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Purification and characterization of alkaline protease from *Lysinibacillus fusiformis*

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Abstract

A novel alkaline protease producing bacterium was isolated from the gut of an estuarine fish *Etroplus suratensis*. The strain was identified by sequencing the fragment of their bacterial 16s rRNA and its homology was 97% closest to the *Lysinibacillus fusiformis.* An extracellular protease from this organism was purified by acetone precipitation, ion exchange chromatography and gel filtration chromatography methods and the specific activity of the purified enzyme was found to be 20.39 U/mg, 169.46U/mg and 352.0U/mg respectively. The molecular weight of the purified enzyme was determined to be 29kDa through SDS/PAGE analysis. The enzyme showed that the maximum at pH 9.0 and temperature at 40ºC. The purified enzyme remains active in the presence of various metal ions and it was strongly stimulated by the addition of Ca^{2+}. Among the tested surfactants, the optimum activity was observed in SDS when compared to the other tested surfactants.

Keywords: Ion exchange chromatography, Acetone precipitation, Gel-filtration chromatography, 16s rRNA sequencing, *Lysinibacillus fusiformis*

Introduction

Micro flora of the digestive tract of fish produces antibacterial material preventing pathogenic bacteria from getting into an organism. Further, it has also been reported that aquatic microbes synthesize exoenzymes identified as inducible catabolic enzymes (Bhaskar et al. 2006). In recent years, proteases from the gut of fishes have received much attention due to microbial proteases are an important group of enzymes that can have application in various industries process (Chi et al. 2007). Moreover, protease production is influenced by some physical factors such as aeration, inoculum size, pH, temperature and incubation time and biological factors such as the genetic nature of the organism. In general, no defined medium has been established for better production of any metabolite, because of the genetic diversity that exists in microbes. Therefore, it is worth to have a detailed investigation on newly isolated microbial strain for production pattern under different environmental conditions and in an optimized pattern to achieve maximum production (Elliah et al. 2002). Hence, the present work was undertaken to investigate the purification and characterization of the fish gut isolate *Lysinibacillus fusiformis*.

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Materials and methods

Isolation and screening of proteolytic bacterial strain

Protease producing bacterial strain was isolated from the gut of *Etroplus suratensis* an estuarine fish from Rajakkamagalam estuary located in Kanyakumari district, southwest coastal of India. The isolated organisms were plated on skim milk agar plates and the plates were incubated at 35ºC for 24h. A clear zone formation of skim milk hydrolysis gave an indication of protease producing organisms. Depending upon the production of haloalkalophilic protease by qualitative and quantitative assay, the candidate species was selected for further experimental studies. The protease production in the liquid medium was assessed first by enriching the bacteria in enrichment medium containing beef extract (0.3 %), peptone (0.5 %), NaCl (1 %) and glucose (0.5 %) at pH 9 for 24 h. The culture was then incubated for 2 days by shaking at 35°C. The cells were then harvested by centrifugation at 10000 rpm for 15 min and the supernatant was used for further protease assay.

Genomic DNA extraction, Cloning and sequencing of 16S rRNA gene

The isolated bacterial strain was grown in 2ml Zobell Marine Broth (HiMedia, India) overnight at 27^{o} C. The culture was centrifuge at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1h at 37°C. To this tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase K from 20 mg/ml were added and incubated at 55^{0}C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water. The amplified product (1,500-bp) was purified using GFX ™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA) according to manufacturer's instruction. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit #K1214, MBI Fermentas). Full-length sequencing of the rRNA gene (about 1500 bp) for the isolated bacteria was carried out in Macrogen (Seoul, Korea). The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST (Altschul et al. 1997).

Protease assay

The assay systems consist of ingredients such as 1.25 ml Tris buffer (pH 7.2), 0.5 ml of 1% aqueous casein and 0.25 ml culture supernatant. Approximate controls were maintained. The mixture was incubated for 30 min at 30˚C and 3 ml of 5% TCA was added to this mixture and placed at 4˚C for 10 min to form precipitate. Then it was centrifuged at 5000 rpm for 15 min. From this, 0.5 ml of supernatant was taken, to this 2.5 ml of 0.5M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of folin phenol reagent and the absorbance was read at 660 nm using UV-Vis Spectrophotometer (TECOMP 8500). The amount of protease was estimated and expressed in microgram of tyrosine released under standard assay conditions.

Enzyme purification and molecular mass determination of protease

Purification was carried out at 4°C. Enzymes (supernatant of fermented broth obtained after centrifugation at 10 000_ g for 10 min) were precipitated by adding two volumes of acetone and kept for 1 h at 0 - 4°C to allow complete precipitation. The resulting precipitate was collected by centrifugation (10 000 rpm, 30 min) and the pellet was air dried and resuspended in a minimal volume of 20 mM Tris HCL buffer, pH 7.2. Finally, to remove the insoluble materials by centrifugation (10 000 rpm 30 min), the supernatant was applied to ion exchange chromatography. Ion exchange chromatography was performed of DEAE-cellulose (2.5 - 30 cm) column, which was preequilibrated with 20 mM Tris HCl pH 7.2. After loading the sample, the column was washed with the same buffer until the optical density of the elution at 280 nm becomes zero. The bound proteins were then eluted with a linear gradient of sodium chloride in the range of 0.1-1M in the equilibrating buffer. Fractions (4.0 ml each) were collected at a flow rate of 40 ml/h. Fractions were collected at a flow rate of 1ml/min and the enzyme activity and protein content of the each fraction were determined. Active fractions showing protease activity from the anion exchange column was centrifuged at 10 000 rpm for 10 min. The collected fractions were dialyzed against the same buffer without NaCl. The residue was dissolved in 20 mM Tris HCL buffer, pH 7.2 applied to gel filtration chromatography (Sephadex G-100). The column was washed and eluted with above mentioned buffer. A fraction of 4ml in sample was collected at a flow rate of 1ml/min and protein content and enzyme activity was assayed in each factions. Equilibration was done using 0.1 M NaCl in 20mM Tris HCL buffer, pH 7.2. SDS-PAGE (12%) was performed according to the method of Laemmli under reducing conditions (1988). The molecular weight was determined from linear semi-logarithmic plots of molecular weight versus the Rf value.

Effect of pH, temperature, surfactants and metal ion of purified enzyme activity and stability

Stability of the purified protease was tested by adjusting the pH of following buffers: sodium acetate, sodium phosphate and Tris HCl. To ensure the pH stability and the enzyme reactions mixture were incubated at 40°C for 1 h and the relative activity was measured. The enzyme stability of the purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 20°, 30°, 40°, 50°, 60°, 70° and 80°C for 1 h and relative activities were assayed at standard assay conditions. The crude enzyme (0.5ml) was pre- incubated with triton X-100, SDS (Sodium Dodecyl Sulphate), PEG (Poly Ethelene Glycol), tween20 and tween80. Then the enzyme activity was then determined at 50°C by using 0.2% casein as a substrate dissolved in 0.2M Carbonate buffer (pH 10.5). The activity of the enzyme without any surfactants was taken as 100% (Doddapaneni et al. 2007). For selection of suitable metal ion source for protease activity, eleven different metal ions were screened.

Effect of EDTA concentration on protease stability

The protease enzyme was reacted with 5ml of casein containing different concentration such as 0.02%, 0.04%, 0.06%, 0.08% and 0.1% of EDTA. It was incubated for 30 mins at 35ºC. Then the protease activity was studied by the standard assay procedure.

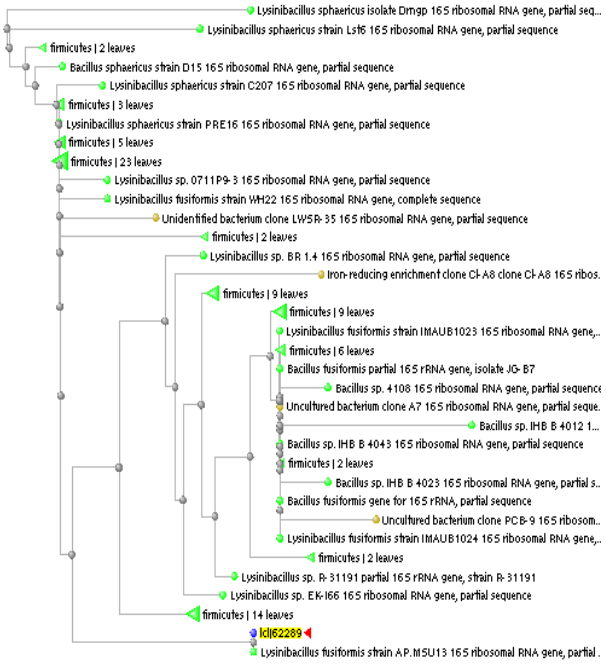
Results and discussion

Characterization of the protease-producing bacteria

Based on the morphological, physiological and biochemical characteristics, the suspected colony was identified as *Lysinibacillus fusiformis* following the standard keys of Bergey's Manual of Determinative Bacteriology and phylogenetic studies revealed that the 16S rRNA gene sequence of the strain *Lysinibacillus fusiformis* show 97% similarity with the nearest match in the Genbank (Fig 1).

Enzyme purification

The protease activity of crude enzyme extract was concentrated by acetone precipitation. Ninety percent protease recovery was achieved with 1.6-fold purification (Table 1). Following acetone precipitation, the resuspended solution was applied to a column of DEAE-cellulose. Fig. 2 shows the anion exchange profile. Most protease activity was observed in a single peak of the eluted fraction with 49.76-fold purification with the specific activity of 169.46 U/mg. The active fraction from the previous step was applied to the gel filtration column. Fig. 3 shows the gel filtration profile chromatography where only a single peak with protease activity was noticed. This step resulted in 17% protease recovery (43.37-fold purification) with a specific activity of 352.0 U/mg of protein. Purified protease from *Lysinibacillus fusiformis* migrated as a single band of 29 kDa in SDS-PAGE under reducing conditions, suggesting that the purified protein was homogeneous (Fig. 4). The results were similar to that of Kim et al. (2001) where the molecular weight of protease was found to be 34 – 45 kDa.



Figur 1: Phylogenetic studies revealed that the 16S rRNA gene sequence of the strain *Lysinibacillus fusiformis*

Table 1 Purification table for protease from *Lysinebacillus fusiformis*

Step	Total Activity (unit)	Total Protein (mg)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Culture Supernatant	520	6.4	81.25	1	100
Ammonium Sulfate	486	2.1	238.09	2.93	93.46
DEAE cellulose	381	1.5	169.46	19.7	78.39
Sephadex G100	225	0.24	937.5	11.53	43.26

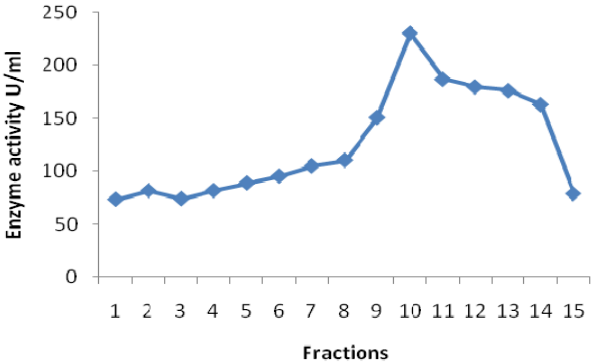


Figure 2: Enzyme activities in purified fractions by DEAE Cellulose

Effect of physical factors on protease activity

Table 2 revealed the effect of temperature on protease stability. The unpurified enzyme, i.e. proteolytic activity of crude extract was thermo stable in the temperature range 30 to 40°C. Irreversible inactivation of the enzyme activity was found to be 60°C. The optimum temperature for protease stability was found in 40ºC where the enzyme was assayed with casein as the substrate. Merkel and Sipos (2005) reported a protease from marine *Vibrio* sp, the temperature optimum was found to be 40 to 50ºC. For determination of optimum pH protease stability was found to be maximum at pH 7.0 using Tris HCL (Table 3). Rashbehari Tunga (2003) also reported that the optimum temperature and pH was 40ºC and 8.0 respectively. Similar behavior was found in the purified *B. cereus* T protease and SP 87 protease (Jadwiga, 1998). Table 4 shows the

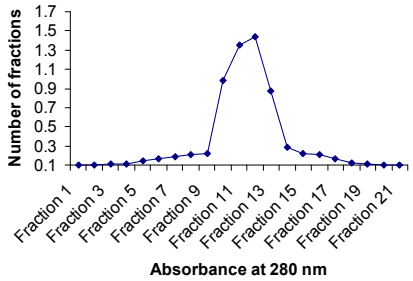


Figure 3 Enzyme activities in purified fractions by Sephadox G 100

Table 2 Effect of various temperatures on protease activity form *Lysinebacillus fusiformis* in reaction mixture maintained for 15mins incubation

S. No	Temperature (^{0}C)	15 min (U/ml/min)	Percentage (%)
1	20	360	66.59
2	30	540.6	100
3	40	528	97.66
4	50	513	94.89
5	60	491.4	90.89
6	70	480	88.79

Table 3: Effect of various pH on protease activity from *Lysinebacillus fusiformis* in reaction mixture containing various pH at 30ºC for 15mins incubation

S. No	pH	U/ml/min	Percentage (%)
1.	5	318	80.06
2.	6	350.4	88.21
3.	7	352.8	88.82
4.	8	356.2	92.44
5.	9	397.2	100
6.	10	367.8	92.59
7.	11	288	72.50

Table 4: Effect of various metal ions on protease activity from *Lysinebacillus fusiformis* in reaction mixture containing various metal ions at 30ºC for 15mins incubation

S. No	Metal ions (5mM) concentrations	U/ml	Relative activity (%)
1	Ca	274.43	100.00
2	Mg	263.31	95.94
3	EDTA	177.43	64.91
4	Fe	206.61	75.28
5	Zinc	228.24	83.16

Table 5: Effect of various surfactants on protease activity from *Lysinebacillus fusiformis* in reaction mixture supplemented with 0.5ml surfactants at 30ºC for 15mins incubation

S.No	Surfactants	U/ml/min	Percentage (%)
1.	PEG	621.6	90.79
2.	SDS	633.6	92.55
3.	Tritonx-100	613.2	89.54
4.	T20	621.6	90.79
5.	T80	682.8	99.73
6.	Control	684.6	100

effect of 5 mM cation on protease activity. The activity of the protease in presence of Ca^{2+} was roughly 100% higher than the control solution and Mg was found to be 93% where Mn^{2+}. Table 5

and 5a revealed that the effect of surfactants on protease activity. The protease was stable in the presence of 0.5ml SDS at 30C for

Table 5a: Effect of various concentrations of SDS on protease activity from *Lysinebacillus fusiformis* at 30ºC for 15mins incubation

S. No	SDS	15 Min (U/ml/min)	Percentage (%)
1.	0.1	501	73.18
2.	0.2	574.8	83.96
3.	0.3	261.6	38.21
4.	0.4	582.6	85.10
5.	0.5	621.6	90.79
6.	Control	684.6	100

Table 6: Effect of EDTA concentrations on protease activity

EDTA concentrations (%)	*Lysinebacillus fusiformis*	
	U/ml	Relative activity (%)
0.02	312.79	79.43
0.04	393.07	100.00
0.06	362.83	92.30
0.08	376.06	95.67
0.1	341.63	86.91

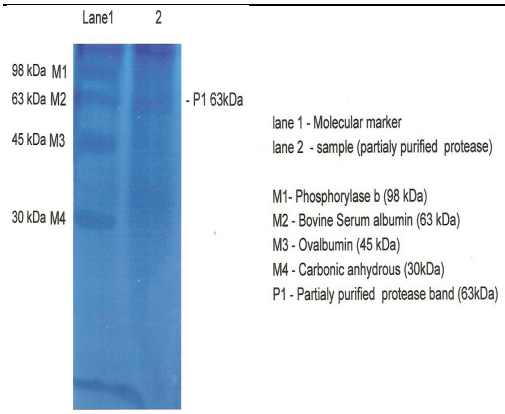


Figure 4: SDS-PAGE profile of purified protease

15mins incubation. The stability of TC4 protease against SDS was greater than ES1 from *Aspergillus clavatus* ES1 (Hajji *et al*., 2007), which retained about 33% after incubation for 1 h with 0.5% SDS, but lower than proteases from *Bacillus* sp. RGR-14 (Oberoi et al. 2001). In this study, the maximum protease activity was recorded in 0.06% EDTA supplemented medium (Table 6). This study was correlated with earlier report of Hoshono et al. (1997) who reported that the maximum protease activity was seen in 48 hours of incubation by bacteria in medium containing EDTA.

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