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Isolation and Optimization of Agarase Producing Bacteria From Marine Sediments

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Abstract: The present study was attempted for Isolation and optimization of agarase producing bacteria from marine sources. Totally 12 morphologically different bacterial colonies were isolated from sediment collected from East coast of Pondicherry. All the isolates were screened for agarase activity by plate method. Among 12 strains E6 strain secrete maximum of agarase enzyme. The partial purification of the enzyme by dialysis, showed over of 29.7 U/ml of agarase activity produced within 48 hours of fermentation in shake flask. The potential strain was identified as *Vibrio sp.,* based on morphological and biochemical characteristics. The optimal medium for agarase production was with glucose (1%), beef extract (1%), while the optimal cultivation conditions for agarase production were pH 8 and 25° C. Findings of the present study showed that marine *Vibrio* will be a potential source for agarase production.
Key words: Agarase, Vibrio, optimization, semi quantitative assay.

Introduction

Agar is abundant biopolymer found in red algae as one of the cell wall component. It is a polysaccharide composed of agarase and agaropectin in which agarose is the neutral fraction of agar which has a linear chain of alternating residues of 3-0-linked β-d-galactopyranose and 4-0linked 3,6-anhydro-α-L-galactose[1]. Agar is an important food additive and solidifying agent for microbiological culture medium. It is one of the most well-known marine polysaccharides, additionally various potential physiological activities of polysaccharides and oligosaccharides derived from agar have been reported, such as antivirus[2], antitumor[3], immune c=enhancement[4], anti-oxidant.[5,6]. Agarose defines the unmodified neutral backbone agars which may hold upto 20% methyl group and sparing sulfate ester group distributes along the polysaccharids chains[7].

Agarase produced by bacteria classified in two groups based on their mode of action; α-agarase, β-agarase which hydrolyzal, α-1, 3 linkage and β-1-4 linkages in agarase respectively. Agarases have applications in the food, cosmetic and medical industries for the production of disaccharides from agar.

Agarases are widely used in food, cosmetic and medical industries for the production of oligosaccharides from agar[8,4]. Agarases can be used to degrade the cell walls of marine algae for extraction of labile substances with biological activities. Agarase enzyme used for liberating cells and DNA entrapped in agarose films for long storage. Bacterial agarases are used in various preparative process namely for detecting agars and removing agar from cell preparations. Agarases used for purifying DNA fragments from agarose gels

in molecular biology. It is also used for preparing protoplast[9]. It is also a useful tool for generating oligosaccharides from polysaccharides which are known to possess various new biological and therapeutic properties.

Several β-agarases have been purified and characterized in the past decade from *Vibrio sp.,* AP-2[9], *Streptomyces coelicolor*[10], *Pseudomonas atlantica*[11], α-agarase have been reported from only from *Alteromonas agarlytices*[12] and *Vibrio sp.,* JT0107[13]. Agarolytic enzymes have been reported for several bacterial genera including cytophage, *Pseudomonas*, *Streptomyces* and *Vibrio*. Most of the bacteria have been isolated from marine environments, although a few species isolated from rivers, soil and sewage[14]. With this view our study was investigated for screening of agarase producing bacteria from Marine sediments.

Materials and Methods

Collection and enrichment of sample

In this present investigation, soil sample for the isolation of agarase degrading bacteria was collected from East coast from Pondicherry were collected. The collected soil sample was dried at room temperature for three days. Five gram of soil sample was taken and added to the 100ml of enrichment media consisted of 0.1% agar in seawater in a 500ml of Erlenmeyer flask. The conical flask was kept in shaker for incubation at 22˚C for 5 days[15].

Isolation of agarolytic organism:

The enriched sample was taken for the isolation of agarolytic organism. 0.1ml of enrichment sample was spreaded onto Czapek dox medium(Sodium nitrate-200mg, Dipotassium hydrogen phosphate-100mg, Magnesium sulphate-50mg, Ferrous sulphate-10mg, Potassium chloride-50mg, Agar-1500mg for Distilled water 100ml) plate containing agar as the only source of carbon energy. The plates were incubated to at 28˚C[16]. The incubated plates were observed daily from second day onwards for agar depression colony. The depression colonies were selected and streaked on Czapek dox medium slants and incubated at 28˚C. All the strains were preserved at 4˚C until further studies.

Selection of potential agarase producing strain by plate method:

All the selected bacterial strain was further screened for confirmation of agrolytic activity by plate method. The bacterial strain were spotted on artificial sea agar medium (Tris base-610/mg, Magnesium sulphate-1230mg, Potassium chloride-740mg, Diammonium hydrogen phosphate-13mg, Sodium chloride-1750mg, Calcium chloride-14mg, Agar-1500mg for Distilled water 100ml). The plates were incubated at 28˚C for 48 hrs. After incubation, the plates were flooded with Logol's iodine and observed for the zone of clearance around the bacterial growth[17]. Strain which showed maximum zone of clearance of their preliminary screening was selected as potential strain for further studies.

Production of agarase enzyme from potent strain

Ten ml of nutrient broth was prepared and inoculated with one loopful of potent bacterial culture and incubated at 28˚C for 18hrs and used as inoculums of production. For the production of agarase[16], the composition of production medium was used (Sodium nitrate-200mg, Dipotassium hydrogen phosphate-100mg, Magnesium sulphate-50mg, Ferrous sulphate-10mg, Potassium chloride-50mg, Agar-750mg for Distilled water 100ml). About 5ml of bacterial inoculums was transferred to 50ml of Production medium supplemented with 0.3% agar as a sole source of carbon was prepared in 250ml Erlenmeyer flask and incubated at 28˚C for 72hrs at 170rpm.

Enzyme recovery and semi quantitative assay of crude agarase enzyme

The fermented broth was centrifuged at 6000 rpm for 30min and the cell free supernatant obtained was collected and used as crude agarase enzyme. The semi quantitative assay of the crude agarase enzyme produced from test strain was done by agar well diffusion method on artificial sea medium plates. About 20µl of crude enzyme was added into the well and incubated at 28˚C for 24hrs[18]. After incubation the plates were observed for zone of clearance around the well as described earlier. The size of zone of clearance was directly proportional to the quantity of crude enzyme present in the crude preparation.

Partial purification of agarase

The separation and dialysis of agarase was carried out by adopting the protocol described[19]. About 50 ml of crude enzyme produced from the potent strain was taken and stirred with magnetic beads. To this 50 ml of 80% ammonium sulphate solution was added and slowly mixed for about one hour. The precipitate was allowed to form at 4ºC for 24 hours. Then the whole solution was centrifuged at 4,000 rpm for 10 minutes at 4ºC. The precipitate obtained after ammonium sulphate precipitation was dialysed using dialysis membrane against phosphate buffered saline (pH-7) for 24 hours. The buffer was changed occasionally. Then the dialysate was tested for agarase assay by Dinitrosalicylic acid method[20].

Assay of agarase

Agarase activity was determined by measuring the increase in the concentration of reducing sugar. The one ml dialysed supernatant was added to 20ml of pH 7.0 phosphate buffered saline solution(PBS) with 0.5% agar substrates and incubated at 33 ºC for 30min. After incubation one ml of reaction solution was mixed with 1.5ml of 3,5-dinitrosalicyclic acid reagent. After incubation, the reaction tubes were kept in a boiling water bath for 10 minutes to stop the enzyme reaction and then cooled to room temperature. The reaction mixture was assayed for reducing sugar as galactose by DNS method[20] by reading the absorbance at 575 nm. The calibration curve was prepared with galactose solutions of known concentration and blanks were run simultaneously with enzyme and substrate solutions. One unit of agarase activity was defined as the amount of enzyme, which produced 1µ mol of galactose under the assay condition.

Optimization of agarase Production

Effect of critical medium components on agarase production was studied by adopting classical one factor at a time method. Factors which are studied include carbon source, nitrogen source, temperature and pH[16].

Characterization and identification of potential strain

Microscopic observation, cultural and biochemical characteristic of potential agarase enzyme producing strain was studied by adopting standard procedure and the potential strain was identified with the help of Bergey's Manual of systematic bacteriology.

Results and Discussion

Table 1. Agarase activity of isolated strains in artificial sea water medium

Strain no.	Zone of clearance in mm
E1	27
E2	21
E3	22
E4	23
E5	27
E6	29
E7	14
E8	17
E9	11
E10	21
E11	18
E12	23

In this present study samples were collected from marine sediment and totally 12 morphologically different bacterial isolates were isolated based on the depression colonies in the screening plate. All the 12 strains were screened for agarolytic activity using artificial sea water medium with agar, and observed using Lugol's iodine. Out of 12 strains the strain E6 showed maximum of 29mm zone of clearance in artificial sea water medium. Agarolytic activity of all the strains was represented in table 1. When compared to the method

described by Ghadi et al.[17] This present study will be a best one for the direct and selective isolation of agarolytic bacteria.

They concluded that the size of zone of colour change around well is that the proportional to the enzymatic activity of the test organism[18]. In this present study, strain E6 showed more zone of clearance then other strains. Dialysate of E6 strain showed 34mm of zone of clearance in semi quantitative assay which shows increase of activity than crude enzyme.

Of this, strain E6 was selected as potent strain that could secrete large amount of agarase into the production medium and the quantity of the enzyme was found to be 20.4 U/ml. Suzuki et al., used *Bacillus sp.,* strain for screening of agarase production and reported the maximum production of agarase activity of 14.1U/ml[21]. In this present study partial purification of the enzyme by ammonium sulphate precipitation and dialysis showed 29.7 U/ml of enzyme activity.

Aoki et al., reported *Vibrio species* with above similar characteristics for the production of agarase[22]. Lakshmikant et al., reported that one of the marine yeast strains, which was identified to be *Acinetobacter sp.,* was able to grow on a wide range of carbon sources and secrete a large amount of agarase into the medium[16]. In this present study the strain E6 identified as *Vibrio sp.,* which produce large amount of agarase into the medium when carbon sources added.

Effects of carbon source on agarase production

The maximum activity 25mm was obtained when glucose (1%) was used as the carbon source and lactose (1%) showed the second maximal enzyme production 23mm. The minimal production 18mm was obtained when fructose (1%) was used as carbon source and sucrose showed the second minimal enzyme production 22mm.

Effects of nitrogen source on agarase production

The maximum activity 34mm was obtained when beef extract (1%) was used as the nitrogen source and peptone (1%) showed the second maximal enzyme production 31mm. The minimal production 28mm was obtained when yeast extract (1%) was used as nitrogen source and malt extract showed the second minimal enzyme production 22mm.

Effects of pH on agarase production

The maximum activity 38mm was obtained when pH 8 was used, 30mm was obtained when pH 7 was used which shows second maximal enzyme production. The minimal production 25mm was obtained when pH 6 was used and 22mm was obtained when pH 5 and 21mm was obtained when pH 4 was used showed the minimal enzyme production.

Effects of temperature on agarase production

The maximum activity 25mm was obtained when temperature was at 25˚C and 24mm was obtained when pH 30˚C was showed maximal enzyme production. The minimal production no zone was obtained when 35˚C and 45˚C was used as temperature.

All these conditions are favorable to industrial application, avoiding microbial contamination and reducing the formation of undesirable products. The agarase produced by the *Vibrio sp.,* E6 isolated in this study will have wide applications. Optimization studies using statistical methods like response surface methodology is in progress to prove its agarase producing potential further.

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