ABSTRACT

The present study was conducted to evaluate the extracellular thermostable and organic solvent tolerant capacity of protease from ascidian associated bacteria *Vibrio* sp., GA CAS2. The bacterium was identified through 16SrRNA and biochemical methods. Its protease producing was evaluated by shake flask experiment. The isolated strain with high protease yield was optimized with respect to pH, temperature, carbon, nitrogen, metal ions, organic solvents and NaCl concentration. Lactose was observed to be the suitable carbon source for maximum protease production over the control. The most suitable nitrogen source for protease production was potassium nitrate and sodium nitrate. Screening the effect of surfactant on protease production revealed that Tween 80 resulted in maximum protease production. Among the tested metal ions, zinc sulphate showed a better production. Chloroform was found to be the best organic solvent in protease production. Testing the effect of various NaCl concentrations, 3% NaCl showed the maximum production compared to other concentrations. The best condition for better activity of the crude enzyme was observed at pH 9 and a temperature of 55°C.

KEYWORDS: Ascidian associated bacteria, protease, *Vibrio* sp., *Polyclinum glabrum*.

INTRODUCTION

Ascidians (phylum Chordata, subphylum Urochordata, class Asciidiacea) are a diverse group of sessile marine invertebrates that attach to natural and artificial substrates in the intertidal and subtidal zones of coastal habitats throughout the world (Van Name, 1945; Monniot et al.)
1991). Currently, about 3000 ascidian species, including both solitary and colonial forms, are known. They are important fouling organisms and as highly efficient filter feeders play an integral role in the benthic community of many coastal regions (Lambert, 2001). Ascidians are prolific producers of secondary metabolites (Ireland et al. 1988; Rinehart, 2000).

The enormous pool of biodiversity in marine ecosystems is an excellent natural reservoir for acquiring an inventory of enzymes with potential for biotechnological applications. Symbiotic bacteria in an animal’s digestive tract often produce complement enzymes for digestion of plant foods as well as synthesize compounds that are assimilated by the host (McBee 1971; Hungate 1975).

Proteases are enzymes that have the ability to degrade protein by the breaking of the hydrogen bond that binds protein into peptides and protein. Proteases are also known as peptidyl-peptide hydrolysases and are industrially useful enzymes which catalyze the hydrolysis of peptide bond from protein molecule. Microbial proteases are acid, neutral or alkaline based on their pH optimum for activity and the active sites viz., metallo, aspartic, cysteine- or sulphydryl- or serine-type. Alkaline proteases are stable in alkaline pH and possess a serine residue at the active site. Microbial proteases have been extensively studied and are used in numerous fields ranging from detergent industry, leather processing, molecular biology, genetic engineering, and medicine and food industry. Proteases constitute 50 – 65% of the global industrial enzyme market, most of which are alkaline protease. Given the wide application of this enzyme, the global proteolytic enzyme demand will increase dramatically. Proteases form 60% of the total global enzyme market estimated to be $6 billion (Gupta et al. 2002).

In this study an attempt is made to identify the bacteria associated with ascidian at molecular level and investigate the protease producing efficiency of the bacteria in optimized culture conditions.

**MATERIALS AND METHOD**

**Sample Collection**

The ascidian, *Polyclium glabrum* (Sluiter, 1895) were collected from Tuticorin coast by SCUBA diving. The collected samples were stored in sterile polythene bags with seawater and brought to the laboratory for further studies.
Strain Isolation
The collected ascidians samples were washed with sterile seawater to remove the loosely attached organisms. A small amount of internal tissue was homogenized with sterilized distilled water. The suspension was serially diluted and spread on Zobell marine agar plates with 1 % NaCl. The plates were incubated at 32° C for 5 days. After incubation, the colonies were isolated based on colony morphology and individual colonies were isolated by repeatedly streaking on Zobell marine agar plates. The colonies were maintained in Zobell marine agar slants at 4°C for further analysis.

Screening of Protease Producing Bacterium
The bacteria, capable of producing a clear zone on skim milk agar plates after incubation was chosen for the present study. The potential protease producing bacteria was identified and characterized based on morphological, physiological, biochemical characteristics and 16S rRNA sequencing.

16s RRNA Gene Sequence Analysis
The bacterial DNA was extracted by phenol chloroform method (Marmur, 1961). Sequencing was carried out by 16S rRNA universal primer. 16S rRNA gene amplification in PCR was done under the following conditions ; 35 cycles consisting initial denaturation at 95°C for 5 min, denaturation 95°C for 30 seconds, annealing at 55°C for 30 seconds and followed by final extension of 5 min at 72°C. Reaction products were electrophoresed on a 1% agarose gel and checked with ethidium bromide under UV light, and then purified and sequenced directly using a Taq Dideoxy Terminator Cycle Sequencing Kit and an ABI Prism 3730 automated DNA sequence (Applied Biosystems). Phylogenetic trees were constructed by the neighbor-joining Method of Saitou and Nei (1987) with Mega the program (version 5.05).

Media Optimization for Protease Production
The media optimization experiment was initiated by enriching the bacterium in Zobell broth with 1% casein powder. Ten percent of the enriched culture was inoculated in 250 ml flask containing 45 ml basal medium (% w/v) (casein-2%, Glucose-2%, peptone -1.0%, yeast extract-1.0%, NaCl-2%, trisodium citrate-0.2%, and potassium dihydrogen orthophosphate-0.1% at pH 7). The culture was then incubated in a shaker at (150 rpm) for 72 hrs at 32°C. The cells were harvested by centrifugation at 10000 rpm for 15 min. The supernatant was further used for protease assay.
Protease Assay
The proteolytic assay system was carried out according to Genckal and Tari (2006) based on the tyrosine released under standard assay conditions. The amount of protease produced was measured with the help of a tyrosine standard graph (Takami et al. 1989).

Effect of Nutritional Factors
The effects of nutritional factors such as carbon sources, nitrogen sources, surfactants and various concentrations of NaCl, metal ions, various organic solvents, pH and temperature on protease production were studied. All experiments were carried out in triplicates and average values are presented.

Effect of Various Carbon Sources
Carbon sources such as glucose, sucrose, fructose, lactose, maltose, sorbitol, xylose, mannitol and corn starch were individually added to the carbon and nitrogen optimized basal medium at a concentration of 0.5%. The basal medium without carbon sources was maintained as a control.

Effect of Various Nitrogen Sources
Nitrogen sources such as ammonium sulphate, ammonium chloride, ammonium hydrogen carbonate, sodium nitrate, potassium nitrate, urea, beef extract, casein, skimmed milk, yeast extract, meat extract, peptone and soybean meal were used. They were tested individually by replacing 1% peptone in the basal medium.

Effect of Different Surfactants
Surfactants effect on protease production was detected by adding seven different surfactants such as Tween 20, Tween 40, Tween 60, Tween 80, Poly Ethylene Glycol (PEG), Sodium Dodecyl Sulphate (SDS) and Triton X 100. They were added individually at 0.1% concentration.

Effect of Various Concentration of Nacl
For determination of suitable NaCl concentrations for protease production using various concentrations (1 to 7 % of NaCl) was used as supplements in the basal medium and replacing 2% NaCl in the basal medium and without addition of NaCl as a control.
Effect of Different Trace Elements (Metal Ions)
Nine different elements i.e., copper sulphate, potassium chloride, magnesium sulphate, barium chloride, zinc sulphate, ferric chloride, manganese sulphate, magnesium chloride and mercuric chloride were added individually into the basal medium at a concentration of 0.1%.

Effect of Various Organic Solvents
Different organic solvents including petrol, kerosene, methanol, chloroform, hexane and DMSO, were individually added in to the basal medium at a concentration of 2% to test their ability to tolerate organic solvents on production of protease. The basal medium without these solvents was maintained as a control.

Effect of pH And Temperature
Optimum pH for higher protease activity was determined by using different pH buffers during the assay. The assay was carried out individually at various pH from 2 to 13. The effect of temperature on protease activity was studied by incubating the enzyme and substrate solution at various temperatures from 10 to 70 °C at an interval of 5 °C and the assay was carried out individually.

RESULTS AND DISCUSSION
In the present study, the potential protease producing bacterium was isolated from the ascidian Polyclinum glabra (Sluiter, 1895). The strain was identified as Vibrio sp., by morphological and biochemical assays and it showed that the strain is a gram-negative, motile, rod shaped with catalase enzyme activity (Table 1). Further it was confirmed by molecular level through 16S rRNA gene sequence analysis confirmed the candidate bacteria as a Vibrio sp. GA CAS2 (Gene bank accession no. JX627402) with 100% similarity to reference sequences. Phylogenetic results (Fig. 1) revealed that the candidate bacteria coming under the genera of Vibrio sp. and belongs to the phylum proteobacteria, subclass gamma proteobacteria.

Influence of Carbon Sources
Carbon sources were considered as an important nutrient factor in protease production for Vibrio sp. GACAS2. In the present study, most of the carbon sources enhanced the protease production over the control with the exception of sucrose and maltose. Maximum protease production (212.31±0.04 U ml⁻¹) was observed in lactose (Fig. 2). Suggesting that lactose is an excellent carbon source for protease production. The influence of different carbon sources on protease production was found to be statistically significant (P<0.0001) by the one way
ANOVA. Interestingly, several studies have reported that the use of carbohydrate in the medium has the capacity for enhancing protease production in bacteria. Prakasham et al. (2006) reported high protease production from Bacillus sp. by the use of carbon sources such as xylose and maltose. Similarly, Esakkiraj et al. (2011) reported that the carbon sources have enhances the maximum production of protease in Serratia proteamaculans AP-CMST.

**Effect of Nitrogen Sources**

Nitrogen sources act as an important nutrient factor in protease production for Vibrio sp. GACAS2. The present study revealed that all the inorganic nitrogen sources had maximum protease production over the organic nitrogen sources. Among these, maximum extracellular protease production (196.03±0.27 U ml⁻¹) was obtained in potassium nitrate (Fig. 3). Peptone added medium expressed poor protease production (71.4±0.14 U ml⁻¹) than the other nitrogen sources. The one-way ANOVA analysis revealed that the difference between different carbon sources on protease production was statistically significant (P<0.0001). The reason may be that sea water that contains rich inorganic nitrogen compounds helps the candidate bacteria for better utilization of those inorganic nitrogen sources for protease production. Consistent with present study, Gupta et al. (2007) reported that the optimization of protease production in Pseudomonas aeruginosa PseA by using complex nitrogen sources. Our results also comparable with the complex nitrogen sources induced protease production in Aspergillus tamari (Anandan et al. 2007).

**Effect of Surfactants**

The protease production by the candidate bacteria was high with surfactant Tween 80 (150.62 ± 0.07 U ml⁻¹). All other surfactants showed lesser protease production than the control (Fig.4). One-way ANOVA revealed a difference in the effect of various surfactants on protease productions was statistically significant (p<0.001). Generally known to the surfactants are encourage the extracellular bacterial enzyme production (Reddy et al. 1999). In the present study also it was observed that the surfactant stimulates protease production in Bacillus sp. (Nascimento and Martins 2006). Similar observation has been reported by Esakkiraj et al. (2011) where maximum protease production was in triton x100 and Tween 80 by marine Serratia proteamaculans.

**Effect of Metals**

Optimization study for the suitable metal ions for protease production by Vibrio sp. GACAS2 revealed that zinc sulphate to be the paramount source for maximum (247.71 ±0.06 U ml⁻¹)
production (Fig. 5). Mercury chloride (203.42 ± 0.028 U ml⁻¹) and potassium chloride (186.26 ± 0.0014 U ml⁻¹) indicated that maximum protease production, followed by zinc sulphate compared to the control (155.17 ± 0.02 U ml⁻¹). One-way ANOVA of protease production by different sources of metal ions were significantly varied (p<0.001). Metal ions are one of the important cofactors for protease production in microbial cells. Rahman et al. (2005) observed that protease production was higher by Pseudomonas aeruginosa in metal ions mediated culture. The present observation is in agreement with the earlier study reported by Krishnaveni et al. (2012) where the magnesium sulphate and manganese sulphate enriched medium enhanced the protease production in Bacillus subtilis.

**Effect of Organic Solvents**
Six different organic solvents were screened for protease production in the present study. Among these all the organic solvents expressed maximum protease production (Fig. 6) compared to the control. Chloroform showed high production (808.69 ± 0.22 U ml⁻¹) than methanol (608.86±0.19 U ml⁻¹) followed by other solvents. One-way ANOVA revealed that all organic solvents were statistically significant (p<0.001). Significance of organic solvents in extracellular enzyme production has been well established in many marine bacterial species. Liu et al. (2010) reported the optimization of organic solvent for stable protease production by Bacillus sphaericus DS11. Organic solvents tolerant lipase production was constant with, hexane, supplemented medium by Pseudomonas aeruginosa Gaur et al. (2008).

**Effect of NaCl Concentration**
Testing the effect of NaCl concentrations for either increase or decrease the protease production revealed that maximum protease production was obtained with 3% NaCl (250.64 ± 0.20 U ml⁻¹) (Fig. 7). The candidate bacteria have ability for salt tolerant due to its adaptation with the ascidian, which survives purely in the marine environment. Consistent with present study, Esakkiraj et al. (2009) reported maximum protease production in 3% NaCl concentration in Bacillus cereus. Similar result result was observed in 1M NaCl concentration by Bacillus sp. VITP4 isolated from Indian coastal area (Pooja and Jayaraman, 2009).

**Effect of pH And Temperature**
pH is an important physical factor for the microbes in fermentation process. Maximum activity was registered at pH 9 (153.81 ± 0.14U ml⁻¹). There was a decline in the protease
production at acidic pH ranging from 2 to 6 (Table 2). Similarly, protease from Bacillus cereus VITSN04 had optimum production at pH 8 (Shakilanishi et al., 2005). Maximum Protease production at pH 9 was also reported in Bacillus sp. (Rajesh et al., 2005). Regarding the effect of various temperatures on protease production, maximum activity was registered at 55°C and minimum at 70°C. High specific activity was also observed at a temperature range of 45-55°C (Table 2). Bayoumi and Bahobil (2011) reported maximum protease production at 50°C in Shewanella putrefaciens-EGKSA21. Similarly, Singhal et al. (2012) reported a higher protease production by Bacillus sp. at 50°C and Halobacillus karajensis strain MA-2 (Hamid et al., 2009).

Table 1 Morphological and biochemical characteristics of strain Vibrio sp. GA CAS2

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td></td>
</tr>
<tr>
<td>Gram stain Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Spore formation</td>
<td>–</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>--</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>–</td>
</tr>
<tr>
<td>Urease</td>
<td>–</td>
</tr>
<tr>
<td>Lipase</td>
<td>–</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
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<td>Amylase</td>
<td>+</td>
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<tr>
<td>Catalase activity</td>
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<tr>
<td>Indole</td>
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</tr>
<tr>
<td>Citrate</td>
<td>+</td>
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</tbody>
</table>
Table 2 Effect of incubation temperature and pH on protease activity by Vibrio sp. GA CAS2.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Protease activity (U/ml)</th>
<th>pH</th>
<th>Protease activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>65.24± 0.47</td>
<td>2</td>
<td>102.08± 0.67</td>
</tr>
<tr>
<td>25</td>
<td>73.16± 0.44</td>
<td>3</td>
<td>118.12± 0.89</td>
</tr>
<tr>
<td>30</td>
<td>81.19± 0.80</td>
<td>4</td>
<td>116.29± 0.57</td>
</tr>
<tr>
<td>35</td>
<td>98.16± 0.91</td>
<td>5</td>
<td>121.19± 0.52</td>
</tr>
<tr>
<td>40</td>
<td>102.11± 1.02</td>
<td>6</td>
<td>129.11± 0.87</td>
</tr>
<tr>
<td>45</td>
<td>109.08± 0.60</td>
<td>7</td>
<td>135.13± 0.53</td>
</tr>
<tr>
<td>50</td>
<td>113.21± 0.69</td>
<td>8</td>
<td>141.20± 0.81</td>
</tr>
<tr>
<td>55</td>
<td>121.15± 0.90</td>
<td>9</td>
<td>143.04± 1.03</td>
</tr>
<tr>
<td>60</td>
<td>104.02± 0.54</td>
<td>10</td>
<td>109.07± 0.90</td>
</tr>
<tr>
<td>65</td>
<td>84.06± 0.47</td>
<td>11</td>
<td>86.13± 0.54</td>
</tr>
<tr>
<td>70</td>
<td>67.14± 0.39</td>
<td>12</td>
<td>81.11± 0.39</td>
</tr>
</tbody>
</table>

Fig. 1 Neighbor-joining phylogenetic tree showing the taxonomic position of Vibrio sp. GA CAS2 and related strains from NCBI (BLAST) relatives based on 16S rRNA gene sequences. Bootstrap values calculated from 1000 resamplings. Bar represents 0.5 substitutions per nucleotide position.
Fig. 2 Effect of carbon sources on protease production

Fig. 3 Effect of various nitrogen sources on protease production
Fig. 4 Effect of different surfactants on protease production

Fig. 5 Various metal ions concentration on protease production
Fig. 6 Effect of different organic solvents on protease production

Fig. 7 concentration on protease production NaCl
CONCLUSION

To the best of our knowledge it is the first report regarding the molecular level identification and optimization of protease production from ascidian associated bacteria. The result of the present study, reported that the Vibrio sp. GA CAS2 have ability to produce the significant level of extracellular protease isolated from the ascidian Polyclinum glabrum. The protease has some special property such as organic solvent tolerance and thermostability. Based on the results of the present study it may be suggested that the candidate bacteria will be useful in many industries.

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REFERENCE


