An agar degrading diazotrophic actinobacteria from hyperalkaline meteoric lonar crater lake - a primary study

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Abstract

There are very few reports on agarases being produced by actinobacteria, *Streptomyces coelicolor* being the only one known since decades for its agar degrading property. Here we report an agar degrading diazotrophic actinobacterium other than *Streptomyces coelicolor*, isolated from the littoral soil of Lonar Lake situated in Buldhana district of Maharashtra, India, a lake characterised by high alkalinity, carbonates, bicarbonates, and algal blooms. The lake has a mean diameter of 1800 meters. The Gram-positive filamentous rod grew in a simple medium of pH 10.5 containing agar as a sole source of carbon. The agar degrading property was detected by the appearance of depressions around each colony after 48 h of growth. The enzyme responsible for this degradation, agarase was also detected and estimated. The isolate also grew on Ashby's Nitrogen free Mannitol Medium aerobically and fixed nitrogen. Morphological, physiological and biochemical characteristics of the isolate are presented in this paper.

Introduction

Agar, a complex polysaccharide is extracted from marine red algae belonging to the family Rhodophyceae, mainly from *Gelidium* and *Gracilaria* species. It is composed of two fractions agarose and agarpectin. 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.¹ It is widely employed as a gelling agent for microbiological culture media. Agarases are enzymes that hydrolyse the polymer agarose and have potential applications in the food, cosmetic and medical industries for breaking down agar components.²

Agar is attacked by relatively few species of microorganisms, including bacteria and actin-

omycetes. Most of the previously reported agar degrading bacteria such as *Alteromonas species*, *Bacillus cereus*, *Cytophaga*, *Pseudoalteromonas*, *Pseudomonas*, *Vibrio sp.* and *Zobella sp.* have quite been isolated from marine environment.²⁻¹⁴ Since agar is a polysaccharide produced by marine red algae, it is natural that most agar degrading bacteria are inhabitants of marine habitats. However, there are very few reports of nitrogen fixing bacteria degrading agar except for an agar degrading marine nitrogen fixing bacterium placed in the family *Vibrionaceae*¹⁵ There are also no reports of any actinobacteria other than *Streptomyces coelicolor* reported by Stanier.¹⁶

Lonar Lake situated in Buldhana district of Maharashtra, India is known to be formed due to a meteorite impact some 52,000 years ago and has a mean diameter of 1800 meters Figure 1.

It is an alkaline closed basin lake with large masses of algal blooms floating on the water and on the banks. Hence, the occurrence of agar degrading bacteria was a distinct possibility in this environment. During the course of a study on nitrogen fixing bacteria from this environment we came across an agar degrading organism. We report the morphological, physiological and biochemical characteristics of this organism in this paper.

Materials and Methods

Enrichment and isolation

Soil samples collected from the littoral zone of Lonar Lake were subjected to enrichment of nitrogen fixing bacteria on Ashby's Nitrogen free Mannitol broth with pH-10.5 and NaCl-2% amended as per the chemical characteristics of Lonar Lake soil. Tubes were incubated at 40°C for 48 h in a shaking incubator. After incubation, loopfuls of enriched medium from each tube were streak inoculated on Ashby's Nitrogen free Mannitol solid medium containing 2.0% agar, pH-10.5 and incubated at 40°C for 48 h.

Colony characteristics and morphological characters of isolates

Isolate was studied for its colony characters, Gram nature by Hucker and Conn modified Gram's staining method. Mycelium arrangement was observed under microscope at by coverslip culture technique.¹⁷

Agar degrading ability

The organism was initially isolated as a nitrogen fixing actinobacterium from Lonar Lake. It was detected to be agar degrading by the appearance of depressions in agar around the colonies after 48 hours incubation.

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Key words: actinobacterium, agarase, Lonar Lake, algal blooms, depressions.

Acknowledgements: the authors would like to thank the Management and Principal of Yashwantrao Chavan College of Science, Karad for the facilities provided in the laboratory. They also would like to thank Dr. Yogesh S. Shouche, Scientist, and Mr. Girish Kulkarni, Senior Research Fellow of Molecular Biology Unit, National Centre for Cell Sciences, Pune for the phylogenetic analysis of the isolate.

Received for publication: 11 June 2011.

Revision received: 29 July 2011.

Accepted for publication: 29 July 2011.

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Microbiology Research 2011; 3:e10
doi:10.4081/mr.2011.e10

Confirmation was done by growing the isolate on a simple medium of composition Agar-20.0 g/L, NaCl-20.0g/L and incubated at 40°C for 48 h. Agarase activity of the isolate was also determined by the method of Shieh *et al.*¹⁸ on semi solid PY medium of composition 2.0 g Peptone, 0.5 g yeast extract and agar 15.0g in 1 litre Lonar Lake water (instead of 90% sea water), with pH 10.5.

Phylogenetic analysis of the 16s rRNA gene

The partial 16s rRNA sequence was determined in the Molecular Biology Unit of National Centre for Cell Sciences (NCCS), Pune using Universal Eubacteria-specific primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1525XP (5'-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3').¹⁹ Its analysis was done using the web based SeqMatch of RDP-II (<http://www.rdp.cme.msu.edu>). The derived sequence was submitted to DDBJ database and an accession number was obtained for the same sequence.

Agarase assay

This assay was performed in triplicates according to the procedure of Miller *et al.*²⁰ Fifty (50) μ L of the enzyme was added to 0.8% agarose solution (in carbonate buffer, pH 10.5) and allowed to react at 40°C for 30 minutes. The reaction was then stopped by addition of 3.0 mL DNS (3, 5-dinitrosalicylic acid) reagent.

The samples were then boiled for 15 min, cooled and centrifuged to remove gel residues. The absorbance was read at 540 nm on UV visible spectrophotometer of Systronics Make at visible light and values for reducing sugar were expressed as galactose equivalents, one unit of agarase activity corresponding to formation of 1 μ mol galactose equivalent/min. The protocol used for conducting this assay is depicted in Table 1.

Nitrogen fixing activity

The nitrogen fixing potential of the organism was tested in triplicates by growing it on Ashby's nitrogen free mannitol broth and estimating total nitrogen in the cell mass as well as the cell free medium by MicroKjeldahl's method.²¹ An uninoculated broth was run as a control during the experiment.

Studies on biochemical characteristics of the isolates

Isolate was studied for various biochemical characteristics using appropriate methods.¹⁷ The biochemical characterization was performed using Ashby's N₂ free broth with pH 10.5 and NaCl concentration 2% amended as per the chemical composition of Lonar Lake soil.

Enzymatic studies

Catalase test

Growth of isolate from Ashby's N₂ free agar plate was picked with sterile nichrome wire loop and dipped into 10% H₂O₂ solution. Evolution of gas bubbles was observed as positive reaction.

Nitrate reduction test

A loopful of suspension of each isolates was inoculated separately in tubes containing

5 mL of sterile nitrate broth with pH 10.5. Tubes were incubated at 37°C for 48 h. The reduction of nitrate to nitrite was detected by adding few drops of sulphanic acid followed by α naphthyl amine. Development of red colour indicates positive test.

Phenylalanine deamination test:

A loopful suspension of the isolate was streaked on separate phenylalanine agar slant and incubated at 37°C for 48 h. After incubation 4-5 drops of 10% ferric chloride reagent was added on the surface on the agar. Development of intense green colour gives positive test.

Carbohydrate utilization test

Utilization of carbohydrates was studied for testing the ability of isolate to utilize carbohydrates as carbon and energy source. The test was carried out by using peptone water with pH 10.5 with phenolphthalein as indicator and 1% solution of Glucose, Mannitol, Sucrose, lactose, inositol, ribose and xylose. A control was also run during each experiment. A loopful suspension of each isolate was inoculated in sugar broth tubes containing respective sugars. The tubes were incubated at 40°C (+/-2°C) for 48-72 h. The tubes were observed for acid and gas production indicated by change in colour by medium and bubble formation in Durham's tube.

Studies on physiological characteristics of isolates

Effect of temperature on growth of isolate:

A loopful suspension of the isolate was inoculated on Ashby's N₂ free mannitol broth and incubated at different temperature namely 0°C, 10°C, 27°C, 40°C and 55°C for 6 days. After inoculation initial reading was taken on UV Visible Spectrophotometer of Systronics

Make at 600 nm, then after every day readings were noted down. Control was run along with every temperature.

Effect of NaCl concentration on growth of isolate

Loopful suspension of the isolate was inoculated on Ashby's N₂ free mannitol broth with different NaCl concentration from 0, 2, 4, 6, 8 ...20 and incubated at 28°C for 6 days. After inoculation initial reading was taken on UV Visible Spectrophotometer of Systronics Make at 600nm, then after every day readings were noted. A control was run along with each NaCl concentration tube.

Figure 1. A view of Lonar Crater Lake showing algal blooms on the bank.

Figure 2. Actinomycete Colonies showing depressions on agar surface.

Table 1. Protocol for agarase assay.

Tube type	Substrate (mL)	Enzyme (μ L)	Distilled water	Incubate at 40°C for 30 minutes	DNS (mL)	Hold in boiling water bath for 15 minutes	Cool at room temperature	Centrifuge at 3000 rpm for 10 minutes	Absorption at 540 nm
Substrate control	0.45	-----	50		3				
Test	0.45	50	-----		3				

Table 2. Morphological characteristics of the isolate NW-II A.

Isolate code	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram Nature	Motility
NWII A	2 mm	Circular	Gray	Irregular	Concave	Opaque	Dry	Gram positive filaments	Non motile

Table 3. Enzyme Activities for the isolate NW-II A.

Isolate code	Catalase	Enzyme activity Nitrate Reduction	Phenylalanine deamination
NW II A	-	+	+

Effect of pH on growth of isolate

A loopful suspension of the isolate was inoculated on Ashby's N2 free mannitol broth with different pH from 0, 2, 4, 6....14 and incubated at 40°C for 6 days. After inoculation initial reading was taken on UV Visible Spectrophotometer of Systronics Make at 600 nm, then after every day readings were noted down. Control was run along with each pH.

Results and Discussion

Morphological characteristics

Morphological characters indicated that it is an actinobacterium Table 2, Figure 2 and Figure 3. Partial 16s rRNA analysis identified the isolate as a member of the genus *Slackia* with similarity to *Slackia exigua*. The sequence of this isolate has been submitted to DDBJ under the accession number AB602923 (Figure 4).

Agarase assay

The agarase assay done in triplicates by DNS method showed that 1600 µg galactose mL⁻¹ was formed in the PY semi-solid medium. This was translated to agarase units of 5.9259 units mL⁻¹. The values could actually be higher as there is a possibility of some of the galactose being consumed by the organism and not being detected here.

Nitrogen fixing ability

The isolate was also observed to fix 0.23055% total nitrogen when grown in Nitrogen free broth. At the same time the uninoculated control sample fixed 0.000341% total nitrogen. The isolate was subjected to some enzymatic studies and the results are presented in Table 3. Isolate NW-II A was tested for the utilization of various carbohydrates and the results are depicted in Table 4. An experiment on Effect of Temperature on the isolate was done whose results are shown in Table 5 and Figure 5. Effect of NaCl on the growth of the isolate was monitored colorimetrically and the values obtained are represented in Table 6 and Figure 6. Effect of pH on the growth of the isolate was performed whose results are shown in Table 7 and Figure 7. A total number of 10 nitrogen fixing actinomycetes were obtained from Lonar Lake during the study but only one isolate namely NW-IIA was found to have agar degrading property according to the agarase assay performed in the laboratory. The obtained isolate fixed considerable amount of nitrogen.

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Figure 3. Microscopic observation of the isolate.

Figure 4. Neighbourhood joining tree of the isolate NW-II A.

Figure 5. Effect of temperature on isolate NW II A.

Figure 6. Effect of NaCl concentration on isolate NW II A.

Figure 7. Effect of pH on isolate NW II A.

Table 4. Carbohydrate utilization by the isolate.

Isolate code	Glucose	Lactose	Sucrose	Mannitol	Inositol	Xylose	Ribose
NW II A	+	-	+	+	+	+	-

Table 5. Effect of temperature on isolate NW II A.

NW II A	Initial	Day 1	Day 2	Day 3	Day 4
	0.01	0.02	0.01	0.01	0.02
	0.03	0.04	0.07	0.11	0.15
	0.01	0.02	0.26	0.34	0.39
	0.01	0.02	0.3	0.43	0.56
	0.02	0.04	0.08	0.11	0.13

Table 6. Effect of NaCl concentration on isolate NW II A.

	0	2	4	6	8	10	12	14	16	18	20
Initial	0.02	0.02	0.03	0.01	0.01	0.01	0.01	0.02	0.03	0.04	0.02
Day 1	0.06	0.09	0.05	0.04	0.02	0.01	0.02	0.03	0.02	0.03	0.01
Day 2	0.12	0.16	0.08	0.06	0.03	0.02	0.01	0.04	0.02	0.03	0.03
Day 3	0.21	0.24	0.09	0.09	0.05	0.03	0.03	0.03	0.01	0.04	0.01
Day 4	0.28	0.33	0.16	0.12	0.08	0.02	0.02	0.03	0.04	0.03	0.01

Table 7. Effect of pH on isolate NW II A.

	0.03	0.04	0.03	0.04	0.02	0.04	0.04
Initial	0.01	0.03	0.09	0.12	0.14	0.06	0.03
Day 1	0.02	0.02	0.13	0.13	0.19	0.09	0.03
Day 2	0.03	0.03	0.2	0.26	0.35	0.12	0.04
Day 3	0.03	0.04	0.32	0.42	0.58	0.19	0.04
Day 4							

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