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Isolation, Purification and Characterization of An Extracellular Agarase from *Alteromonas* sp.

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A marine agarolytic bacterial strain of *Alteromonas* producing an extracellular agarase was isolated from the sea water from Marina and characterized. The optimum growth requirements of the organism for production and activity of the enzyme was determined. The agarase enzyme was purified by the ammonium sulphate precipitation method and dialysis. The molecular weight of the partially purified enzyme was estimated to be 56kDa by NATIVE-PAGE.

Keywords: Agarase, *Alteromonas sp,* PMSF.

Agarase specifically digests the agar polysaccharide core into oligosaccharides (Yaphe, 1957). Agarolytic enzymes have been reported from several bacterial genera, including *Cytophaga, Pseudomonas, Streptomyces* , and *Vibrio*. Most of these bacteria have been isolated from marine environments, although a few species isolated from rivers, soil, and sewage have also been described (Oscar Leon *et al*, 1992). Hydrolytic enzymes which degrade agarose are classified into two groups in terms of the mode of action on agarose; α-agarases cleave the α-1,3 linkage of agarose , and β-agarases cleave the α-1,4 linkage of agarose (Yasushi Sugano *et al*, 1993). In coastal seawater,where the red tide occurs frequently marine bacteria play an important role for the interactions of phytoplanktons, in the sense of reducing and preventing harmful algal blooms. Algicidal bacteria that can kill and lyse causative phytoplanktons of red tides could be of great use in reducing and preventing harmful algal blooms (J H Sohn, 2004).

Agarase can be used for gentle yet efficient recovery of DNA or RNA fragments from low melting point agarose. The recovered nucleic acids can be directly used for amplification, cloning, sequencing, etc. It is also used for softening agar media for culturing plant cells and extract useful products from sea weeds. India has a large coastline and huge marine resources which have not been fully explored. The objective of this study was to obtain a novel indigenous agarolytic bacteria and characterize it for future use.

MATERIAL AND METHODS

Isolation and characterization of the organism

The isolation of agarolytic bacteria was done from samples of seawater obtained from the Marina Beach (Chennai) and spread on plates containing Medium A. The plates were incubated at 25°C for 48hr.

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Medium A (Oscar Leon *et al*, 1992)

Casein hydrolysate	0.25%
Yeast extract	0.05%
Sodium chloride	2.5%
Sodium dihydrogen phosphate	0.06%
Magnesium sulphate	0.5%
Ferrous sulphate	0.002%
Calcium chloride	0.01%
Agar	1.5%
pH	7.25

Bacterial colonies forming pits in the above medium were taken and further characterized using conventional biochemical tests (Table 1.)

Table 1. Biochemical tests

S.No.	Biochemical test	Test results
1	Oxidase	Positive
2	Catalase	Positive
3	Indole production	Negative
4	H_2S production	Negative
5	Urease	Negative
6	Sodium for growth	Positive
7	Citrate	Positive
8	Methyl Red	Negative
9	Voges Proskauer	Negative
10	Gelatin hydrolysis	Negative
11	D-glucose utilization	Positive
12	D-maltose utilization	Positive
13	D-lactose utilization	Negative

Growth curve

100 ml of nutrient broth was inoculated with culture. The zero hour reading was taken at 540 nm soon after inoculation. The culture was kept in a shaker at 100 rpm. The optical density value at 540nm was measured every one hour. The growth curve was drawn by taking time in hours in x- axis and OD value in y-axis.

Antibiotic sensitivity assay (Kirby-Bauer Method)

The antibiotic sensitivity test was performed using the Kirby-Bauer method using antibiotics viz., (Cephalexin- 30mcg, Cefuroxime- 30mcg, Cefazoline- 30mcg, Nalidixic acid- 30mcg, Neomycin- 30mcg). The plates were incubated at 37°C for 24hrs.

Estimation of proline

The amount of proline in the culture filtrate was estimated using the method of Bates, 1973 (Table. 2)

Estimation of protein- Lowry's method (Lowry *et al*.,)

0.2 to 1 ml of standard solution is pipette out into different tubes and the volume is made upto 2ml with distilled water. A blank is also taken. 5 ml of alkaline copper reagent is added to each of the tubes and was left at room temperature for 10min. Then 0.5 ml of Folin's Ciocalteau reagent was added. After 20 min, the colour was read at 620 nm using a colorimeter. A graph was drawn and the amount of protein in the solution was calculated.

Purification of enzyme

Purification of the enzyme was carried out by ammonium sulphate precipitation method and dialysis. The optimum pH and temperature of the purified enzyme was determined.

Ammonium sulphate precipitation

500ml of Medium A was inoculated with bacterial culture and the cells were grown in an orbital shaker at 140 rpm, 25°C till they reached the stationary phase. Phenyl methyl sulphonyl fluoride(PMSF) was added to a final concentration of 0.1mM. The cells were then centrifuged at 12,000 rpm for 30 min. The supernatant was taken in a beaker and brought to 75% saturation with solid

Table 2. Estimation of Proline

	Reagent	B	S_1	S_2	S_3	S_4	S_5	U_1	U_1'
1.	Amount of proline (µg)	0	200	400	600	800	1000	-	-
2.	Volume of proline (ml)	-	0.2	0.4	0.6	0.8	1	-	-
3.	Volume of culture filtrate (ml)	-	-	-	-	-	-	0.2	0.2
4.	Volume of glacial acetic acid (ml)	2	2	2	2	2	2	2	2
5.	Volume of ninhydrin (ml)	2	2	2	2	2	2	2	2
6.	Volume of toluene (ml)	4	4	4	4	4	4	4	4
7.	Optical density at 520nm	0	0.61	0.75	0.80	0.95	1.1	0.63	0.60

ammonium sulphate for 1 hr. The ammonium sulphate was added slowly till it completely dissolves in the supernatant. This procedure was carried out by placing the beaker on a magnetic stirrer with bead. After complete saturation occurs, it was left for another hour in the magnetic stirrer. The contents of the beaker were transferred to centrifuge tubes and the tubes were centrifuged at 12,000 rpm for 30min.

Dialysis

The pellet obtained was resuspended in 40 ml of 20 mM Tris-HCl (pH 7.5) - 0.1 mM EDTA containing 0.1 mM PMSF. It was then loaded into a dialysis bag and dialyzed against the same buffer. This was left to dialyze overnight. The dialysis bag was then transferred to 50% sucrose containing beaker and dialyzed for 17 hrs. The resulting dialysate was taken for further characterization.

Assay for Agarase

4.5 ml of 0.05% agar was taken in 50mM sodium phosphate buffer (pH 6.5). The tube was heated at 100°C for 2 min then cooled to 30°C. The reaction was started by adding 0.5ml of the enzyme and 0.5ml aliquots were taken at intervals. To this 0.5 ml of copper reagent was added to stop the reaction then 0.5ml of neocuproine-HCl was added and incubated in boiling water bath for 5 minutes. Reducing sugar production was monitored as apparent release of D-galactose.

Estimation of reducing sugars (Miller, 1972)

The amount of reducing sugars was done by taking a series of 8 test tubes to which was pipetted out 100-500mg of galactose standard solution (1mg/ml stock). The volume was made upto 3ml by adding distilled water. Blank containing water was taken. Add 3 ml of DNS reagent into each test tube. Test tubes were incubated at 80°C for 5 min. To this solution, 1 ml of potassium sodium tartarate was added. The solution was mixed well and OD was taken at 510 nm (Table 4). A standard graph was drawn by taking concentration on x-axis and OD on y-axis.

pH optimum

Four sets of test tubes in duplicates were taken and marked as T_1, C_1, T_2, C_2, T_3, C_3, T_4,C_4. To

Table 3. Estimation of proteins- Lowry's method (After Dialysis)

	Reagents	B	S1	S2	S3	S4	S5	U1	U1'
1.	Volume of standard (ml)	0.0	0.2	0.4	0.6	0.8	1.0	-	-
2.	Concentration (µg)	-	20	40	60	80	100	-	-
3.	Volume of unknown (ml)	-	-	-	-	-	-	0.2	0.2
4.	Volume of water (ml)	2	1.8	1.6	1.4	1.2	1.0	1.8	1.8
5.	Alkaline copper reagent	← 5 ml →							
		Leave for 10 mins at room temperature							
6.	Folin s reagent	← 0.5 ml →							
		Leave for 20 min at room temperature							
7.	Optical Density at 620 nm	0.00	0.02	0.04	0.06	0.08	0.09	0.08	0.09

Table 4. Estimation of reducing sugar (D-galactose)

S.No.	Reagents	B	S_1	S_2	S_3	S_4	S_5	U_1	U_1'
1.	Standard D-galactose solution (ml)	0	0.1	0.2	0.3	0.4	0.5	-	-
2.	Concentration of D-galactose (µg)	0	100	200	300	400	500	-	-
3.	Volume of dialysate (ml)	-	-	-	-	-	-	0.6	0.6
4.	Distilled water (ml)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.4
5.	DNS reagent	← 3.0ml →							
		Incubate at 80°C for 5 min							
6.	40 % sodium potassium tartarate	← 1.0 ml →							
7.	Optical density at 510 nm	0.0	0.2	0.5	0.6	1.2	1.3	0.7	0.7

these 0.5 ml of phosphate buffer of pH (6.0, 7.0, 8.0, 9.0, 10.0) was added. 1 ml of substrate and 1 ml of sodium chloride activator (0.8%)was added to all the test tubes. The test tubes were pre-incubated at 37°C for 15 min. In the test tubes, T_1-T_5, 0.2 ml of the enzyme solution was added and incubated for 15 min. The reaction was arrested by adding 1.0 ml of 2M sodium hydroxide. To the four control tubes (C_1-C_4) enzyme was added and the controls were mixed well. 0.5 ml of DNSA was added. The tubes were boiled in a water bath for 5 min, cooled. The colour intensity was measured at 540 nm against the blank. The graph is plotted with optical density against pH and the optimum pH is determined.

Temperature optimum

Five sets of test tubes were taken as duplicates and were marked as T_1,C_1,T_2,C_2,T_3,C_3, T_4,C_4,T_5,C_5. 2.5 ml of phosphate buffer was added to each of the test tube. 1 ml of the substrate and 1 ml of NaCl activator (0.8%) was added to all the test tubes. These were then pre-incubated at 37°C for 15 min. In the test tubes, T_1-T_5, 0.2 ml of the enzyme solution was added and incubated at differing temperatures, such as 10°C, 25°C, 37°C, 60°C for 15 min. The reaction was arrested by adding 0.5 ml of 2M sodium hydroxide and then 0.2ml of the enzyme was added to all the control tubes. 1 ml of DNSA was added to all the test tubes and mixed well and heated for 10 min in a boiling water bath. The test tubes were then cooled and the orange red colour developed was read at 540 nm.

Effect of Agarase on Agar

0.5 ml of the purified enzyme was added to the plate containing Medium A. The plates were incubated at 37°C for 1 hr.

RESULTS AND DISCUSSION

Isolation and characterization of the organism

The isolated strain had the following characteristics, namely it produced pits on the agar surface with irregular borders showing haloes of clearing. This organism had the following characteristics: Gram-negative thin rods, motile, catalase and oxidase positive and indole negative. The organism was ascertained via the biochemical tests to be an *Alteromonas* sp. (Fig. 1)

Growth curve

From the growth curve (Fig. 2) it was seen that the highest level of agarase production was seen during the stationery phase. At longer incubation periods, the level of agarase decreases, a trend probably due to the presence of proteases.

Antibiotic sensitivity

The bacterium does not show sensitivity to any of the antibiotics used. Hence the bacterium is resistant to the antibiotics used.

Estimation of proline

The amount of proline present in the given sample was found to be 260µg/ml.

Purification of agarase

The enzyme purified through ammonium sulphate precipitation (75%) saturation and dialysis (Fig. 3) was stable when stored in buffer containing Tris-HCl (pH 7.5) and 0.4 M NaCl at 4°C. The enzyme activity remained constant after one hour of incubation at temperatures below 30°C.

Assay for agarase

Estimation of reducing sugar

The amount of reducing sugar (D-galactose) was estimated and was determined to be 320mg/ml.

Protein determination

The amount of protein in the dialysate was estimated via the Lowry's method using bovine serum albumin as standard (Table 3). The concentration of the unknown was plotted within the standard graph and the amount of protein in the given sample was found to be 80mg/ml.

pH optimum

The optimum pH at which the enzyme is stable was determined using phosphate buffers of varying pH, the optimal activity was observed at pH 7.0 (Fig. 4)

Temperature optimum

The stability of the enzyme was examined at different temperatures in phosphate buffers of constant pH. The enzyme activity was maximum at 35°C but declined after 40°C due to loss in activity (Fig. 5)

Native PAGE

Native PAGE was performed using 15% acrylamide gel and the proteins were stained using silver staining. The band for the sample was found to correspond with marker at 56kDa.

Effect of Agarase on agar

The plates containing agar showed depression and pitting after incubation for 24 hrs. This was consistent with the reports of Agbo *et al*, 1979. (Fig. 6).

Fig. 1.

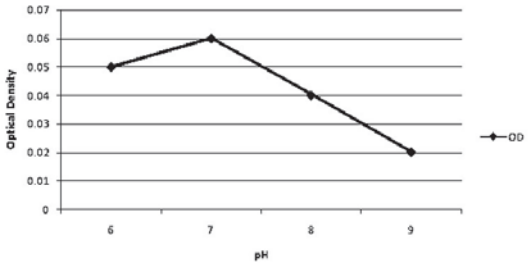

Fig. 4. Effect of pH on Agarase

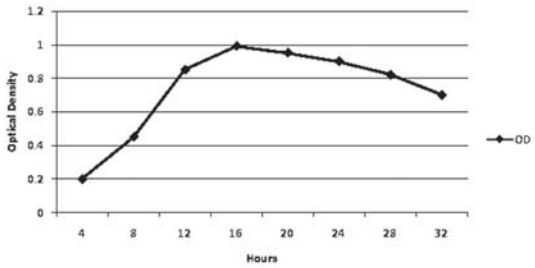

Fig. 2. Growth curve

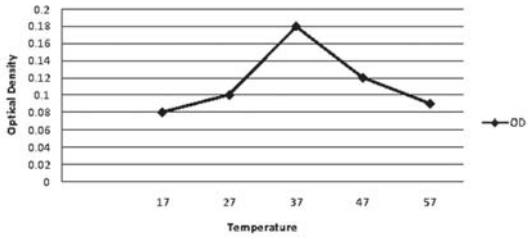

Fig. 5. Effect of temperature on Agarase

Fig. 3.

Fig. 6.

Application of the enzyme

The agarase enzyme has wide potential for use in industry and in laboratories.

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