



Isolation and Characterization of Agarase Producing Bacteria from Marine Ecosystem of South Gujarat

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Received: 12 Jan 2019 / Accepted: 10 Mar 2019 / Published online: 1 Apr 2019

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Abstract

Marine ecosystem is the least explored ecosystem of the earth which contains microbes capable of producing different enzymes. This study was focused on isolation and characterization of agarase producing microorganisms from marine water, as many marine algae are great source of agar-agar. In present study marine samples were collected from Tithal, Danti and Daman beaches from South Gujarat, India. Enrichment and plating were done on Minimal mineral salt (MMS) agar medium, 30 isolates were obtained from primary screening. Secondary screening was performed for confirmation of Agarase production and 3 maximum activity giving isolates C-17, C19 and C-29 were selected for further characterization study. During characterization all 3 isolates gave optimum activity at pH 5, temperature 45°C, and 4% NaCl concentration. The partial purification of crude enzyme was performed by ammonium sulphate precipitation and good activity was found at 80% saturation.

Keywords

Agarase, MMS medium, Characterization, Marine ecosystem

INTRODUCTION:

The world's oceans cover more than 70% of our planet's surface; countless marine microorganisms contain biochemical secrets which can provide new insights and understanding of enzyme. Marine microorganisms have been attracting more and more attention as resource for novel enzymes, because these enzymes are more stable and active than derived from plant and animal [1]. In 1965, at the Fifth International Seaweed Symposium, Araki (1966) reviewed the chemistry of agar [2]. Agar is present as polysaccharides in the cell wall of seaweeds. Many types of seaweed like *Gelidium* and *Gracilaria* belong to *Rhodophyceae* family contain agar in their cell

wall. Here, some agarolytic bacteria present around cellwall of sea weeds or red algae or brown algae. Agarolytic bacteria utilize agar as sole source of carbon and energy. *Gracilaria* is good source for agar production, because it is easily harvested and cultivated. Agar is composed of agarose and the other was agaropectin. It is a linear polymer structure consisting of D-galactose and 3, 6 anhydro-L-galactose and D-glucuronic acid in its structure. In short agar can be considered to consist mainly of alternating unites of β -(1-3)-D and α -(1-4)-L linked galactose residues.

Agarase is enzyme which degrades agar and produce oligosaccharides and neoagarooligosaccharides.

Agarase is mainly found on cell wall of red algae because red algae contain agar in their cell wall. Two types of agarases are mainly found. α -agarase which cleaves α -L-(1-3) linkage from series of 3,6 anhydro-L-galactopyranose, and produce oligosaccharides. β -agarase which cleaves β -D-(1-4) linkages from D galactopyranose and produce neoagarooligosaccharides. Agarases are classified into other two groups also. Group 1 softens agar and forms depression around colonies, whereas group 2 cause extensive liquification. Applications of agarases include enzymes like neoagarobiose, teraose and hexose, which are used as skin whitening agent. It also acts as antioxidative, antitumour, antimutagenic and immunomodulating agent. Due to its stabilizing and gelling properties they are widely used in food industry, used in genetics for DNA recovery and also used to isolate protoplast from seaweeds [3].

MATERIALS AND METHODS:

Material used:

Crystal violet, Saffranin, Gram's iodine, Alcohol, Minimal mineral salt agar medium, Lugol's iodine, Fermentation broth, DNSA (3,6 dinitrosalicylic acid) reagent, (0.1%) Rochelle salt, NaCl salt, Waterbath, pH meter, Ammonium sulphate powder, Glycine - NaOH buffer (pH 9), Sodium acetate buffer (pH 5), Phosphate buffer (pH 7).

Methods:

3 marine water samples were collected from Tithal, Danti and Daman beaches of South Gujarat, India. All samples were enriched in 0.1% agar containing Minimal Mineral Salt (MMS) medium. After 48 hours enrichment dilutions were made, and plating was performed on MMS agar plates by spread and/or streak plate method for each sample. Inoculated MMS agar plates were incubated at 37°C for 24 – 48 hours. After incubation plates were flooded with Lugol's iodine. Colonies showing zones were observed and preserved for further study. Colony characteristics were recorded, and gram staining was performed from purified isolates [4].

0.1% agar containing Minimal Mineral Salt medium tubes were prepared and inoculated with each purified isolate, incubated at 37°C for 24 hours. On the next day, spots of centrifuged broths from various isolates containing crude agarase enzyme were kept onto MMS agar plate medium and incubated at 37°C for 24 hours. Lugol's iodine was flooded over the MMS agar plate. 5 isolates giving highest zone in secondary screening were further assessed for agarase activity. For 5 selected isolates agarase enzyme was produced by fermentation in

fermentation broth (0.1% agar containing MMS medium) and enzyme activity was performed by DNSA method described elsewhere [5]. Optical density was measured at 540 nm.

From the outcome of secondary screening and agarase activity 3 best isolates finally selected for enzyme activity characterization. Optimization of enzyme activity was done, and activity was measured from fermentation broth incubated at different pH 5, 7, 9; temperatures at 37°C, 45°C, 55°C and NaCl concentrations 3%, 4%, 5%. Crude enzyme was obtained by centrifugation and enzyme activity was performed as above. Enzymes from each isolate were partially purified by ammonium sulphate at 70% and 80% salt saturation [4, 5]. The precipitates formed were dissolved in Glycine-NaOH buffer and enzyme activity was performed by DNSA method. The amount of enzyme that released 1 μ mol of galactose per minute under the assay conditions was defined as 1 unit of agarase.

RESULTS AND DISCUSSION:

Agarases producing microorganism were screened and isolated from marine water samples and 30 isolates were obtained during the study. Out of these 30 isolates, 8 were gram positive and 22 were gram negative. One of the plates after incubation is shown in figure 1 and figure 2 shows appearance of Agarase producing colonies after flooding the plates with Lugol's iodine. Results of secondary screening are shown in Figure 3 and Table 1. Table 1 indicates results of secondary screening in terms of zone diameter in mm. This test also made confirmation of agarase production.

5 isolates were selected on the basis of zone size for further analysis by enzyme activity. Out of 5 isolates, 3 isolates C-17 (19 mm, 4.69 U/ml), C-19 (20 mm, 5.67 U/ml), and C-29 (19 mm, 4.93U/ml) were selected on the basis of zone size and enzyme activity by secondary screening for further optimization (Figure 4).

On the basis of gram staining out of 3 isolates, 2 isolates (C-17, C-19) were confirmed as gram positive bacteria and another isolate was gram negative bacterium(C-29). For these three isolates optimization process was carried out at three different pH (5, 7, 9); temperature (37°C, 45°C, 55°C) and NaCl concentration (3%, 4%, 5%). All isolates gave best activity, when incubated at pH 5. Moreover, at pH 5 maximum agarase activity was given by isolate C-29 (13.347 U/ml). At 45°C temperature C-29 (12.345 U/ml) gave best agarase activity and overall 45°C temperature was found optimum for all 3 isolates. At 4% NaCl concentration

C-17 (17.7 U/ml) showed optimum activity. Other 2 isolates were also producing high Agarase activity at 4% NaCl concentration. During partial purification at

70 and 80%; good results were obtained at 80% saturation, but further optimization was required as activity obtained was poor. (Figure 5, 6, 7 and 8).

Table 1: Secondary screening of agarolytic isolates

Isolate No.	Zone Diameter (in mm)	Isolate No.	Zone Diameter (in mm)	Isolate No.	Zone Diameter (in mm)
C - 1	17	C - 11	---	C - 21	13
C - 2	11	C - 12	---	C - 22	---
C - 3	17	C - 13	10	C - 23	17
C - 4	18	C - 14	11	C - 24	---
C - 5	14	C - 15	15	C - 25	11
C - 6	17	C - 16	11	C - 26	11
C - 7	16	C - 17	19	C - 27	14
C - 8	11	C - 18	17	C - 28	---
C - 9	---	C - 19	20	C - 29	19
C - 10	10	C - 20	15	C - 30	17



Figure 1: MMS agar plate containing agarolytic bacteria

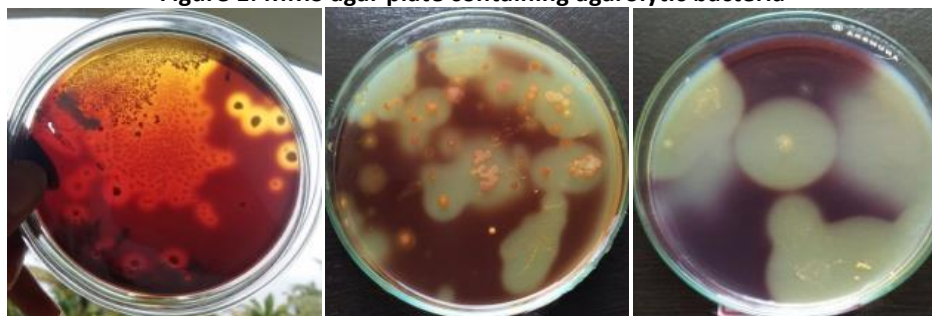


Figure 2: Results of primary screening after flooding with Lugol's iodine

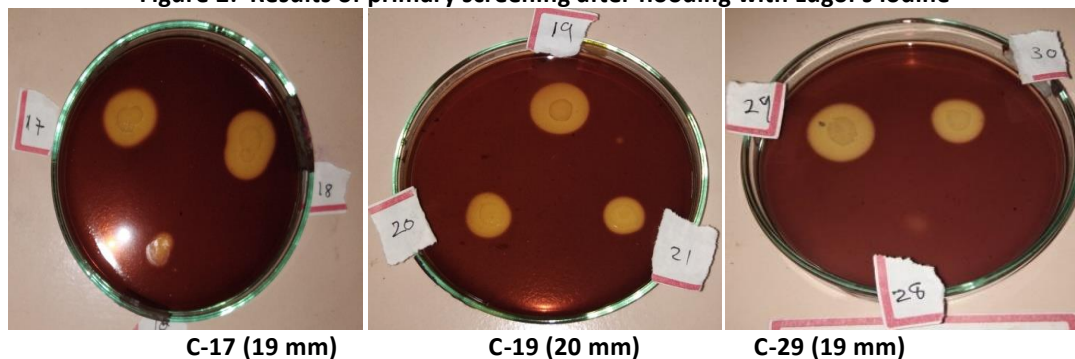


Figure 3: Results of secondary screening

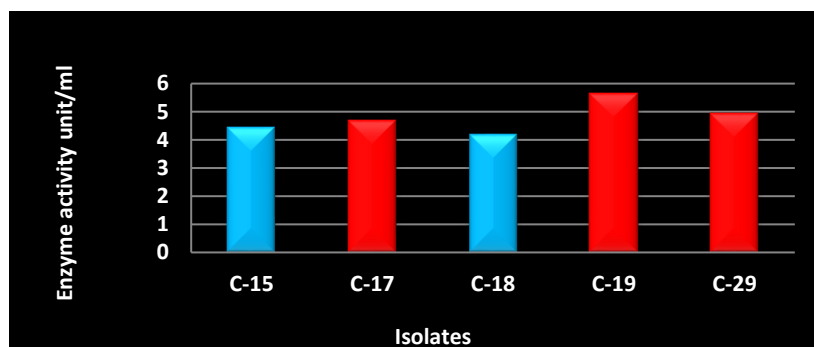


Figure 4: Enzyme activity given by isolates C-15, C-17, C-18, C-19 and C-29

In one study researchers observed growth of isolate in broad range of pH 5-11, but the optimum pH was 8 [4]. Some researchers found that optimum pH for growth and agarase activity was pH 8 for *Bacillus subtilis* [5]. According to one research agarase had an optimal pH at 7.6 [6]. According to one study report

most of the agarolytic bacteria grow at pH range 6.5-7.8 and *Pseudomonas aeruginosa* grew at the broad pH range of 5-11 [7]. Marine bacterium, *Vibrio sp.* strain JT0107 gave best activity at pH 8 in one research [8]. In our study optimum enzyme activity was obtained, when medium was incubated at pH 5.

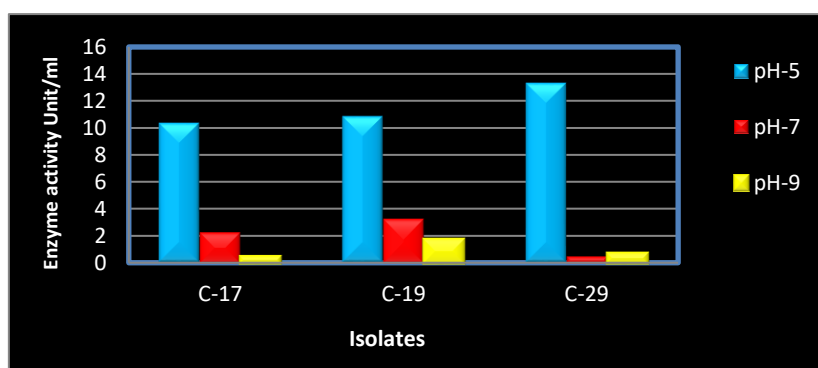


Figure 5: Effect of pH on agarase activity

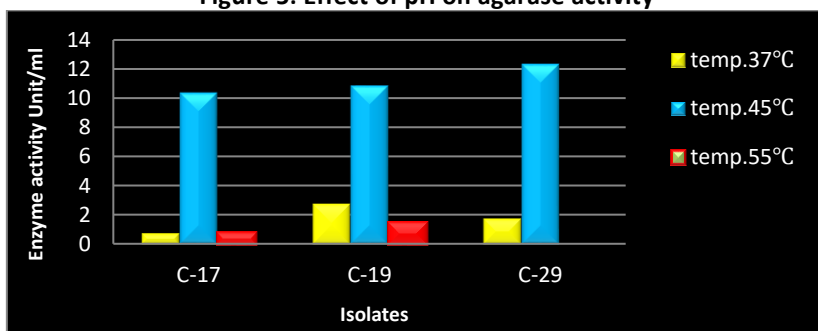


Figure 6: Effect of temperature on enzyme activity

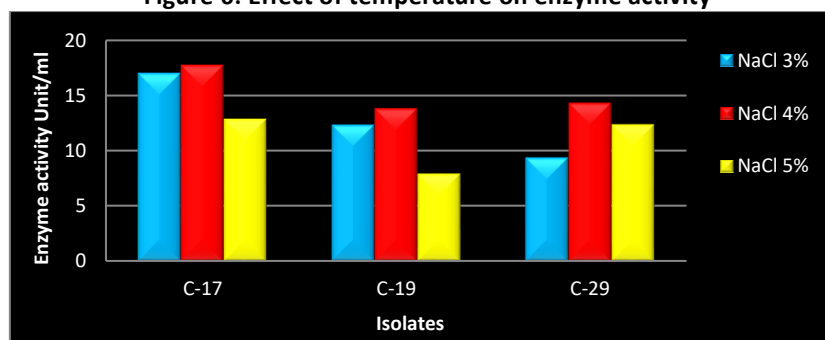


Figure 7: Effect of NaCl concentration on enzyme activity

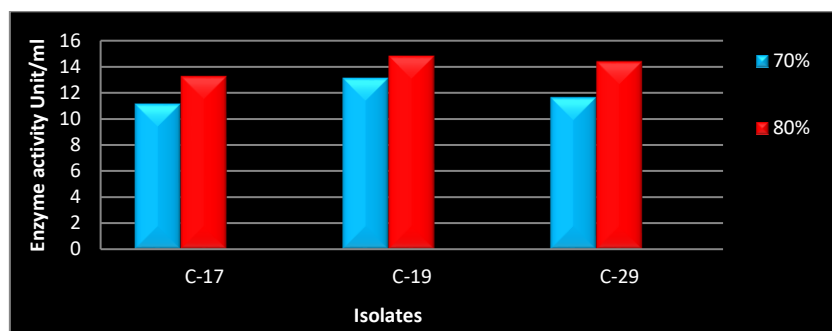


Figure 8: Results of ammonium sulphate partial purification

Enzyme activity Unit/ml

In present study C-17 (10.37 U/ml), C-19 (10.86 U/ml) and C-29 (12.345 U/ml) gave optimum agarase activity at 45°C. Temperature plays great role in activation and inactivation of enzyme. From figure 6 it is clear that all isolate gave best activity at temperature 45°C. But C- 29 inactivated at 55°C. Generally, agarolytic bacteria grow at temperature range 20°C to 40°C with optimum around 30°C [4]. According to previous research 35°C found to be an optimum temperature for the growth and activity of agarase enzyme [5].

In present study, the effect of NaCl concentration in agarase activity was determined at 3%, 4%, 5% concentration and best activity was obtained at 4% NaCl concentration and they were C-17 (17.77 U/ml), C-19 (13 U/ml), C-29 (14.3209 U/ml). According to the study of M. Ziyaddin et al. (2010), 2% NaCl concentration was optimum (7.285 U/ml) for *Pseudomonas aeruginosa*. Present study is in agreement to previous research done [9]. Researches obtained optimum result at 2-4% NaCl concentration by *Alteromonas sp.* GNUM-1. *Bacillus subtilis* gave optimum activity at 3% NaCl concentration in one reaserch⁵. 0.5% NaCl concentration found to be optimum for isolates in one study [10]. Therefore, efforts to isolate good microbial sources of Agarase may be helpful to hydrolyse the agar found in plentiful amount in marine ecosystem.

CONCLUSION:

Marine environment is a plentiful resource for agarolytic bacteria as marine ecosystem contain good amount polysaccharide agar. This ecosystem contains huge amount of unexplored Microflora including agarolytic bacteria. 3 marine isolates C-17, C-19 and C-29 were found best 13garose producer out of total 30 isolates obtained. Isolates gave maximum enzyme activity at pH-5, temperature 45°C and 4% NaCl concentration. Ammonium sulphate

partial purification at 80% saturation was better than 70% but needs further optimization.

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