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Research Article

ISOLATION, PURIFICATION, AND OPTIMIZATION OF THERMOPHILIC AND ALKALIPHILC PROTEASE ORIGINATING FROM HOT WATER SPRING BACTERIA

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ABSTRACT

Objective: The main objective of this study is to investigate the industrial applications of a thermophillic alkaline protease from a hot water spring bacterial isolate "A" and to study its production, optimization, and purification.

Methods: The alkaline protease was produced using shake flask studies maintaining a pH of 9.0 and a temperature of 50°C. Optimization studies of the enzyme were carried out using variable pH, temperature, organic carbon, and nitrogen sources followed by purification of the enzyme using DEAE-cellulose ion exchange chromatography technique. Stability of the enzyme was analyzed in the presence of organic solvents and surfactants. The efficiency of the enzyme in the removal of proteinaceous stains in the presence of strong detergents under extreme conditions was assessed. The fibrinolytic activity of the enzyme in dissolving the blood clot was confirmed.

Results: The isolated alkaline protease was purified to homogeneity with a 16-fold increase. Media optimization studies revealed that 1% glucose and 1 % casein-induced the production of alkaline protease. The purified enzyme retained stability in the presence of ethanol, methanol, and acetone and surfactants such as 0.5% (w/v) sodium dodecyl sulfate (SDS) and 0.5% (v/v) Triton-X-100. The isolated alkaline protease successfully removed the proteinaceous stains and showed significant results in the dissolution of blood clot.

Conclusion: The above experimental results confirm that the isolated enzyme has both thermophilic and alkaliphilic protease properties. Thereby the enzyme finds promising industrial applications even in extreme conditions.

Keywords: Protease, Thermophillic, Alkaliphillic, Purification, Applications.

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INTRODUCTION

Proteases (EC 3.4.2.1), are the protein hydrolyzing enzymes and constitute a large group of industrially important enzymes. They catalyze the cleavage of peptide bonds in proteins. Based on their mechanism of action proteases are divided as endopeptidases and exopeptidases. The exopeptidases act only near the terminal amino or carboxyl position. Endopeptidases are further categorized as serine, cysteine, aspartate, and metallopeptidases based on their catalytic mechanism. Bacterial proteases are the most significant [1] and account for nearly 60% of the total worldwide enzyme sales [2,3]. *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Halomonas*, *Pseudomonas,* and *Serratia* are the major bacterial genera which contribute to proteases. Bacterial alkaline proteases, are specialized due to their higher activity under alkaline pH range (6-13) specialized and broad substrate specificity. Most of the alkaline proteases have active serine center, whereas some others are of a metallo-type where metal ions contribute to enhanced activity and stability at higher temperatures [4]. Enhanced thermostability was observed for alkaline proteases from *Bacillus* sp., *Streptomyces* sp., and *Thermus* sp. after the addition of $CaCl_2$ [5]. Serine proteases find industrial importance [6]. Thermal stability and activity at alkaline pH ensures applications of alkaline protease in detergent formulation [7]. Studies have revealed that bacteria capable of producing alkaline proteases have keratinolytic activity [8]. Alkaline proteases have wide-scale industrial applications including food processing, leather processing as a dehairing agent, textile industry, diagnostic reagents, household waste management, recovery of silver from X-ray film, and bioremediation [9,10]. Proteases have wide applications in many industries, namely, detergents, and food processing, especially for cheese ripening, meat tenderization, animal nutrition, pharmaceutics, paper industry, and food industry [11].

Microbial proteases have immense applications as compared to other hydrolytic enzymes in various spheres of science and industries and there is a high demand of alkaline protease in detergent industries which make it as a potent enzyme [12]. Insoluble fibrous keratin protein is the primary substance found in chicken feathers, and cannot be degraded by common proteases. Keratinases belong to the group of extracellular serine proteases capable of degrading keratin [13].

Thermophilic bacteria from hot water springs produce unique thermostable enzymes where proteases originating from such bacterial species are of particular interest due to their wide range of commercial applications [14-16].

Very few reports are available on the bacterial enzymes showing both thermophilic and alkaliphilic nature. Therefore, the present work aims to study the biotechnological and industrial applications of a thermophilic alkaline protease isolated from hot water spring in the removal of proteinaceous stains and its medicinal importance as a fibrinolytic agent. Production of enzyme to a significant extent was achieved using wheat bran as a cheaper agro based residues. Entire study aims to carry out production, optimization, and purification of the isolated alkaline protease enzyme.

METHODS

Isolation of thermophilic strain

Water and soil samples were collected from hot spring region near Mumbai (Maharashtra, India) in sterile containers and transported to laboratory and maintained at 4°C microorganisms were isolated from collected soil and water samples using nutrient agar plates (pH 7.0) by serial dilution technique. Plates were incubated at 37°C and 50°C for

24-48 h. Pure cultures for seven bacterial isolates (A, B, C, D, E, F, and G) obtained as individual colonies were screened for the production of alkaline protease using skim milk agar (pH 9.0) which were incubated at 50°C for 24-48 h. Isolates showing maximum zone of hydrolysis, maximum pH, and temperature tolerance were further selected for production of alkaline protease by submerged fermentation process. The biochemical characterization was performed for selected bacterial isolates.

Production of protease by submerged fermentation process

The production media (pH 7.0; pH 9.0) comprising glucose 0.1%, peptone 1%, yeast extract 0.02% (w/v), $MgSO_4$ 0.01% (w/v), $CaCl_2$ 0.5% (w/v), and $FeSO_4$. $7H_2O$ 0.01% (w/v), were inoculated using all seven bacterial isolates (A, B, C, D, E, F, and G) and incubated at 37°C and 50°C in a shaking incubator (Remi, Thane, Maharashtra, India) maintained at 150 rpm. Media were harvested after 24, 48, and 72 h and centrifuged (± 4°C) at 8000 rpm for 15 min. Supernatant derived from the bacterial culture served as a source of extracellular protease.

Selected bacterial isolates with higher activity were used further for optimization of enzyme production under variable pH and temperature conditions.

Protease assay

Protease activity was determined by following the regular assay method using casein as a substrate [17]. The protease activity was assayed by incubating 1 ml of the enzyme with 2.5 ml of 1% (w/v) casein (prepared in 100 mM sodium phosphate buffer; pH 7.0 and 100 mM Tris-HCl buffer; pH 9.0) at 37°C for 30 minutes. Reaction was terminated by adding 2.5 ml tricarboxylic acid (TCA). TCA soluble fraction containing soluble peptides was measured using the Lowry method [18] by referring the standard curve of tyrosine (0-100 µg/ml).

One unit activity of alkaline protease is the amount of enzyme required to liberate 1.0 µM of tyrosine under optimized conditions of pH and temperature.

Optimization of pH and temperature for the production of alkaline protease

The effect of variable pH (7.0, 8.0, 9.0, and 10.0) on the production of alkaline protease was analyzed using selected bacterial isolates "A" and "D" at 37°C and 50°C. Isolates were grown in the production media for 24-72 h incubation period. The caseinolytic activity of enzyme was measured after every 24 h using the standard assay procedure mentioned as above.

Optimization of enzyme production using agricultural residues

Production of alkaline protease was analyzed using agricultural residues as wheat bran, rice husk and maize, and chick pea powder collected from local markets. Minimal media (disodium hydrogen phosphate - 3%, potassium dihydrogen phosphate 1.5%, ammonium chloride 0.5%, sodium chloride 0.25%, and calcium chloride 0.001%) supplemented with 1% (w/v) different agricultural were used for bacterial growth using isolates (A, B, C, D, and F) and production of enzyme. The inoculated media (pH 9.0) were incubated further at 50°C for 24, 48, and 72 h and the activity of enzyme was analyzed.

Optimization of enzyme production using variable carbon sources

The production of protease was analyzed using variable carbon sources such as glucose, lactose, maltose, dextrose, and starch at 1% (w/v) concentration. The carbon sources were autoclaved separately and then added to the production medium. Growth of bacterial isolates and subsequent production of enzyme was monitored at pH 9.0 and a temperature of 50°C after 24, 48, and 72 h. The caseinolytic assay was performed and the activity was recorded.

Optimization of enzyme production using variable nitrogen sources

The production of protease was analyzed using variable organic nitrogen sources such as yeast extract, peptone, beef extract, and casein at 1% (w/v) concentration in the production medium. Growth of bacterial isolates and subsequent production of enzyme was monitored at pH 9.0 and a temperature of 50°C after 24, 48, and 72 h. The caseinolytic assay was performed and the activity was recorded.

Effect of variable buffers on enzyme activity

The stability of protease was analyzed using variable buffers (tris NaOH, Carbonate-bicarbonate, Glycine-NaOH, and Borax) with pH = 9.0 at 50°C for 24 h. The enzyme was preincubated along with buffers for 1 h. The activity of protease without buffer was considered 100%. The residual proteolytic activity was measured.

Effect of organic solvents and surfactants on the activity of alkaline protease

The stability of alkaline protease from isolate "A" was analyzed in the presence of 5% (v/v) of different organic solvents (hexane, methanol, isopropanol, ethanol, toluene, dimethyl sulfoxide (DMSO), and acetone), 0.5% and 1% (w/v) of surfactants such as (sodium dodecyl sulfate [SDS], Triton X-100, and Tween-80) and hydrogen peroxide as an oxidizing agent (H_2O_2). The enzyme was preincubated along with solvents, surfactants, and oxidizing agents for 1 h. The activity of protease without solvent, surfactant, and the oxidizing agent was considered 100%. The residual proteolytic activity was measured.

Purification of alkaline protease

The cultural supernatant derived from isolate "A" containing alkaline protease activity was subjected to partial purification using 80% ammonium sulfate fractionation. The precipitated proteins were separated by centrifugation of extract at 10,000 rpm for 20 minutes. Resultant precipitate was dissolved in Tris buffer (0.02 M, pH 9.0) and dialyzed extensively against the same buffer for 48 h. The resultant dialysate was used for estimation of enzyme activity and specific activity.

Alkaline protease was further purified by ion exchange chromatography using DEAE-cellulose and CM-cellulose. The activated ion exchanger was repeatedly washed with buffer. Both DEAE-cellulose and CM-cellulose columns were equilibrated with 50 mM Tris-HCl of pH 9.0.

Dialyzed enzyme solution was applied to the DEAE-cellulose (1.5 cm × 50 cm) and eluted by applying continuous stepwise gradient of 0.5-1 M NaCl using same equilibration buffer. Fractions each of 4 ml were collected along with a flow rate of 25 ml/h. Fractions containing enzyme activity were pooled together, dialyzed and analyzed for enzyme activity and total protein content.

Applications of alkaline protease

Compatibility with commercial detergents

The compatibility of the isolated alkaline protease was analyzed using a blood stained cloth with dimensions 2" × 2". Blood stained cloth was treated with 1 ml of isolated alkaline protease along with detergent solution (7 mg/ml) in one container and only with detergent solution separately in another one. A control set was prepared by treating blood stained cloth only with distilled water in a third container.

Blood clot dissolution studies

Blood sample was collected from the blood bank. A volume of 0.5 ml of venous blood sample was added in preweighed sterile tubes and allowed to stand at for 45 min at 37°C for the formation of blood clot. Serum was removed, and the tubes were again weighed to determine the actual weight of the clot. Clots were further subjected to the action of 0.5 ml and 1.0 ml of partially purified enzyme. A control set was prepared by replacing the enzyme solution with distilled water. The tubes were further incubated for 90 min at 37°C to observe the lysis of blood clots. The remnant fluid was removed, and the tubes were weighed. The difference in weight taken before and after lysis of the clot was measured in terms of % of clot lysis.

RESULTS AND DISCUSSION

Isolates showing maximum zone of inhibition

Morphologically distinct bacterial colonies isolated from soil and water samples from hot water spring through repeated streaking on nutrient agar plates were subjected to extensive screening for the production of alkaline protease using skim milk agar (pH 9.0). Five bacterial isolates (A, B, C, D, and E) showing maximum zone of hydrolysis revealed production of extracellular alkaline protease. Growth of bacterial isolates at alkaline pH of 9.0 and at a temperature of 50°C indicated their thermophilic and alkaliphilic nature.

Selected isolates were used further for the optimization of alkaline protease production using liquid medium provided with carbon and nitrogen source (Table 1).

Optimization of alkaline protease production

The production of alkaline protease is affected by fermentation parameters such as pH and temperature, incubation time, and media composition with variable source carbon in the form of metabolizable sugars, organic solvents, and surfactants. Maximization in the production of alkaline protease from selected alkaliphilic and thermophilic bacterial isolates is essential to reveal their industrial applications. Further work was carried out for optimization of alkaline protease production using media with variable compositions and cultivation conditions.

Effect of incubation period (pH 7.0; 37°C)

Incubation period affects the production of alkaline protease by bacterial isolates and is simultaneously influenced by other parameters including physical factors as pH, temperature, inoculum size, and metabolic state of the cell. The data regarding the production of protease under variable incubation period from 24-72 h is revealed in Fig. 1. All the bacterial isolates produced a maximum titer of protease after an incubation period of 72 h. Maximum protease production was recorded for isolates "A" and "D" (250 U/ml and 218 U/ml). Isolates "B" and "C" have been reported to show maximal protease activity of 172 U/ml and 202 U/ml after 72 h of incubation. The protease production recorded for isolates "E," "F," and "G" was 174 U/ml, 143 U/ml, and 134 U/ml, respectively. Maximum growth and enzymes production were recorded after 72 h of incubation [19-21].

Production of alkaline protease was carried out at 37°C in the medium comprising glucose 0.1%, peptone 1%, yeast extract 0.02% (w/v), $MgSO_4$ 0.01% (w/v), $CaCl_2$ 0.5% (w/v), $FeSO_4$. $7H_2O$ 0.01% (w/v), at pH 7.0 for variable time intervals of 24, 48, and 72 hrs. Values are presented as mean ± standard deviation (SD) of three independent experiments.

Effect of incubation period (pH 9.0; 37°C)

Fig. 2 also displayed similar results where enzyme production increased linearly with increase in incubation time from 24-72 h. Maximum enzyme production was observed for all isolates at 72 h incubation period. Higher yield of the enzyme was revealed for all isolates after growth in the production media maintained at an alkaline pH of 9.0. The activity of alkaline protease was maximal for isolates "A" (223 U/ml) and "D" (208 U/ml) followed by "B" (186 U/ml) and "C" (199 U/ml). Thus, bacteria produce higher enzyme during stationary growth phase while the enzyme produced by them is alkaline in nature. Similar observations were made for protease produced from *Bacillus* sp. using wheat bran and groundnut cake [22].

Table 1: Promising isolates showing maximum protease activity on skim milk agar

Isolates	Zone of hydrolysis (cm)
Isolate A	2.5±0.2
Isolate B	1.5±0.4
Isolate C	1.8±0.7
Isolate D	2.3±0.3
Isolate E	1.6±0.6
Isolate F	1.5±0.7
Isolate G	1.8±0.6

Zone of hydrolysis indicates the protease producing ability of bacterial isolates.
All values are expressed as mean±SD of three independent experiments.
SD: Standard deviation

Production of alkaline protease was carried out at 37°C in medium comprising glucose 0.1%, peptone 1%, yeast extract 0.02% (w/v), $MgSO_4$ 0.01% (w/v), $CaCl_2$ 0.5% (w/v), $FeSO_4$. $7H_2O$ 0.01% (w/v), at pH 9.0 for variable time intervals of 24, 48, and 72 h. Data represent mean ± SD of three independent sets of observations.

Effect of incubation period (pH 9.0; 50°C)

The influence of the higher temperature of 50°C and alkaline pH of 9.0 on the production of alkaline protease by all six bacterial isolates was evaluated at 50°C and pH 9.0. Maximal production of alkaline protease was observed for the bacterial isolates "A" and "D" as 232 U/ml and 191 U/ml, respectively, at 72 h incubation. This was followed by isolates "B" and "C" with a subsequent yield of protease 163 U/ml and 151 U/ml, respectively. Results explicit the alkaline and thermophilic nature of bacterial isolates where "A" and "D" being promising one were further selected for purification and biochemical characterization. This suggested that the bacterial strain was alkaliphillic in nature and optimum pH range between 9.0 and 10.0 for growth and enzymes production is a common feature among alkaliphillic organisms (Fig. 3) [11].

Production of alkaline protease was carried out at 50°C in medium comprising glucose 0.1%, peptone 1 %, yeast extract 0.02% (w/v), $MgSO_4$ 0.01 % (w/v), $CaCl_2$ 0.5 % (w/v), and $FeSO_4$. $7H_2O$ 0.01%

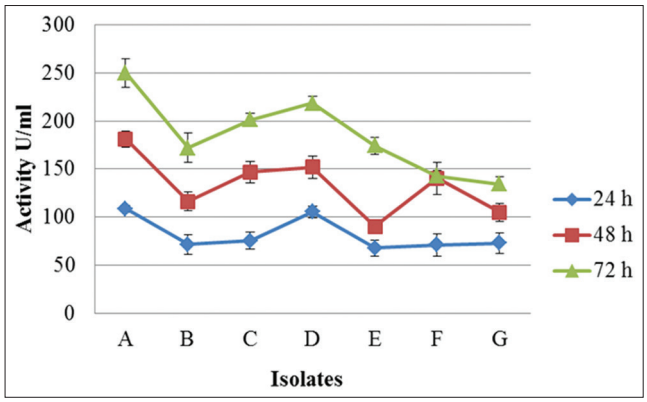

Fig. 1: Effect of incubation period on protease production (pH 7.0; 37°C)

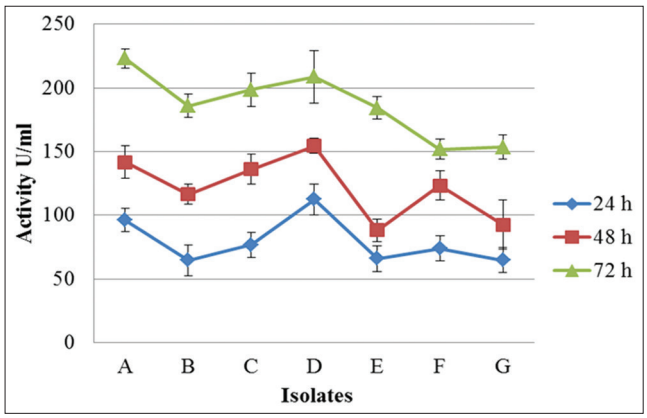

Fig. 2: Effect of incubation period on protease production (pH 9.0; 37°C)

(w/v), at pH 9.0 for variable time intervals of 24, 48, and 72 h. Values for the enzyme activity are presented as mean ± SD of three sets of observations.

Effect of agricultural residue

For the commercial scale production of protease on an industrial level, use of the cheaper source is equally important apart from other physical and chemical parameters. Therefore, wheat bran, rice husk and maize powder were used as a substrate for the production of alkaline protease. The analysis of results showed that agricultural residues used alone also led to the production of the enzyme. Among them, wheat bran showed comparatively higher level of alkaline protease production. All isolates showed maximal activity at 72 h incubation period where isolate "A" produced higher enzyme activity (35 U/ml) using wheat bran as a sole source of the nutrient. The corresponding production of alkaline protease for "C" and "D" isolates was (32 U/ml) and (27 U/ml), respectively. Comparatively lower activity of the enzyme was noted for isolates "B" (23 U/ml) and "F" (18 U/ml). No significant activity of alkaline protease was observed using rice husk, chickpea, and maize powder, thereby concerned results are not shown (Fig. 4).

Production of alkaline protease was carried out by cultivating the isolates using minimal media (disodium hydrogen phosphate 3%, potassium dihydrogen phosphate 1.5%, ammonium chloride 0.5%, sodium chloride 0.25%, calcium chloride 0.001%) with 1.0% (w/v) of wheat bran, as a sole carbon source at 50°C for 72 h, pH = 9.0. Values for the enzyme activity are presented as mean ± SD of three independent experiments.

Effect of variable pH

Isolate "A"

pH is an important physical parameter required to maintain ionization status for active site amino acid residues thereby affecting the growth and subsequent yield of alkaline protease.

Influence of variable pH (7.0-10.0) on the production of alkaline protease was investigated using isolate "A" and "D." The production level of the enzyme was observed to increase with corresponding increase in the pH of production media from 7.0 to 9.0. In all cases, the maximal activity of the enzyme was obtained at pH 9.0 for an incubation period of 24 h (129 U/ml), 48 h (187 U/ml), and 72 h (243 U/ml). These results indicate alkaliphilic nature of protease originating from isolate "A." The yield of the enzyme was decreased with further increase in pH to 10.0. Thus, isolate "A" can grow and produce alkaline protease over a wide pH range from 7.0 to 10.0 (Fig. 5).

The activity of the enzyme from isolate "A" was measured at variable pH (7.0, 8.0, 9.0, and 10.0) at 50°C for 24, 48, and 72 h using casein as the substrate. Buffers used were acetate (4.0-6.0), phosphate (6.0-7.0), Tris-HCl (7.0-9.0), and glycine-NaOH (10.0-12.0). Values are presented as mean ± SD of triplicates.

Effect of carbon source

Since isolate "A" gave maximal activity of alkaline protease under alkaline pH of 9.0 and at a higher temperature of 50°C indicating its alkaliphilic and thermophilic nature; further studies were carried out by selecting the same isolate.

Carbon plays a prime role during growth and production of desired metabolites by the microbial cell. Both types of carbon source as well as its concentration added to the production medium significantly affects the production of the extracellular enzyme. The effect of different carbon sources including mono-, di- and poly-saccharides such as maltose, glucose, starch, and lactose on the production of alkaline protease was analyzed. All the carbon sources supported growth and production of alkaline protease by selected isolate "A." The isolate responded to different carbon sources by showing variable yield of the enzyme. Among various carbon sources, maximum production of the protease was observed in the presence of glucose (95 U/ml) and

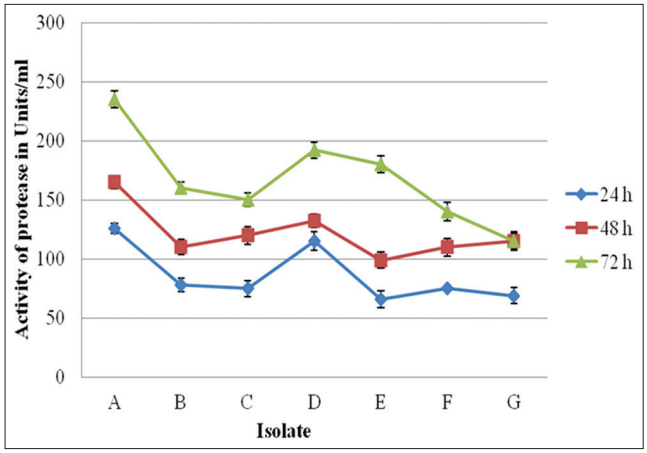

Fig. 3: Effect of incubation period on protease production (pH 9.0; 50°C)

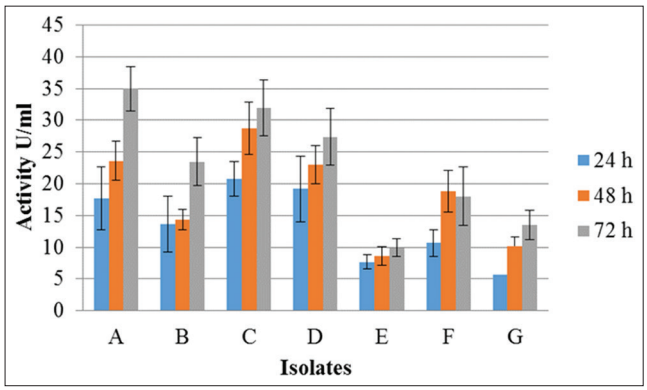

Fig. 4: Effect of wheat bran on the protease production (pH 9.0; 50°C)

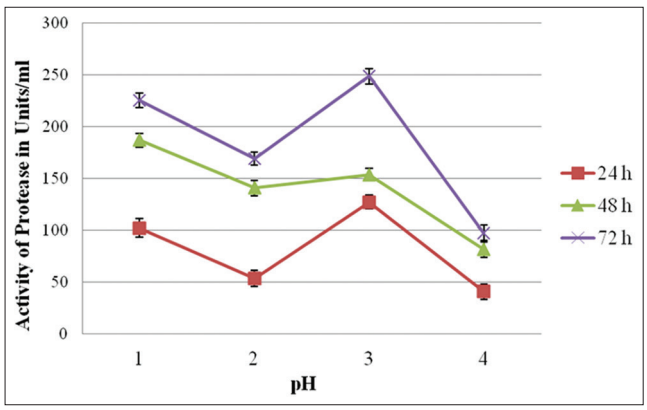

Fig. 5: Effect of variable pH on protease production from isolate "A"

maltose (91 U/ml). The production of enzyme was further decreased for starch and lactose by 10% showing 85 U/ml activity. Thus, glucose and maltose being simple and easily metabolizable sugars support maximal production. These results are in accordance with the results obtained for *Bacillus* sp. from different wastes [23]. The decrease in enzyme production in case of lactose and starch was observed due to their structural complexity accompanied by catabolite repression of protein biosynthesis (Fig. 6).

Production of alkaline protease was carried out by cultivating isolate "A" in the growth medium with 1% (w/v) of different sugars at 50°C for 24, 48, and 72 h. Values are given as mean ± SD of triplicates.

Effect of nitrogen source

Nitrogen source also affects metabolic processes of the cell significantly. In accordance with this, the effect of nitrogen on the production of alkaline protease was analyzed by growing bacterial isolate "D" in the production medium supplemented with variable nitrogen sources at 0.5% (w/v) concentration. Bacterial cells grown using casein as a nitrogen source exhibited the higher activity of alkaline protease (223 U/ml) followed by yeast extract (160 U/ml). Production of the enzyme was reduced significantly for beef extract and peptone to 51% and 43%, respectively. Thus results indicated that casein being an organic nitrogen source enhances maximum protease and also serves as an inducer for the production of the enzyme. Similar results were observed for alkaline protease isolated from *Bacillus* sp. (Fig. 7) [24,25].

Production of alkaline protease was carried out by cultivating isolate "A" in the growth medium with 1% (w/v) of different organic nitrogen sources at 50°C for 24, 48, and 72 h. Data represent mean ± SD of three sets of observations.

Effect of variable buffers on the enzyme activity (pH 9.0)

Buffers components as weak acids and their corresponding salts of weak base can also affect the enzyme activity. From graph 8, it revealed that alkaline protease displayed higher activity for Tris NaOH buffer (154 U/ml). Comparatively, lower activity was observed in the presence of glycine-NaOH buffer (75 U/ml) followed by Borax buffer (67 U/ml). Use of carbonate-bicarbonate buffer, significantly lowered the activity to 43 U/ml. Thus, buffer components interact variably with proteins causing proton transfer or buffer component binding thereby inducing conformational changes resulting in the denaturation of protein (Fig. 8) [26].

Production of alkaline protease was carried out by cultivating isolate "A" in the growth medium with different buffers, pH = 9.0 at 50°C for 24 h. Data represent mean ± SD of three sets of observations.

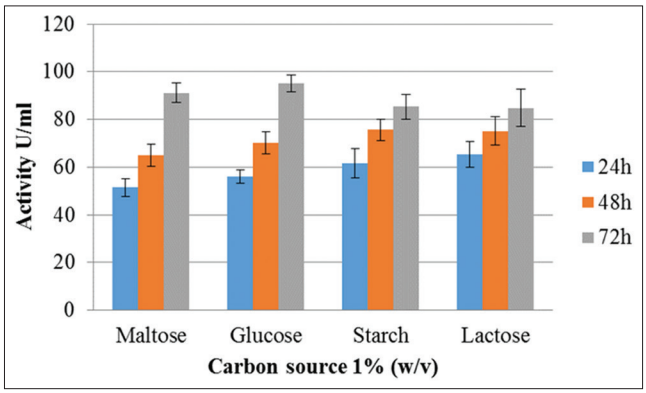

Fig. 6: Effect of carbon source on protease production from isolate "A"

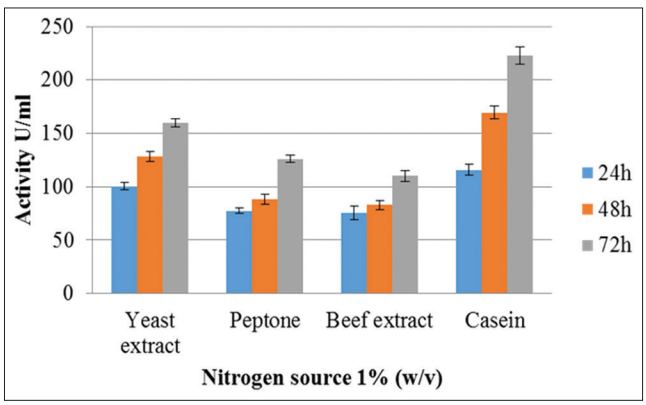

Fig. 7: Effect of nitrogen source on protease production from isolate "A"

Partial purification of enzyme by ammonium sulfate purification

Alkaline protease present in the crude extract was purified by 80% saturation with ammonium sulfate leading to enhancement in the activity (190 U/ml) by 4% (Fig. 9). The protease produced by Trichosporon japonicum was partially purified by the ammonium sulfate precipitation method with 60% saturation giving highest protease activity [27].

Purification of the enzyme was carried out using 80% saturation with ammonium sulfate followed by dialysis using Tris buffer (0.02 M, pH 9.0). Data represent mean ± SD of three sets of observations.

The observed specific activity of alkaline protease after ammonium sulfate purification was 169 U/mg indicating 35% purification fold. Wide-ranging results of protease purification (4.25-200-fold) with various specific activities (13.34 U/mg of protein) and % recovery (2-21%) have been described for different microbial species such as *Bacillus licheniformis, Bacillus subtilis,* and *Bacillus megaterium* (Fig. 10) [28-30].

Specific activity of the alkaline protease was analyzed before and after dialysis: Data represent mean ± SD of three sets of observations

The alkaline protease was purified using DEAE-cellulose ion exchange chromatography technique. The bound proteins were eluted with 0.5 M NaCl in phosphate buffer of pH 7.0. The alkaline protease enzyme bound to DEAE-cellulose and eluted in fractions numbers 25-35 by applying step gradient of 0-0.5 M NaCl. Thereby elution profile of enzyme displayed a single peak resulting in the formation of peak containing protease activity. The enzyme was purified up to 16-fold as bound fraction of DEAE-cellulose column with 1928 U/mg^{-1} specific activity (Fig. 11). As per previous results regarding protease purification, the values of specific activities observed for different microbial species such as *B. licheniformis, B. subtilis,* and *B. megaterium* were 13.33-159381 U/mg of protein with 4.25-200 purification fold [28,29].

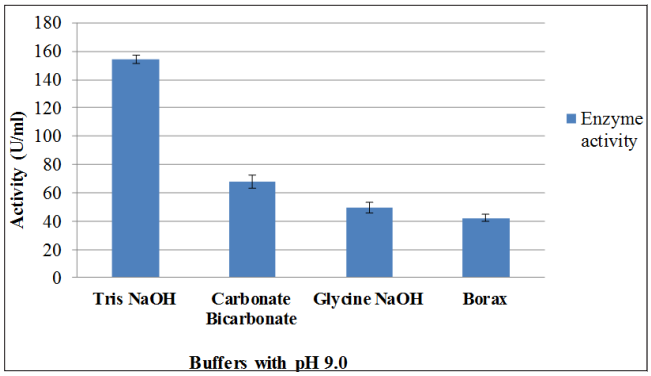

Fig. 8: Effect of buffers with variable pH on protease activity from isolate "A"

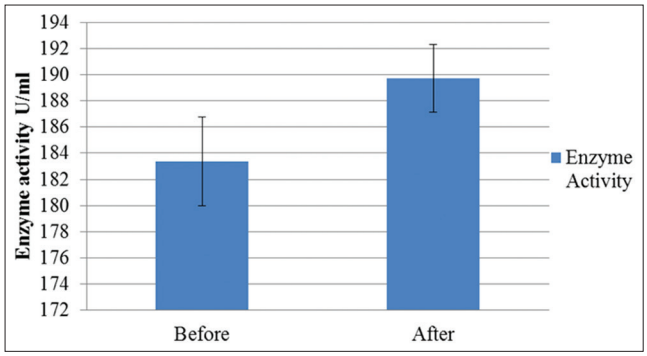

Fig. 9: Activity of enzyme before and after ammonium sulfate purification

Chromatogram of the purified enzyme produced by isolate "A" using DEAE-cellulose ion exchange column with dimensions 1.5 cm × 50 cm and a flow rate of 25 ml/h. Fractions 25-35 containing the eluted enzyme confirmed alkaline protease activity (Table 2).

Effect of organic solvents

Stability of alkaline protease toward organic solvents revealed their commercial significance on industrial scale. In view of this effect of different water miscible and immiscible organic solvents on the activity of alkaline protease produced by isolate "A" was analyzed by incubating the enzyme along with organic solvents for 1 hr. The alkaline protease was found to be comparatively more stable in the presence of ethanol, methanol, and acetone with residual activity of 61.92%, 53.78%, and 44.42%, respectively. N-hexane and toluene caused greater inhibitory effect showing 25% and 29% residual activity for the enzyme. Organic solvents alter the catalytic mechanism by interfering the hydrogen bonding and hydrophobic interactions among active protein leading to unfavorable changes in dynamics and conformation [31]. Previous studies have reported organic solvent-tolerant strains from the genus *Pseudomonas* and *Bacillus* producing solvent stable enzymes (Fig. 12) [32,33].

Figure indicates stability of alkaline protease in the presence of organic solvents at 37°C for 1 h. Residual protease activity was measured at 50°C for 30 minutes with pH 9.0 using caseinolytic assay. The proteolytic activity measured without organic solvent was taken as control. Values are presented as mean ± SD of three independent experiments.

Effect of surfactants

Stability of alkaline proteases toward surfactants enhances their potential in washing and destining technology making them compatible in the detergent manufacturing process. Stability of alkaline protease was analyzed by preincubating the enzyme in the presence of various surfactants for 1 h and the residual activity is presented in Fig. 13. The alkaline protease remained comparatively stable in the presence of 0.5% (w/v) SDS and Triton-X-100 with the relative residual activity of 35% and 30%, respectively. The reduction of the activity and stability of enzyme observed in the presence of SDS, a nonionic surfactant with 8% of residual activity as revealed for several enzymes due to disruption of their structures after interaction with SDS [34]. The precipitation and subsequent inhibition of alkaline protease were observed in the presence of SDS observed. The results obtained are in accordance with Komarov *et al*. [35] where inhibition of pepsin by SDS was observed due to precipitation of the enzyme at isoelectric point. A reduction of 43% and 67% in the activity of pepsin after incubation with 1.4% SDS for 2 and 4 h, respectively indicated unfolding of enzyme leading to its inactivation [36].

The alkaline protease from isolate "A" was preincubated with 0.5-1% (w/v and v/v) of various surfactants (SDS, Triton-x-100 and Tween-80) at 37°C for 1 h. The residual enzyme activity was measured at 50°C for 30 minutes with pH 9.0. The proteolytic activity measured without surfactant and oxidizing agent was taken as control. Values are presented as mean ± SD of three sets of observations.

Role of alkaline protease enzyme in the destaining process

Alkaline protease is primarily employed as cleansing additives [37]. One of the major applications of alkaline protease includes removal of proteinaceous stains. The enzyme shows much reduction in the stain intensity compared to the detergent. Similarly, when the enzyme was applied along with the detergent, the effect of destaining was enhanced as compared to the treatment given using only distilled water or distilled water with detergent. As per earlier reports addition of alkaline protease to commercial detergents significantly increases washing performance and removal of blood stains [38]. The significant role of alkaline protease produced by isolate "A" in washing performance in addition to higher proteolytic activity and stability indicates its application as an additive in commercial detergents (Fig. 14).

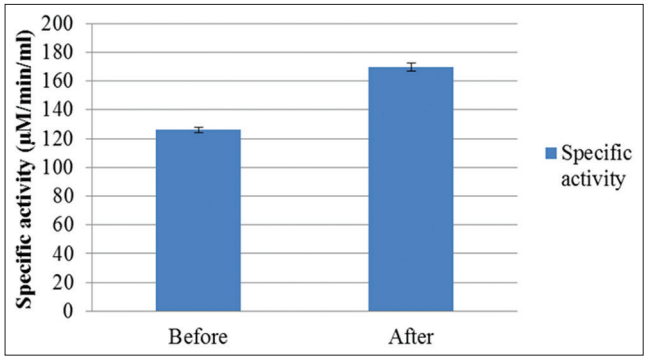

Fig. 10: Specific activity of alkaline protease

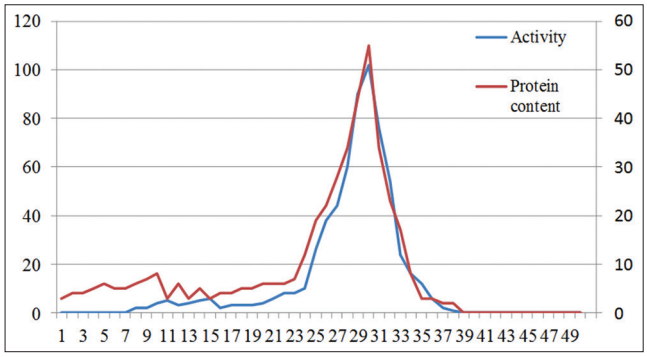

Fig. 11: Ion exchange chromatography of the alkaline protease using DEAE-cellulose

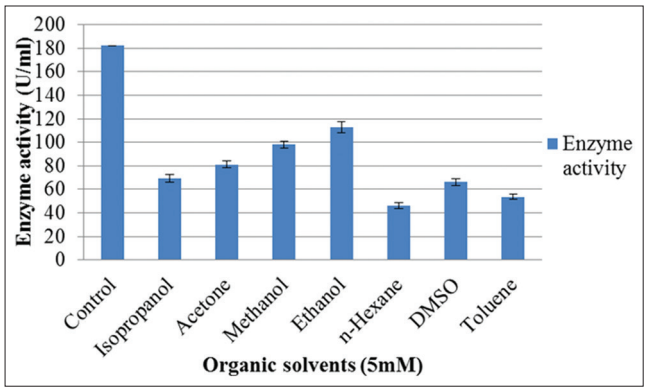

Fig. 12: Effect of solvents on the protease production from isolate "A"

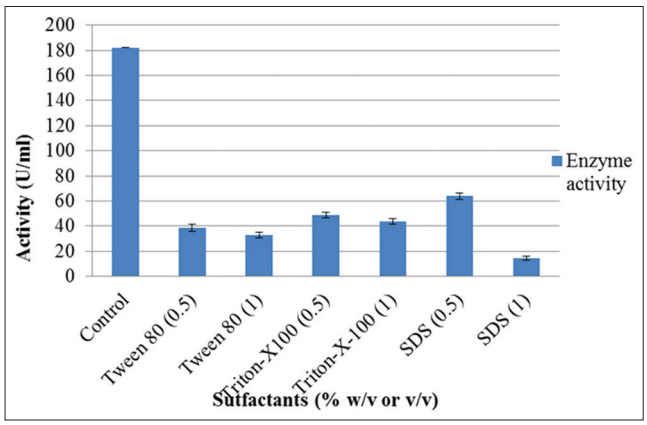

Fig. 13: Effect of surfactants on protease production from isolate "A"

Table 2: Purification steps for the alkaline protease

Purification steps	Total activity	Total protein (U/mL)	Specific activity (mg)	Purification fold (U/mg)	Yield (%)
Crude enzyme	2749	22.4	127.72	1	100
Ammonium sulfate precipitation	1897	11.11	170.74	1.4	69
DEAE-cellulose ion exchange	2650	1.4	1928	16	96

The alkaline protease was purified by 16-fold with increase in specific activity

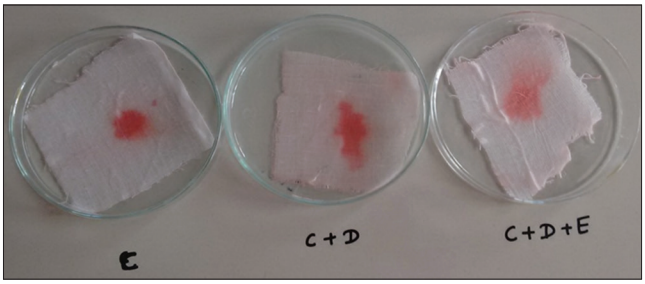

Fig. 14: Destaining of blood on cloth [C= Distilled water; C+D= Detergent solution (7 mg/ml) and distilled water; C+D+E=Detergent solution along with alkaline protease]

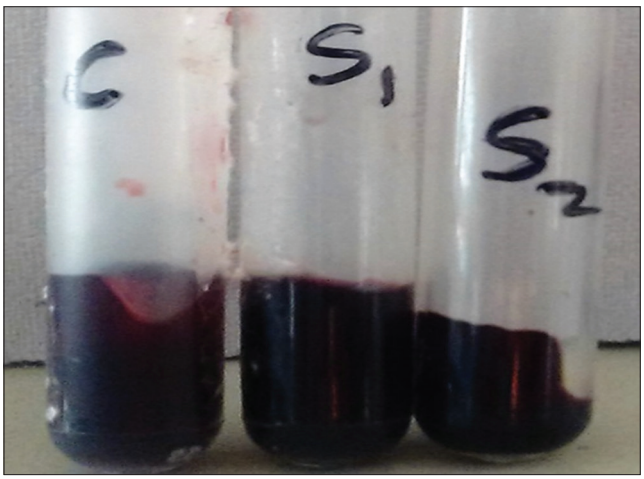

Fig. 15: Fibrinolytic action of the alkaline protease on blood [C = Blood clot + distilled water, S1 = Blood clot + 0.5 ml enzyme, S2 = Blood clot + 1 ml enzyme]

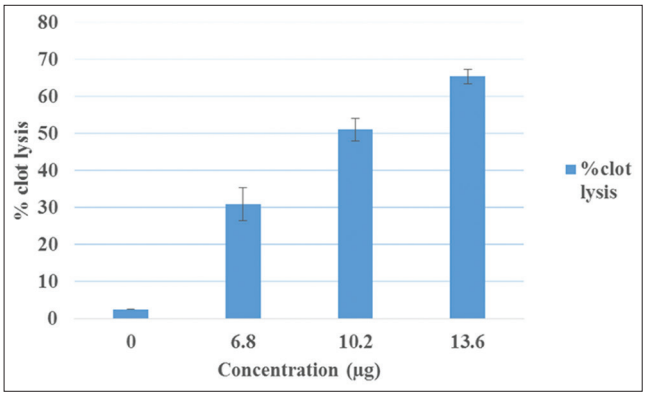

Fig. 16: Percent of clot lysis by alkaline protease enzyme from isolate "A"

C = Control containing distilled water showing negligible stain removal (without protease), C + D = Detergent solution (7 mg/ml) and distilled water showing partial stain removal, C + D + E = Detergent solution along with alkaline protease showing considerable stain removal.

Blood clot dissolution studies

The alkaline protease showed positive results for the dissolution of blood clot where the significant results were observed using higher concentration of enzyme (Fig. 15). Dissolution of blood clot indicates the fibrinolytic action of the enzyme. The mechanism involves hydrolysis of cross-links between fibrin molecules with disruption of the structural integrity of blood clot due to proteolytic action of the enzyme. The ability of alkaline protease in converting insoluble forms of blood clot to soluble form designates its clinical and medicinal applications in the thrombolytic drug. Similar results were obtained for the alkaline protease from *B. licheniformis* B18 for dissolution of blood clot [39,40]. Fibrinolytic enzymes from microbial sources, like, nattokinase, offer great promise in the management (7-8 h) and support of healthy blood circulation. It lyses fibrin directly ad changes prourokinase to urokinase and increase tissue plasminogen activator, which increases our plasmin. Nattokinase lessens excessive coagulation and thus improves circulation, increasing oxygen flow to tissues (Fig. 15) [41].

Percentage of clot lysis corresponds to the amount of the enzyme added. Data presented reveals mean ± SD of three sets of observations.

CONCLUSION

Seven thermophilic bacterial isolates derived from soil and water samples collected from hot water springs exhibited extracellular protease production as observed from zone of clearance on skim milk agar (pH 7.0). Optimal time duration for the protease production by all thermophilic isolates observed was 72 h. The ability of these isolates to produce protease at pH 9.0 and at a higher temperature of 50°C also verified their alkaliphilic and thermophilic nature. Isolate "A" was found to be promising one that exhibited higher level of alkaline protease production (235 U/ml) at pH 9.0 and at a temperature of 50°C. Use of glucose as a carbon source decreased the protease production showing 95 U/ml activity. However using casein as a source of nutrient resulted in significant increase in the activity of alkaline protease (231 U/ml). Thus, casein acts as an ideal inducer for the production of alkaline protease. Using wheat bran as a sole nutritional source, isolate "A" displayed considerable enzyme activity of 32 U/ml. The enzyme was purified by using anion exchange chromatography, eluted as a single peak by applying stepwise gradient of 0.5 M NaCl. The enzyme was purified by 16-fold with 1928 U/mg^{-1} specific activity. The activity of alkaline protease was found to be comparatively stable in the presence of organic solvents such as acetone and methanol showing 44%, 53%, residual activity, respectively. Similarly, enzyme was also stable in the presence of 0.5% (w/v) SDS as a surfactant showing 35% residual activity. These results indicate industrial significance for isolate alkaline protease.

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