JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ISOLATION AND CHARACTERIZATION OF A NOVEL AGAR-DEGRADING BACTERIUM, *Microbacterium barkeri* sp. SELA 4, FROM SOIL ENRICHED WITH LABORATORY AGAR

*Shomini Parashar, Narendra Kumar**

Address(es): Narendra Kumar,
IMS Engineering College, Department of Biotechnology, NH-24 Adhyatmik Nagar, Ghaziabad, India- 201015.

*Corresponding author: narendra.kumar@imsec.ac.in

doi: 10.15414/jmbfs.2019.9.1.78-83

ARTICLE INFO

Received 8. 9. 2018
Revised 15. 3. 2019
Accepted 15. 3. 2019
Published 1. 8. 2019

Regular article

OPEN ACCESS

ABSTRACT

An agar-degrading bacterium, designated as strain SELA 4, was isolated from agriculture soil enriched with laboratory agar. The isolated strain was Gram-positive, rod-shaped, aerobic bacterium with off-white colonies showing marked depression on Mineral Salt Agar (MSA) medium. A similarity search was done based on its 16S r DNA sequence and the analysis revealed that the strain SELA 4 shared 99% similarity with the genus *Microbacterium*, showing its affiliation with *Microbacterium barkeri* strain which is known to be famous as biocontrol agents. On the basis of results obtained after biochemical analysis, physiological and phylogenetic analysis this strain was named *Microbacterium barkeri* sp. SELA 4. The strain SELA 4 is able to utilize agar as the only carbon source when grown at 37°C, 5 pH and produced the enzyme, β-agarase. Highest activity of 2.04 U/ml was observed at 1.5% agar concentration in the growth media. The enzyme activity at 37°C was 2.09 U/ml and at 5 pH was 1.96 U/ml respectively. Initially, the activity was qualitatively determined by iodine clear zone assay and later confirmed by activity staining. Activity staining showed the secretion of agarase enzyme by the strain whose molecular weight was found approximately 55.6 kDa. The enzyme hydrolysis product of agar was analyzed using Thin Layer Chromatography, indicating that the agarase enzyme from SELA 4 was β agarase which is known for cleaving agarose into Neoagarohexaose as the final product.

Keywords: Activity staining, Biocontrol, *Microbacterium*, MSA, Neoagarohexaose, Agarase, Hydrolysis

INTRODUCTION

The great utility of the agar hydrolyzed oligosaccharides has attracted some researchers in this area. The bacterial isolate, *Microbacterium sp.* SELA 4 mentioned in the current study, produced agarase enzyme which was isolated from soil enriched with laboratory agar and possess the ability to degrade agarose. Some red algae like *Gelidium* and *Gracilaria* are most popularly known for the location of two major components of agar, namely Agarose and Agaropectin in their cell walls. Both the components are polysaccharides in nature. The former component is a neutral molecule with linear chains of repeatedly alternating units of β-1,3- linked-D-galactose and α-1,4 linked 3,6 anhydro L-galactose **(Duckworth and Turvey, 1969)**has gelling abilities while the later component, is a non-gelling fraction of sulfated polysaccharide. Agarase enzymes act on the substrate, agarose of agar and break its polysaccharide into oligosaccharides. The agarases are of two types: α-agarases (EC 3.2.1.158) and β-agarases (EC 3.2.1.81) on the basis of their cleavage pattern **(Chi *et al.*, 2012)**.The α-agarase break α-1,3 linkages to produce agaro-oligosaccharides while β-agarase enzyme breaks β-1,4 linkages to produce neoagaro-oligosaccharides **(Suzuki *et al.*, 2002)**. Agarase enzyme is used in biochemistry lab, to retrieve DNA from agarose gel **(Sugano *et al.* 1993)** while it's hydrolytic products are utilized in food industry as food additives and gelling agent **(Fu and Kim, 2010)**, as anti-oxidants by scavenging the reactive free radicals **(Wang *et al.*, 2004)**, in cosmetics, as moisturizers for skin fairness**(Kobayashi *et al.*, 1997)**, in medicine industry, as immunity booster by exhibiting macrophage stimulating activity **(Yoshizawa *et al.*, 1995).** Agar-degrading bacteria are known to utilize agar for carbon requirement, unlike other bacteria for which agar just acts as a solidifying agent. Such bacteria were usually isolated from marine sources as the substrate, agar is available to them in the cell wall of red algae. However, few bacterial isolates have known to be reported from non - marine environments like *Asticcacaulis* sp.SA7 from rhizosphere soil **(Hosoda *et al.*, 2006)**, *Paenibacillus* sp.SSG-1 from soil **(Song *et al.*, 2014)**, *Ammonibacillus agrariperforans* FAB2(T) from sewage sludge compost **(Sakai *et al.*, 2015)** and the endophytic bacteria isolated from the plant source **(Song *et al.*, 2015)**. Therefore, our study focuses to know the significance and ecology of non-marine agar-degrading bacteria.

Till now, no reports are there on agarolytic bacterial isolates, that belong to *Microbacterium* species either from marine or non-marine sources. Our current study describes the identification of a novel agar degrading bacterial strain, SELA 4, molecular weight determination of its agarase enzyme, detection of enzyme hydrolysis products and the effect of different parameters (agar conc., temperature, pH) on the production of extracellular agarases. There are reports about a few strains of *Microbacterium* sp. that cause diseases in plants **(Kaku *et al.*, 2000)** animals **(Hodgkin *et al.*,2000)** and human beings **(Alonso-Echanove *et al.*, 2001)** but recently few isolates from soil have also shown to possess biocontrol activity **(Sartori *et al.*, 2012)** production of bio-ethanol**(Kawaroe *et al.*, 2018).**Through this article, we are for the first time mentioning about a novel agar-degrading bacterium, *Microbacterium barkeri* sp., SELA 4 which extracellularly secretes β agarase enzyme with molecular weight 55.6kDa and has the potential to produce Neoagarohexaose.

MATERIAL AND METHODS

Isolation of agar degrading bacterium and its culture medium

An agarolytic bacterium was isolated from the agriculture soil sample on Mineral Salt Agar plates. The compositions for MSA agar medium were (gram per liter): $CaCl_2$ 0.1; $MgSO_4.7H_2O$ 0.5; $(NH_4)_2SO_4$ 0.5; K_2HPO_4 0.5; NaCl 0.5; 1.5% agar. To inhibit the growth of fungi on the plates, 5g/l of Nystatin was added to culture media whose pH was adjusted to five. One gram soil was taken from the pot containing agriculture soil in which laboratory agar wastes were regularly dumped and mixed. Then this one gram soil thoroughly mixed with 100ml distilled water and through serial dilution method; 0.1 ml of the suspension was taken from each dilution tubes 10^{-4}, 10^{-5}, 10^{-6}, spread on Petriplates containing MSA. The Petri plates were then incubated at 37°C temperature for several days and were daily checked for agarolytic activity. After three days of incubation, colonies forming depression and zones around them on the smooth agar surface were selected and sub-cultured on fresh MSA plates for further purification. Total 4 different bacterial colonies appeared on the smooth agar plates. Out of these only one which showed marked depression and zone of clearance, was picked and examined for colony characteristics.

Morphological, physiological and biochemical characteristics

After getting the pure culture of the agarolytic bacterium after 3 days of incubation at 37°C, the culture was observed for its colony morphology like margins, elevation, pigmentation, opacity, the texture on solid and smooth agar plates. The Morphology of the bacterium was examined with a light microscope. Physiology analysis and biochemical tests were conducted as described by **Li and Gao (1997).**

Qualitative and Quantitative assay for agarase activity

For the qualitative assay, one culture plate was taken and poured on with Lugol's iodine solution. The iodine solution stains the agar polysaccharide dark brown while the degraded polysaccharide portion of the agar plate shows a zone of light color. For the quantitative estimation of enzyme activity, DNSA method **(Miller, 1959)** was used. In this test the agarase activity was measured as the enzyme releases the reducing sugar equivalent using DNSA (3,5- di-nitrosalicylic acid) and the increasing concentration of reducing sugar was determined with the help of spectrophotometer at 540nm wavelength, keeping D-galactose as standard.

Partial purification of Agarase enzyme

MSA broth supplemented with 0.3% (w/v) agar was prepared and the isolated culture was inoculated into it. The flasks having 50 ml of culture media was inoculated with 1 ml of culture in its log phase. The flasks were incubated at 37˚C for 24 hours on a rotary shaker at 170 rev min-1. After 24 hours of incubation, the cultures were centrifuged at 10,000 rev min-1 for 10 min. at 4˚C. The pellet was discarded and the supernatant was used for assaying the enzyme activity.

Agarase activity detection

For calculating the enzyme activity, the galactose stock solution was prepared by mixing 250 mg of galactose in 100 ml of distilled water. From this stock solution, 1 ml solution was taken and mixed with 9 ml of distilled water so that the working solution could be prepared. In 7 dry and clean test tubes, standard sugar solution was pipette out in the range of 0 to 3 ml and the final volume of each test tube was adjusted to 3 mL with distilled water. Finally, every tube was added with 1 ml DNSA reagent and by putting the cotton plug, the tubes were kept in boiling water bath for 5 minutes. After allowing the tubes to cool at room temperature, OD was taken at 540 nm against the blank. The reducing sugars data were expressed as galactose equivalents. For measuring the enzyme activity, 100 µl sample of enzyme supernatant (described above) was added to 900 µl Phosphate Buffer Saline (pH, 7.0) with 0.25% agar substrate and incubated for 15 minutes at 37˚C. Initial OD was recorded. Then 1 ml of this reaction mixture was added with 2 ml of DNSA reagent and heated for approximately 5 minutes at 100˚C in the water bath. After allowing the mixture to cool, it is diluted with 10 ml of distilled water and optical density reading was taken at the above-mentioned wavelength. The enzyme activity was measured in Units per ml. One unit is that quantity of the enzyme which released 1µmol reducing sugar (galactose) per minute from agar substrate under defined conditions **(Suzuki *et al.*, 2002).**

Effect of agar concentration, Temperature, and pH on the activity of agarase enzyme

For calculating the agarase activity at various conditions, 0.2ml of exponential growth phase culture was inoculated into a clean Erlenmeyer flask of 100ml, containing 20ml MS broth with varying concentration of agar. The different agar concentrations taken were 0.05%, 0.1%, 1.5% and 2.0% agar. Cultures were incubated in an incubator shaker at 170 revs. The growth of bacterium at different agar concentration, temperature and pH was measured by the spectrophotometer and the enzyme activity was recorded. For agarase activity assay, the bacterial cells and the residual gel in the flask were removed after centrifugation at 10,000 rev min^{-1} for 10 min. at 4 ˚C. The supernatant so obtained, was consumed for quantifying the agarase enzyme activity. The activity was plotted graphically to determine the optimal growth at varying agar concentration, temperature, and pH. The temperature variations taken were 30˚C, 35˚C, 37˚C, 40˚C, and 45˚C. Agarase activity was taken at these different temperatures and plotted against it. The pH variations taken were 5, 7, 9 and 11. The buffer solution of these different pH was prepared. 0.1ml of the partially purified agarase enzyme was added to 0.9 ml of a buffer solution containing 0.5% agar for enzyme activity to be assayed for each different pH.

Identification of the bacterium by 16S rDNA sequencing

The identification of bacterial isolate, SELA 4was performed by 16S rDNA sequencing. The extracted DNA was used as a template for PCR amplification of the 16S rRNA gene sequence of length 1466bp. Universal primers complementary to the conserved regions were used so that the region could be amplified using 27F - 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R - 5' TACGGYTACCTTGTTACGACTT 3'. PCR was performed. For initial denaturation, in the 1st cycle, the temperature kept was 94°C for 2 minutes. The PCR machine was set for 25 cycles of annealing and extension of DNA (98°C for 10 sec; 53°C for 30 sec; 68°C for 1min). The Standard protocols were used for PCR products purification, which was further directly sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). A 16S rDNA sequence of length 1466bp obtained from SELA 4was submitted to GenBank. Phylogenetic tree construction and analysis were done using MEGA 6.0 software.

Partial purification of agarase from the culture broth

The extracellular agarase enzyme was extracted from the flask after 24 hours of growth in the culture medium. The culture was cooled at 4°C for 30 min. After which, the cells were separated by centrifugation at10,000 rev/min for 10 minutes at 4°C temperature. Solid ammonium sulfate at 80% saturation was calmly added to the supernatant containing the enzyme by constant stirring for an hour. The precipitate obtained was further purified by the process of centrifugation at 12,000 rpm for 30 min at 4 ^{0}C. The supernatant was carefully separated from the tube leaving behind the pallet. The pallet having the enzyme was then dissolved in Tris HCl buffer and dialyzed according to **Lakshmikanth *et al.*, (2006).**

SDS –PAGE and activity staining

The agarase enzyme which was partially purified by the above process was separated using an SDS-PAGE system. 12% SDS Polyacrylamide gel was prepared and performed similarly to **Laemmli (1970)**. From the partially purified agarase enzyme, 10µl sample was mixed with equal volumes of SDS loading buffer and incubated at 40^{0}C for 40 minutes to reduce and denature the protein for effective separation. In another well, protein molecular weight markers were also allowed to run with the sample. The process of electrophoresis was carried out at 30mA for 3.5 hours at room temperature. After electrophoresis, several protein bands obtained were stained with Coomassie brilliant blue R 250 for overnight and then de-stained to visualized proper blue agarase bands over visible light. The activity staining was done according to **Lakshmikanth *et al.*(2006)** with some modifications. After SDS PAGE, the gel was soaked in 20mM Tris- HCl buffer pH 7 for 30 minutes to remove SDS. To hasten the process, the soaking buffer changed thrice. The gel was then overlaid onto the plate containing 1.0% (w/v) agarose gel in 20mM Tris-HCl buffer with pH 7 and incubated for 30 minutes at 40°C. The gel was separated from the gel plate and then flooded with Gram's iodine to visualize the agarase activity.

Identification of enzyme-hydrolyzed products

Thin layer chromatography (TLC) was performed for identifying these enzyme hydrolysis products of agarose **(Kim *et al.*, 1999)**. LMP-agarose was enzymatically hydrolyzed in 200ml of 20mM Tris–HCl buffer pH 7.0. The reaction mixture was incubated for about 2 hours at 40^{0}C. After 2 hours, the reaction of the enzyme was stopped after heating the samples in a boiling water bath for a minute. The lysate mixture was later analyzed for hydrolysis products. The lysates were applied to activated silica gel-G-coated TLC glass plates. The Petri plates were chromatographed with n-butanol: acetic acid: water (2:1:1, v/v) as a solvent system. The resolved compounds were visualized by spraying with Naphthoresorcinol reagent followed by heating at 100^{0}C in a hot air oven for 10 min.

RESULTS

Isolation of agar degrading bacterium and its culture medium

A novel bacterial strain that degrades agar degrading, was isolated from the agriculture soil enriched with laboratory agar. The strain produced depression on the plates and showed a remarkable zone of clearance around them. The bacterial strain SELA 4 was grown at 37°C, pH 5 and sub-cultured regularly on MSA media slants containing agar only for carbon and energy source. Fig1 shows the recently isolated strain SELA 4 that showed depression on the smooth agar surface.

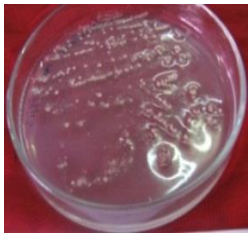

Figure 1 Culture plate ofthe SELA4 strain isolated from Agriculture soil enriched with laboratory agar

Qualitative assay for agarase activity

After selecting the colonies that degraded agar, the streaked plate was taken and poured on with Lugol's iodine solution. After a few seconds, the entire culture plate having the culture showed a light-colored translucent zone as shown in Fig 2.

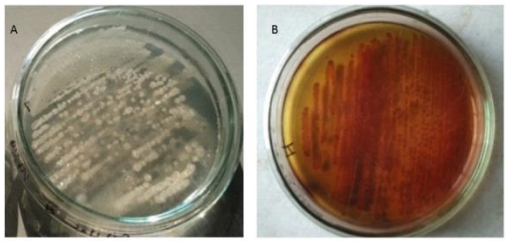

Figure 2 SELA 4 Plate showing translucent zone (B)
A: Before adding Iodine solution; B: After adding Iodine solution

Morphological, physiological and biochemical characteristics

The morphological, physiological and biochemical features of the strain SELA 4 were investigated and compiled in Table 1. The cells of the strain SELA 4, cultured for 3 days at 37°C were gram-positive, rod-shaped, capsular negative, aerobic, non-motile and formed smooth, off-white, flat colonies with entire margin on Mineral Salt Agar media plates. The biochemical analysis revealed that the strain SELA 4, hydrolyzed gelatin and utilized Arabinose, Glucose, Inositol, and Sucrose.

Table 1 Biochemical and physiological characteristics of strain SELA 4

Characteristic	Result
Cell shape	Rod
Margin	Entire
Elevation	Flat
Texture/Surface	Smooth
Colour	Off-white
Gram stain	+
Capsule	-
Cilium	-
Polar flagellum	-
Catalase	-
Oxidase	-
Growth at	
30°C	++
35°C	++++
37°C	+++++
40°C	++++
45°C	+++
Urea hydrolysis	-
Gelatin hydrolysis	+
Acetoin production(Voges-Proskauer test)	-
Indole production	-
Citrate utilization	-
Arabinose utilization	+
Glucose utilization	+
Inositol utilization	+
Sucrose utilization	+
Fructose utilization	-

+ positive/utilizable, -negative/non-utilizable, ++, +++,++++, +++++ increasing amount of growth

Effect of different agar concentration, temperature, and pH on the agarase activity

Fig 3(A) showed the effect of various agar concentrations on the activity of partially purified agarase from *Microbacterium* sp. SELA 4. The results showed that the agarolytic activity of 2.04 U/ml was observed as highest when the agar concentration in the media was 1.5% while it slightly decreased to 1.9 U/ml when the agar concentration was increased to 2%. This shows that increasing the level of the substrate has no effect on the activity above a limit of 1.5%. The reason may be saturation of active sites with the substrate. The results of Fig 3(B) indicated that the activity of agarase enzyme reached the maximum, 2.09 U/ml at 37°C and decreased to 1.57 U/ml and 1.26 U/ml with increasing temperature 40°C and 45°C respectively. This may be due to the denaturation of enzymes caused by structural deformity after incubation at high temperatures. For investigating the optimum pH for agarase activity, enzymes were incubated at 37°C in buffer solutions with pH 5,7,9,11. Results in Fig 3(C) indicated that the enzyme showed the maximum activity of 1.96U/ml at pH 5 which decreased to 1.42 U/ml, 1.14 U/ml and 0.96 U/ml at pH 7, 9 and 11 respectively. Increasing pH might have caused enzyme inactivation due to the formation of an ionic form of enzyme active sites.

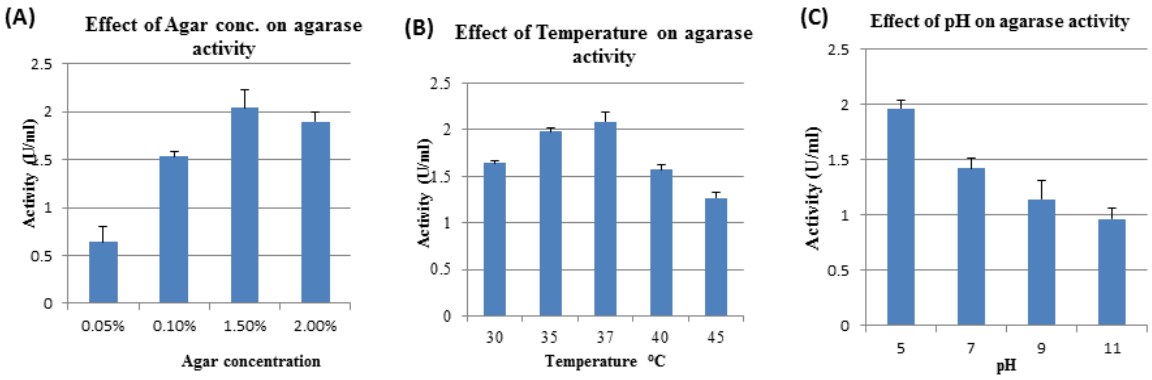

Figure 3 The above figure presents the bar graph showing (A) the effect of different Agar concentrations, 0.05%, 0.10%, 1.50% and 2.0% (B)Effect of temperature, 30°C, 35°C, 37°C, 40°C and 45°C (B) effect of pH 5,7,9 and 11, on agarase activity of SELA 4. The error bar shows the standard deviation.

Phylogenetic affiliation of the strain SELA4 through 16S rDNA sequence

The sequence of the PCR product of length 1466bp from 16S rDNA of SELA 4 was used as a query sequence for an initial similarity search against the sequences of NCBI's Gen Bank database. BLASTn tool was used for homology search and the search results of the 16S rDNA for the strain SELA 4 showed its close relationship to *Microbacterium barkeri* strains with 99% sequence similarity. A Phylogenetic tree of the Strain SELA 4 was constructed(Fig 4) using 16S rDNA sequences. The query sequence matched 99% in similarity with *Microbacterium* sp. through the results obtained by BLASTn. Multiple Sequence Alignment and the construction of the phylogenetic tree was done by Maximum Likelihood statistical method using MEGA 6: Molecular Evolution Genetics Analysis Version 6.0. A bootstrap confidence analysis was cried out with 1000 replicates. Heuristic search tree was obtained when we take the parameters as Neighbor-Joining and BioNJ algorithms for a matrix of pairwise distances estimated using a JTT model. The topology was selected having superior log-likelihood value. 10 sequences were taken for analysis. The positions of sequence alignment having gaps and missing data were ignored. The results of the Phylogenetic analyses suggest that the strain SELA 4 is closely affiliated to *Microbacterium barkeri* strain DSM 20145, Accession No. NR 026164.1 (99%), *Microbacterium barkeri* M3, Accession No. KY928100.1 (99%) as they share a common ancestor. This cluster was found related to the other cluster of closely related sequences of *Microbacterium* genus, *Microbacterium oryzae*, and *Microbacterium gilvum.* Thus it was concluded that SELA 4 is a member of genus *Microbacterium,* species *barkeri* belonged to the class *Actinobacteria.* Among the existing agar degrading bacteria, none has been reported to belong to the genus *Microbacterium.* The 16S rDNA sequence of *Microbacteriumbarkeri* sp. strain SELA 4 has been deposited in GenBank database of NCBI under the accession no**.** MG203882.1

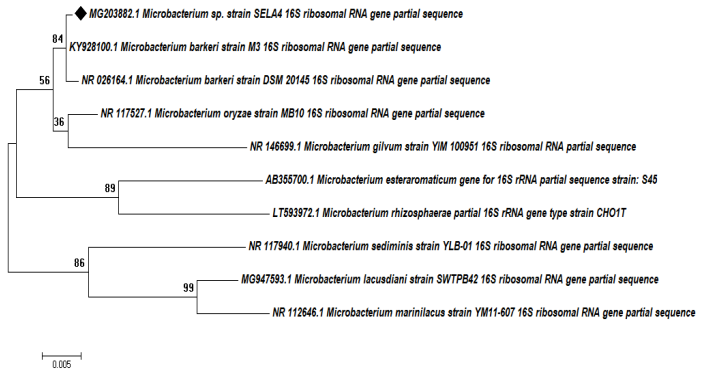

Figure 4 Phylogenetic tree of strain SELA 4, constructed from 16S rDNA nucleotide sequence using the Maximum Likelihood Method of statistical analysis. The tree shows the position of strain SELA 4 and the other species of the genus Microbacterium. The % of replica trees in which the associated clustered taxa in the bootstrap test (1000 replica) are shown above the branches. Accession number of the 16S rRNA gene partial sequences are given in front of the species name. The scale bar of 0.005 shows substitution per nucleotide position.

SDS –PAGE and activity staining

For detecting the molecular weight of the enzyme produced by the bacterium, SDS-PAGE was run and the activity of the enzyme was detected by Activity staining (Fig 5). After Electrophoresis, four distinct bands were observed in the SDS gel in Lane 2 while Lane 1 showed the bands of Protein Molecular weight marker(G-Biosciences PAGEmark Blue Plus Protein marker) that ranged in size from 27 to 158 kDa. The positions of these markers are indicated on the left side of the gel. Out of the four bands in Lane 2, Band 2 showed a clear zone which corresponds to the enzymatic activity with Lugol's iodine solution. Thus a single band showing agarase activity was detected and the molecular weight was estimated to be about 55.6 kDa, corresponding to the molecular marker in Lane 1.

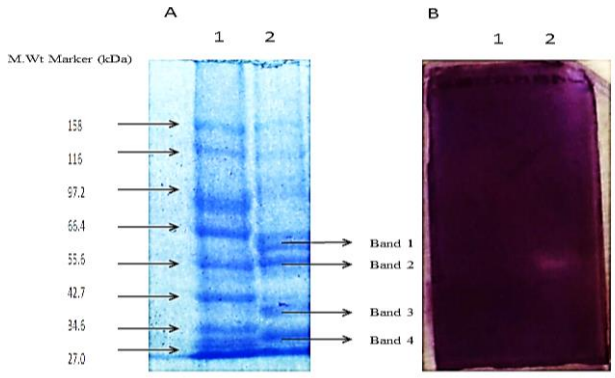

Figure 5 (A) SDA-PAGE profile and (B) Activity staining.
(A) Lane 1-Molecular marker; Lane 2-Enzymes from SELA 4.
(B) Band 2 in Lane 2 indicates putative agarase with the molecular wt. approximately 55.6kDa

Identification of enzyme-hydrolyzed products

Neoagarohexaose was the oligosaccharide detected in the agarose hydrolysate after 120 min of incubation. (Fig 6) The results suggest that the agarase purified from the strain, SELA 4 belongs to β agarase which specifically cleaves β-1,4 linkages found in agarose. The agaro oligosaccharides were also allowed to run along with agarose hydrolyzed products by the agarase on TLC but none of the product produced by the purified agarase matched the Rf values of the acid hydrolysis product of agarose (data not shown here). However, the Rf values correlated with the standard Neoagarohexaose after 120 min. of incubation. Thus, it becomes clear that the agarase secreted by the bacterium, *Microbacterium barkeri* sp. SELA 4 is a β agarase.

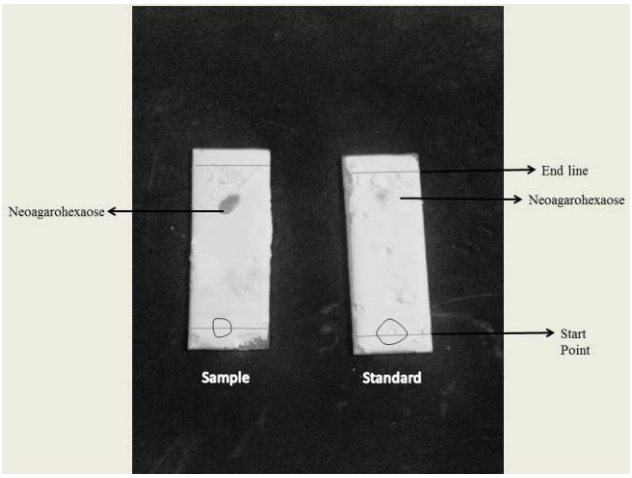

Figure 6 TLC of agarose hydrolyzed products.
(A) Agarose hydrolyzed product after 120 min.
(B) Standard- Neoagarohexaose

DISCUSSION

Strain SELA 4, isolated from agar enriched agriculture soil has the agarolytic activity which showed marked depression on an agar plate. It is rod-shaped, Gram-positive bacterium utilizing agar for carbon along with other mineral compounds. The biochemical characteristics exhibited by the isolate indicates it to be a member of class *Actinobacteria*. This class is well known for its potential to degrade a variety of stable organic compounds **(Lewin *et al.*,2016)**including natural and synthetic substances **(Solvanikova and Golovleva, 2015)**. The strain required 1.5% of agar in the media for optimum enzyme activity of 2.04 U/ml. Temperature and pH are considered important parameters regarding enzyme activity. It was noticed that the activity of SELA 4 consistently increased from 30-37^{o}C, with optimal activity of 2.09 U/ml, at 37^{o}C. With increasing temperature, there was a decrease in the activity of the enzyme. When compared to the other agar degrading bacteria, SELA 4 has slightly lower optimum temperature requirement than many agarases **(Kirimura *et al.*, 1999; Suzuki *et al.*, 2003; Temuujin *et al.*, 2012;Lakshmikanth *et al.*, 2006).** The enzyme showed maximum agarase activity, 1.96U/ml at pH 5.0 which is similar to many other agarases **(Oh *et al.*, 2010;Ghazi *et al.*,2013;Temuujin*et al.*, 2012).** While agarases active at high pH have also been reported **(Sakai *et al.*, 2015;Kim *et al.*,2012).**16S rDNA gene sequencing confirms that the isolate belongs to the genus *Microbacterium* of class *Actinobacteria*, by showing 99% sequence similarity with *Microbacterium barkeri* strain DSM 20145**(GenBank: NR 026164.1)**and *Microbacterium barkeri* M3 **(GenBank: KY928100.1).**
Currently, most of the reported bacterial isolates producing agarase enzymes, are from marine sources **(Fu and Kim, 2010)**. By far none of the research reports talks about the isolation of agarase enzyme producing bacteria belonging to genus *Microbacterium.* A relatively closer study is about the agarolytic isolates from the surface of macroalgae, affiliating to eleven different genera including *Microbacterium* **(Alvarado *et al.*,2017)**. Recently this class has been exposed to possess good cellulose degrading activity **(Rajagopal and Kannan, 2016)**. A few studies have been reported about the degradation of polysaccharide, xanthan by *Microbacterium* sp. Strain XT 11 **(Qian *et al.*, 2007)** and the degradation of xylan by *Microbacterium xylanilyticum sp.***(Kim *et al.*, 2005)**. The utilization of Arabinose, Glucose, Inositol and Sucrose, hydrolysis of gelatin and other physiological characteristics further ascertain the affiliation of SELA4 to *Microbacterium* sp.
In the current study, we isolated the agarase enzyme from *Microbacterium barkeri* sp. SELA 4, having a molecular weight of about 55.6kDa which is closer to β agarases with molecular weight 52 kDa **(Leon *et al.*, 1992)** produced by gram-negative *Alteromonas* sp. C-1, *Rhodococcus* sp. Q5, 54kDa **(Feng and Li, 2013)**, *Bacillus* sp.BI 3, 58kDa **(Li *et al.*, 2014)** isolated from soil. According to **Leon *et al.*, 1992** the agarase enzymes present in agarolytic bacteria are of two types; Group I and Group II. Softening of agar and formation of depressions at the colonies is the characteristic feature of Group I while that of Group II is extensive liquefication. **Vera *et al.*** (1998)divided β-Agarases into 3 groups based on the size of the agarase enzyme. The molecular weight of group 1 is approximately 30 kDa, group 2, approximately 50 kDa and group 3 approximately 100 kDa. Hence, the isolated enzyme belongs to group 2 β agarases. The isolate *Microbacterium barkeri sp.* SELA 4 was grown at 37^{o}C with pH 5 which is similar to other agarolytic bacterial isolates like *Streptomyces coelicolor* A3(2) **(Temuujin *et al.*, 2012)** and *Acinetobacter* sp. AGLSL-1 **(Lakshmikanth *et al.*, 2009)** from the soil environment. The degradation of agar requires the sequential breakdown of agarolytic products by enzymes like β agarase, which breaks down β-1,4 glycosidic bonds resulting in the production of neoagaro oligosaccharides (NAOs). Its major products are Neoagarobiose and Neoagarotetraose **(Fu *et al.*, 2009),** Neoagarohexaose **(Ohta *et al.*,**

2004).Neoagarobiose is further converted to the products 3,6 anhydro L-galactose and D-galactose which has skin fairness and anti-inflammatory effect on human **(Kim *et al.*, 2017)**. The agarolytic enzyme, α agarase cleaves α-1,3 linkages to produce agaro-oligosaccharides (AOs). The present study showed that *Microbacterium barkeri* sp. SELA 4 hydrolyzed agarose to Neoagarohexaose. The same type of product was also produced by non- marine bacteria *Paenibacillus sp. SSG-1***(Song *et al.*, 2014)***Bacillus sp. BI-3* **(Li *et al.*, 2014)***Streptomyces coelicolor A3(2)***(Temuujin *et al.*, 2012)**.

CONCLUSION

Based on our present study, it is summarized that *Microbacterium barkeri sp.* SELA 4 is the first report on an agarolytic bacterium that produce the enzyme β agarase, isolated from soil enriched with laboratory agar. The enzyme showed the highest activity of 2.04 U/ml 1.5% agar in the culture media. The optimal temperature was 37^{o}C as maximum activity was seen at this temperature while optimum pH was found to be 5. It produces Neoagarohexaose, could prove to be a better skin moisturizer than neoagarobiose and neoagarotetraose, as the viscosity of Neoagarohexaose is higher than that of smaller oligosaccharides. Hence *Microbacterium barkeri p. SELA 4* could be considered as one of the potential sources of Neoagarohexaose for industrial application.

Acknowledgment: We are grateful to the Director, IMS Engineering College Ghaziabad, for providing all the essential facilities for conducting the research work. I also feel thankful to the Director, Codon Biotech Pvt. Ltd., for necessary help regarding Sequencing.
The authors declare that no conflict of interest exists.

REFERENCES

Alonso-Echanove, J., Shah, S.S., Valenti, A.J., Dirrigl, S.N., Carson, L.A., Arduino, M.J., Jarvis, W.R. (2001). Nosocomial outbreak of *Microbacterium* species bacteremia among cancer patients. *J Infect Dis*, 184(6), 754-60. https://doi.org/10.1086/323080
PMid:11517437

Alvarado, R., Leiva, S. (2017). Agar-degrading bacteria isolated from Antarctic macroalgae. *Folia Microbiol (Praha)*, 62(5), 409-416 https://doi.org/10.1007/s12223-017-0511-1

Chi, W.J., Chang, Y.K., Hong, S.K. (2012). Agar degradation by microorganisms and agar-degrading enzymes. *Appl Microbiol Biotechnol*,94(4), 917-30.https://doi.org/10.1007/s00253-012-4023-2

Duckworth, M., Turvey, J.R. (1969). An extracellular agarase from a *Cytophaga* species. *Biochemical Journal*, 113(1), 139-142.https://doi.org/10.1042/bj1130139

Feng, Z., Li, M. (2013). Purification and characterization of agarase from *Rhodococcus sp*. Q5, a novel agarolytic bacterium isolated from printing and dyeing wastewater. *Aquaculture*, 372–375, 74–79. https://doi.org/10.1016/j.aquaculture.2012.10.026

Fu, X.T., Kim, S.M. (2010). Agarase: review of major sources, categories, purification method, enzyme characteristics and applications. *Mar Drugs*, 8(1), 200-18.https://doi.org/10.3390/md8010200

Fu, X.T., Pan, C.H., Lin, H., Kim, S.M. (2009). Gene cloning, expression, and characterization of a beta-agarase, AgaB34, from *Agarivorans albus* YKW-34. *J Microbiol Biotechnol*, 19, 257–264. PMid:19349750

Ghazi, M., Aziz, Hala, Ali, M. (2013). Purification and Characterization of Agarase from *Bacillus* sp., H12. *Current Research Journal of Biological Sciences*, 5(1), 13-18.

Hodgkin, J., Kuwabara, P.E., Corneliussen, B. (2000). A novel bacterial pathogen, *Microbacterium nematophilum*, induces morphological change in the nematode *C. elegans*. *Curr Biol*, 10, 1615–1618.https://doi.org/10.1016/S0960-9822(00)00867-8

Hosoda, A., Sakai, M. (2006). Isolation of *Asticcacaulis sp*. SA7, a novel agar-degrading *Alphaproteo bacterium*. *Bioscience, Biotechnology, and Biochemistry*, 70(3), 722-725. https://doi.org/10.1271/bbb.70.722 PMid:16556992

Kaku, H., Subandiyah, S., Ochiai, H. (2000). Red stripe of rice is caused by a bacterium *Microbacterium sp*. *J. Gen Plant Pathol*, 66, 149–152.https://doi.org/10.1007/PL00012937

Kawaroe, M., Pratiwi, I., Sunudin, A. (2018). Isolation and characterization of marine bacteria from macroalgae Gracilaria salicornia and Gelidium latifolium on agarolytic activity for bioethanol production. In International Conference on Biomass: Technology, Application, and Sustainable Development IOP Publishing IOP Conf. Series: *Earth and Environmental Science*, 65 (2017) 012025 https://doi.org/10.1088/1755-1315/65/1/012025

Kim, B.C., Poo, H., Lee, K.H., Kim, M.N., Park, D.S., Oh, W., Lee, J.M., Shin, K.S. (2012). *Simiduiaareninigrae* sp. nov., an agarolytic bacterium isolated from sea sand. *Int. J. Syst. Evol. Microbiol*, 62(4), 906-11. http://doi: 10.1099/ijs.0.031153-0. PMID: 21669928.

Kim, B.J., Kim, H.J., Ha, S.H., Hwang, S.H., Byun, D.S., Lee, T.H., Kong, J.Y. (1999). Purification and characterization of β agarase from marine bacterium *Bacillus cereus* ASK202. *Biotechnology letters*, 21, 1011-1015.https://doi.org/10.1023/A:1005422000171

Kim, J.H., Yun, E.J., Yu, S., Kim, K.H., Kang, N.J. (2017). Different Levels of Skin Whitening Activity among 3,6-Anhydro-l-galactose, Agarooligosaccharides, and Neoagarooligosaccharides. *Mar Drugs*, 15(10), 321. https://doi.org/10.3390/md15100321

Kim, K.K., Park, H.Y., Park, W., Kim, I.S., Lee, S.T. (2005).*Microbacterium xylanilyticum sp. nov*., a xylan-degrading bacterium isolated from a biofilm. *Int J Syst Evol Microbiol*, 55, 2075–2079.https://doi.org/10.1099/ijs.0.63706-0 PMid:16166712

Kirimura, K., Masuda, N., Iwasaki, Y., Nakagawa, H., Kobayashi, R., Usami, S. (1999). Purification and characterization of a novel β-agarase from an alkalophilic bacterium *Alteromonassp.* E-1. *Journal of Bioscience and Bioengineering*, 87, 436–441. https://doi.org/10.1016/S1389-1723(99)80091-7

Kobayashi, R., Takisada, M., Suzuki, T., Kirimura, K., Usami, S. (1997). Neoagarobiose as a novel moisturizer with whitening effect. *Biosci Biotechnol Biochem*, 61(1), 162-3. https://doi.org/10.1271/bbb.61.162

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.https://doi.org/10.1038/227680a0 PMid:5432063

Lakshmikanth, M., Manohar, S., Souche, Y., Lalitha, J. (2006). Extracellular β-agarase LSL-1 producing neoagarobiose from a newly isolated agar-liquefying soil bacterium, *Acinetobacter sp*., AG LSL-1. *World J Microbiol Biotechnol*, 22(10), 1087-1094.https://doi.org/10.1007/s11274-006-9147-z

Lakshmikanth, M., Manohar, S., Lalitha, J. (2009). Purification and characterization of β-agarase from agar-liquefying soil bacterium, *Acinetobacter sp*., AG LSL-1. *Process Biochem*, 44(9), 999-1003.https://doi.org/10.1016/j.procbio.2009.04.025

Leon, O., Quintana, L., Peruzzo, G., Slebe, J.C. (1992). Purification and properties of an extracellular agarase from *Alteromonas sp. strain C-1*. *Appl Environ Microbiol*, 58, 4060–4063. PMid:16348832 PMCid:PMC183228

Lewin, G.R., Carlos, C., Chevrette, M.G., Horn, H.A., McDonald, B.R., Stankey, R.J., Fox, B.G., Currie, C.R. (2016). Evolution and Ecology of Actinobacteria and their Bioenergy Applications. *Annu Rev Microbiol*, 70, 235-54. doi: 10.1146/annurev-micro-102215-095748. https://doi.org/10.1146/annurev-micro-102215-095748

Li, J., Sha, Y., Zilda, D.S., Hu, Q., He, P. (2014). Purification and Characterization of Thermostable Agarase from *Bacillus sp. BI-3*, a Thermophilic Bacterium Isolated from Hot Spring. *J Microbiol Biotechnol*, 24(1), 19–25.https://doi.org/10.4014/jmb.1308.08055
PMid:24064915

Li, X., Gao, P. (1997). Isolation and partial properties of cellulose-decomposing strain of *Cytophaga sp.* LX-7 from soil. *J Appl Microbiol*,82(1), 73 – 80. https://doi.org/10.1111/j.1365-2672.1997.tb03299.x

Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 1959; 31(3): 426–428. http://pubs.acs.org/doi/abs/10.1021/ac60147a030

Oh.,C., Nikapitiya, C., Lee,Y., Whang, I., Kang, D.H., Heo, S.J., Choi, Y.U., Lee, J. (2010). Molecular cloning, characterization and enzymatic properties of a novel Beta-agarase from a marine isolate *Pseudoalteromonas* SP. AG52. *Brazilian Journal of Microbiology*, 41(4), 876-889. http://dx.doi.org/10.1590/S1517-83822010000400006

Ohta, Y., Hatada, Y., Nogi, Y., Miyazaki, M., Li, Z., Akita, M., Hidaka, Y., Goda, S., Ito, S., Horikoshi, K. (2004). Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from a novel species of deep-sea Microbulbifer. *Appl Microbiol Biotechnol*, 64(4), 505-14. https://doi.org/10.1007/s00253-004-1573-y PMid:15088129

Qian, F., An, L., Wang, M., Li, C., Li, X. (2007). Isolation and characterization of a xanthan-degrading *Microbacterium* sp. strain XT11 from garden soil. *J Appl Microbiol*, 102(5), 1362-71. https://doi.org/10.1111/j.1365-2672.2006.03215.x PMid:17448171

Rajagopal, G., Kannan, S. (2016). Systematic characterization of potential cellulolytic marine actinobacteria *Actinoalloteichus sp.* MHA15. *Biotechnol Rep*, 13, 30-36. https://doi.org/10.1016/j.btre.2016.12.003

Sakai, M., Deguchi, D., Hosoda, A., Kawauchi, T., Ikenaga, M. (2015).*Ammoniibacillus agariperforans* gen. nov., sp. nov., athermophilic, agar-degrading bacterium isolated from compost. *Int J Syst Evol Microbiol*, 65(2), 570-577.https://doi.org/10.1099/ijs.0.067843-0PMid:25404482

Sartori, M., Nesci, A., Etcheverry, M. (2012). Production of Fusarium verticillioides biocontrol agents, *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*, using different growth media: evaluation of biomass and viability after freeze-drying. *Food Addit ContamPart A Chem Anal Control Expo Risk Assess*, 29, 287–292. https://doi.org/10.1080/19440049.2011.563369 PMid:21547798

Solvanikova, I.P., Golovleva, L.A. (2015). Physiological and biochemical properties of actinobacteria as the basis of their high biodegradative activity (review). *Prikl Biokhim Mikrobiol*, 51(2), 132-9. https://doi.org/10.7868/S0555109915020208PMid:26027348PMid:26027348

Song, T., Cao, Y., Xu, H., Zhang, W., Fei, B., Qiao, D., Cao, Y. (2014). Purification and characterization of a novel β-agarase of *Paenibacillus sp.* SSG-1 isolated from soil. *J Biosci Bioeng*,118(2), 125-

129.https://doi.org/10.1016/j.jbiosc.2014.02.008 PMid:24631192

Song T, Zhang W, Wei C, Jian, T., Xu, H., Cao, Y., Qiao, D. (2015). Isolation and characterization of agar-degrading endophytic bacteria from plants. *Curr Microbiol*, 70(2), 275-281.https://doi.org/10.1007/s00284-014-0713-6PMid:25331792

Sugano, Y., Terada, I., Arita, M., Noma, M. (1993). Purification and characterization of a new agarase from a marine bacterium, *Vibrio sp.* Strain JacT0107. *Appl Environ Microbiol*, 59, 1549-1554. PMid:8517750 PMCid:PMC182117

Suzuki, H., Sawai, Y., Suzuki, T., Kawai, K. (2002). Purification and characterization of an extracellular alpha-neoagarooligosaccharide hydrolase from *Bacillus sp.* MK03. *J Biosci Bioeng*, 93(5), 456-63. https://doi.org/10.1016/S1389-1723(02)80092-5

Suzuki, H., Sawai, Y., Suzuki, T., Kawai, K. (2003). Purification and characterization of an extracellular β-agarase from *Bacillus sp.* MK03. *Journal of Bioscience and Bioengineering*, 95(4), 328-334. https://doi.org/10.1016/S1389-1723(03)80063-4

Temuujin, U., Chi, W.J., Chang, Y.K., Hong, S.K. (2012). Identification and biochemical characterization of Sco3487 from *Streptomyces coelicolor* A3(2), an exo- and endo-type β-agarase-producing neoagarobiose. *J Bacteriol*, 194(1), 142-9.https://doi.org/10.1128/JB.05978-11 PMid:22020647 PMCid:PMC3256618

Vera, J., Alvarez, R., Murano, E., Slebe, J.C., Leon, O. (1998). Identification of a Marine Agarolytic *Pseudoalteromonas* Isolate and Characterization of Its Extracellular Agarase. *Appl Environ Microbiol*, 64(11), 4378–4383. PMid:9797294 PMCid:PMC106656

Wang, J., Jiang, X., Mou, H. (2004). Anti-oxidation of agar oligosaccharides produced by agarase from a marine bacterium. *J Appl Phycol*, 16(5), 333-340.https://doi.org/10.1023/b:japh.0000047944.40463.e6

Yoshizawa. Y., Ametani, A., Tsunehiro, J., Nomura, K., Itoh, M., Fukui, F., Kaminogawa, S. (1995). Macrophage stimulation activity of the polysaccharide fraction from a marine alga (*Porphyra yezoensis*): structure-function relationships and improved solubility. *Biosci Biotechnol Biochem*, 59(10), 1933-7. https://doi.org/10.1271/bbb.59.1933PMid:8534986