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Effect of Critical Medium Components on Protease and Agarase Production from Pigmented Marine *Pseudoalteromonas* species

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Abstract: The present study was attempted for bioprospecting of pigmented marine *Pseudoalteromonas* species with special reference to enzymes. Totally 36 pigmented bacterial colonies were isolated from sediment and water samples collected from Pichavaram, Parangipettai and Muttukadu coastal areas and characterized. Based on the studied phenotypic characteristics 10 isolates were identified as *Pseudoalteromonas* species. All the isolates were screened for enzymatic activities such as lipase, protease, amylase, cellulase, agarase, L-asparaginase and L-glutaminase. Most of the isolates showed protease (7), amylase (7) and agarase (7) activity. Protease and agarase from the active isolates were produced using submerged fermentation and its activity was semi quantitatively assayed at different time period. Effect of critical medium components on protease and agarase production from two potential strains S2B17 and S6B1 was tested by adopting one factor at a time method. Almost all the tested variables, except pH 10, influenced protease production by the strain S2B17. Agarase production from strain S6B1 was influenced by glucose, casein, 0.4% agar and pH 10. Dialysate of protease and agarase showed 25 mm and 19 mm zone of clearance in semi quantitative assay. The present study evidenced that the existence of enzyme producing pigmented *Pseudoalteromonas species* in Indian oceans and this report is an inventory for bioprospecting of marine *Pseudoalteromonas.*
Key words: marine *Pseudoalteromonas*, protease, agarase, optimization, semi quantitative assay.

Introduction

The genus marine *Pseudoalteromonas* is a Gram negative rod shaped bacteria, motile with sheathed polar and unsheathed for lateral flagella; catalase and oxidase positive and possess a strictly aerobic and chemoheterotrophic metabolism. All members of this genus require Na+ ions. One interesting feature of *Pseudoalteromonas* is that the genus can be divided cleanly in to pigmented species and non pigmented[1].

Analysis of some pigmented isolates lead to the general conclusion that pigmented *Pseudoalteromonas species* possess a broad range of bioactivity associated with the secretion of extracellular compounds, several of which include pigment compounds[2]. This realization provided greater evidence to earlier studies of *Alteromonas* that this group of marine bacteria represent a rich source of biologically active substances. Non pigmented species of *Pseudoalteromonas* do not appear to share the same extensiveness of bioactive compound synthesis. With in the limits of existing knowledge non-pigmented species tend to possess unusual and diverse enzymatic activities (carrageenases, chitinases, alginases, and cold active enzymes) with broader environmental tolerance ranges (temperature, water activity and pH)

and substantially greater nutritional versatility compared to the pigmented species[3]. Protease is one among the various microbial enzymes which has greater applications in the detergents, food, pharmaceuticals, chemical and leather industries[4]. Most of the commercially available proteases are produced from the genus Bacillus. A survey of literature showed that extracellular proteases were largely responsible providing nutrients to the bacteria. Agarases are the enzymes which catalyze the hydrolysis of agar. They are classified into α-agarase (E.C. 3.2.1.158) and β-agarase (E.C. 3.2.1.81) according to the cleavage pattern. Several agarases have been isolated from different genera of bacteria found in seawater and marine sediments, as well as engineered microorganisms. Agarases have wide applications in food industry, cosmetics, and medical fields because they produce oligosaccharides with remarkable activities[5].

Optimization of medium components is the pre-requisite for the better production of microbial enzymes. One factor at a time is a classical, simple method to study the effect of individual medium components on enzymes production. This is very useful to select the critical medium components for further statistical based optimization such as response surface methodology. With this view, the present study was attempted for bioprospecting of pigmented *Pseudoalteromonas species* with special reference to their enzymatic potentials.

Experimental

Sediment and sea water samples were collected from Pichavaram, Parangipettai and Muttukadu coastal area during the year 2008- 2009 using sterile plastic bottles and polythene covers, respectively and transported immediately to laboratory for further processing. About 1ml of seawater sample was serially diluted up to 10^5 dilution using sterile distilled water blanks. About 0.1ml of aliquot from 10^5, 10^6 and 10^7 dilution was plated on Zobell marine agar medium 2216 and nutrient agar plates supplemented with filter sterilized nystatin (20 µg/ml). Plating was done in triplicate and all the plates are incubated at 28^0C for 48-72 hours. The same procedure was adopted for the isolation of marine bacteria from sediment samples[6]. Morphologically different pigmented bacterial colonies were selected from isolation plates and purified using nutrient agar prepared in natural seawater. Phenotypic characteristics such as Gram's staining, motility, cultural characteristics, catalase, oxidase and IMViC test of all the marine bacterial isolates were studied by adopting standard procedures. The characteristics studied include antibiotic susceptibility pattern of selected marine bacterial isolates using gentamicin and polymixin were tested by adopting Kirby-Bauer disc diffusion method[7]. Effect of sodium chloride on growth was tested using nutrient agar medium supplemented with different concentration of sodium chloride (0, 1, 2.5, 5, 7.5 and 10%). All the plates were incubated at 28^0C for 5 days[6]. All the tests were performed by adopting standard procedures.

All the marine bacterial isolates were screened for enzymatic activities such as lipase, amylase, protease, cellulase, agarase, asparaginase and glutaminase by plate method using Tween 80 agar, starch agar, skim milk agar, congo red agar, czapek Dox agar, asparagine agar and glutamine agar, respectively. Marine bacterial isolates were spot inoculated on all the enzyme screening medium and incubated at 28^0C for 3-5 days[8].

Based on the results of preliminary screening, protease enzyme was produced from the active isolates by submerged fermentation using casein broth. About 10% of inoculum (18 hours old) was transferred in to casein broth and kept at 28^0C in rotary shaker with 120 rpm for 96 hours. For every 24 hour, about 2 ml of production medium was collected and separated by centrifugation at 10,000 rpm for 10 minutes. The clear supernatant was collected in sterile vials and used as crude protease enzyme.

For semi quantitative assay by agar well diffusion method[9], about 50 µl of crude enzyme was loaded on 5 mm diameter well on the skim milk agar plates and incubated at 28^0C for 24 hours. The zone of clearance around the well was measured as diameter in millimeter. The size of zone of clearance is directly proportional to the quantity of crude enzyme present in the culture supernatant[10].

Production of agarase enzyme from active isolates was carried out by submerged fermentation using agarose broth. About 10% of inoculum (18 hours old) was transferred in to agarose broth and kept at 28^0C rotary shaker with 120 rpm for 96 hours. For every 24 hours, about 2 ml of production medium was collected and separated by centrifugation at 10,000 rpm for 10 minutes. The clear supernatant was collected in sterile vials and used as crude agarase enzyme.

For semi quantitative assay by agar well diffusion method, about 50 µl of crude enzyme was loaded on 5 mm diameter well on the agar plates and incubated at 28^0C for 24 hours. The zone of clearance around the well was measured as diameter in millimeter.

Based on the results of semi quantitative assay, one potential strain was selected to study the effect of critical medium components on protease production by one-factor-at-a-time method[11]. Basal medium used for this study consists of soybean meal

(1%), dipottassium hydrogen phosphate (0.02%), magnesium sulphate (0.05%), peptone (1%)[10].

Basal medium was prepared and supplemented with different variables in respective concentration (table 4). About 10% of bacterial inoculum was transferred in to all the media and incubated in rotary shaker at 28^{0}C with 120 rpm for 48 hours. After fermentation, culture medium was separated by centrifugation and the culture supernatant was semi quantitatively assayed for protease activity by agar well diffusion method as described earlier.

Based on the results of semi quantitative assay, one potential marine bacterial strain was selected to study the effect of critical medium components on agarase production by one-factor-at-a-time method. Basal medium used for this study consists of agarose (0.3%), potassium chloride (0.74%), diammonium hydrogen phosphate (0.0013%), sodium chloride (1.75%)[12]. Effect of various factors on agarase production was studied by the method as described for protease.

Finely powdered ammonium sulphate was slowly added into enzyme preparation so as to reach 40% saturation. The whole content was stirred at 4 ^{0}C with a magnetic stirrer. The precipitated protein was removed by centrifugation at 10,000 rpm for 20 minutes at 4 ^{0}C. Fresh ammonium sulphate was added to the supernatant to increase the concentration to 60 %. The obtained precipitate was resuspended in a minimal volume of 0.01M phosphate buffer, pH 7.5. Precipitated protein was removed by centrifugation as described earlier. Once again the fresh ammonium sulphate was added to the supernatant to increase the concentration to 80%. The obtained precipitate was resuspended in a minimal volume of 0.01M phosphate buffer (pH 7.5). The precipitate obtained after ammonium sulphate precipitation was dialysed against 0.01 M phosphate buffer, pH 7.5, for 24 hours at 4 ^{0}C with continuous stirring. The buffer was changed occasionally. Finally the protease activity of dialysate was semi quantitatively assayed by the method described earlier. The same procedure was adopted for partial purification of agarase from potential *Pseudoalteromonas* species. Activity of partially purified protease and agarase was semi quantitatively assayed.

Table-1. Characteristics of pigmented marine *Pseudoalteromonas* species isolated from marine samples

Strain No	Colony color	Microscopic		Biochemical tests						Salt tolerance %						Antibiotic susceptibility	
		Gram stain	Motility	C	O	I	MR	VP	C	0	1	2.5	5	7.5	10	G	P
S1B1	Pale yellow	G^- rods	+	+	+	-	+	-	-	-	+	+	+	+	+	+	-
S2B2	Pale red	G^- rods	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-
S2B17	Pale yellow	G^- rods	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+
S3B1	Reddish orange	G^- rods	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-
S3B4	Yellow	G^- rods	+	+	+	-	+	-	-	-	+	+	+	+	+	-	-
S4B6	Orange	G^- rods	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
S5B9	Yellowish orange	G^- rods	+	+	+	-	-	-	-	-	+	+	+	+	+	+	-
S6B1	Red fluorescent	G^- rods	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
S6B2	Yellowish orange	G^- rods	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
S6B5	Orange	G^- rods	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+

+ - Positive; - - Negative

C-Catalase; O-Oxidase; I-Indole; MR-Methyl red; VP-Vogus prousaker; C-Citrate; G-Gentamicin; P-Polymixin B

Table 2. Semi quantitative assay of protease produced from marine bacteria

Strain No.	Incubation period (hours)			
	24	**48**	**72**	**96**
S1B1	6	8	13	13
S2B17	7	13	12	12
S3B1	-	-	-	-
S3B4	10	11	-	-
S4B6	-	-	10	12
S6B9	-	-	-	-
S6B2	-	-	-	-
S6B5	10	13	-	-

- - No activity
*Zone of clearance measured as millimetre in diameter

Table 3. Semi quantitative assay of agarase produced from marine bacteria

Strain No.	Incubation period (days)		
	7	**8**	**9**
S1B1	12	12	12
S2B2	-	-	-
S2B17	-	-	-
S3B1	12	12	12
S3B4	10	12	12
S4B6	10	12	12
S5B9	13	13	13
S6B1	15	15	15
S6B2	12	12	12
S6B5	12	12	14

- - No activity
*Zone of clearance measured in millimetre in diameter

Table 4. Effect of critical medium components on protease and agarase production from marine *Pseudoalteromonas* species

Factors	Semi quantitative assay (zone of clearance in millimetre in diameter)	
	Protease (S2B17)	**Agarase (S6B1)**
Carbon source		
Glucose	14	18
Fructose	13	13
Sucrose	15	13
Lactose	14	10
Glycerol	12	7
Nitrogen source		
Peptone	14	13
Malt extract	15	10
Beef extract	12	16
Yeast extract	16	16
Casein	15	18
Agar concentration (%)		
0.1	NT	10
0.2	NT	12
0.3	NT	14
0.4	NT	16
0.5	NT	12
pH		
4	15	15
5.5	14	10
7	14	14
8.5	16	15
10	-	18

- - No activity ; NT – Not Tested

Results and Discussion

Totally 44 bacterial isolates were isolated from the marine samples. Among the 44 isolates, 36 were pigmented strains and 8 were non-pigmented. All the pigmented strains were selected for characterization and identification. Based on Gram's reaction, among the 36 strains, 17 strains were identified as gram negative rods in which 11 isolates showed positive results for motility. Out of 11 isolates 10 isolates were both catalase and oxidase positive. In salt tolerance studies, all the isolates showed growth at 1% to 10 % NaCl concentration. None of the isolates showed growth in the absence of sodium chloride. Except the strain S3B4 all the isolates are sensitive to gentamicin. Strains S2B17, S4B6, S6B1, S6B2 and S6B5 were found to be polymixin B sensitive (Table 1). Based on the results of studied phenotypic characteristics, all the 10 isolates were tentatively identified as *Pseudoalteromonas* sp. Diversity and antimicrobial activity of pigmented *Pseudoalteromonas species* were well documented from various marine samples and marine organisms. But reports on enzymes from pigmented *Pseudoalteromonas* species is scanty[13]. For the various enzymes screened, most of the isolates showed amylase (7), protease (7), agarase (7) and cellulase (7) activity. None of the isolates showed lipase, asparaginase and glutaminase. Pigmented *Pseudoalteromonas sp.* possesses broad range of bioactive compounds with the secretion of extra cellular compounds than non pigmented species. Within the limits of existing knowledge, non-pigmented species tend to possess unusual and diverse enzymatic activities than pigmented species[13]. But the present study reported pigmented marine *Pseudoalteromonas* species with special reference to enzymes.

Of the 10 isolates tested, about 5 isolates namely S1B1, S2B17, S3B4, S4B6 and S6B5 showed protease activity in semi quantitative assay in which, except S4B6, all other four isolates showed protease production with in 24 hours of production period (Table 2). In particular, strain S2B17 produced maximum enzymatic activity in 48 hours of incubation, which produced 13 mm zone of clearance in semi quantitative assay. Vazquez *et al.,*[10] reported extracellular protease from Antarctic marine *Pseudoalteromonas* species P96-47 strain.

Totally 8 marine bacterial isolates, except S2B2 and S2B17, showed agarase activity in semi quantitative assay. All the isolates showed protease production on seventh day of production period. In particular strain S6B1 showed maximum agarase activity in 7 days of incubation which produced 15 mm zone of clearance (Table 3). Previously Vera *et al.,*[14] reported agarolytic *Pseudoalteromonas* species from algae. In most of the studies potential strains were selected based on enzymatic activity while inoculating the strains on agar media supplemented with respective substrate[11]. In this method, the quantity of inoculums may vary from one strain to another. To overcome this screening problem, agar plate based semi quantitative assay was adopted in this study. The enzyme from active strains was produced under same conditions and known quantity of crude enzyme was added in to well with known size made on screening agar plate. The semi quantitative method adopted in this study is a simple, time saving, sensitive, and reliable method for the detection of enzymatic activity and selection of potential strain before performing quantitative assay. Strains S2B17, S6B1 which showed maximum protease and agarase activity, respectively in short incubation period, was selected as potential strains for further investigations.

Of the various factors tested, except pH 10, almost all the factors and variables influenced protease production. At the end of fermentation in all the medium pH was decreased to 4. Of the various factors tested maximum agarase production was observed on medium supplemented with glucose (carbon source), casein (Nitrogen source), 0.4% agar concentration and pH 10. At the end of fermentation in all the medium pH was decreased to 4 (Table 4).

Effect of medium components on enzyme production from marine bacteria was reported previously[15,16]. A single culture media and culture conditions are not kind enough to produce same enzymes from different bacterial species and even strains. The optimization of the medium by the conventional research technique involves classical one factor at a time method involves changing one independent variable (such as carbon source, nitrogen source, etc) while fixing other at constant levels. In the present study, effect of culture conditions on protease, and agarase production was studied by adopting classical one factor at a time method of the various culture conditions tested. Further statistical optimization was needed to determine the interaction of medium components on protease and agarase production. Partial purification of protease, and agarase from potential strains were carried out by ammonium sulphate precipitation and dialysis. Dialysate of protease and agarase showed 25 mm and 19 mm zone of clearance in semi quantitative assay. Further purification of these enzymes by PAGE and Column chromatography was needed for its characterization.

Among the various enzymes, microbial proteases found to have wide range of applications particularly in detergent industries. Agarase enzyme has its application in food, cosmetic industries and also in molecular biological research. Strain S2B17 and S6B1 reported in this study will be a potential source

for protease and agarase, respectively. In addition, both potential strains were recovered from sediment samples of Muttukadu coastal area a less explored ecosystem for microorganisms when compared to Pichavaram and Parangipettai coastal area. The study also suggested to extensively investigating Muttukadu coastal area for microbial bioprospecting. Both the *Pseudoalteromonas* species will be a potential source for protease and agarase enzyme, respectively. The present study evidenced that the existence of pigmented *Pseudoalteromonas* species in Indian oceans and this report is an inventory for bioprospecting of marine *Pseudoalteromonas* from our country.

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