IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 13, Issue 3 Ver. III (May. – June. 2018), PP 01-07
www.iosrjournals.org

Isolation and Identification of Agar Degrading Bacteria from Marine Environment

Neeraja Kolhatkar[1], Dr. Seema Sambrani[2]
[1](Department of Microbiology, K. J. Somaiya College of Science and Commerce, vidyavihar-77, Mumbai, India)
[2](Department of Microbiology, K. J. Somaiya College of Science and Commerce, vidyavihar-77, Mumbai, India)
Corresponding Author: Neeraja Kolhatkar

Abstract: *Agarolytic bacteria use agarase to utilize agar as sole source of carbon. It is usually observed in life sciences labs that lot of agar medium needs to be thrown after its usage. The media gets solidified and clogs the drainage systems. Hence there is a need to isolate agarolytic bacteria and use them to solve this pertinent problem for efficient and eco-friendly disposal of agar in labs. Further the enzyme that these organisms produce would also help to harness its several applications in food, cosmetic and medical industries as agarase produces oligosaccharides with remarkable activities after breaking down agar. In this study agarolytic bacteria were isolated from marine water source. The three isolates were able to grow on a media that only had agar as a carbon source. Agarase activity was visualized by using Lugol's solution. By DNSA method it was found that the organisms produced reducing sugars. Effect of various parameters was also checked. Immobilization of isolates was carried out and consortium of organisms was also made to see its effect on agar degradation. MALDI characterization of the two isolates was carried out and 16S rRNA analysis of one of the isolates was carried out.*

Keywords: *Agar, Agarase, Agarolytic Bacteria, Consortium, Enzyme.*

Date of Submission: 24-05-2018 Date of acceptance: 11-06-2018

I. Introduction

Agar is the main component of the cell wall of some species of algae that belongs to the phylum Rhodophyta. These algae are commonly known as agarophytes [1]. The percentage of agar present in the cell wall of the algae varies according to the growth and environmental conditions, it is found to be that around 60% of the dry weight of the red algae consists of agar [2]. Agar is made up of agarose and small molecules called agaropectin. Generally red algae are the one from which agar is extracted, *Gelidium* and *Gracilaria* are the one that are used commonly [1]. *Agarivorans sp., Cytophaga sp., Alteromonas sp., Bacillus cereus, Pseudoalteromonas sp., Pseudomonas sp., Vibrio sp.,* are various bacteria found to produce agarase and have been isolated from various environment [1], [3]. Agarases catalyze agar hydrolysis and are of two type α-agarase and β-agarase depending on the pattern of its cleavage [1]. Agarooligosaccharides is produce when α- Agarases cleave α-1, 3 linkages of agar polymer and neoagarooligosaccharides is produce when β-agarases cleave β-1, 4 linkages of agar polymer [1], [2]. Agarases are produced extracellularly accept few that are been produce intracellularly [1]. As far as the knowledge many bacteria produces this enzyme agarase and these bacteria are isolated from various environments like seawater, soil, marine sediments [1].

Agarase have many applications [1]. They have been used for agar hydrolysis for production of oligosaccharides, which have essential physiological and biological activities that are helpful for human health. Even protoplasts from seaweeds have been obtained by using agarase [1], [4] and for DNA recovery from agarose gel [1], and for understanding the composition and structure of cell wall of seaweeds [1]. Recent advances like cloning and sequencing of these enzymes has made it possible for its structure-function analysis [1]. This in turn will provide valuable understanding of this enzyme and also understanding use of this enzyme [1].
So isolating organisms producing agarase would be of great importance in medical, cosmetic, life sciences and industrial fields.

II. Materials And Methods

1. Source
Agar degrading bacteria are found mostly in marine water, so water samples were collected from Arabian Sea and Indian Ocean.

2. Enrichment

Enrichment media used was sea water (sample water) and 0.5% agar was added to the media as carbon source for agar utilizing bacteria. 0.25% of agar was added to 12.5ml of distilled water and autoclaved and to this 37.5ml of the sample was added, total volume of 50ml was used. The medium after inoculation was kept on shaker for 48hrs at 30^0-31^0C under aerobic conditions.

3. Isolation of Organism

Isolation of organisms was carried out on media that had agar as the only carbon source. Medium B, Zobell's agar media, basal salt media are been used for isolation of agar degraders in the research till now [5, 6], but media used for isolation was Kadota's media it was observed that all the required nutrients are present in the media. One set for isolation was made by media modifications were instead of distilled water artificial sea water was made and used for preparing the media to mimic sea water conditions. On both this medium spread plate method was used to get isolated colonies.

4. Screening of Organism

Literature survey suggests that 25^0C- 40^0C is the temperature suitable for the growth of agar degrading organisms. Temperature preferred for isolation of agar degrading bacteria was 30^0C. Hence, inoculated plates were incubates at 30^0C for 48hrs. All the colonies that were observed were isolated on the same media in duplicates. One set was used to perform Lugol's iodine test.

Iodine test for selection of potential agarase producing strain by plate method

The test is performed to shows that agarase diffuses out from the colonies, and that the properties of the gel are changed before it is degraded by the bacteria. Zone of clearance around colonies on agar plates treated with iodine shows that the properties of the gel are changed by the action of agarase diffusing out from the bacterial colonies [5]. The binding of iodine to agar gels to give a specific color depends on the structure of the gel matrix [5]. This test is basically used to detect agarase activity [1].

All the bacterial strain that were observed after 48hrs of incubation were further screened for confirmation of agarolytic activity by plate method. After incubation for 48hrs at 30^0C, the plates were flooded with Lugol's iodine and observed for the zone of clearance around the bacterial colonies. Colonies which showed zone of clearance were selected as potential strain for further studies.

5. DNSA Method for identification of reducing sugars This method can be used for the detection of reducing sugars that are produce due to degradation of agar [5].

As agar is a polysaccharide it cannot be detected by DNSA method. But when organism degrades agar large amount of oligosaccharides are produced with galactose at one end which can be detected by DNSA method. D-galactose was taken as standard [1].

6. Physical Parameters

pH range that were checked were from 7-9. 28^0C, 30^0C, 37^0C and 55^0C were the temperature at which growth of organism was checked.

8. Gram nature

Gram staining and KOH test were carried out.

9. Growth on other Media

Nutrient agar
Basal salt media

Immobilization

Immobilization of cells and enzyme is carried out for many industrially important cultures and enzymes as this process helps in long term utilization of these cultures and enzymes. Hence, immobilization of all the three isolates was carried out using sodium alginate and calcium chloride.

10. Consortium

Many a times effect produce by single organism increases when the organism works in consortium with other organism, similarly the effect can be reduced by other organism, so to check if such effect increases agar degradation consortium was of all the three isolates was made and this was used to see whether organisms degrade agar when used together or is there ability inhibited by the other organism. Consortium of all the three

isolates was made by using suspensions of all the three cultures. 0.5ml of each culture suspension was used in the consortium.

11. MALDI Analysis

Matrix- assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) coupled to analysis software is one of the method which identifies and classifies the organism according to its ribosomal protein spectral profile. The method is well known as MALDI biotyping [7]. For Identification isolates designated as S2 and ISO were given for MALDI analysis at NCMR, Pune.

12. 16S rRNA

Isolate designated S1 was identified by 16S rRNA analysis.

III. Results And Discussion

1. Result

1.1. Source

Sample that was collected from of Arabian Sea (sample 1) and Indian Ocean (sample 2) had a pH of 7.2 and 7.1 respectively. The sample was directly used for enrichment without any treatment.

1.2. Enrichment

Enrichment of the sample was done for 48hrs at 30^0C-31^0C. From this enrichment using spread plate method, isolation of agar-degrading bacteria was carried out.

For all the media that were used spread plate technique was used to get isolated as well as separated colonies of agar degrading bacteria if present.

1.3. Isolation of organisms

Growth was observed on modified medium that is Kadota's media made using artificial sea water. Plate was incubated at 30^0C for 48hrs.

Figure 1: Plate showing growth of colonies isolated from sample 1

Two colonies were observed on the media inoculated with sample 1 and one colony was observed on plate inoculate with sample 2. These three colonies were given designation as S1, S2 and IOS respectively. All the three colonies were isolated on same media for carrying out iodine test to observe agarase activity.

Table 1: Colony characteristics of the three isolates:

Sample	Type	Size	Shape	Color	Margin	Elevation	Opacity	Consistency	Gram nature
1	S1	Small	Circular	Off white	Irregular	Concave	Opaque	Sticky	Negative rods
	S2	Small	Circular	Off white	Entire	Concave	Opaque	Smooth	Negative rods
2	IOS	Small	Circular	Pale yellow	Entire	Flat	Translucent	Hard	Negative rods

1.4. Screening of organism

Iodine test for selection of potential agarase producing strain by plate method

After incubation for 48hrs at 30^0C, the plates were flooded with Lugol's iodine and observed for the zone of clearance around the bacterial growth. As mentioned before agarase enzyme change the gelling properties of agar due to which its property to bind with iodine and form a color complex changes due to which

a clear zone is observed around the colonies which produce the enzyme. Hence, colonies which showed zone of clearance were selected as potential strain for further studies.

Fig 2 (a)

Fig 2 (b)

Figure 2: Iodine test for testing agarase activity: a) Clearance zone observed in plate inoculated with IOS and no zone of clearance observed in control plate. b) Clearance zone observed for both S1 and S2 culture.

1.5. DNSA method for identification of reducing sugars

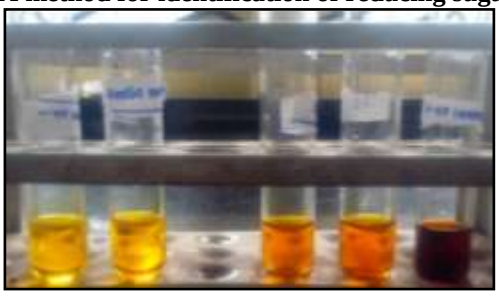

Figure: 3 a) S1 and S2

Fig: 3 b) IOS

Figure 3: a and b) DNSA Method to check for the production of reducing sugars

All the three isolates S1, S2 and IOS produce reducing sugar. Reducing sugar is absent in media control which confirms that agar in the test is degraded by the organism to produce different oligosaccharides (having reducing ends). These isolates that were able to produce reducing sugar were used for further analysis and experiments. Only qualitative analysis can be done using DNSA method for quantitative analysis methods like HPLC and ESI-MS [8] are used using standards. These methods were not carried out due to unavailability of standards.

Table 2: Characteristics and tests

Characteristics and tests	Organisms		
	SI	S2	IOS
Catalase	+	+	+
Oxidase	+	+	+
KOH	+	+	+
Physical parameters			
Temperature	Growth		
	S1	S2	IOS
28^0C	+	+	+
30^0C	+	+	+
37^0C	-	+	+
55^0C	-	-	-
pH	Growth		
	S1	S2	IOS
7	+	+	+
8	+	+	+
9	+	+	+
Growth on other media			
Nutrient agar	+	+	+
Basal salt media	+	+	+

1.6. Immobilization

Immobilization of all the three isolates was carried out using sodium alginate and calcium chloride. It was for the first time that immobilization of agar degrading bacteria was carried out.

For the immobilization process 20 beads were transferred to 50ml of semi- solid media. Production of reducing sugar was to be detected. After 2 days of incubation DNSA assay was carried out for detection of reducing sugar, no reducing sugar was produce in 48hrs so further incubation of 48hrs was carried out.

It was seen that immobilization of cell was not effective in agar degradation. Other method of immobilization of cells needs to be developed where enzyme or the substrate can move easily through the immobilization matrix. Another method that can be used is that enzyme can be purified and then this purified enzyme can be immobilized and checked for effective agar degradation and its long term usage.

1.7. Consortium

Consortium of all the three isolates was made to see whether organisms degrade agar when used together or is there ability inhibited by other organism. Consortium of all the three isolates was made by using suspensions of all the three cultures. 0.5ml of each culture suspension was used.

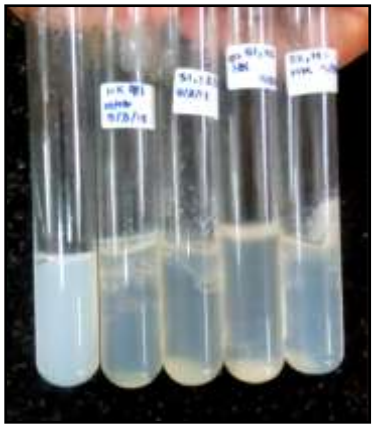

Figure 4: Consortium of all the three isolates

It was observed that consortium of *Alteromonas marina SW*-47(T) and *Pseudomonas stutzeri* showed less degradation as compared to other three tests.

1.8. MALDI analysis

Recently it is mentioned by Neelja Singhal *et al* that matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has emerged as a potential tool for microbial identification and diagnosis. During the MALDI-TOF MS process, intact cells or cell extracts are used for identification [9]. The process is rapid, sensitive, and economical in terms of both labor and costs involved [9]. Nowadays the technology has been readily used by Microbiologists.

Isolates S2 and IOS were identified using MALDI at NCMR, Pune. S2 was identified to have best match with *Vibrio alginolyticus* CCM 7037 CCM and second match with *Vibrio alginolyticus* CCM 2578T CCM and IOS was found to have best match with *Pseudomonas stutzeri* B367 UFL and second match with *Pseudomonas stutzeri* 040_W09 NFI.

1.9. 16S rRNA analysis

S1 sample was sent for 16S rRNA analysis to NCMR, Pune. The culture was identified to be *Alteromonas marina SW*-47(T).

1.10. Deposition at NCMR depository

All the three isolates were deposited in general depository at NCMR, Pune.

IV. Discussion

Three isolates able to produce agarase were isolated from marine environment.

Agar is considered as a "generally recognized as safe" (GRAS) food additive and it is widely used as gelling agents, in diet foods, for icings, glazes, processed cheese, jelly sweets, and marshmallows [1]. It is also been used in microbiological media preparation and for agarose gel electrophoresis and in various chromatographic techniques. The oligosaccharides that are produce from agar degradation have several biological functions [2]. Agarooligosaccharides (AOs) produced by hydrolysis of the α-1, 3 glycosidic bonds of

agar, were reported to have anti-tumor promoting activities against mouse skin carcinogenesis, antioxidant activity, and hepato-protective potential. Neoagarooligosaccharides (NAOs) produced by hydrolysis of the β-1,4 glycosidic bonds of agar, were reported to inhibit the growth of bacteria, inhibit the degradation of starch, and function as low-calorie additives for improving food quality. These NAOs also show moisturizing and whitening effects in melanoma cells. Therefore, these oligosaccharides produced from agar have wide applications in the food, pharmaceutical, and cosmetic industries [10]. So agarase enzyme can be used to produce these oligosaccharides in an eco-friendly way.

It is clear that alga-associated microorganisms are a major resource for the discovery of novel specific enzymes and secondary metabolites [11]. So isolating these organisms and using these organisms to degrade the agar waste that is generated and produce oligosaccharides which have wide applications can be carried out.

The organisms (S1, S2 and IOS) that were isolated in this study were showing agarase activity which was confirmed by Lugol's iodine test. S2 and IOS was identified by MALDI analysis to be *Vibrio alginolyticus* and *Pseudomonas stutzeri* respectively, S1 was identified by 16S rRNA analysis and was found to have % similarity of 99.84 to its closest neighbour *Alteromonas marina SW*-47(T). M. Lakshmikanth *et al* have reported isolation of *pseudomonas sp* able to degrade agar and tolerage pH range of 7-9. All the three isolates were able to tolerate pH range from 7-9 which is observed in many agar degrading bacteria. It was even reported that Anashi *et al* have isolate agar degrading bacteria that tolerate alkaline pH, Fangyuan Cui *et al* isolated organism that can tolerate pH range of 7-9 and was able to degrade agar at this pH range, so the isolated organisms can be used for production of oligosaccharides from agar waste generated in life sciences laboratories, this oligosaccharides can be identified and can be used in various industrial, cosmetics and medical applications. It was seen that *Pseudomonas stutzeri* showed rapid growth when peptone was added to the media. Organisms were able to grow in the temperature range from 28^0C, 30^0C and 37^0C but were unable to grow at 55^0C.

Immobilization was carried out of these agar degrading bacteria. But it was seen that no reducing sugars were produce, there can be various reason that agar was not degraded, first reason could be the enzyme that is produce is membrane bound due to which there was no enzyme substrate interaction as agar was not penetrated through the pores in the bead, second reason could be that very minute quantity of reducing sugar were produced which were not detectable by DNSA method but could have be detected by HPLC (if standards were available). Third reason could be that organisms were not viable after immobilization for long time enough to degrade agar. Hence, methods like surface immobilization of cells on matrix can be used or enzyme can be purified and used for immobilization studies. Immobilization of agar degrading bacteria was carried for the first time as far the knowledge. Consortium of all the three isolates was made and it was seen that media containing 1% agar was not solidified at the top layer of the tube when consortium was used and the organisms used in the consortium were also viable when grown on plate, the cells also showed lateral and vertical growth in the media, it concludes that the organisms are motile and turbidity of the media was also reduce. Though for consortium of *Alteromonas marina SW*-47(T) and *Pseudomonas stutzeri* , media was solidified at the top layer, it can be due to inhibitory effect of either *Alteromonas marina SW*-47(T) on *Pseudomonas stutzeri* activity or vice versa.

V. Conclusion

Three agar degrading organisms (*Alteromonas marina SW*-47(T), *Vibrio alginolyticus* and *Pseudomonas stutzeri*) were isolated from different marine environments. All three isolates were able to grow on alkaline pH from range 7-9 and all were utilizing agar as the carbon source. The three isolates were able to produce extracellular neoagarooligosaccharide. Two of the isolate S2 and IOS were identified to be closely related to *Vibrio alginolyticus* CCM 7037 CCM and *Pseudomonas stutzeri* B367 UFL respectively, *Vibrio alginolyticus* CCM 2578T CCM and *Pseudomonas stutzeri* 040_W09 NFI respectively. Isolate S1 was identified by 16S rRNA analysis and it had close % similarity of about 99.84 with *Alteromonas marina SW*-47(T). Immobilization method was carried out for the first time and it was seen that it is not effective as there was no degradation of agar. It may be due to semi-solid nature of agar that the substrate was not able to penetrate through the pores. As an alternative method immobilization of enzyme can be carried out to see whether agar can be degraded, instead of using porous beads for immobilization surface immobilization of enzyme can be carried out, so that the immobilized enzyme can be used for long duration of time. Consortium of organisms was made and it was seen that media in the tubes was not solidified at the top layer. Hence, consortium of agar degrading bacteria can be used to reduce solid waste generated in life sciences laboratories at the same time care should be taken that the organisms are separated when disposing the liquefied waste.

VI. Future Prospects

The organisms that were isolated can be used in future studies aimed at enhancing the production of agarase in order to carry out complete degradation of agar so that solid waste generation in all life sciences laboratories can be reduced. The neoagarooilgosaccharieds produced from the waste can be used by food, pharmaceutical, and cosmetic industries. It was seen in the studies carried out that immobilization of cell was

not effective in agar degradation. Other method of immobilization of cells needs to be developed where enzyme or the substrate can move easily through the immobilization matrix. Another method that can be used is that enzyme can be purified and then this purified enzyme can be immobilized and checked for effective agar degradation and long term usage. If immobilization of agar degrading bacteria is achieved then filters can be designed so that blockages of pipe due to agar disposal can be prevented. Even these organisms along with other agar degrading organisms can be used in consortium to increase its effect for agar degradation. This consortium can be used to increase production of agarase. SDS-PAGE needs to be carried out to find out the type of agarase that is been produce by the organisms isolated. Zymogram analysis needs to be carried out.

Acknowledgement

We would specially acknowledge National Centre for Microbial Resource (NCMR) for their help in identification of cultures by MALDI analysis and 16S rRNA analysis.

References

[1]. Xiao Ting Fu and Sang Moo Kim (2010). Agarase review of major sources , categories, purification method, enzyme characteristics and applications. *Marine drugs* 2010, 8, 200-218.
[2]. Won-Jae Chi and Yong-Keun Chang and Soon-Kwang Hong. Agar degradation by microorganisms and agar- degrading enzymes. *Appl Microbiol Biotechnol* (2012) 94:917–930.
[3]. Linda J. Ross. *The Enzymic hydrolysis of Neoagarotetraose*, a thesis submitted to the Facu1ty of Graduate Studies and Research in partial fulfi1lment of the requirements for the degree of Masters of Science Department of Microbio1ogy and Immuno1ogy McGi11 University Montreal June, 1970.
[4]. L.C.-M Chenl, J.S. Craigiel & Z.K. Xie (1993). Protoplast production from prophyra linearis using a simplified agarase producer capable of commercial application. *Journal of Applied Phycology* 6: 35-39, 1994.
[5]. B. V. Hofsten and M. Malmqvist (1974). Degradation of Agar by a Gram-negative Bacterium. *Journal of General Microbiology* Printed in Great Britain.
[6]. Kim, Jonghee and Soon-Kwang Hong (2012). Isolation and Characterization of an Agarase-Producing Bacterial Strain, *Alteromonas* sp. GNUM-1, from the West Sea, Korea. *J. Microbiol. Biotechnol.* (2012), 22(12), 1621–1628.
[7]. Maria -Theresia Gekenidis, Patrick Studer, Simone Wüthrich, René Brunisholz, and David Drissner. Beyond the Matrix-Assisted Laser Desorption Ionization (MALDI) Biotyping Workflow: in Search of Microorganism-Specific Tryptic Peptides Enabling Discrimination of Subspecies. Appl Environ Microbiol. 2014 Jul; 80(14): 4234–4241.
[8]. Jianfeng Mei and Jinting Shao and Qi Wang and Hong Wang and Yu Yi and Guoqing Ying. Separation and quantification of neoagaro oligosaccharides. *J Food Sci Technol* 2011.
[9]. Neelja Singhal, Manish Kumar, Pawan K. Kanaujia, and Jugsharan S. Virdi. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front. Microbiol.6:791. doi: 10.3389/fmicb.2015.00791. August 2015, Volume 6, Article 791.
[10]. Min-Kyung Jang, Seung Woo Lee, Dong-Geun Lee, Nam-Young Kim, Ki Hwan Yu, Hye Ji Jang, Suhkman Kim, Andre Kim, Sang-Hyeon Lee (2010). Enhancement of the thermostability of a recombinant b-agarase, AgaB, from *Zobellia galactanivorans* by random mutagenesis. *Biotechnol Lett* (2010) 32:943–949.
[11]. Roxana Alvarado and Sergio Leiva1. Agar-degrading bacteria isolated from Antarctic macroalgae. *Folia Microbiol* 2017, 1-8.
[12]. M. Lakshmikanth, S. Manohar, J. Patnakar, P. Vaishampayan, Y. Shouche and J. Lalitha(2005). Optimization of culture conditions for the production of extracellular agarases from newly isolated *Pseudomonas aeruginosa* AG LSL-11. *World Journal of Microbiology & Biotechnology* (2006) 22: 531–537.
[13]. Anashi, kiran and bhupendra (2015). Isolation , characterization and purification of an extracellular enzyme agarase from agarolitic bacteria. *International Journal of Current Research Vol. 7, Issue, 08, pp.19345-19349, August, 2015.*
[14]. Fangyuan Cui, Sujie Dong, Xiaochong Shi, Xia Zhao and Xiao-Hua Zhang (2014). Overexpression and Characterization of a Novel Thermostable β-Agarase YM01-3, from Marine Bacterium *Catenovulum agarivorans* YM01T. *Marine. Drugs* 2014, *12*, 2731-2747.

IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) is UGC approved Journal with Sl. No. 5012, Journal no. 49063.

Neeraja Kolhatkar, "Isolation and Identification of Agar Degrading Bacteria from Marine Environment." IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 13.3 (2018): 01-07.