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Short communication

Production, purification and partial characterization of a novel protease from marine *Engyodontium album* BTMFS10 under solid state fermentation

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Abstract

Engyodontium album isolated from marine sediment produced protease, which was active at pH 11. Process parameters influencing the production of alkaline protease by marine *E. album* was optimized. Particle size of <425 μm, 60% initial moisture content and incubation at 25 °C for 120 h were optimal for protease production under solid state fermentation (SSF) using wheat bran. The organism has two optimal pH (5 and 10) for maximal enzyme production. Sucrose as carbon source, ammonium hydrogen carbonate as additional inorganic nitrogen source and amino acid leucine enhanced enzyme production during SSF. The protease was purified and partially characterized. A 16-fold purified enzyme was obtained after ammonium sulphate precipitation and ion-exchange chromatography. Molecular weight of the purified enzyme protein was recorded approximately 38 kDa by SDS-PAGE. The enzyme showed maximum activity at pH 11 and 60 °C. Activity at high temperature and high alkaline pH suggests suitability of the enzyme for its application in detergent industry.
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Keywords: *Engyodontium album*; Alkaline protease; Detergent enzyme; Solid-state fermentation; Enzyme purification

1. Introduction

Proteases, one among the three largest groups of industrial enzymes, account for about 60% of the total worldwide sale of enzymes and is widely used in several industries that include detergent, leather processing, meat processing, diary, preparation of organic fertilizer, as digestive aid, silk industry [1] and also for the recovery of silver from used X-ray films [2]. Now proteases are added as key ingredients in detergents, which accounts for approximately 25% of the total worldwide sales of enzymes and all detergent proteases currently used in the market are serine proteases produced using *Bacillus* sp. [1]. Reports on protease production by fungi are limited to the study of virulence factors that are contributed by these proteases in entomopathogenic fungal infection [3,4]. Recently protease production by a few fungal sources such as *Conidiobolus coronatus* [5], *Penicillium* sp. [6] and *Aspergillus parasiticus* [7] were studied for application in commercial detergent industry, whereas, marine fungi which have immense potential as source of exoenzymes, are yet to be harnessed as source of proteases for commercial application [8].

In the production of microbial exoenzymes, solid state fermentation (SSF) has several economic advantages over conventional submerged fermentation such as use of agro industrial wastes as simpler substrates, minimal requirement of water, production of metabolites in a more concentrated form and making the downstream processing less time consuming and less expensive. Among the various groups of microorganisms used in SSF, filamentous fungi are the most widely exploited owing to their ability to grow and produce a wide range of extracellular enzymes on complex solid substrates [9].

In the present communication, we report the potential of a marine *Engyodontium album*, an alkalophilic and salt tolerant fungus, as yet another source of protease. To the best of our knowledge this is the first report on alkaline protease production by any marine fungus that has probable industrial application.

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2. Materials and methods

2.1. Microorganism and inoculum preparation

E. album BTMFS10, isolated from marine sediment of Cochin, available as stock culture at the author's laboratory, was used in the present study. It was maintained on Bennet's agar slants prepared in 50% of aged seawater, subcultured periodically, grown at 28 °C for 14 days and stored at 4 °C [10].

Conidial inoculum was prepared from a freshly raised 14-day-old Bennet's agar slant by dispersing the spores in 0.1% Tween 80. One milliliter of this inoculum (2×10^8 cfu/ml) was used unless otherwise mentioned.

2.2. Solid state fermentation

Solid state fermentation studies with *E. album* was performed using commercially available wheat bran (WB) as the solid substrate [10]. Ten grams of WB of particle size <425 μm taken in 250 ml Erlenmeyer flasks was moistened with aged seawater, so that the final moisture content was 60% (w/v) after inoculation unless otherwise specified. The contents were mixed thoroughly and autoclaved at 121 °C for 30 min, cooled to room temperature, inoculated with 1 ml of conidial inoculum, mixed thoroughly and incubated in a slanting position at 28 °C. The humidity inside the incubator was maintained using distilled water. Enzyme was extracted from the moldy wheat bran (MWB) after 120 h of incubation.

2.3. Optimization of process parameters for protease production

Various process parameters, which influence protease production by *E. album* were evaluated to effect maximal enzyme production using wheat bran as solid substrate and aged seawater incorporated with various nutrients (as detailed below) as moistening medium. Strategy adopted for the optimization was to evaluate the effect of each parameter on protease production under SSF and later to optimize the significant parameter using statistical methods. However, a time course experiment was conducted under optimized condition. Particle size of <425 μm, moisture content of 60% and incubation temperature of 28 °C were taken arbitrarily. The parameters studied included initial moisture content of the medium (30–100%), incubation time (0–144 h), particle size (<425–1400 μm), incubation temperature (20–35 °C), initial pH of the medium (2–13), proteinaceous substrates (gelatin and casein 1%, w/w), additional carbon source (arabinose, dextrose, fructose, galactose, maltose, mannose, mannitol, lactose, ribose, sucrose, sorbitol and xylose at 0.1 M level) and additional nitrogen sources (organic nitrogen sources viz., yeast extract, beef extract, peptone, soybean meal, tryptone and urea at 0.5% (w/w) level and inorganic nitrogen sources viz., ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium hydrogen carbonate, ammonium acetate, ammonium oxalate, ammonium hydrogen phosphate, ammonium iron sulphate, sodium nitrate and potassium nitrate at 0.1 M level) including different amino acids (viz. alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at 1% level). Time course experiment was conducted with the various optimized variables (wheat bran with <425 μ particle size, 60% moisture content, 0.1 M sucrose, 0.1 M ammonium hydrogen carbonate, pH 10, 2 ml inoculum with spore count of 2×10^8 cfu/ml and incubation temperature of 25 °C).

2.4. Extraction and recovery of enzyme

Protease from the MWB was extracted by the simple contact method of extraction using distilled water as extractant [10]. Ten volumes of distilled water per gram MWB (based on initial dry weight of the substrate) was added to the fermented media and the extraction was performed by agitation at room temperature in a rotary shaker for 30 min at 150 rpm. The slurry was then squeezed through cheese cloth and clarified by centrifugation at 10,000 rpm at 4 °C for 15 min. The clear supernatant was used as crude enzyme for protease assay and protein estimation.

2.5. Enzyme assay

Protease activity was determined by caseinolytic method of Kunitz [11] with some modification. One milliliter of diluted enzyme solution was incubated with 2 ml of 1% (w/v) Hammerstein casein in 0.05 M carbonate–bicarbonate buffer (pH 10.0) at 40 °C for 30 min. The reaction was arrested with 2.5 ml of 0.44 M trichloroacetic acid (TCA) solution. The precipitated protein was removed by centrifugation at 10,000 rpm for 15 min and the absorbance of the supernatant was measured at 280 nm against tyrosine as reference compound. One unit of protease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions. Enzyme activity was expressed as units per gram initial dry substrate (U/gIDS) in the case of SSF and as U/ml for purification and characterization.

2.6. Protein estimation

Protein was determined according to the method of Lowry et al. [12].

2.7. Protease purification

Protease produced under SSF was extracted and purified employing ammonium sulphate precipitation, followed by dialysis and ion-exchange chromatography.

Various concentrations of ammonium sulphate (20, 40, 60, 80 and 90%) were used to standardize the precipitation of protease enzyme from the fermented broth. The precipitated protein was resuspended in 0.1 M phosphate buffer (pH 7), dialysed against 0.01 M solution of the same buffer for 24 h, at 4 °C, with six changes of buffer and assayed for protease activity and protein content.

Further purification was done by ion-exchange chromatography using DEAE-cellulose. The pH at which the enzyme binds at its maximum to DEAE-cellulose was standardized to pH 7. Dialysed sample was applied to DEAE-cellulose column (XK16 column from Amersham Biosciences) that had been pre-equilibrated with phosphate buffer (pH 7, 0.01 M). Unbound proteins were washed with the same buffer and stepwise elution was done at a flow rate of 2 ml/min using 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl in the same buffer. Five-milliliter fractions were collected and protein content was estimated by measuring the absorbance at 280 nm. Peak fractions from the column were pooled and assayed for protease activity and protein content.

2.8. Electrophoretic methods

2.8.1. Estimation of the molecular weight of protease

Active fractions collected after ion-exchange chromatography was electrophoresed by native and SDS-PAGE in a 10% polyacrylamide gel according to Laemmlli [13]. SDS-PAGE of purified enzyme was carried out under reductive and non-reductive conditions, i.e. with and without β-mercaptoethanol, respectively. One millilitre of sample was lyophilized and resuspended in 0.1 ml of sample buffer (0.0625 M Tris–HCl, 2% SDS, 10% sucrose, pH 6.8) with (0.1 M) or without β-mercaptoethanol and subjected to electrophoresis. Low molecular weight markers of Amersham Pharmacia was used as molecular mass standard and molecular weight of protease was determined using Quantity One Software of Biorad.

2.8.2. Zymogram

Proteolytic activity of enzyme protein band was confirmed by zymogram analysis on X-ray film according to the method of Cheung et al. [14]. To prepare a zymogram, sample was mixed with electrophoresis sample buffer, (sample buffer of SDS-PAGE, under non-reducing condition) and electrophoresed in a 10% polyacrylamide gel at 4 °C. After electrophoresis, the gel was washed with 2.5% (v/v) Triton X-100 for 30 min followed by carbonate–bicarbonate buffer (0.05 M, pH 10) and incubated on a fresh X-ray film for 10 min at 40 °C. After incubation the film was rinsed in distilled water. The clear zones on X-ray film indicate the presence of protease bands.

2.9. Determination of optimal temperature and pH of purified enzyme

Optimal pH for maximal protease activity was determined with purified enzyme by checking the enzyme activity in different buffer systems of pH 2–13 which include, 0.05 M solutions of HCl–KCl (pH 2), citrate–phosphate (pH 3–6), disodium phosphate–sodium dihydrogen phosphate (pH 7), Tris–HCl (pH 8), glycine–NaOH (pH 9), carbonate–bicarbonate (pH 10), boric acid/potassium chloride/sodium hydroxide (pH 11), disodium hydrogen phosphate/sodium hydroxide (pH 12) and KCl/NaOH (pH 13) at 40 °C.

The temperature optimum for maximal enzyme activity was determined by assaying the enzyme activity at different temperatures (5–100 °C).

3. Results and discussion

3.1. Enzyme production

Alkaline protease production by *E. album* BTMF S10, under SSF using WB as solid substrate was optimized for various physicochemical parameters. A maximum of 4351 U/gIDS was recorded after 120 h of incubation (Fig. 1) with WB of 60% moisture content. A considerable level of enzyme production was also observed at 70% moisture content. In SSF, the initial moisture content significantly influence hydrolytic enzyme production. In fungal and bacterial SSF, lower moisture content was stated to lead to reduced solubility of the nutrients present in the solid substrate, a lower degree of substrate swelling and higher water tension [15]. Similarly, higher moisture content may cause decreased porosity, loss of particle structure, development of stickiness, reduction in gas volume, decreased exchange and enhanced formation of aerial mycelia [16]. In the present study a similar observation was made at moisture levels above 70%.

Enhanced protease production was recorded with substrate particles of average size <425 μm (12,089 U/gIDS and specific activity 255 U/mg protein). With increasing particle size, the enzyme production was considerably reduced. Meanwhile, wheat bran without sieving also supported considerable level of enzyme activity (10,267 U/gIDS) and specific activity (236 U/mg protein). Particle size has a profound effect on enzyme production. The small particles have more surface area for growth but reduced porosity, leading to lowering of gas diffusion and heat transfer, while the large particles absorb less moisture, swell less and by drying rapidly support only a sub-optimal growth of fungi [17].

Maximal enzyme production (5106 U/gIDS) and maximal specific activity (82 U/mg protein) were observed at 25 °C, whereas, temperatures above 30 °C did not support enzyme production (196 U/gIDS and specific activity 5 U/mg protein). The incubation temperature has a profound effect on the enzyme yield and duration of enzyme synthesis phase [18]. Most of the marine fungi investigated showed optimum growth in the range of 10–20 °C and none appeared to require a temperature above 30 °C [19].

Fig. 2 shows that this fungus prefers both acidic (pH 4 and 5) and alkaline pH (pH 10) for protease production. These dual optima of pH for growth are characteristic of most marine fungi [19] and similar reports are also available in literature [10].

In the present study, the fungus produced protease enzyme even in the absence of any proteinaceous substrate supplemented to WB (5106 U/gIDS). Addition of proteinaceous substrates, casein (1607 U/gIDS) or gelatin (2595 U/gIDS), to seawater-based medium did not promote enzyme production. This could be attributed by the fact that WB is a complex medium containing traces of protein, which could have played the role of inducers and hence addition of casein or gelatin as additional protein substrate did not enhance the production of the enzyme.

Among the different additional carbon sources tested, sucrose (15,912 U/gIDS and specific activity 341 U/mg protein) and mannitol (14,709 U/gIDS and specific activity 334 U/mg protein) have a profound effect on enzyme production. Whereas, maltose supported a marginal enhancement in the level of enzyme yield (13,616 U/gIDS and specific activity 285 U/mg protein) compared to the control (13,317 U/gIDS and specific activity 277 U/mg protein). Excepting this observation, in general, it was noticed that the addition of any carbon source to seawater led to the reduction of both enzyme

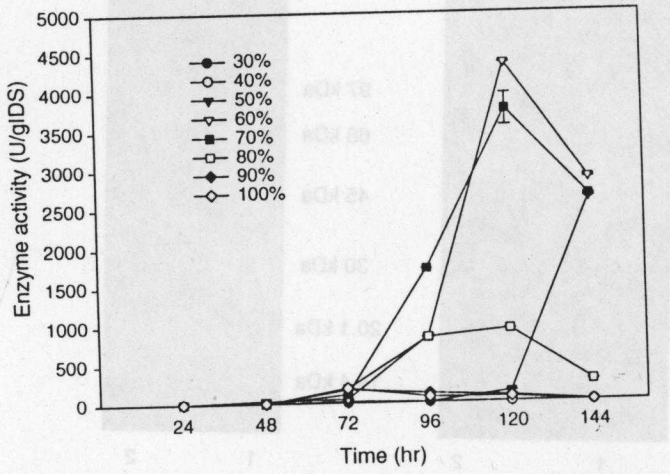

Fig. 1. Protease production by *E. album* under SSF. SSF was conducted using WB of particle size <425 μm moistened to varying levels with seawater and incubated at 28 °C.

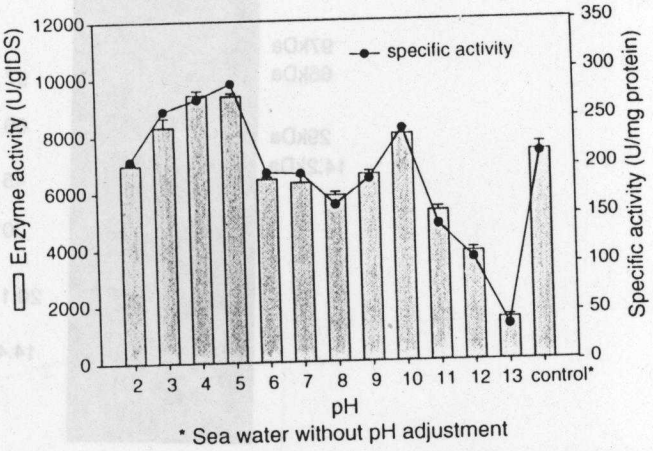

Fig. 2. Optimization of pH for protease production by *E. album*. SSF was conducted using WB of particle size <425 μm moisten to 60%, incubated at 28 °C and activity was assayed at 120 h.

Table 1
Yield and fold of purification

Sample	Total protein (mg/ml)	Total activity (U/ml)	Specific activity (U/mg protein)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude	1345	37120	28	100	100	1
$(NH_4)_2SO_4$ precipitated fraction (40–90%)	107	30455	286	8	82	10
Ion-exchange chromatography (DEAE)	7	3148	438	0.6	9	16

activity and specific activity and particularly addition of ribose and xylose caused a total repression of enzyme synthesis.

Except urea, all the other organic nitrogen sources tested showed a positive effect on the protease production by *E. album*. Although soybean meal (11,433 U/gIDS and specific activity 321 U/mg protein) and tryptone (11,340 U/gIDS and specific activity 289 U/mg protein) supported enhanced level of enzyme production, malt extract supported a maximal enzyme yield (11,487 U/gIDS). Of the inorganic nitrogen sources tested, ammonium hydrogen carbonate had a profound effect on enzyme production with maximal enzyme activity of 15,187 U/gIDS and specific activity of 380 U/mg protein, which was 94% higher than the control (7813 U/gIDS). Ammonium nitrate (9603 U/gIDS) and ammonium hydrogen phosphate (8465 U/gIDS) also promoted enhanced enzyme yield. In literature, protease production was found to be enhanced by nitrogen sources like tryptone, peptone, yeast extract, skim milk and soybean meal [5], whereas, ammonium ions were reported to inhibit enzyme production [20].

Most of the amino acids tested have a positive effect on the enzyme production. Among them, leucine attributed to maximum enzyme activity (12,731 U/gIDS) and specific activity (362 U/mg protein). Arginine, histidine and lysine contributed to 30% enhancement in enzyme yield when compared to the control (7813 U/gIDS). Whereas, methionine and tryptophan led to total reduction in enzyme activity and addition of these resulted in decrease of enzyme production to 10.4 and 7.8% compared to the control.

Time course experiment conducted for 10 days under optimized condition indicate that the enzyme production started on the 2nd day (i.e. 48 h) and reached a peak (11,540 U/gIDS) at 120 h. Further incubation beyond 120 h did not favour enhanced enzyme activity and instead resulted in a decline. Rapid decline in enzyme activity occurred after 168 h.

3.2. Enzyme purification

Result obtained for purification of crude enzyme is summarised in Table 1. The precipitate formed at 40–90% saturation of ammonium sulphate, which showed a 10-fold increase in specific activity compared to the crude sample, was used for further purification employing ion-exchange chromatography.

Elution profile from the DEAE-cellulose column furnished a single peak with protease activity, which could be eluted with buffer containing 0.2 M NaCl. This step resulted in 9% protease recovery (16-fold of purification) with a specific activity of 438 U/mg protein.

Purified protease subjected to native polyacrylamide gel yielded single band, testifying its homogeneity. Further, SDS-PAGE under non-reducing and reducing conditions also yielded a single band, which endorse the single polypeptide nature of the enzyme (Fig. 3A–C). Protease activity of this band was confirmed by a zymogram on X-ray film (Fig. 4A and B). The molecular mass of protease estimated by comparing the electrophoretic mobility of marker protein showed that the *E. album* protease has an apparent molecular mass of 38 kDa.

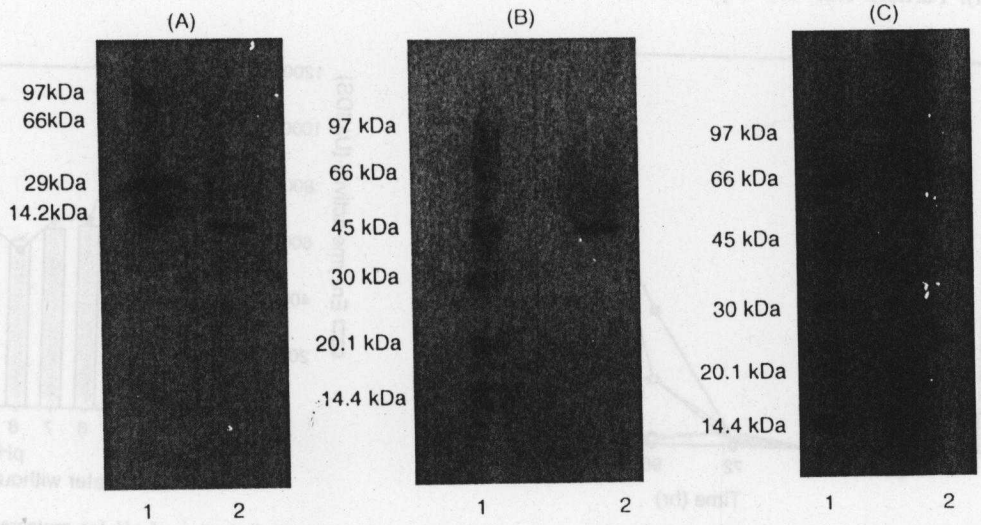

Fig. 3. Polyacrylamide gel electrophoresis of purified sample: PAGE analysis was conducted on 10% poly acrylamide gel. (A) Native-PAGE, (B) non-reductive SDS PAGE, (C) reductive SDS-PAGE. Lane 1: molecular weight marker, lane 2: fraction after ion-exchange chromatography.

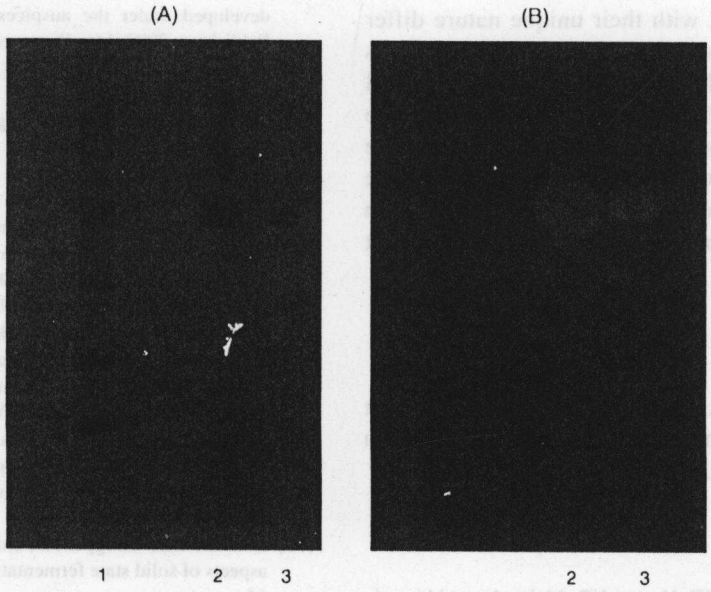

Fig. 4. Electrophoretic and zymographic profile of protease. PAGE was carried out on 10% polyacrylamide gel. (A) Gel on Coomassie staining; (B) zymographic analysis of the gel on X-ray film. Lane 1: molecular weight marker, lane 2: ammonium sulphate precipitated protease sample, lane 3: purified protease enzyme after ion-exchange chromatography.

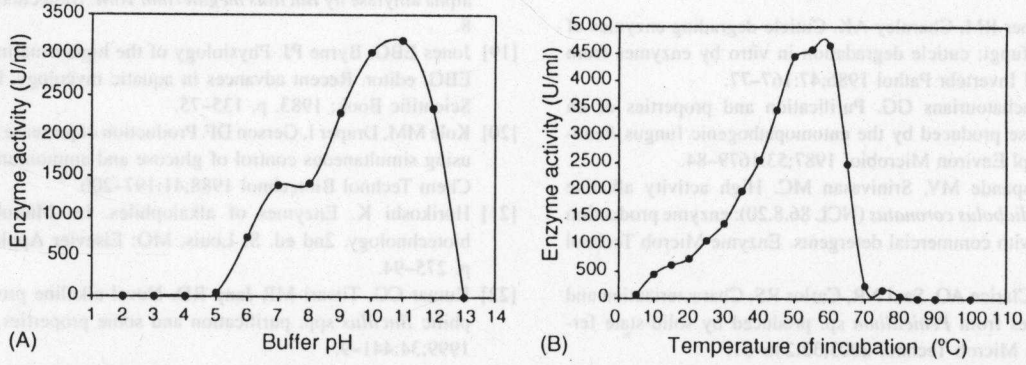

Fig. 5. Activity profile of the enzyme at different pH and temperature. (A) Activity at different pH: enzyme assay was conducted with casein prepared in different buffer systems of pH 2–13 at 40 °C. (B) Activity at different temperature: enzyme assay was carried out at pH 10 with casein as substrate at different temperatures.

3.3. Determination of optimal pH and temperature of purified enzyme

Although optimum pH for activity of purified protease was recorded between 10 (3035 U/ml) and 11 (3186 U/ml), maximal activity was recorded at pH 11 (Fig. 5A). However, more than 80% of the maximal activity was retained in the pH range between 9 and 12 and at pH below 8, enzyme showed only less than 50% of relative activity. Highest optimal pH for activity of alkaline protease reported earlier was between 10 and 10.5 [7] and only few reports are available where the optimal pH of activity was at 11 or above from some *Bacillus* sp. [21,22].

The enzyme was active over a wide range of temperature and maximal activity was recorded at 60 °C (4658 U/ml) (Fig. 5B). More than 90% of the maximal activity was conserved between 45 and 65 °C. A number of alkaline proteases isolated from *Bacillus* sp. have high optimal temperatures [22,23], whereas, there are only few reports on the fungal protease with high temperature optima [24]. This is an important characteristic required for use of these enzymes as detergent additives. Temperature and pH optima of our protease coincide with the two alkaline proteases produced by *Bacillus* sp., which is currently used in detergent industry under the trade name Savinase and Esperase.

4. Conclusion

The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood and other body secretions. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to the detergent are among the major prerequisites for the use of proteases in detergents. Currently, there is a lot of interest in the scientific community around the world in exploiting novel microorgan-

isms. Marine microorganisms, with their unique nature differ very much in many aspects from their terrestrial counterparts and are known to produce diverse spectra of novel useful substances. In this context, the results obtained during the course of this study indicate the scope for utilization of marine fungi for extracellular protease production through solid state fermentation, which is active at highly alkaline pH and at high temperature owing to its probable application in detergent industry.

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