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PRODUCTION AND OPTIMIZATION OF THERMOSTABLE ALKALINE PROTEASE BY *VIBRIO ALGINOLYTICUS*

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Abstract
Vibrio alginolyticus is a gram negative rod shaped bacterium which was enumerated in seawater from Chennai coast, Southeast coast of India. The isolates showed high thermostable alkaline protease activity which exhibited maximum at pH between 7.5 and 8.8 and maintains the stability at pH 8 at 36° C. The maximum activity of protease was found with 200 revolution per minute. The protease content was found to be 89% in *V. alginolyticus*. *V. alginolyticus* was resistance (95%) to Gentamycin and susceptible (100%) to Amikacin and Amoxyclav. The protease production did not show significant differences ($P > 0.05$) among concentrations of 15 µl, 20 µl and 25 µl in semi quantitative assay.

Key Words: *Vibrio alginolyticus*; Thermostable alkaline protease; Protease activity; Quantitative assay; Zone of Inhibition.

Introduction

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Proteases are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management and silver recovery [1]. The protease enzyme constitutes two thirds of total enzymes used in various industries and this dominance in the industrial market increased by the year 2005 [2,3].

Vibrio alginolyticus is a common inhabitant of marine environment in both temperate and tropical waters [4,5]. Some *Vibrio* species are hemophilic (tolerance upto 10% NaCl) and sodium ions stimulate their growth. *V. alginolyticus* is the marine organism which is salt tolerant and it has diverse range of enzymatic activity leads to precipitation of denatured proteins [6]. *Vibrio* is also a photo bacterium which appears as a rod-shaped with light yellow colour. It contained pigment with a tuft sheathed flagella [7]. The mechanism of pathogenicity induced by *Vibrio* infection is still complex and related to several factors including cytotoxins, enterotoxins and lytic enzymes [8,9]. The adhesive properties of *Vibrio* sp are key factor of bacterial pathogenecity [10].

This micro organism produces many extracellular proteases responsible for the interaction between the bacterium and host [11,12,13]. Two extracellular proteases produced by *V. alginolyticus* NCMB 1339 and one metallo protease produced by *V. alginolyticus* is highly efficient in producing thermostable alkaline proteases. Thus the present study was undertaken to examine the production of thermostable alkaline protease from *V. alginolyticus* by conventional methods.

Materials and Methods

Sample collection

The seawater was collected from Neelangarai at Chennai coast (Lat. 13° 04' N; Long. 80° 17' E), Southeast coast of India in August 2009 using new sterile can and subsequently transported to the laboratory for further biological analysis.

Isolation of organism

Thiosulphate Citrate Bile Sucrose (TCBS) agar medium was used for the isolation [14]. TCBS plates were prepared with 8.9 gm of TCBS agar was dissolved in 100ml of marine water. It was slightly heated and temperature was reduced to 55^0 C. Agar medium was poured into 3 petri plates each with 15 – 20 ml. after solidification, 2ml of diluted (10^{-4} x 10^{-6}) samples were transferred to 2 different petri plate for spread plate method. Petri plates were incubated at room temperature

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for the growth of *V. alginolyticus*. This was incubated at lab temperature to allow the growth of *V. alginolyticus*.

Identification

Sub culturing was done for single colony isolation. A loopful of seed culture from overnight sample was streaked on to new TCBS agar plate and was incubated for 24 hrs.

Characterization of isolates

A gram staining test was using the method described by Smibert and Krieg [15]. A loopful of overnight culture was subjected to gram staining using crystal violet and saffranine.

Screening of protease production

V. aliginolyticus produced thermostable alkaline protease in the nutrient basal media was examined from the media consist of preparing 0.5gm of NaCl; 0.5 gm of peptone and 0.3gm of beef extract in 100ml marine water. This was incubated with inoculums in a rotatory shaker with rpm 200 for 24 hrs.

Protease activity was studied by both quantitative and semi quantitative assay. To examine quantitative assay, the protein content in the enzyme solution was measured by Lowry *et al* [16]. Semi quantitative assay was performed by the following procedure,

Skim milk agar was prepared by centrifuged milk without lipid content. This milk was slightly boiled and added to sterile agar medium and wells were cut. The sample was loaded on to these wells and incubated for a time period of 24 hrs. The zone of inhibition was viewed on the next day and it was calculated by using studied formulae 'Activity α $D^2 - d^2$'.

After the production of protease the optimum pH, temperature and rpm were calculated.

Determination of antibiotic susceptibility

The antibiotic susceptibility was determined by using the Muller Hinton agar medium plates supplemented with marine water. *V. aliginolyticus* culture was streaked on the medium with the help of L-rod [5,9]. The antibiotic discs such as Erythromycin (15 µg); Tetracycline (10µg); Chloramphenicol (10 µg); Ampicillin (10 µg); Gentamycin (10µg) and Streptomycin (10µg) were placed on the medium. After incubation at 37°C for 18-24h, the diameter of the inhibition zone was measured with 1 mm flat rule and the diameters were interpreted and classified as sensitive (S), intermediate (I) or resistance (R).

Statistics

Statistical analyses were carried out with SPSS package (windows version 12.0). Data were subjected to one-way analysis of variance (ANOVA) and differences between means were assessed by Duncan's multiple range test (DMRT). All statistical tests were considered significant at 5% level ($P < 0.05$).

Results

Vibrio alginolyticus isolates produced yellow color colonies without pigmentation and showed the faster growth within a day. Then it was identified through series of sub culturing and analyzing enzymatic properties. Characterization of isolates showed gram negative red color, rod shaped bacteria as *Vibrio alginolyticus.*

The protease content was found to be 89% in *V. alginolyticus*. The protease production did not show significant differences ($P > 0.05$) among different concentrations of 15µl, 20µl and 25µl in semi quantitative assay (Table 1). The isolated *V. alginolyticus* screened for thermostable alkaline protease had positive results in semi quantitative assay. The highest zone of inhibition (3.45 cm^2) was observed in 15 µl, 20µl and 25 µl concentrations which showed the thermostable alkaline protease activity.

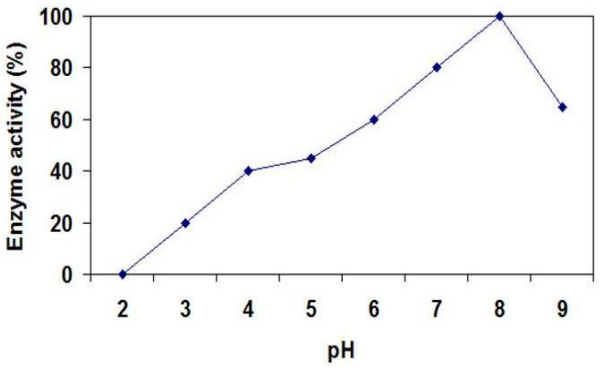
Table 1. Semi quantitative assay for protease production by *Vibrio alginolyticus*

Concentration (µl)	Zone of inhibition (cm^2)
5	2.09 ± 32^a
10	2.73 ± 08^b
15	3.45 ± 11^c
20	3.45 ± 23^c
25	3.45 ± 19^c

Mean values ± S.D. of determinations for duplicate samples
Means in the same column sharing different superscripts are significantly different ($P < 0.05$)

The optimum pH was likely between 7 and 8. The pH activity curve (Fig. 1) showed that the enzyme is reached about 100% of its maximum activity at pH 8. The purified protease showed maximum stability at pH 8. Under the pH 5, the protease loss was about 40% of its activity after holding at 4 hrs. The stability range was 7 to 9.

Fig. 1. Effect of pH on enzyme activity by *Vibrio alginolyticus*



The increasing of aeration speed resulted in increasing of alkaline protease by *V. alginolyticus*. Fig.2 shows that the optimum aeration speed for the production of alkaline protease (mg/l) by *V. alginolyticus* was about 200 rpm.

The effect of temperature on the protease activity (Fig. 3) showed that optimum temperature for the enzyme activity occurred at 36ºC and about 89% of its maximum activity. The protease of *V. alginolyticus* showed 89% stability at 36ºC. But the least residual activities were at 5, 10 and 50ºC. There was no activity observed in 60ºC, 65ºC and above.

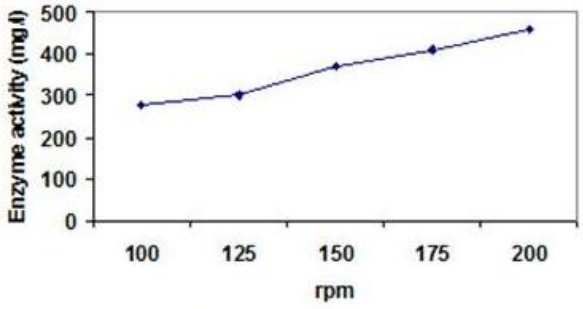


Fig. 2. Effect of rpm on enzyme activity by *Vibrio alginolyticus*

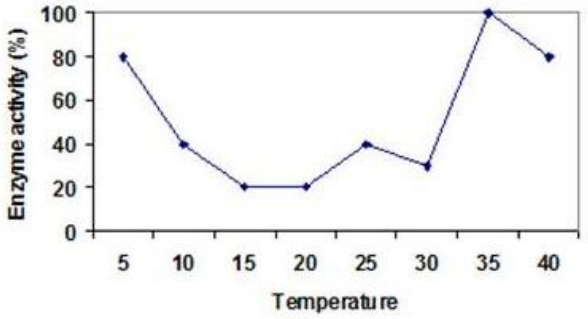


Fig. 3. Effect of temperature on enzyme activity by *Vibrio alginolyticus*

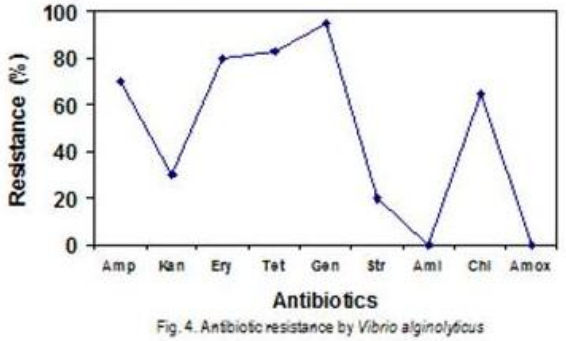


Fig. 4. Antibiotic resistance by *Vibrio alginolyticus*

About 95% of *V. alginolyticus* was resistance to Gentamycin followed by Tetracycline (83%), Erythromycin (80%) and Amphicillin (70%). The lowest percentage of resistance (65%) was noticed when Chloramphericol tested. The intermediate resistance was observed in Kannamycin and Streptomycin. The organism was susceptible (100%) to Amikacin and Amoxyclav (Fig.4).

Discussion

In the present investigation, *V. alginolylicus* was isolated from seawater which collected from Neelangarai beach at Chennai coast using TCBS agar plates. The similar kind of work was carried out by Ellaiah and Reddy [17] and Rathnakala and Chandrika [18] who have isolated the actinomycetes in starch casein agar plates from Vishakapatnam and Cochin sea waters respectively.

The *V. alginolylicus* isolate produced yellow colour colonies without pigmentation and analyzing showed the faster growth within a day which was identified through series subculture and analyzing enzymatic properties. But Balagurunathan *et al* [19] identified *Actinomycetes* produced grey and white colour colonies without pigmentation and showed the fast growth within 2 days.

Dhevagi and Poorani [20] characterized gram positive ash colour spore producing *Actinomycetes* from marine sample. In the present investigation, gram negative red colour rod shaped bacteria were obtained as a *V. alginolyticus*.

The isolated *V. alginolyticus* screened for themostable alkaline protease had positive results in semi quantitative assay. In contrast to above, Nair *et al* [21] and Dhevagi and Poorani [20] who have isolated *Streptomyces* species screened for L asparaginase with positive results in rapid plate assay.

In the present study, the protein content in protease showed the maximal activity at pH 8.0 at 50º C. The thermostable alkaline protease was characterized and exhibited maximum activity between pH 7.5 and 8.8 at 36º C. The protease was quite stable at pH 7 and 8 except pH 10 where it showed the maximum loss of activity. The maximum activity of protease was found with 200 rpm. These results correlate the findings of earlier studies. The purified L- asparaginase was characterized for its activity 85% [22]. It exhibited maximum activity between pH 8 and 8.5 with 85% of activity. The protease was quite stable at pH 8 and 9.

All other *Vibrio* species, except *V. alginolyticus*, was produced many extracellular enzymes such as amylase, gelatinase, chitinase and DNAase [11,23,24]. In the present investigation, *V. alginolyticus* found to have the capability to produce protease enzyme from seawater. Normally marine environment found to be salt tolerant and maintains the thermostability. These properties intended to the production of thermostable alkaline protease by *V. alginolyticus* in the present study.

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