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ORIGINAL RESEARCH ARTICLE

Isolation and Characterization of Agarolytic Microorganisms and Purification of an Extracellular Enzyme Agarase

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ABSTRACT

In the present study, nine samples were collected along the coast of Chennai in three places namely, Marina, Mahabalipuram and Pudhucherry, to study the agarolytic microbial flora of this marine environment. On screening of the samples for agar-degrading bacteria, after enrichment, all the samples were found to contain agarolytic bacteria. In each sample, various patterns of agar degradation were seen indicating different bacteria present in the sample. Four isolates were obtained from the 9 samples. They were checked for reproducibility of the agarolytic activity by culturing on the same media mentioned above. They were identified to be belonging to the genera *Streptomyces*, *Pseudomonas*, and *Cytophaga*. A versatile agarobacterium has been described which grows on varies oligo and polysaccharides adaptively forms enzymes which will cleaves these carbohydrates. An enzyme, agarase has purified about 10 fold from the culture medium and appears to release oligosaccharide units, but not free galactose, from agar. Therefore, it is evident that the agarase specifically cleaves β- I, 4 linkages between D-galactose and 3, 6-anhydro-lactose of the agarose structure.

Key words: Agarase, Agarolytic activity, *Streptomyces*, *Pseudomonas* and *Cytophaga.*

1. INTRODUCTION

Agar-agar is prepared from certain marine algae. It is used as food in orient and for medicinal purposes in Europe and America. It is also employed for preparation of culture media. It was first mentioned in the bacteriological literature by Robert Koch in 1882, and its introduction into bacteriological technique was due to the suggestions of Frau Hesse, the wife of one of Koch's early co-workers. It was introduced to overcome the difficulty encountered with gelatin due to its liquefaction at relatively low temperature.

Composition of agar varies with nature of the plant from which it is obtained. Japanese agar is prepared from *Gelidium corneum*, while other species of *Gelidium* and species of *Gracilaria*, *Focus* and *Eucheuma* are also employed in parts of the world. Although agar contains a considerable amount of ash, the major part of it consists of one or more hemicelluloses or carbohydrates. The most important constituent of agar is a galactan which was previously referred to as gelose by Payen. The inorganic constituents are chiefly calcium and magnesium sulphates.

Several bacteria capable of liquefying agar have been isolated. They are *Bacillus gelaticus*, *Microspira liquefaciens*, *Vibrio andoi Zobellia Galactanivorans dsij, Cytophaga sps, Pseudomonas atlantica, Pseudomonas segne, Pseudomonas lacunogenes, Achromobacter pastinator, Alteromonas sps and Streptomyces coelicolor.* A highly purified form of agar called agarose is a hydrophilic polysaccharide found in the cell wall of marine red algae (Rhodophyceae) where it naturally occurs in the form of a pseudocrystalline matrix associated with cellulose. It consists of a linear backbone of galactopyranose residues linked by alternating a-1, 3- and β-1, 4-linkages. The β-linked residues are in D configuration and the a-1, 3-linked galactose units are in rare L configuration. They are further modified by a 3, 6-anhydro bridg.

The agar- degrading bacteria are also found to degrade agarose by secretion of an enzyme called agarase. Agarase are glycoside hydrolases (GH) that hydrolyze agarose. They cut a-1, 3- and β-1, 4- linkage of agarose's galactoanhydrogalacto repeat units. They are classified into two groups

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based on their mode of action as alpha-agarase and beta-agarase. Those that enable to cleave that a-1, 3-anhydro-L-galactosidic bond are called alpha-agarase and those that enable to cleave β-1, 4- galactosidic bonds are called beta-agarase. Bacteria that secretes α -agarases are *P. atlantica* and *Cytophaga flavensis*. Bacteria producing β-agarase are *Alteromonas, Cytophaga, Streptomyces, Vibrio, Pseudomonas and Zobellia galactanivorans.*

2. MATERIALS AND METHODS

2.1. Sample collection

Nine samples of seawater from varies places along the east coast of Chennai namely Marina, Mahabalipuram and Pudhucherry were collected and transfer to laboratory very safely.

2.2. Enrichment of the samples:

Samples of 1.5ml seawater was added to 75ml of enrichment media consisted of 0.1% agar in seawater in a 250ml Erlenmeyer flask as a shallow layer and incubated at 22°C for 5 days [1].

2.2. Isolation and identification of the agarolytic organism:

The enriched water samples were streaked onto Minimal Medium plates having peptone as the source of nitrogen and incubated at 22°C for 24 hrs. The enriched water spread onto Seawater Peptone Agar plates using a sterile swab and incubated at 22°C for 48 hrs [1]. After incubation, colonies were isolated. Colonies from seawater peptone agar were picked up and streaked onto nutrient agar plates and incubated at 37° C for 48 hrs. Gram's staining was performed by picking up each of the colonies from all samples to find out the nature of organism. The isolated colonies from nutrient agar were plated onto blood agar to study the haemolytic property. Standard biochemical test were performed for each isolate and they were identified using Bergey's classification of bacteria.

2.3. Production of extracellular enzyme agarase:

The isolated potential strain producing agarase were picked up and inoculated into the culture medium. The inoculums were kept in shaker (140rpm) for 48 hrs at 35°C [1].

2.4. Agarase purification:

All the procedures were carried out at below 4°C. The cultures were centrifuged at 9000 rpm for 20 minutes and 100ml of the supernatant was obtained. The culture supernatant was adjusted to 70% saturation with solid ammonium sulfate and stirred over night. The precipitate collected by centrifugation at 15,000g 30 minutes. The collected precipitate was dissolved in 50 ml of 30 mM Tris-Hcl buffer (pH 7.5) containing 0.1mM EDTA (buffer A) and dialyzed against buffer A for overnight [2].

2.5. Column chromatography:

The dialysate was applied onto DEAE-cellulose column (Geni Lab Bangalore) equilibrated with buffer A, and the column was washed with 70ml of buffer and then with 70ml of the same buffer with 0.13M NaCl. The active fraction were eluted with a linear gradient of 0.13M NaCl to 0.4M NaCl in buffer A. active fraction were pooled and concentrated to 5ml and stored at 4°C [3].

2.6. SDS-PAGE:

SDS-PAGE was performed on an 8% acrylamide gel by the method of (Lammeli) Protein standard (Geni Lab Bangalore) were used as molecular marker standard. The proteins were stained with coomassie Brilliant Blue R-250.

3. RESULTS

3.1. Isolation, identification and distribution of the agarolytic bacteria:

All samples were screened for agarolytic organisms after enrichment by plating them onto seawater enriched agar and minimal salt peptone agar. Standard biochemical tests showed that the agarolytic organisms present in the above 4 isolates belongs to genus *Streptomyces, Pseudomonas, Bacillus* and *Cytophaga* respectively.

When plated onto Minimal agar medium, a gradual degradation of agar was seen. The white colour of the medium turned colourless. Minute pin point colonies were seen. On culturing in Seawater enriched agar, different kinds of growth were observed in different samples. A crater-like depression was formed around the white colony indicating agar degradation. Colonies that formed a characteristic iridescence were observed when the UV light is allowed to pass through them. A thin layer of growth of organisms was observed that caused softening of the agar beneath and the agar became sunken. The reproducibility of the agarolytic property was confirmed as they were degrading agar when cultivated again on the above media.

3.2. Agarase Purification:

Agarase was purified from the culture supernatant of organism by the Ammonium sulphate precipitation, dialysis, and column chromatography on a DEAE-Cellulose (**Fig 1**). The table summarizes the results of each step.

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3.3. Properties of purified agarase:

The purified agarase had a molecular mass of approximately 90-110KD estimated by SDS-PAGE shown in (**Fig 2**).

Table 1: Distribution of agarolytic bacteria

Location	Sample	Degradation of agar
Marina	1	+
	2	+
	3	+
Pudhucherry	1	+
	2	+
	3	+
Mahabalipuram	1	+
	2	+
	3	+

Table -2: Enzyme Activity on 1% Agar

Steps	Total Activity/Unit(mm)
Culture supernatant	14.1
Ammonium sulfate precipitation	8.11
Dialysis	3.03
DEAE-Cellulose	1.47

Table 3: Enzyme Activity on 0.1% Agarose

Steps	0.1% Agarose (u/mg)
Culture supernatant	0.11
Ammonium sulfate precipitation	0.19
Dialysis	1.57
DEAE-Cellulose	4.90

Fig 1: Enzyme Activity on 1% Agar

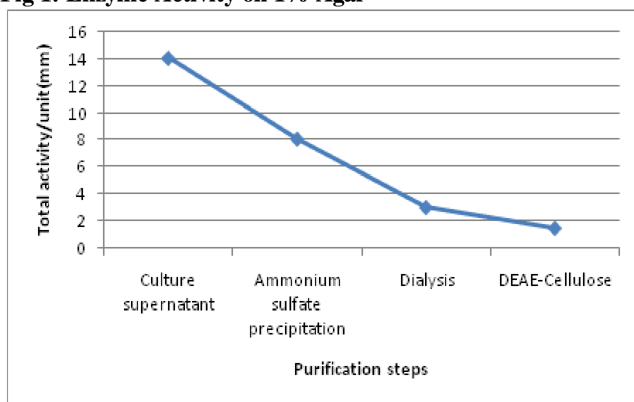
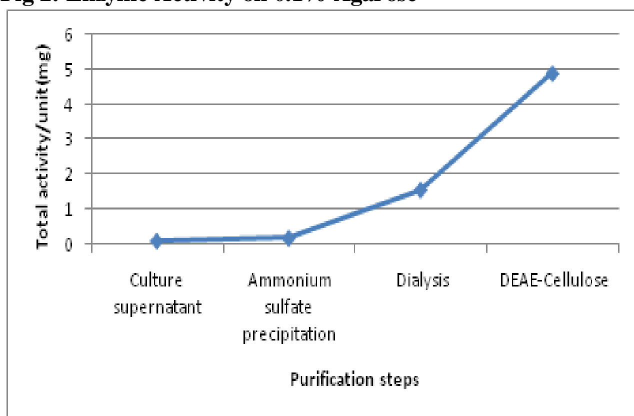


Fig 2: Enzyme Activity on 0.1% Agarose



4. DISCUSSION

Agarolytic bacteria were first isolated early in 1902 by Gran who reported the bacterium *Bacillus gelaticus*. Later other bacteria belonging to various genera were isolated from various parts of the world and also from varied sources. Marine environment has always been an abundant resource for agarolytic bacteria. Goresline [4] isolated 3 groups of bacteria from trickling filter, of which 2 belonging to the genus *Pseudomonas*. Stanier [1] first reported an actinomycete (*Actinomuces coelicolor)* having agarolytic activity. Later it was identified as *Streptomyces coelicolor*. He also reported a bacterium under the genus *Cytophaga* that degraded agar. In India, agar digesters were isolated from the coastal areas of Puri, Vishakapatnam and Mumbai [5]. They were predominantly belongining to the genus *Pseudomonas*. In Karnataka, Lakshmikanth *et al.* [6] isolated *Pseudomonas* that was agarolytic. But very less or no work has been carried out in the coastal area of Tamilnadu to study the agarolytic flora. Thus in this study attempt was made to isolate four kinds of agarolytic bacteria belonging to the genera *Streptomyces, Pseudomonas, Bacillus* and *Cytophaga.*

Seawater sample was enriched for the isolation of 9 isolates, later out of 9 potential strains were subjected for further processing like Ammonium sulphate precipitation, dialysis and column chromatography. The agarase secreted into the liquid culture and the enzyme was purified to homogeneity on the SDS-PAGE. The enzyme respectively indicating that the enzyme is a monomer. The enzyme cleaves β, 1- 4 linkage of agarose to produce neoagarotetrose as the predominant product. As India has a very rich marine resource, intensive research on the agar-digesters will help production of the important enzyme, agarase, in a commercial scale and in a cost-effective manner so that India no longer needs to import this enzyme thereby saving the valuable foreign exchange to a greater extent. Its widely useful properties can then be applied by the molecular biologists at lesser cost and made beneficial to the common people.

REFERENCES

1. Stanier, R. Y. 1941. Studies on marine agar-digesting bacteria. In part a thesis submitted in partial fulfillment for the degree of Master of Arts at the University of California, Los Angeles.
2. Suzuki, H., Y. Sawai, T. Suzuki, T and K. Kawai, 2002. Purification and Characterization of an extracellular α-neoagarooligosaccharide hydrolase from *Bacillus sp.* Mko3. *J. Biosci*. 93: 456-463.
3. Kawai, J. H. J. M., P.W. Postma, 1994. Glucose kinase has a regulatory role in carbon catabolite repression in

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Streptomyces coelicolor. *J. Bacteriol*. 176: 2694-2698.

4. Goresline, H. E. 1933. Studies on agar-digesting bacteria. A thesis submitted to the graduate faculty of Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
5. Safdar Jawaid, 2004. Screening, isolation and identification of marine bacteria for agarase activity and characterization of agarase from a selected strain, A thesis submitted to the Jamia Millia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
6. Lakshmikanth, M., S. Manohar, Y. Shouche, J. Lalitha, 2006. Extracellular β-agarase LSL-1 producing neoagarobiose from a newly isolated agar- liquefying soil bacterium *Actinebacter sp*. AG LSL-1. *W. J. Microbiol and Biotech*. 22: 1087-1094.

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