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Isolation and characterization of protease producing actinobacteria from marine crab

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ABSTRACT

For many years, actinobacteria were best known as a source of large numbers of antibiotics. More recently, they have been found to be a promising source of a wide range of important enzymes. Studies on proteolytic marine actinobacteria especially in the southeast coast of Tamilnadu have not been carried out adequately. Actinobacteria were isolated from the flesh content of the Mud crab, *Scylla serrata*. This is first report on isolation of protease producing actinobacteria from crab. These crabs have a high tolerance to both nitrate and ammonia. Partial purification of the enzyme by DEAE Cellulose yielded 2.16-fold purity. *Streptomyces nigellus* showed highest enzyme activity at pH7 and 40°C. Out of the 7 morphological different strains, the strain LK-3 which was tentatively identified *as Streptomyces nigellus* showed protease activity. Therefore, the present study was undertaken to isolate the proteolytic actinobacteria from the crab.

Introduction

The biological and chemical diversity of the marine environment has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, enzymes, fine chemicals, and agrichemicals (Ireland et al., 1993). The oceans represent a virtually untapped resource for the discovery of even more novel compounds with useful activity. Although the commercial success stories in biotechnology are familiar, such stories in marine biotechnology are far less familiar and far fewer (Zilinskas et al, 1995, Karthik et al., 2010a). In the last decade, there has been a continuous effort to learn more about the still largely unexplored realm of marine enzymes.

A marine enzyme may be a unique protein molecule not found in any terrestrial organism or it may be a known enzyme from a terrestrial source but with novel properties. Beside microorganisms like bacteria, fungi, and actinobacteria, many other marine organisms such as fishes, prawns, crabs, snakes, plants, and algae have also been studied to tap the arsenal of the marine world. Properties like high salt tolerance, hyperthermostability, barophilicity, cold adaptivity, and ease in large-scale cultivation are the key interests of scientists. These properties may not be expected in terrestrial sources as marine organisms thrive in habitats such as hydrothermal vents, oceanic caves, and some areas where high pressure and absence of light are obvious (Ghosh, 2004, Karthik et al.,2010c).

Actinobacteria occur in wide range of environments producing a variety of scientifically interesting and commercially useful high value metabolites. Marine actinobacteria have recently emerged as rich source for the isolation of industrial enzymes. Enzymes, after antibiotics, are the most important product of actinobacteria. For many years, actinobacteria were best known as the source of large numbers of antibiotics. More recently, they have been found to be a promising source of a wide range of important enzymes. Recently, while most of the studies on actinobacteria have focused on antibiotic production, only few reports are on their enzymes potential (Tsuchiya, 1997, Chaphalkar and Deys, 1998, Schippers et al., 2002). The present study was designed to isolate and purify the protease from marine actinobacteria and also identify the potential protease producing strains through conventional and chemotaxonomical methods.

Materials and Methods

Isolation of actinobacteria

In the present study, Mud crab, *Scylla serrata* was collected using cast net from the south east coast of India (Fig 1). Sample was kept in sterile polyethylene bags and transported to the laboratory under ice for microbiological analysis. The body surface was wiped with 70% ethanol. The flesh content was removed aseptically and then one gram of the squeezed out flesh contents were taken and homogenized separately in a sterile mortar and pestle. The sample was serially diluted with filtered and sterilized 50% seawater. One ml of the serially diluted sample was plated in petriplates containing Kuster's agar medium and incubated at 35°C for seven days. The leathery colonies of actinobacteria that appeared on the petriplates were counted from the 5th day onwards upto 28th day (Karthik et al., 2010b).

Fig. 1 Study area

Screening of Proteolytic actinobacteria

Protease activity of the strains was screened qualitatively in Gelatin agar medium. After inoculation, the plates were

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incubated at 36±1°C for five days. After incubation, the plates were flooded with 20% aqueous solution of salphosalycylic acid. The clear zones around the colony were indicated as the protease activity. The experiment was performed in triplicates (Cruickshank et al., 1975).

Preparation of protease enzyme

Actinobacteria isolated from the crab flesh were screened for protease activity. The packed cells were suspended in distilled water and this was inoculated into 5 ml of gelatin broth and incubated for seven days at 37°C temperatures. After the 7th day, the broth was centrifuged at 10,000 rpm for 15 min. and cell free supernatant was used for protease assay.

Enzyme assay

The original casein assay was first described by Kunitz (1947) and later modified by Detmar and Vogels (1971). It involved TCA precipitation of the undigested substrate, followed by photometric quantification of the released aromatic amino acids, using L-tyrosine as a standard.

Optimization of temperature and pH

The fermentation was carried out at various temperatures such as 32°C, 34°C, 36°C, 38°C, 40°C and 42°C to study their effect on enzyme production. Different pH concentrations viz. 6, 7, 8, 9, 10 and 11 of the gelatin broth were prepared using buffer solution. All the experiments were carried out in triplicate at optimum temperature and average values were reported.

Partial purification of enzyme

The partial purification of the protease was carried out by using standard protocol. The packed cells were suspended in distilled water and these were inoculated into 5 ml of gelatin broth and incubate for 7 days at 37°C. After the 7th day, the broth was centrifuged at 10,000 rpm for 15 min and cell free supernatant was used for protease assay.

Supernatant and the pellet suspended in a mixture of volume of buffer were used for the enzyme assays. The crude extract was treated with potamine sulphate and centrifuged at 27,000 rpm for 10 min.

This supernatant was partially purified using the following three sub-sequential steps.

The supernatant was brought to 60% saturation by mixing ammonium sulphate (pH 8.5) slowly with gentle agitation and allowed to stand for 24 hrs at 4°C in the cold room. The pH of the supernatant was maintained at 8.5. After the equilibration, the precipitate was removed by centrifugation (10,000 rpm at 4°C for 20 min). The precipitate obtained was dissolved in 10 ml of 0.5 M Tris-HCL Buffer (pH 8.5) and the protein content was estimated.

The precipitate was desalted by dialysis. One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, dialysis bag was suspended in a beaker containing 0.5 M Tris-HCL buffer (pH 8.5) for 24 hrs and then it was transferred to 5% sucrose solution.

The dialyzed enzyme solution obtained from the previous step was loaded into a DEAE cellulose column. Pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.5).After loading the column, it was washed with the same buffer. Proteins were eluted with 10 mM Tris-HCl and 0 to 2.0 M NaCl gradient. Eluted fractions (3 ml) were collected and absorbance was measured at 280 nm. The protease activity and protein concentration of fractions was measured and specific activity calculated.

Zymography

a. Zymography was performed as described by Schmidt et al (1988) with minor modifications. Gelatin (10 mg/ml) was incorporated in 10% separating gel and 5% gel without gelatin was used as stacking gel.

b. The sample was mixed with an equal volume of a buffer (non reducing reagent) of 4% SDS, 20% Glycerol, 0.25 M Tris-HCL, pH=6.6 and 0.02% bromophenol blue without heat treatment and required volume was added to the well and run at 100 volts.

After electrophoresis, the gels were placed in individual polystyrene petridish and washed successively with 2.5% (v/v) triton X-100 (2 times, 10 min each) at room temperature. Further, the gel were washed for 30 minutes in 50 mM Tris-HCL buffer, pH= 7.6, containing 2.5% triton X-100 with gentle agitation, in order to remove the excess of SDS. After washing was completed, 50 mM Tris-HCL buffer pH= 7.6 was poured into dishes containing the gels and dishes were covered and incubated at 37°C for 1.5 hrs. Following incubations, gels were fixed for 10mins, stained with Commassie brilliant blue and destained to reveal zones of substrate lysis.

Statistical analysis

All analyses were performed in triplicates and expressed as mean ± standard deviation (SD). Excel was used for the statistical evaluation of the present study.

Results and Discussion

In the present investigation, the population density of actinobacteria was recorded 7 X 10^3 CFU/g from Marine crab. A total of 7 morphologically different actinobacterial strains were selected to test the protease activity.

Earlier Sahu et al (2008) recorded higher population density of actinobacteria from marine shrimps than the present study. The lower population in the present study was may be because the sample was collected and analyzed one time only.

Further, repeating sampling could provide more density. During the present study, among the 7 strains, 4 strains showed the protease activity (Table 1). Among the 4 strains, only one strain viz. LK-3 showed more activity, which was selected for further study. The ability to produce a variety of proteolytic enzyme is a well known phenomenon in mesophilic actinobacteria isolated from terrestrial sources. Few reports on proteolytic enzymes isolated from marine origin have been reported. Sahu et al. (2008) reported protease producing actinobacteria from the gut content of the shrimp.

Total protein, Total activity, Specific activity, Fold purification and Yield was estimated and presented in the Table 2. Temperatures ranging from 32ºC - 42ºC were examined for the detection of optimum temperature for the protease production and found that a temperature of 40ºC (Fig. 2) was required for better production of protease and it was also found that in lower temperature the activity was low.

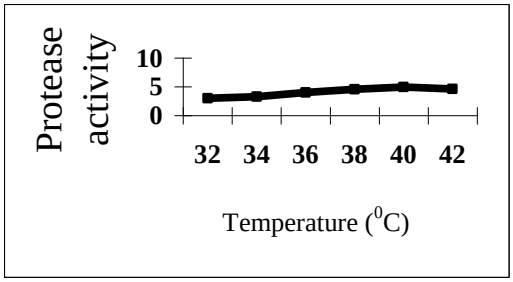

Fig 2. Effect of temperature on protease activity of the strain LK-3

The pH ranging from 6 –11 were studied for the detection of optimum pH for high protease production and the results reveals that the production of enzyme was maximum at pH of 7 and minimum at pH 6 (Fig. 3).

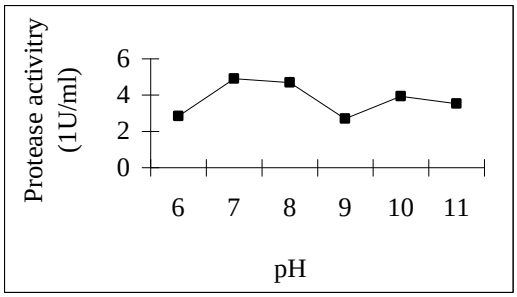

Fig 3. Effect of pH on protease activity of the strain LK-3

The maximum enzyme activity of protease was temperature of 40ºC and pH 7. The protease from *S.clavuligerus* exhibited greater thermal stability than the enzymes from other fungal sources, suggesting possible biotechnological and industrial detergents use, properties such as pH and temperature stability of the crude and partial purification extract were determined (Moreira, 2001). A novel alkaliphilic Actinomycetes *Nocardiopsis alkaliphila* sp. Nov. was reported to grow with an optimum pH of 9.5-10 (Wael et al, 2004). Like in the case of temperature and pH, the carbon and nitrogen utilization also varies with other works for better enzyme production. Sahu et al. (2008) have reported temperature optima of 40ºC, pH 9 as optimum conditions for better production of protease by actinobacterial strain PE 9.

The results show that the enzyme was purified 1.35-folds with a specific activity of 0.35 U/mg protein after ammonium sulfate precipitation. The enzyme solution was then purified using dialysis membrane. This purification step showed 1.73-fold enzyme purification with a specific activity of 0.45 U/mg proteins. The dialyzed enzyme was then purified using a DEAE-Cellulose column. The final purification step presented 2.16-fold enzyme purification with a specific activity of 0.56 U/mg proteins. These results indicated the effectiveness of purification method applied in this research. However, the yield of the enzyme after purification was found to be low. This might be due to the result of autolysis of the enzyme in each purification step.

The seventh peak contains the highest protein concentration (0.13 mg/ml). However, Ogundero and Osunlaja (1986) reported 26.2% recovery by using DEAE cellulose and Sephadex G-200 columns for purification of alkaline protease from *Aspergillus clavatus*. The elution pattern of the enzyme and a broad active peak was obtained when the dialyzed sample was passed through a DEAE Cellulose column with 0.6 M Tris-HCI buffer with NaCl pH 8.5 (Fig. 4). *Streptomyces alboniger* producing enzymes were purified by precipitation with ammonium sulphate and ion exchange chromatography and the SDS-PAGE showed a single band for the purified enzyme, with an apparent molecular weight of 80 (amylase), 66 (cellulase) and 97 KDa (protease) (Manivasagan et al., 2010).

The strain LK-3 possesses LL-DAP and contains glycine in its cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype I (Table 3).The genera belonging to the wall type - I are *Streptomyces, Streptoverticillium, Chainia, Actinopycnidium, Actinosporangium, Elyptrosporangium, Microellobosporia, Sporichthya* and *Intrasporangium* (Lechevalier and Lechevalier, 1970). The micromorphological observations of the strain LK-3 reveal that the strain belongs to the genus *Streptomyces*. The physiological characteristics of the strain LK-3 was given in Table 4 and compared with those of the *Streptomyces* species given in the key of Nonomura (1974).

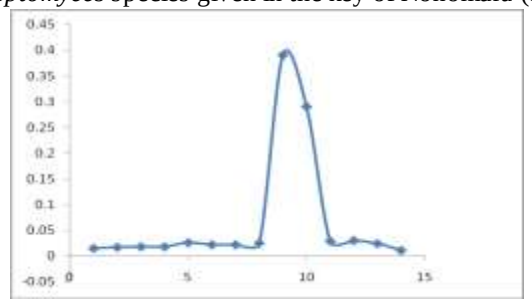

Fig.4 Purification of protease using DEAE-Cellulose column

Strain LK-3 showed more similarity when compared to the reference strain, *S. nigellus* (Table 5). The strain LK-3 showed good growth in the presence of 3% of NaCl. It also showed good growth at 40^0C and pH 7 and hence this temperature and pH can be considered as the optimum range for this strain.It utilized the nitrogen sources *viz*. L-phenylalanine and L-hydroxyproline and L-asparagine and L-histidine. Most of the physiological and biochemical features of the test species were showing similarity with that of the reference species *S. nigellus*. Hence, the strain LK-3 is tentatively identified as *Streptomyces nigellus*. However, molecular approach towards the identification of the strain LK-3 will help confirm the species identity.

The present study reveals that the mud crab may be considered as a potential source for the isolation of novel actinobacteria. The present study indicates that the tentatively identified species, *Streptomyces nigellus,* possess good protease activity and may be considered as an ideal organism for the industrial production of proteases.

Fig 5. Zymograph of partially purified protease

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Table.1 Screening of proteolytic bacteria

Strain no.	Zone of inhibition Mean ±SD
LK-1	1.33±0.577
LK-2	21±1
LK-3	28.33±0.577
LK-4	23.33±0.577
LK-5	20±1
LK-6	6.33±0.577
LK-7	1.33±0.577

Table 2. Purification of protease from the strain LK-3

Purification steps	Total protein (mg/ml)	Total activity (IU/ML)	Specific activity	Fold purification	Yield
Crude extract	9	2.4	0.26	1	100%
Ammonium sulphate precipitation	3.4	1.2	0.35	1.35	50%
Dialysed sample	2.6	0.6	0.45	1.73	25%
Ion exchange chromatography	0.13	0.4	0.56	2.15	12.5%

Table 3. Cell wall amino acids and whole cell sugars of the strain LK-3

Strain No.	LL-DAP	Meso-DAP	Glysine	Whole cell sugars	Wall type
LK-3	+	-	+	-	I

Table 4. Physiological characteristics of the strain LK-3

Parameter	Range					
NaCl requirement (%W/V)	0.5	1	1.5	2	2.5	3
	PG	PG	PG	MG	MG	GG
Temperature (°C)	32	34	36	38	40	42
	NG	PG	MG	MG	GG	NG
pH range	4	5	6	7	8	9
	NG	PG	MG	GG	GG	NG

PG – Poor growth; GG – Good growth; MG – Moderate growth; NG – No growth

Table 5. Comparison of morphological characteristics of strain LK-3 and *Streptomyces nigellus*

Characteristics	Strain LK-3	*S. nigellus*
Colour of aerial mycelium	Grey	Grey
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-
Sporechain morphology	Spiral	Spiral
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+