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Deep-sea fungi as a source of alkaline and cold-tolerant proteases

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Abstract

Fungi from coastal environments have been widely studied with respect to the production of secondary metabolites and biotechnologically useful lignocellulolytic enzymes. A few studies on mycology of deep-sea sediments, however, have been carried out. This paper reports a study on alkaline, cold-tolerant proteases from deep-sea fungi. A total of 221 deep-sea isolates of fungi from 5000 m in the Central Indian Basin were screened for the enzyme. Many of these grew and produced alkaline protease at 5 and 30 °C and 1 bar pressure. *Aspergillus ustus* (NIOCC #20) producing the highest amounts of the enzyme was selected for further studies. The growth yield was substantial at 30 and 5 °C at 1 bar and elevated hydrostatic pressures. The fungus produced alkaline, cold-tolerant protease when grown at 30 °C and 1 bar pressure. The enzyme was active at combinations of 30, 5 °C and 50 and 300 bar pressure. However, protease production was negligible when the fungus was grown at 5 °C, under 1 bar or elevated hydrostatic pressures. The enzyme produced at 30 °C and 1 bar pressure was further characterized. The fungus produced a maximum of 1639 ACU mL^{-1} of protease by day 7. The enzyme, with molecular mass of 32 kDa and p*I* values of 6.6 and 6.9 showed several interesting properties. It had a broad pH range of 6–10, with an optimum at pH 9. The optimum temperature for protease activity was 45 °C and approximately 10% of the activity was retained at 2 °C. The enzyme was totally inhibited in the presence of 2 mM PMSF suggesting it to be a serine protease. It was active in the presence of several commercial detergents at 2 g L^{-1} concentration and in the presence of 0.5 M NaCl, equivalent to 29 parts per thousand salinity. In the presence of stabilizing agents such as glycerol, $CaCl_2$ its thermostability at 60 °C was enhanced. Heavy metal ions Cu, Hg, Fe, Ni and Zn did not inhibit the enzyme activity considerably. This study indicates that fungi from deep-sea sediments could be a useful source of proteases.

Keywords: Central Indian Basin; Sediments; Hydrostatic pressure; Deep-sea fungi; Proteases

1. Introduction

Fungi from marine habitats have received much attention in recent years for the production of useful secondary metabolites [1–3]. Research on marine fungi and biotechnologically useful enzymes produced by them, however, has been restricted to those isolated from coastal habitats and to lignocellulose degrading enzymes for application in bioremediation and paper industries [4,5]. Fungi and their enzymes from the deep-sea environment have received scant attention. Proteins and peptides constitute a substantial portion of the organic nutrients present in the deep-sea sediments as well as suspended particulate matter [6,7]. Therefore, extracellular proteases would play a pivotal role in the physiology of deep-sea fungi and in remineralization processes. Raghukumar and Raghukumar [8] reported production of protease enzyme under simulated deep-sea conditions by two filamentous fungi isolated from deep-sea calcareous sediments. About 25 and 75% of fungi isolated from the deep-sea sediments of the Central Indian Basin showed hydrolysis of casein and gelatin [9]. These studies suggested that deep-sea fungi could be a potential source of proteases.

Proteases are of immense interest in food, dairy, detergent, pharmaceutical and leather industries [10]. More than 25% of the worldwide sale of enzymes is contributed by proteases alone, where mainly alkaline proteases are used [10]. Alkaline proteases of bacteria from a variety of marine substrates, such as the hemolymph of a polychaete [11], the stomach of Antarctic krill [12], marine crab [13] and deep-sea sediments [14,15] have been described in recent literature. Extremophiles are an important source of enzymes and their specific properties are expected to be useful for novel applications [16]. In the light of the above, we have carried out a study on the production of alkaline, cold-tolerant proteases from fungi isolated from deep-sea sediments of the Central Indian Basin. The enzyme produced by one of the

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isolates was characterized in detail, and the results are presented in this paper.

2. Materials and methods

2.1. Isolation of fungi

Sediment samples were obtained from depths of around 5000 m in the Central Indian Basin (9–16°S and 73–76°E) on board the Russian research vessel AA Sidorenko, using USNEL-type box corer of 50 cm^3 size. Using alcohol-sterilized PVC cylinders of 5 cm diameter, subcores of sediments were collected from the center of the box corer. Subsections of 2 cm were extruded from these sediment cores of ~30 cm length directly into sterile plastic bags to avoid any aerial contaminants. The bags were closed with rubber bands and transported to the laminar flow hood in the laboratory on board. A portion of the sediment from the middle of each sub sample that had not been in contact with the walls of the PVC cylinder was removed using an alcohol flame sterilized spatula and placed in sterile vials for isolation of fungi [17]. Fungi were isolated using different techniques [18].

For a comparison between deep-sea fungal isolates and shallow-water ones, fungi were also isolated from sediments collected from 30 m depth of a coral lagoon of the Lakshadweep Islands in the Arabian Sea (11°N and 71°E) using similar technique [18].

2.2. Protease production

All the 221 deep sea and 22 shallow water fungal cultures isolated during the study were tested for their ability to produce alkaline protease using a qualitative plate assay on Czapek Dox agar (CDA) supplemented with 1% skimmed milk powder (Trade name Sagar, India). Clearance zone produced around the fungal colonies in plates indicated protease positive reaction [19]. The protease positive cultures were used for further quantification of the enzyme. This was done by growing the cultures in CD broth (Czapek Dox broth without agar) with 0.3% skimmed milk powder. Protease production was compared at 5 and 30 °C by growing the cultures at these temperatures. The culture supernatants collected by centrifugation at 10,000 rpm and 5 °C were used to assay the protease enzyme.

2.3. Protease assay

The protease activity was assayed with 150 μL of crude culture filtrate and 250 μL of the substrate azocasein (Sigma Chemicals, USA) at 2% concentration prepared in 0.1 M boric acid–borax buffer at pH 9. Protease activity was measured by incubating the reaction mixture at 30 and 5 °C for 30 min. The reaction was stopped by addition of 1.2 mL of 10% trichloroacetic acid solution. The contents were centrifuged at 8000 rpm for 10 min. To the supernatant, 1.4 mL of 1N NaOH was added and the absorbance read immediately at 440 nm against appropriate blanks in a spectrophotometer (Shimadzu, Model 1210, Japan). One ACU (Azocasein Digestion Unit) is defined as the increase in absorbance by 0.001 min^{-1} under the assay conditions [14]. One tyrosine unit is equivalent to μg tyrosine released mL^{-1} min^{-1} [20]. Accordingly, 1 ACU is equivalent to 0.05 tyrosine units.

Protease activity under elevated hydrostatic pressures was assayed in Eppendorf tubes of 0.5 mL capacity. The lids of the tubes were snapped off and sealed with parafilm after adding the reaction mixture, which contained the substrate azocasein, buffer at pH 9 and appropriately diluted enzyme. Care was taken to avoid trapping of air bubbles while sealing. The tubes were suspended in deep-sea culture vessel pressurized to the desired hydrostatic pressure and incubated under desired temperature for 30 min. At the end of the incubation period, the vessels were depressurized and without any delay the enzyme activity was arrested by adding TCA. Protease activity was measured as described above.

2.4. Characterization of enzyme

One of the cultures NIOCC #20, identified as *Aspergillus ustus* (Bain.) Thom and Church (deposited at the Institute for Microbial Technology, Chandigarh, India under the reference number MTCC 5102) showed the highest protease activity at pH 9, both at 5 and 30 °C temperatures among all the deep-sea fungal cultures and therefore was selected for further studies. The conditions for obtaining maximum protease production were partially optimized with reference to the pH of the medium, protein sources and the time of harvesting. On the basis of these results, the culture filtrate was collected by filtering first through glass fibre (GF/F) filters (Whatmann, USA) and then by durapore 0.22 μm (Millipore, USA) on day 7 and was concentrated in a speed vacuum concentrator (Biotron, Korea). The concentrate was passed through an anion exchange column 'Resource Q' (Amersham Biosciences, Uppsala, Sweden). It was eluted using a gradient of NaCl (0–0.25 M) prepared with 10 mM phosphate buffer at pH 7. The flow rate was adjusted to 0.1 mL min^{-1} and the eluted fractions of 1 mL each were collected. Fractions showing protease activity were pooled, concentrated and was further subjected to gel filtration column chromatography using Superdex 200 column (Amersham Biosciences, Uppsala, Sweden). The enzyme was eluted with 0.2 M NaCl prepared in 100 mM acetate buffer at pH 4.5. The flow rate was adjusted to 0.5 mL min^{-1} and the eluted fractions of 2 mL each were collected. Fractions showing protease activity were pooled and used for the characterization of the enzyme. The homogeneity of the fractionated enzyme was confirmed by running a native PAGE with 10% acrylamide at 60 V. The gel was blotted on 1.5% agarose containing 1% casein for 1 h. The agarose blot was stained with 0.15% amido black (Sigma Chemicals, USA) and 0.3% coomasie blue in methanol:acetic acid:water at a ratio of 4:1:5 [21]. The molecular weight of the purified enzyme was determined using SDS-PAGE. The electrophoresis was carried out on 12% resolving gel at a constant voltage of 60 V. The protein was detected using silver staining method [22]. The p*I* of the purified enzyme was estimated by running an isoelectric focussing tube gel. Broad range ampholytes of pH 3–10 were used. The pH gradient was formed by running the gel at 250 V for 30 min following which the sample was loaded onto the gel. The isoelectric focussing was carried out at 500 V for 4 h.

The optimum pH of the purified enzyme activity was determined using eight different buffers (100 mM): sodium acetate (pH 5), citrate phosphate (pH 5–7), phosphate (pH 6–8), Tris–HCl (pH 8 and 9), borax–boric acid (pH 8 and 9), glycine–NaOH (pH 9 and 10), borax–NaOH (pH 10) and carbonate–bicarbonate (pH 10 and 11) at 45 °C. Optimum temperature of the protease activity was determined at pH 9 by assaying at 2, 10, 15, 30, 45, 50, 60, 70, 80 and 90 °C. The K_m constant and V_{max} were determined from Lineweaver Burke plot at pH 9 and 45 °C using the substrate azocasein with purified enzyme. Thermostability of protease at different temperatures was assayed by incubating the enzyme samples at 40, 50, 60, and 70 °C for 10 min at pH 9 and then the residual activity was measured. Thermostability of purified enzyme at its optimum temperature (45 °C) was estimated by incubating the enzyme at 45 °C and the residual activity was assayed at an interval of 10 min. The stability of purified enzyme at different pHs was carried out by incubating 25 μL of enzyme with 75 μL of corresponding buffer for 1 h and then the residual protease activity was measured. Different additives were used to increase the thermostability of the enzyme at 60 °C. The effect of various inhibitors, heavy metals, reducing agents, ionic strength, bleaching agents and detergents on protease activity of this isolate was carried out at pH 9 and 45 °C. All chemicals used were of ultrapure quality (Sigma Chemicals from USA, Merck and Qualigens from India).

2.5. Growth and protease production under elevated pressures

NIOCC #20 was also tested for its ability to grow and produce protease at elevated hydrostatic pressures. Sterile plastic pouches containing 45 mL of Czapek Dox broth with 0.3% skimmed milk solution were inoculated with 5 mL of culture suspension (finely broken mycelia and spores in seawater). These were suspended in deep-sea culture vessels (Tsurumi & Seiki Co., Japan) filled with sterile distilled water. These vessels were pressurized at 50 and 100 bar (10 bar = 1 MPa) and incubated at 30 and 5 °C for 20 days. Three replicates were maintained for each treatment. After 20 days, the biomass was separated from culture broth by centrifugation and further filtered through sterile 0.22 μm hydrophilic durapore membrane filters (Millipore, USA). Protease activity of the filtrate was estimated using azocasein as substrate. The assay was carried out at the optimal conditions of the enzyme (pH 9; 45 °C and 1 bar pressure). The activity was also assayed under 50 and 100 bar pressure at pH 9 and 30 °C.

The extracellular protease produced by the culture when grown under atmospheric pressure (1 bar pressure) and 30 °C and 50 bar/30 °C was compared by assaying its activity under elevated hydrostatic pressure of 50, 100, 200 and

Table 1
Growth and alkaline (pH 9) protease production by deep-sea and shallow water isolates of fungi

Source of fungi	Total number isolated	Culture condition	Number and (%) isolates showing growth	Cultures with protease activity at 30 °C[a]	Cultures with protease activity at 5 °C[a]
Deep sea	221	1 bar and 30 °C	221 (100%)	105 (48%)	73 (33%)
		1 bar and 5 °C	113 (51%)	15 (13%)	12 (11%)
Shallow water	22	1 bar and 30 °C	22 (100%)	10 (45%)	3 (14%)
		1 bar and 5 °C	7 (32%)	2 (29%)	2 (29%)

[a] Percentage of cultures with protease activity was calculated from the total number of cultures showing growth (numbers in the 4th column).

300 bar at pH 9 and 30 °C. The K_m constants of the protease activity from cultures grown at 30 °C and 1 bar pressure were measured at 1 bar/5 °C and 50, 100, 200 and 300 bar/45 °C using the substrate azocasein at pH 9.

3. Results

A total of 221 and 22 fungi from deep-sea and shallow depth sediments respectively were obtained by employing different techniques of isolations (Table 1). Among these, 33% of the deep-sea and 14% of shallow water fungal isolates when grown at 30 °C and 1 bar pressure produced protease, that was active at alkaline pH and low temperature of 5 °C. Up to 51% of the deep-sea isolates and only 32% of the shallow water isolates showed ability to grow at low temperature of 5 °C. Among the cultures showing growth at low temperature, 11% of the deep-sea isolates and 29% of the shallow water isolates produced protease that was active at alkaline pH (9.0) and 5 °C (Table 1). The number of isolates showing protease activity at 30 °C was much higher than those showing activity at 5 °C. A large number of isolates showed alkaline protease activity within a range of 1–10 ACU mL^{-1} (Fig. 1). Measurement of protease activity using azocasein was found to be very sensitive because as low as 1 ACU could be detected.

A culture of *A. ustus* (NIOCC #20) from deep-sea sediments, with maximum protease production initially in the range of 30–40 ACU mL^{-1} when assayed at 30 °C and 1 bar pressure, and showing growth at elevated hydrostatic pressure and 5 °C was selected for further characterization of the enzyme. The culture showed growth under elevated hydrostatic pressures at 30 and 5 °C (Table 2). Protease production by this fungus was assayed both at 30 and 5 °C. The latter temperature revealed negligible or non-detectable levels of protease in all the assays. Therefore further comparisons of protease production under different growth conditions of elevated hydrostatic pressures were made by assaying at 30 °C.

Table 2 presents that only 12.35% of protease activity was detected when the fungus was grown at 5 °C and 1 bar pressure, with respect to the amount produced at 30 °C and 1 bar pressure. At 50 and 100 bar of pressure, a very low amount of protease was produced both at 5 and 30 °C. The protease synthesized by the culture grown at 1 bar pressure and room temperature, retained 100% of the activity when assayed at elevated hydrostatic pressure (up to 300 bar of pressure). Similarly the enzyme synthesized at 50 bar and room temperature retained 100% of its activity up to 300 bar pressure (Table 3).

NIOCC #20 showed maximum biomass production on day 3, when grown at 30 °C and atmospheric pressure while the maximum protease production was observed on day 7 in Czapek Dox broth with 0.3% skimmed milk powder (Fig. 2). The enzyme production was maximum on day 7 whether assayed at 5 or 30 °C

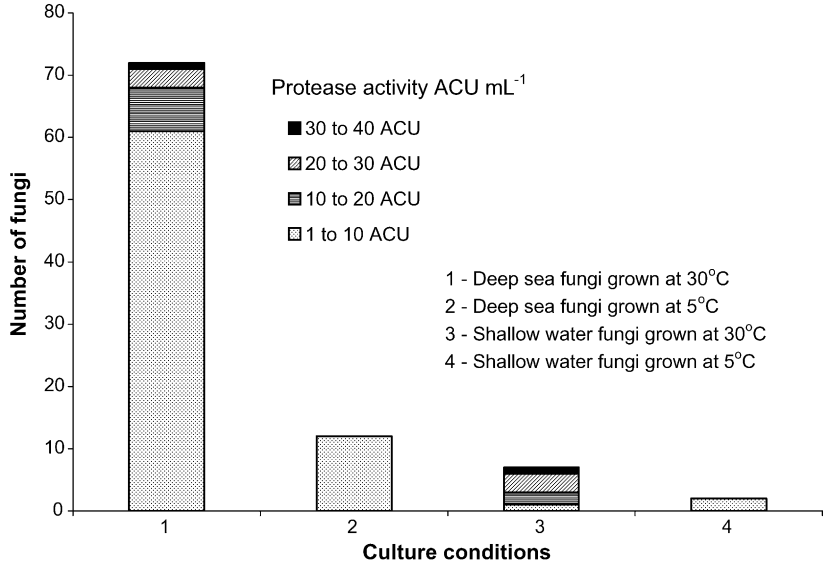

Fig. 1. Number of fungi isolated from deep-sea and shallow-water sediments showing protease activity in the range of 1–40 ACU mL^{-1} when grown at 5 and 30 °C. The NIOCC #20 belonged to the group showing 30–40 ACU mL^{-1}.

Table 2
Characteristics of the deep-sea isolate NIOCC #20 when grown for 20 days in CD broth with 0.3% skimmed milk powder under various pressure and temperature conditions

Growth temperature (°C)	1 bar	50 bar	100 bar	Parameters
30	208.2	69.4	44.1	Biomass produced (mg dry weight)
5	121.4	93.7	85.8	
30	36.02	3.67	0.78	Protease activity (ACU mL^{-1}) assayed at 30 °C and 1 bar
5	4.45	0.45	0.22	
30	100	10.2	2.17	Percentage activity against the highest value
5	12.35	1.25	0.61	
30	0.173	0.15	0.014	Protease activity/mg dry biomass
5	0.037	0.013	0.007	

The biomass represents the dry weight of the fungal mycelium. The liquid culture was filtered over a pre-weighed Whatmann No. 1 filter paper, dried to a constant weight at 50 °C and the weight determined. The values are mean of two replicates.

Table 3
Activity of the protease of the deep-sea fungus NIOCC #20 assayed under elevated hydrostatic pressures and 30 °C

Pressure (bar)	Protease activity (ACU mL^{-1})	
	A	B
1	427.33 ± 4.51	ND
50	356 ± 2.12	3.67 ± 0
100	363.33 ± 4.72	3.33 ± 0
200	417.33 ± 10.24	4.33 ± 0.24
300	428.33 ± 4.95	4.67 ± 0

A: protease produced when grown at 1 bar pressure/30 °C; B: protease produced when grown at 50 bar pressure/30 °C.

(Fig. 3). The culture showed maximum enzyme production in the medium adjusted to pH 9. Protease was produced in Czapek Dox medium without an organic nitrogen supplement (Fig. 4). Addition of milk, soy meal, malt extract, Tween 80, and corn steep liquor (CSL) increased the enzyme production by several folds (Fig. 4).

The protease was purified from 980 mL of culture supernatant using a three-step procedure that included speed vacuum concentration, anion exchange and gel filtration column chromatography. The enzyme eluted out as unbound fractions from Resource Q anion exchange column with 0.27-fold purification (Table 4). After gel filtration on superdex-200 column, the purity of the enzyme was 4.05-fold (Table 4). The homogeneity of the enzyme was further confirmed by activity staining on non-denaturing-PAGE. One distinct protease band was visualized on native gel (Fig. 5a). The molecular weight of the purified enzyme fraction obtained by gel filtration chromatography was 32 kDa as determined by SDS-PAGE (Fig. 5b). The purified enzyme had p*I* of 6.6 and 6.9 (Fig. 5c). This might be isozymes of protease which were resolved due to the presence of ampholytes in the gel. They might have been merged in the native gel where only one activity band was noticed.

The enzyme exhibited pH optima of 9 with >80% of activity between pH 6 and 10 at 45 °C (Fig. 6a). The protease activity was tested at temperatures ranging from 5 to 90 °C at pH 9 (Fig. 6b). The maximum activity was at 45 °C and about 45% of the maximum activity was detected at 20 °C and about 10% at 2 °C (Fig. 6b). Thermostability studies showed that the enzyme retained 80 and 55% of its activity at 50 and 60 °C respectively for 10 min at pH 9 (Fig. 6c). At its optimum temperature of activity (45 °C), the enzyme retained 100% of its activity for 30 min after which it steadily decreased (data not shown). The

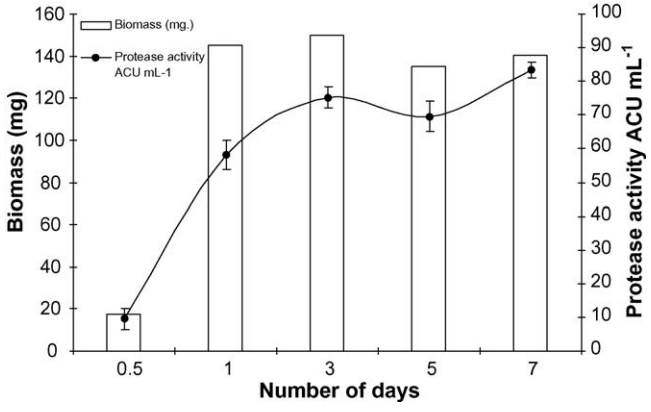

Fig. 2. Temporal production of biomass and protease by NIOCC #20 grown in CD broth with 0.3% skimmed milk powder. The protease assay was carried out at pH 9 and 45 °C with azocasein as substrate. The biomass was determined by filtering the cultures (in triplicates) on specified days over pre-weighed Whatmann filter papers and dry weight of the fungal mycelium was determined.

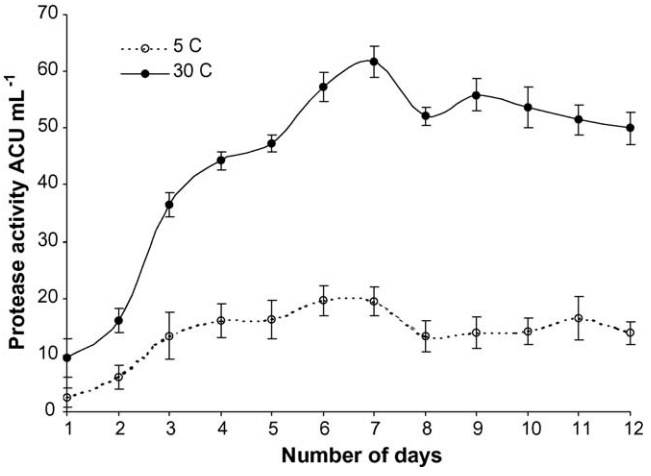

Fig. 3. Protease production by NIOCC #20 when grown at 5 °C (broken line) and 30 °C (solid line). Protease was assayed as described in Fig. 2.

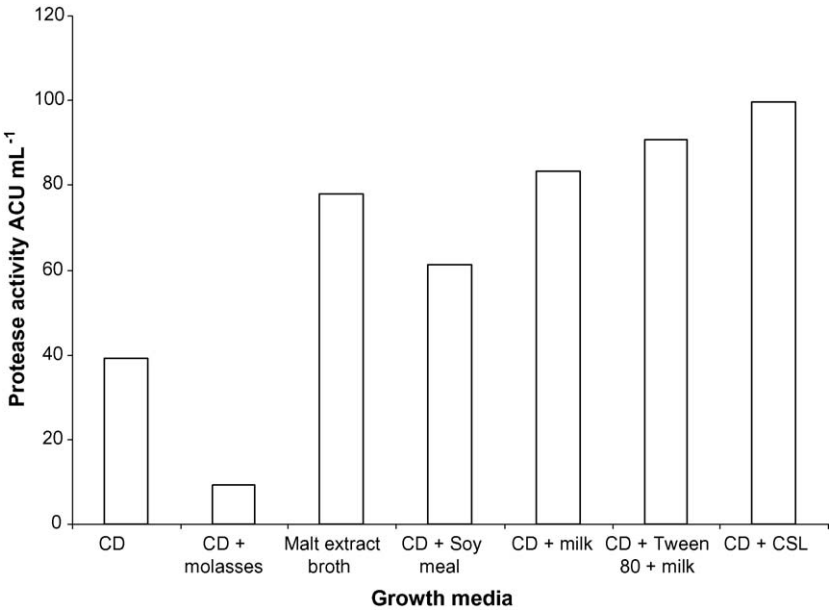

Fig. 4. Protease production by NIOCC #20 when grown at 30 °C in different media. CD = Czapek Dox broth, CSL = corn steep liquor.

Table 4
Purification of protease of the deep-sea fungus NIOCC #20 grown at pH 9.0 and 30 °C

	Total enzyme units (ACU)	Total protein (mg)	Sp. activity (ACU mg^{-1} protein)	Purification (fold)	Recovery (%)
Crude filtrate	981000	131.08	7484	1	100
Vacuum concentrated	205800	93.06	2212	0.3	21
Resource Q	166786	82.61	2019	0.27	17
Superdex-200	206800	6.82	30323	4.05	21

Protein was estimated using Folin phenol reagent [28].

enzyme was most stable at pH 4.5 in the absence of the substrate (Fig. 6d).

The protease activity was totally inhibited by 2 mM PMSF suggesting it to be a serine protease. EDTA at 5 and 100 mM concentration hardly inhibited the protease activity (Table 5) indicating that it was not a metalloprotease. The protease of NIOCC #20 is thiol-independent because reducing agents as DTT and mercaptoethanol did not affect the activity considerably (Table 5). At 1 mM concentration of Hg only 45% inhibition of the enzyme was observed. Addition of Fe, Ni and

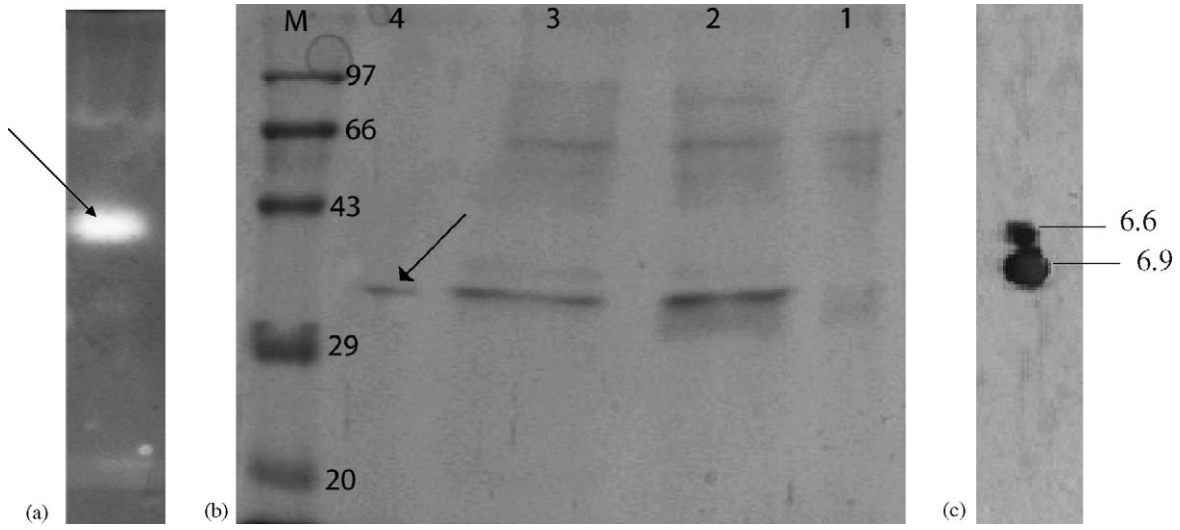

Fig. 5. (a) Zymogram of the active fraction on native page showed activity staining for protease. The clear zone in casein as a result of protease activity appears as a band. (b) The SDS-PAGE of the protease: lane 1 = crude culture filtrate; lane 2 = vacuum-concentrated culture filtrate; lane 3 = Resource Q-pooled fraction; lane 4 = pooled active fraction obtained by gel filtration using Superdex 200 showing single band of 32 kDa (arrow). (c) IEF gel showing isozymes with p*I* values of 6.6 and 6.9.

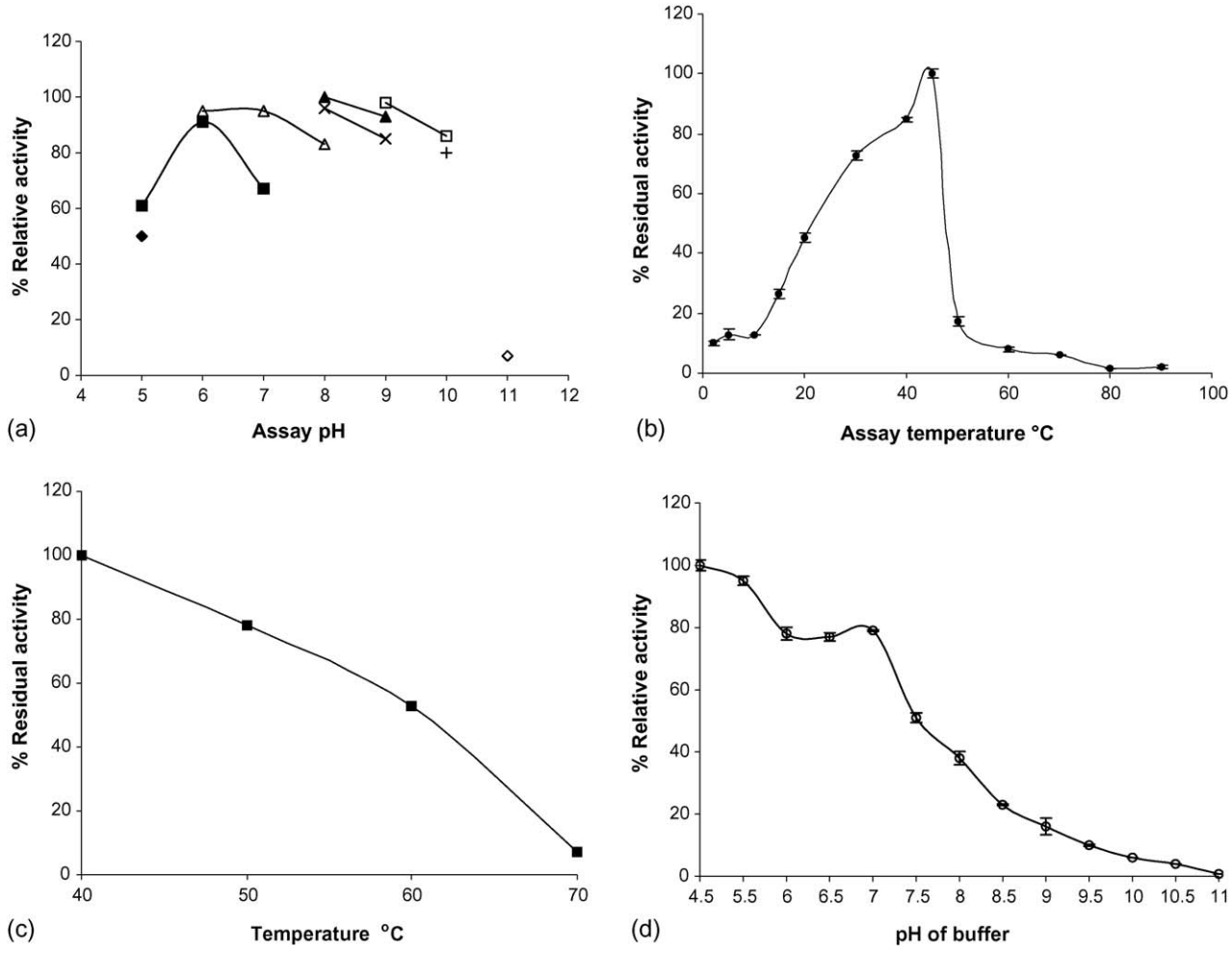

Fig. 6. (a) Relative activity of protease of NIOCC #20 at different pHs and 45 °C. Protease activity was measured as mentioned under Section 2: (▲) acetate buffer; (■) citrate phosphate buffer; (△) phosphate buffer; (×) Tris–HCl buffer; (▲) boric–borax buffer; (□) Glycine–NaOH buffer; (+) borax–NaOH; (◊) carbonate-bicarbonate buffer. (b) Relative activity of protease at different temperatures measured at pH 9. (c) Residual activity of protease was measured after incubating the enzyme sample at pH 9 for 10 min at different temperatures. (d) Stability of protease at different pHs was carried out by incubating the enzyme at specified pHs without the substrate for 60 min. Activity was measured at its optimum temperature (45 °C) and expressed as percent residual activity.

Zn at 1 mM hardly inhibited the enzyme activity. On the other hand, Cu at 1 mM concentration inhibited the protease activity totally (Table 5). About 70% of the activity was retained in the presence of sodium chloride of 0.5 M concentration which equals to 29 ppt salinity of seawater (Table 5). Chemical surfactants like Triton X-100 and Tween 80 (both at 1.0%) and commercial detergents at 2 g L^{-1} concentrations did not reduce the protease activity, although SDS (0.1%) totally inhibited its activity (Table 6). About 50% of the enzyme activity was lost after treatment at 60 °C for 10 min. However, thermostability of the enzyme at 60 °C increased on addition of glycerol, $CaCl_2$, PEG 6000, sucrose, mannitol, sorbitol and starch during enzyme assay (Table 7). The protease of the culture grown at 1 bar pressure showed a K_m constant of 2 mg mL^{-1} when measured at pH 9 and 45 °C and this increased marginally at elevated hydrostatic pressures of 50 and 100 bar (Table 8). There was a marked increase in K_m constant at 5 °C/1 bar, 45 °C/200 and 300 bar pressure. The V_{max} values were not much affected by the elevated hydrostatic pressures except a substantial drop at 50 bar pressure from that at 1 bar pressure (Table 8).

4. Discussion

The major aim of this study was to explore deep-sea fungi for production of cold-tolerant and alkaline protease. The isolate NIOCC #20 obtained after the screening produced substantial growth at 5 °C, under 1 and 100 bar pressure (corresponding to 1000 m). Under the above two conditions, it attained a biomass equivalent to 58 and 41% respectively of that produced at 30 °C/1 bar pressure (Table 2). However, when the fungus was grown at 5 °C under 1, 50 or 100 bar and the enzyme activity assayed at 5 or 30 °C under different hydrostatic pressures, little or negligible amount of cold-tolerant protease was detected. In contrast to the above, the enzyme produced by the fungus at 30 °C and 1 bar was much more versatile and retained about 10% of activity at 2 °C at 1 bar compared to its activity at optimum temperature (Fig. 6b). The enzyme produced at

Table 5
Effect of various inhibitors, heavy metals, varying ionic strengths (sodium chloride) and reducing agents on protease activity of the deep-sea fungus NIOCC #20

Inhibitors	Residual activity (%)
Protease inhibitors (mM)	
None (control)	100
PMSF (2)	0
EDTA (5)	99
EDTA (100)	93
Heavy metals (mM)	
$NiCl_2$ (1)	72
$ZnCl_2$ (1)	81
$HgCl_2$ (1)	55
$CuCl_2$ (1)	0
$FeCl_3$ (1)	74
Sodium chloride (M)	
0	100
0.1	88.6
0.25	80
0.50	70
0.75	61.4
1.0	40
Reducing agents (%)	
None	100
DTT dithiothereitol (0.1)	91
DTT (0.5)	73
β-Mercaptoethanol (0.1)	77
β-Mercaptoethanol (0.5)	75
Sodium thioglycolate (0.1)	45
Sodium thioglycolate (0.5)	27
Sodium thioglycolate (1.0)	27
Glutathione (0.1)	0

30 °C under 1 or 50 bar also retained its activity when assayed under elevated hydrostatic pressures up to 300 bar (Table 3). However, the increasing K_m constants of protease at elevated hydrostatic pressures of 200 and 300 bar (Table 8) indicates low enzyme-substrate affinity under these extreme conditions. The total enzyme activity per se is not affected by elevated pressures (Table 3), which is also indicated by the almost constant V_{max} values (Table 8).

Table 6
Effect of detergents and bleaching agents on protease activity of the deep-sea fungus NIOCC #20

Compound (concentration in %)	Residual specific activity (%)
None	100
Tween 80 (1.0)	142
Triton X-100 (1.0)	139
SDS (0.1)	0
Ala (commercial fabric bleach) (2.0)	20
Sodium hypochlorite (1.0)	2
H_2O_2 (2.0)	40
Commercial detergent wheel (2 g L^{-1})	91
Mr. White	85
Rin Shakti	64
Tide	62
Surf Excel	161
Ariel Compact	73

Table 7
Effect of additives on thermostability of the crude enzyme of the deep-sea fungus NIOCC #20 with reference to 10 min of heat treatment at 60 °C without any additive

Additive present during the heat treatment	Residual activity (%)
Control (untreated)	100
No additives	51
Glycerol (1%)	101.5
$CaCl_2$ (1 mM)	108.5
$CaCl_2$ (5 mM)	110.6
$CaCl_2$ (10 mM)	102.4
PEG 6000 (1%)	111.1
Sucrose (1%)	84.8
Mannitol (1%)	79.2
Sorbitol (1%)	85.6
Starch (1%)	98.8

Thus, although growth was fairly unaffected by elevated pressure and low temperature, protease production under similar conditions was reduced 10–100 folds. This appears to be a general trend with all the deep-sea isolates that we obtained (Table 1). Thus, a higher percentage of deep-sea isolates (51%) were capable of growing at 5 °C, when compared to those isolated from shallow waters (32%). Besides, a high number of deep-sea isolates produced low-temperature active protease when grown at 1 bar and 30 °C, but very few of these did so when grown at 1 bar and 5 °C (Table 1). Compared to the deep-sea isolates, fewer isolates from shallow water produced cold-tolerant protease when grown at 1 bar pressure and 30 °C and their growth was also poor at 5 °C. NIOCC #20 appears to be a strain adapted to deep-sea conditions, since it grew better at 5 °C/100 bar than at 30 °C/100 bar, although its production of protease under the former conditions was low (Table 2). This is in contrast to the report on a deep-sea bacterial strain DB6705, which grew better at higher rather than low temperatures when cultured at 500 bar hydrostatic pressure [23]. Growth and metabolic activities under extreme conditions are known to be low [24]. Our results with NIOCC #20 also show similar trends.

Enzymes from cold-adapted organisms have been classified into three groups [10]. Group I enzymes are heat-sensitive, but the other enzymatic characteristics are similar to mesophilic enzymes. Group II enzymes are heat-sensitive and relatively more active than mesophilic enzymes at a low temperature. Group III enzymes have same thermostability as mesophilic enzymes but are more active than mesophilic enzymes at a low

Table 8
K_m constant and V_{max} values of the purified protease of the deep-sea fungus NIOCC #20 measured under different conditions

Enzyme activity assayed at	K_m constant (mg mL^{-1} azocasein)	V_{max} (ACU mL^{-1})
1 bar and 45 °C	2.0	26.3
50 bar and 45 °C	2.2	19.2
100 bar and 45 °C	2.4	24.4
200 bar and 45 °C	3.3	25.0
300 bar and 45 °C	5.0	25.0

Table 9
Comparison of NIOCC #20 with other alkaline and low-temperature active proteases

Organism	Source of isolation	Substrate used	Maximum U	Definition of U	Optimum pH	Optimum temperature (°C)	Other properties	Reference
Alteromonas haloplanktis	Marine bacterium	MCA		nmol substrate released min^{-1}	8–9	20	74 kDa, thiol protease	[15]
Vibrio sp.	Deep-sea sediments	Casein	353 U mL^{-1}	μg tyrosine min^{-1}	8.5–9.0	40	35 kDa	[14]
Azospirillum sp.	Cold mountain soil	Casein	2 U mL^{-1}	Increase in OD of 0.1 min^{-1}	8.5	40	48.6 kDa, cysteine	[21]
Vibrio sp.	Cold marine source in Iceland	Azocasein					47 kDa, serine	[13]
Paecilomyces marquandii (fungus)	Not known	Casein, haemoglobin	0.016–0.018 U mL^{-1}		12	45	38 kDa	[29]
Nocardiopsis dassonvillei	Not known	Casein, haemoglobin	0.053 AU mL^{-1}	CPU = mM serine min^{-1}	8–9	60	Serine	[30]
Sphingomonas paucimobilis	Stomach of Antarctic krill	A variety of substrates		Tyrosine released	6.5–7.0	30 (47% of the activity at 0 °C)	Metalloprotease	[12]
Conidiobolus coronatus	Soil	Casein	30 U mL^{-1}	1 Abs mL^{-1} min^{-1} = U	9.7	37		[31]
Rhizophus oryzae	Not known	Azocasein	3370 U mL^{-1}	0.1 Abs h^{-1}	10.5	60		[32]
Bacillus mojavensis	Soil	Casein		μg mL^{-1} min^{-1} of tyrosine	10.5	60		[20]
Nocardiopsis sp.	Not known	Azocasein	50 U mg^{-1} protein	Abs h^{-1}	8.0	50	Serine protease	[33]
Paecilomyces lilacinus	Biocontrol agent	Azocasein						[34]
Bacillus sp.	Korean polychaete	Casein		1 μg tyrosine min^{-1}	10.0	45–50	Serine protease	[11]
A. ustus NIOCC #20	Deep-sea sediment	Azocasein	1639 ACU mL^{-1}	Abs 0.001 min^{-1}	6.5–10	45 (45% of the activity at 20 °C)	32 kDa, serine protease	The present work [35]

temperature. Accordingly, the protease of our isolate falls into the group II. The protease of mesophilic *Penicillium* sp. did not show any activity below 20 °C [25]. The alkaline protease from another mesophilic species of *Penicillium* did not show any activity below 35 °C [26]. On the other hand, NIOCC #20 showed about 45, 26 and 12% of the maximum activity at 20, 15 and 5 °C. It looses about 50% of its activity at 60 °C (Fig. 6c). Moreover, at its optimum temperature of activity (45 °C), it is stable only for 30 min after which it rapidly looses activity (data not shown). The enzyme is stable for more than 24 h at 5 °C and pH 4.5. Its half-life under these conditions is about 196 h. These results suggest its placement in the group II. The effect of temperature on the enzyme activity is determined by the temperature coefficient Q_{10} [27]. It is the factor by which the rate increases when the temperature is raised by 10°. If the rate of the reaction is completely temperature-independent, the resulting Q_{10} will be 1.0. If the reaction rate increases with increasing temperature, Q_{10} will be greater than 1. Thus, the more temperature-dependent a process is, the higher will be its Q_{10} value. The calculated Q_{10} value of 1 between 2 and 10 °C in the present work indicates temperature-independent enzyme activity at lower temperatures. On the other hand, the increased Q_{10} values (above 1) at 15–45 °C indicate direct relation to increasing temperatures. Above 45 °C, the Q_{10} value is less than one suggesting negative effect of temperature on the enzyme activity.

The present strain produced a maximum of 1639 ACU mL^{-1} of the enzyme, as estimated by the azocasein method. Application of different methods and the definition of enzyme units to estimate the enzyme by various authors using different substrates such as casein, haemoglobin, methylcoumarylaminoacid (MCA), azocasein or gelatin make the comparison difficult. However, our estimations have shown that the protease activity per mL in the crude culture filtrate of NIOCC #20 corresponds to 84 μg of tyrosine released. The protease production by NIOCC #20 appears to be as good as or even better than those reported (Table 9).

The protease of NIOCC #20 is a serine protease because it was totally inhibited by PMSF which is a known inhibitor of serine protease. It was not inhibited by EDTA and therefore it is not a metalloprotease. It was not substantially inhibited by the heavy metals Ni and Zn which are the inhibitors of cysteine protease. Its activity did not increase in the presence of thiol compounds, indicating that it was thiol-independent serine protease. Protease produced by this isolate showed several special features. It was active in the presence of several commercial detergents when assayed under its optimum conditions. The enzyme was active in the presence of 0.5 M NaCl equivalent to 29 ppt of seawater salinity. Its stability at 60 °C increased in the presence of various stabilizing agents. Such features in proteases are often much sought after for use as additives in detergents for low-temperature wash [10]. Most alkaline proteases have been reported to be significantly stabilized in the presence of additives such as those used by us [20].

NIOCC #20 secreted maximum extracellular protease among all the deep-sea fungi isolated and when grown in a medium prepared with seawater at pH 8 and 9 and thus is truly alkalophilic. Protease production occurred in Czapek Dox medium without an organic nitrogen source also and thus the enzyme appears to be constitutive (Fig. 4). Its production, however, could be enhanced in the presence of milk, soy meal, and corn steep liquor.

Protease of *A. ustus* (NIOCC #20) with an optimum pH of 9 and showing about 45 and 25% of its activity at 20 and 15 °C respectively is an alkaline protease. Its performance in terms of activity can be further improved by optimization of growth and other parameters. Terrestrial isolate of *A. ustus* (isolate # MTCC 2200) obtained from IMTECH culture collection showed extremely low protease production (23 ACU mL^{-1}) under similar assay conditions. This study shows that deep-sea fungi are useful candidates in the search for alkaline proteases.

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