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Studies on agar degrading *Salegentibacter* sp. and characterization of its agarase

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Abstract

The phenotypic and agar degrading features of an unidentified marine bacteria was investigated. The strain was Gram-negative, obligatory aerobic and non motile. On the basis of several morphological features and a phylogenetic analysis of the genes coding for the 16S rDNA, this strain was identified as *Salegentibacter* sp. On solid agar medium, this isolate produced extracellular agarase which causes agar liquefaction around the colonies. An extracellular agarase was purified by ammonium sulfate precipitation, gel filtration and ion-exchange chromatography on DEAE-cellulose. The purified protein migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and it had a molecular weight of 28 kDa. The enzyme exhibited maximal activity at pH 7.5. The kinetic parameters of the enzyme were Km = 3 ± 0.19 mM, Kcat = 160 ± 10 S^{-1} and Kcat / Km = 53 ± 10 $S^{-1}.mM^{-1}$. The purified agarase may be used for application in protoplast formation of agarophytes which can subsequently be used in algal biotechnology to evolve a superior strain of *Gracilaria* sp. yielding superior quality agar.

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Introduction

Several types of agarase-producing bacteria that degrade and utilize agar have been isolated. Generally, most previously reported agar-degrading bacteria were isolated from marine environments. A wide range of agar-degrading bacteria, including *Alteromonas* sp. (Leon *et al*., 1992; Potin *et al*., 1993), *Bacillus cereus* (Kim *et al*., 1999), *Cytophaga* sp. (Van *et al*., 1975), *Pseudoalteromonas* sp. (Ivanova *et al*., 1998; Chiura *et al*., 1983), *Pseudomonas* sp. (Morrice *et al*., 1983a; Kong *et al*., 1997) and *Vibrio* sp. (Sugano *et al*., 1993) have been isolated from marine environments. Since agar is a polysaccharide produced by marine red algae, it is natural that most agar-degrading bacteria are inhabitants of marine habitats. Agar-degrading bacteria are considered to utilize agar as a carbon and energy source to inhabit marine environments.

Agar is composed of two fractions, agarose and agaropectin. Agarose, the main constituent, is a neutral polysaccharide that forms a linear chain structure consisting of repeating units of agarobiose which is an alternating polymer of D-galactose and 3 ,6-anhydro-L-galactose linked by alternating β-(1 ,4) and α-(1 ,3) bonds (Hirase, 1969). The agar-degradation processes have been studied on several agar-degrading bacteria. The agarase was identified in studies on *Pseudoalteromonas atlantica* ATCC 19292 (Morrice *et al*., 1983a; Morrice *et al*., 1983b;). *P. atlantica* hydrolyzes agarose by extracellular β-agarase. This enzyme cleaves the β-(1, 4) linkage between D-galactopyranose and 3, 6-anhydro-L-galactose to give a series of neoagaro-oligosaccharides. Then, neoagarotetraose, one of the major end products, is cleaved at the central β-linkage by neoagarotetraose hydrolase to yield neoagarobiose. Finally, neoagarobiose is degraded by periplasmic a-neoagarobiose hydrolase to the D-galactose and 3, 6-anhydro-L-galactose, which are metabolized by intracellular enzymes. The other type of agar-degradation process involves the cleavage of α-(1, 3) linkage in agarose by extracellular α-agarase (Potin *et al*., 1993) yielding a series of agaro-oligosaccharides.

In our laboratory, we have isolated an agar-liquefying bacterial strain from the Gulf of Mannar coast to characterize their extracellular agarase in an attempt to contribute to our understanding of the basis of agar hydrolysis. We describe here the identification of a new agarolytic bacterial strain *Salegentibacter* sp and the characterization of an extracellular agarase.

Materials and methods

Agar liquefying bacterium was isolated from decayed seaweed in Gulf of Mannar (Tamilnadu, India). The screening was carried out on agar plates in a medium containing (g/L): 1 KH_2PO_4; 0.5 NH_4Cl; 0.5 $MgSO_4.7H_20$; 0.1 $CaCl_2.2H_2O$ and 20 agar. The plates were incubated at 37°C for 48 h. Colonies that liquefying agar were picked up and purified further by the same plating method.

Phenotypic analysis of the strain

The agar liquefying bacterium was identified by using *Bergey's Manual of Systematic Bacteriology* (Baumann *et al*., 1984). Staining, morphological, biochemical and physiological tests were carried out essentially as described by Sneath (Sneath, 1994). Genomic DNA was prepared by the procedure of Zhou method (Zhou *et al*., 1996).

PCR amplification of the 16S rDNA

Amplification of the 16S ribosomal DNA (rDNA) was carried out as described by Ruimy *et al*. (Ruimy *et al*., 1994). First, 10 to 20 ng of purified genomic DNA was amplified in 50 ml of a reaction mixture consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.12 mM deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase with primers 5'-AAGTCGT AACAAGGTAAC-3' and 5'-CTGAGCCATCAAACTCT-3'

(7 µM concentrations of each). The initial denaturation step was 4 min at 95°C; this was followed by an annealing step at 52°C for 80 s and an extension step at 72°C for 90 s. The thermal profile then consisted of 25 cycles of annealing at 52°C for 80 s, extension at 72°C for 90 s, and denaturation at 94°C for 45 s. A final extension step was carried out at 72°C for 5 min. The single DNA band of approximately 1.5 kb as detected by agarose gel electrophoresis was purified by using the DNA extraction kit spin gel (Genei, India.). The DNA sequence was determined by direct sequencing of the PCR product on an Applied Biosystems sequencer (Ana-Gen Technologies, Inc., Palo Alto, Calif.).

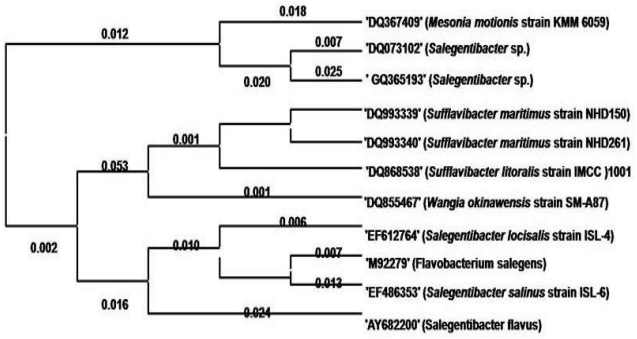


Fig 1. Phylogenetic tree based on 16s rDNA sequences showing the position of *salegentibacter* sp.

Phylogenetic analysis and alignment

The sequence of the 16S rDNA of isolated bacterium was aligned and analyzed essentially as described by Weisburg *et al*. (Weisburg *et al*., 1991).

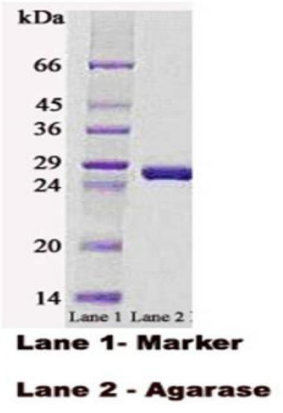
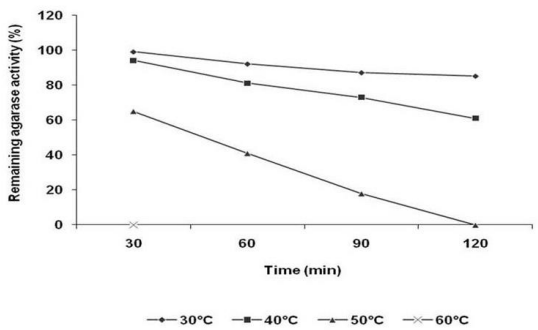


Fig 2. Molecular mass of purified agarase.

Purification of agarase

Unless specified otherwise, all operations were done at 4°C. An overnight culture of isolated colonies of bacterium was prepared in the basal mineral medium containing of (g/L): 1 KH_2PO_4; 0.5 NH_4Cl; 0.5 $MgSO_4.7H_20$; 0.1 $CaCl_2.2H_2O$, 0.2 agar and 0.1 % yeast extract. The cells were grown in a Sciegenics orbital shaker at 140 rpm and 30°C to the stationary phase (30 h). The cells were centrifuged at 6,000 X *g* for 25 min. The supernatant was brought to 75% saturation with solid ammonium sulfate overnight and centrifuged at 6,000 X *g* for 25 min. The resultant pellets were collected by centrifugation at 6,000 X *g* for 25 min, dissolved in 20 mM Tris-HCl buffer (pH 7.5) and dialyzed three times against the same buffer. The dialyzate was loaded onto a DEAE-cellulose column (10 by 1.5 cm) equilibrated with 20 mM Tris-HCl buffer. The protein was eluted batch wise with 90 ml of 1.5 M NaCl in 20 mM Tris-HCl buffer and concentrated by precipitation with ammonium sulfate (75% saturation), dissolved in 2 ml of Tris-HCl buffer, and loaded on a Sephadex G75 column (60 by 2.5 cm) equilibrated with Tris-HCl buffer. Elution was performed with 1.5 M NaCl in 20 mM Tris-HCl (total volume 15 ml).Fractions (3 ml) were collected, pooled on the basis of activity, and then loaded onto the DEAE-cellulose column (10 by 1.5 cm). Under these conditions more than 85% of the enzyme eluted in the flow through. The enzyme was concentrated with polyethylene glycol and dialyzed against Tris-HCl buffer. The enzyme was stored at -20°C.

Fig 3. Thermo stability of *Salegentibacter* agarase.



Agarase assay

The assay system consisted of 2.0 mL substrate [0.25% agarose in 20 mM Tris-HCl buffer (pH 7.5)] and 1.0 mL of enzyme in a total volume of 3.0 mL and was incubated at 30 ± 2°C for 2 h. The reaction was stopped by heating the tubes in a boiling water bath for 15 min (Ghadi *et al.*, 1997). Boiled enzyme was used as blank. The reducing sugar generated was determined by the Somogyi-Nelson method (Nelson, 1994), using D-galactose as standard. The protein content of enzyme preparation was estimated according to the method of Lowry (Lowry *et al.*, 1951). One unit of agarase activity was expressed as µmol of reducing sugars (D-galactose equivalent) released in 1 min under the above conditions.

SDS-PAGE

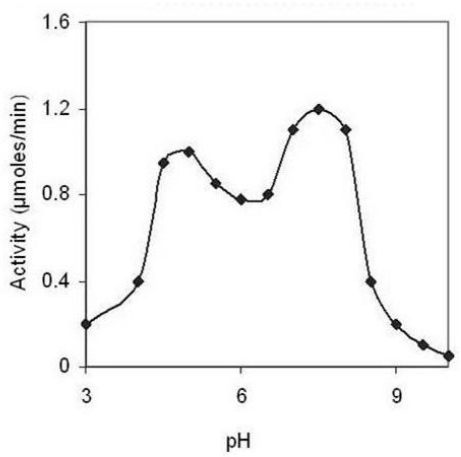
The purified enzyme was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gel as per method by Sambrook and Russel (2001) along with standard molecular weight protein markers. The sample and marker proteins were treated with 2% SDS and 5% 2- mercaptoethanol at 100°C for 5 min just before loading. The gel was stained with Coomassie-brilliant blue R-250 for 4~5 h and destained.

Effects of temperature and pH on agarase activity

The optimum temperature for agarase activity was determined under the standard assay condition by varying the incubation temperature i.e. 10, 20, 30, 40, 50 and 60°C. Enzyme activity at temperatures from 10 to 60°C was determined by incubating the enzyme along with the substrate at the respective temperatures. These studies were carried out in sealed Eppendorf tubes for 2 h by incubating the tubes in water bath at the required temperatures.

The pH profile for agarase activity was obtained using 10 mM phosphate buffer of varying pH ranging from 3.5 to 10. Substrate solution (0.25% agarose) was prepared using the above mentioned buffer. The residual enzyme activity was measured by the following the procedure as described above.

Fig 4. Effect of pH on agarase activity.



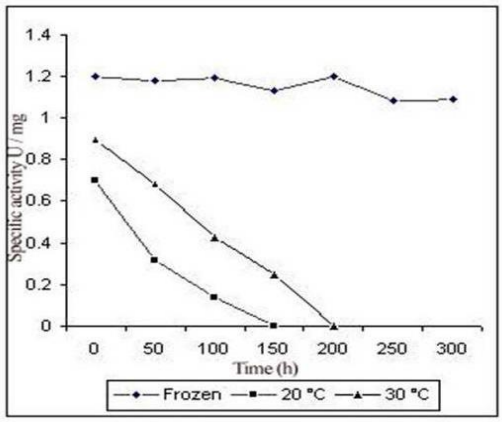
Effect of different cations on enzyme activity

Effect of various cations like Al, Ba, Ca, Fe_3, Mg, Mn, Hg, and K on the reducing sugar releasing activity of agarase was studied by using respective chlorides i.e., $BaCl_2$, $CaCl_2$, $FeCl_3$, $MgCl_2$, $MnCl_2$, $Hg\ Cl_2$, and KCl at 1 mM concentration in the assay system.

Storage stability

The storage stability of agarase with respect to temperature was confirmed by incubating the enzyme at 20, 32, and −20°C (frozen temperature) and then measuring its activity at regular intervals.

Fig 5. Storage stability of agarase.



Kinetic studies

Different concentrations of substrates varying from 1~5 mg/ 2 mL prepared in 20 mM Tris-HCl buffer were mixed with 0.129 mg of enzyme protein and incubated at 40°C for 2 h. The kinetic constants Km and Vmax were estimated following the method of Lineweaver and Bruk (Lineweaver and Bruk, 1934).

Results and discussion

Strain properties and identification

The isolated bacterial strain liquefied agar after 24 to 48 hours of incubation at 30°C. At longer incubation times, the colonies produced a red brown pigmentation. The agarolytic strain was a Gram-negative rod shaped bacterium, non-motile; it is also obligate aerobic, phosphatase, oxidase, lysine decarboxylase, methyl red and indole positive and VP and citrate utilization negative. The preliminary identification results showed that the isolated strain was in accordance with *Salegentibacter* sp, according to Bergey's Manual of Systematic Bacteriology. The results of several biochemical and physiological tests for the isolated strain are shown in Table 1.

Phylogenetic analysis of 16S rDNA

The 16s rDNA sequence of the agarolytic strain was compared to sequences available from public databases. Figure1 shows an unrooted tree of the *Salegentibacter* species. The isolated strain and *Salegentibacter* sp. DQ073102 formed a robust clade. Based on these data, we proposed the assignment of our strain as *Salegentibacter* sp.

Purification of agarase

The selected isolate was cultured in basal mineral medium containing 0.1% yeast extract and 0.3% (w/v) agarose at 30° C. The viscosity of the medium was very high before inoculation, decreased with the time of incubation, and reached a minimum after 5 days. These changes indicated that the bacteria secreted some agar degrading enzymes into the culture medium. Therefore, isolation of the one of the enzymes from the 5th day broth was attempted. The enzyme was purified about 8.3fold, with a final specific activity of 1.09U/mg. The overall recovery of the purification was 5.9%, which is the moderate activity among all the reported literature on agarase (Table 2).Vera *et al*., have reported specific activity of crude agarase to be 2.3 units/mg protein, which, after subjecting to 75% ammonium sulfate precipitation increased to 13.4 units/mg protein indicating almost 5.82-fold purification (Vera *etal*., 1998). A maximum agarase activity 292 units/mg protein have been reported resulting in 4.49 fold purification after following three steps (Vera *et al*., 1998).

Table 1. Differential morphological, physiological and biochemical characteristics of *Salegentibacter* sp.

S.No	Characteristics	MW4
1	Cell morphology	Rods
2	Color of colonies	Yellow
3	Motility	-
4	Methyl red	+
5	VP	-
6	Indole	+
7	Oxidase	+
8	Lysine decarboxylase	+
9	Phophatase	+
10	H_2S	+
11	Citrate utilization	-
12	Agar	+
13	Casein	-
14	Cellulose	-
15	Gelatin	+
16	Tween 80	+
17	Starch	+
18	Nitrate reduction	+
19	Catalase	+
20	Phosphatase	+
	Acid production from:	
21	Galactose	+
22	Glucose	+
23	Maltose	+
24	Arabinose	-
25	Cellobiose	-
26	Melibiose	-
27	Rhamnose	-
28	Sorbose	-
29	Sucrose	-
30	Xylose	-
31	Succinate	-
32	Glycerol	-
33	Mannitol	-
34	H_2S production	+
35	Growth at pH 6	+
36	Growth at 10% NaCl	W
	Growth temperature:	
37	Maximum	40°C
38	Optimum	32°C

(+, Positive reaction; -, negative reaction; W, weakly positive reaction)

Table 2. Purification of agarase from *Salegentibacter* sp.

Purification step	Total amount of protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture fluid	124	16.8	0.13	100	1
Dialyzed sample	32	5.1	0.16	30.3	1.2
DEAE-cellulose fraction	3.4	1.38	0.40	8.2	3
Sephadox G-75 fraction	0.91	1.0	1.09	5.9	8.3

Molecular mass

Agarase from *Salegentibacter* sp had a molecular mass of 28 kDa, as determined by a comparison with the mobility of protein standards (Fig2). This is the lowest molecular weight among all the reported values. Since the isolated enzyme appeared as a single band, it was concluded to be monomeric. Izumi *et al.* (1991) reported that the molecular weight of *Pseudomonas* sp. PT-5 agarase is 31 kDa and Morrice *et al.* (1983) reported that the molecular weight of *Pseudomonas atlantica* β-agarase I is 32 kDa. From the reported data, it can be concluded that agarase enzyme varies in its MW depending upon the organism from which it is isolated.

Biochemical properties of the enzyme

The purified agarase from *Salegentibacter* sp. retained only 61% enzyme activity after being heated at 40°C for 120 minutes, where as significantly lower and higher temperatures adversely affected its activity (Fig3). This enzyme was completely inactive when heated at 60°C for 30 minutes. This result was comparable to that of Yasushi *et al.*, where maximum agarase activity was reported at 30°C (Yasushi *et al.*, 1993).

The pH profile of agarase from *Salegentibacter* sp. strain was bell shaped, with a maximum at pH 5 and 7.5 (Fig4). Hence the enzyme can be used both in acidic as well as in alkaline pH based on its application. Maximum agarase activity at pH 8.5 has been reported from *Pseudomonas* sp. (Izumi *et al.*, 1991). The agarase from *Vibrio* sp. exhibiting maximum activity at pH 8.0 has also been reported (Sugano *et al.*, 1993). No reports are available where agarase was shown to be active in acidic as well as alkaline conditions.

Agarase activity was assayed in the presence and absence of metal ions. It was observed that none of the cations exhibited activation in the enzyme activity. On the contrary, there was a 51~61% reduction in the activity with cations like Hg and Mg and 41~33% reduction with Al, Fe, Mn, and K. Hence it can be concluded that agarase of present study is cation independent (Table3). Izumi *et al.* (1991) have reported an inhibition in the enzyme activity by NaCl at concentration more than 0.2M. However, in the present studies it is difficult to conclude the mechanism for enzyme inhibition.

Table 3. Effect of metal ions on agarase activity from *Salegentibacter* sp.

Metal ions	Specific activity (%)	Reduction in specific activity (%)
Blank	100	-
$AlCl_2$	58.57	41.43
$BaCl_2$	67.18	32.82
$CaCl_2$	52.16	47.84
$FeCl_2$	58.52	41.48
$HgCl_2$	48.23	51.77
KCl	56.54	43.46
$MgCl_2$	38.44	61.56
$MnCl_2$	56.11	43.89

Storage temperature was found to have a profound effect on the agarase activity. The agarase stored at 20°C exhibited a decline in agarase activity by 20% within three days, which was totally lost within 6 days whereas the one stored at room temperature (30°C) exhibited a 48% decline in enzyme activity within 3 days which was completely lost within 6~7 days of incubation. In frozen condition (- 20°C), the agarase remains stable even after 10 days of incubation period (Fig5). The agarase which was stored in frozen

condition undisturbed remained stable for almost 30 days.

Kinetic parameters

The kinetic parameters were Km = 3 ± 0.19 mM, Kcat = 160 ± 10 S^{-1} and Kcat / Km = 53 ± 10 $S^{-1}.mM^{-1}$. However, Km = 2 ± 0.15 mM, Kcat = 150 ± 10 S^{-1} and Kcat / Km = 75 ± 10 $S^{-1}.mM^{-1}$ for agarase from *Zobellia galactanivorans* have been reported (Murlelle *et. al*, 2005).

Conclusion

Seaweeds, in addition to phycocolloid contain other useful substances such as vitamins, betain, carotenoid, etc. In order to recover these useful substances, enzymatic degradation may be a promising alternative to chemical methods because of the mild conditions produced during degradation process. Additionally, agarase isolated in the present investigation will also may be useful in the isolation of protoplast from *Gracilaria* sp.

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