearly depicted the mesophilic nature of enzyme as compared to thermophilic nature (23). The difference between thermostability and thermophilicity is the resistance to the unfolding is assessed at various temperature. While in case of thermophilicity, the ability of enzyme to carry out the reaction at higher temperatures are assessed. The activation energy for denaturation of protease (Fig. 5) was found to be less than that was obtained from xylanases reported by Bokhari et al., (23). The enzyme inactivation at higher temperatures is denoted by the breakdown of non-covalent interactions resulting in increase in enthalpy. The denaturation of enzyme and resulting expansion in the enzyme structure results in increase in entropy (40, 41). The higher free energy and

lower enthalpy values depicted that the enzyme is only meagerly thermostable at the given temperatures.

In the present work, *G. candidum* QAUGC01 strain isolated from indigenously fermented milk product was found suitable for production of an extracellular protease. This protease has interesting biochemical, thermodynamic and kinetic properties. The main characteristics features of this protease are: Serine type, alkaliphilic, and thermostability are the main characteristics of this protease from *G. candidum* QAUGC01.

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