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ISOLATION OF AGAR DEGRADING BACTERIUM *PSEUDOMONAS AERUGINOSA* ZSL-2 FROM A MARINE SAMPLE

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ABSTRACT
A gram negative bacterium forming orange colonies and designated ZSL-2 was isolated from sea-shore. ZSL-2 was selected for its ability to actively degrade both agar and carrageenans. The cells occurred singly or in pairs as long rods. The temperature range for growth was 20- 40ºC with an optimum at 30ºC, the pH range for growth at 30ºC was from 5.0 to 11, with an optimum around 8.0. The NaCl concentrations required for growth at 30ºC and pH 8.0 ranged from 0 to 5%, with an optimum around 2%. Phylogenetic analysis of 16S rDNA sequences indicated that strain is closely related to *Pseudomonas aeruginosa*. Based on phenotypic and phylogenetic features the new bacterium designated as *Pseudomonas aeruginosa* ZSL-2. The bacterium did not utilize other polysaccharides like, alginate, cellulose, xylan and CMC. But utilize agar and carrageenan as sole source of carbon and energy.

INTRODUCTION

Seventy percent of the earth's surface is covered by the sea and in general, marine microorganisms are considered to be a major component of global nutrient cycle (Arrigo, 2005). The algal polysaccharides constitute a crucial carbon source for many marine bacteria which degrade the cell wall by secreting specific glycoside hydrolases (Michel *et al*., 2006). Marine red algae (*Rhodophyta*) are characterized by abundance of sulfated polysaccharides, which have no equivalent in land plants (Kloareg and Quatrano, 1988). These anionic polymers are laid out in the cell wall at a high density and can constitute up to 50% of the dry mass of seaweed (Craigie, 1990). This large family of hydrocolloids, agar and carrageenans are well known for their gelling properties and are traditionally used as high quality ingredients in food and cosmetics (McHugh, 2003; Renn, 1997) and in variety of laboratory and industrial applications (Rudolph, 1986). They are made up of linear chains of galactose with alternating α–(1→3) and β–(1→4) linkages. In all these galactans the β–linked galactose units are in the D-configuration (G unit), but the α-linked galactose units are in the L-configuration as in agar (L unit) and D-configuration in carrageenans (D unit). Agar comprised of the gelling component agarose, with zero sulfate substitution**.** Carrageenan, the sulfated galactans are further classified according to the number and the position of sulfated ester(s) and by the occurrence of 3,6-anhydro-bridges in the α-linked residues (DA unit) found in gelling carrageenans (Knutsen *et al*. 1994). The three most industrially exploited carrageenans, namely κ-(DA-G4S), τ-(DA2S-G4S) and λ-(D2S, 6S-G2S) carrageenan, are distinguished by the presence of one, two or three ester-sulfate groups per repeating disaccharide unit respectively. The biotechnology of enzymes and enzyme degraded products of agar and carrageenans is still in infancy compared to that of alginate, starch (Guzman and Paredes**,** 1995) or pectin (Beldman, 1996). These hydrocolloids are widely used as thickening and gelling agents in structural and functional applications and generally regarded as safe (GRAS) by the Food and Drug Administration in the United States (Mao-hong Zhou *et al*., 2008). There has been great interest in agarases (E.C.3.2.1.81) since they are used for single step recovery of oligonucleotides / nucleic acids from agarose gels fairly and quantitatively. The agarase hydrolysed products agar-oligosaccharides, which are increasingly used as functional food ingredients (Kono 1993). κ-Carrageenase (E.C. 3.2.1.83), which hydrolyzes β-1,4 linkages in κ-carrageenan to a series of homologous, even numbered oligosaccharides (Weigl and Yaphe, 1966) is a useful tool for the structural analysis of the cell walls and protoplast isolation from red algae (Le Gall *et al*., 1990; Ostgaard *et al*., 1993). The degraded carrageenans have drawn considerable interest (Mou *et al*., 2003), since these sulfated oligosaccharides have diverse biological and physiological activities, including anticoagulation (Alban *et al*., 2002), anti-inflammation (Arfors and Ley, 1993), anti-thrombosis, antitumor activity (Hiroishi *et al*., 2001), and viral inactivation (Caceres *et al*., 2000), which depend on structural parameters such as carbohydrate structure, molecular mass, degree of sulfate esterification, and the linking position of sulpho groups (Liu *et al*., 2000).

Reports on agar and carrageenan degrading bacteria are plenty; while the bacteria degrading both are only few and more over, to our knowledge, they were enteric bacteria. Erasmus *et al*., (1997) have reported few enteric bacteria capable of degrading agar as well as carrageenan from the digestive system of

Halioteis medae; *Vibrio Y8, Pseudomonas Y5, Alkaligenes S4, Vibrio U4 and U.*

The present investigation was initiated to isolate and characterize the agar and carrageenan degrading bacterium from a marine sample, the reports of which are only few to our knowledge.

MATERIALS AND METHODS

Isolation of agarolytic bacterium

Various agar degrading bacterial strains (Lakshmikanth *et al.*, 2006a, 2006b) were isolated in our laboratory. These microorganisams were isolated by enrichment culture technique method in a mineral salts medium. Five ml sea water was used to inoculate 250mL Erlenmeyer flasks containing 50mL sterilized mineral salt medium supplemented with agar (0.1%, w/v) as the only source of carbon and energy. The flasks were incubated on rotary shaker at 170 rev. min^{-1}, 30ºC, for 1-3 days. The culture grown in agar (0.1%, w/v) limiting mineral-salts medium, after several sub-cultures, was serially diluted and spread on to mineral-salts-agar plates. The plates were incubated for 3 days, and 10 different colonies that appeared on the plate were picked up aseptically and individual bacterial colonies were sub-cultured on different plates. The bacterial cultures were designated as ZSL-1 to 10. Of these, a bacterial colony (ZSL-2) that had exhibited extensive agar degrading capacity on agar plates was selected for further studies. The following minimal mineral-salts medium (MMS) was used for standardizing the culture conditions. The medium contained (g/L^{-1}) the following composition, K_2HPO_4, 0.38; $MgSO_4$, 0.20; $FeCl_3$, 0.05; NH_4NO_3, 1.0. The pH of the medium was adjusted to 7.0 and this was supplemented by agar (0.1%, w/v) as the only source of carbon and energy. The bacterial culture were maintained on minimal salt agar plates/ slants or LB agar/ slants and sub-cultured fortnightly.

Cell morphology, phenotypic characterization and electron microscopy

Gram's staining; motility tests and various biochemical tests were performed on strain ZSL-2 in order to identify the bacterium as described in Bergey's manual of systematic bacteriology. (Ivanova *et al.*, 1998). For scanning electron microscopy (SEM), bacterial samples were prepared from 24 hr grown culture as described by Junko Kai *et al.*, (1999) and observed under scanning electron microscope (Philips XL-30 ESEM, USA).

Culture conditions

The bacterial strain ZSL-2 was inoculated into the MMS medium supplemented with agar (0.3%, w/v) and incubated. A 1mL exponential growth phase culture was inoculated into a fresh 250mL Erlenmeyer flasks containing 50 mL MMS medium, supplemented with agar (0.3%, w/v). The flasks were incubated on rotary shaker at 170 rev min^{1} and operated at different temperature such as 20, 22, 24, 26, 28 up to 40ºC to test the optimum growth temperature of the bacterium. Similarly, the MMS media was maintained at different pH values (pH 5.0-11.0) and at different ionic strengths (0-5% NaCl, w/v) to optimize the growth conditions of the strain ZSL-2. The growth of the bacterium at different incubation periods was measured spectrophotometrically by monitoring increase in the optical density at 660 nm. All growth experiments were performed in triplicate.

The utilization of polysaccharides (0.1%, w/v) like agar, carrageenan, alginate, cellulose, carboxymethylcellulose (CMC) and starch by bacterium was also tested in order to check the substrate specificity.

Antibiotic sensitivity test

The double layer method was used for the antibiotic sensitivity test. The diameter of the inhibition zone around the antibiotic disc was calculated after 48hr of incubation. The following antibiotic discs were used: Ampicillin, Streptomycine, Erythromycin, Methycillin, Tetracycline and Ciproflaxin.

Production of agarase and carrageenase

One ml of exponential phase culture of ZSL-2 was inoculated into 250mL Erlenmeyer flask containing 50mL of sterilized MMS medium supplemented by agar (0.3%, w/v) as sole and limiting carbon source. The flasks were incubated on a rotary shaker at 170 rev. min^{-1} and 30ºC for 24hr. The bacterial cells were harvested by centrifugation at 8000 rev. min^{-1} for 10 min at 4ºC. The clear supernatant obtained was used as source of enzyme.

Assay of carrageenase and agarase activity

Carrageenase and agarase activities were determined by neocuproine method as described by Dygert *et al.*, (1965). The assay mixture of 1mL contained 20mM Tris-HCl buffer (pH 8.0), 0.5mL of carrageenan or agrose (0.1% w/v in Tris-HCl buffer) and 50 μL suitably diluted enzyme at 40ºC. For carrageenase assay it was necessary to add ethanol (50%, v/v) to reaction mixture before measuring the absorbance. One unit of the enzyme activity was defined as the amount which liberates 1μmol galactose equivalent per minute under assay condition. The protein content of the enzyme solution was determined by the Lowry *et al.*, (1951) method using Bovine serum albumin as standard protein.

RESULTS AND DISCUSSION

Isolation and Identification of agarolytic bacterium

By enrichment culture technique 10 different agar utilizing bacterial colonies were obtained. The bacterial colony (ZSL-2), which outgrew other potential growers and also had shown orange pigmentation and produced soft pits on the surface with clear haloes around the bacterial colonies, which was obvious mark indicating high ability of depolymerizing polysaccharide (agar/carrageenan) was chosen for further investigations.

The identification of strain ZSL-2 was based on the morphological and physiological characteristics. The bacteria were helical in shape with varying length (1.25-2.0 μm) and width (643-776 nm) as observed under electron microscope (Fig. 1). The colonial appearance of strain ZSL-2 on the agar plate was colorless transparent round colonies. The strain was Gram-negative rod, motile and was positive for oxidase, gelatinase, arginine dehydrolase, urease, Voges-Proskauer, nitrate reduction and citrate utilization tests. Furthermore, it

was very active in the degrading casein, indicating the presence of proteases among its hydrolytic enzymes. The strain was negative for catalase, and indole production tests. Carbohydrate utilization at 37ºC for 48 h was positive for fructose, galactose, mannose, maltose, sucrose. The preliminary identification results showed that the morphological and physiological characteristics of this strain were in accordance with *Pseudomonas* according to Bergey's Manual of Systematic Bacteriology (Table 1) and the strain ZSL-2 was assigned to the genus *Pseudomonas*. Further 16 S rDNA sequences of strain and BLAST results showed the isolate had similarity with *Pseudomonas aeruginosa* (data not shown). Hence the strain ZSL-2 was designated as *Pseudomonas aeruginosa* ZSL-2.

The antibiotic resistance assay was carried out in order to check its pathogenic activity on agar plates and was found be sensitive to the antibiotics like Ampicillin, Streptomycine, Erythromycin, Methycillin, Tetracycline and Ciproflaxin (Table 2)

Determination of growth conditions

The bacterium was able to grow at temperature ranging from 20ºC to 40ºC, with an optimum around 30ºC. While no growth was detected below 20ºC or above 40ºC after 30h of

Table 1: Morphological and Biochemical features of *Psuedomonas aureginosa* ZLS-2

Characteristic(s)	Results
Growth on nutrient or LB broth	+ +
Growth temperature	
5° C	-
4° C	-
pH range of growth	5.0 -11.0
Relation to free oxygen	
Morphological	Aerobic
Form	Rods
Cell grouping	Short chains or inndividuals
Gram's staining	Gram -ve
Motility	Motile
Cultural	
Agar cultures	Ropy transparent
Pigment production	Orange color on different medium
McConkeys agar	Lactose not fermented
Hydrolysis of	
Casein	+
Starch	+
Gelatin	+
Urea	+
Indole	-
MR	+
VP	-
Arginine	+
Cystein desulfurase/H_2S production	-
Oxidase	+
Catalase	+
Utilization of	
D-fructose	+
Galactose	+
Mannose	+
Maltose	+
Sucrose	+
Citrate	+

Table 2: Antibiotic sensitivity fo the isolate *Psuedomonas aureginosa* ZSL-2

Antibiotics	Results
Ampicillin	–
Streptomycine	–
Erythromycin	–
Methycillin	–
Tetracycline	–
Ciproflaxin	–

Table 3: Ability of the isolate *Psuedomonas aureginosa* ZSL-2 to hydrolyze a variety of algal and other plant polysaccharides

Polysaccharides	Hydrolysis
Agar	+
Carrageenan	+
Alginate	-
Cellulose	-
Starch	-
CMC	-
Chitin	-
Xylan	-

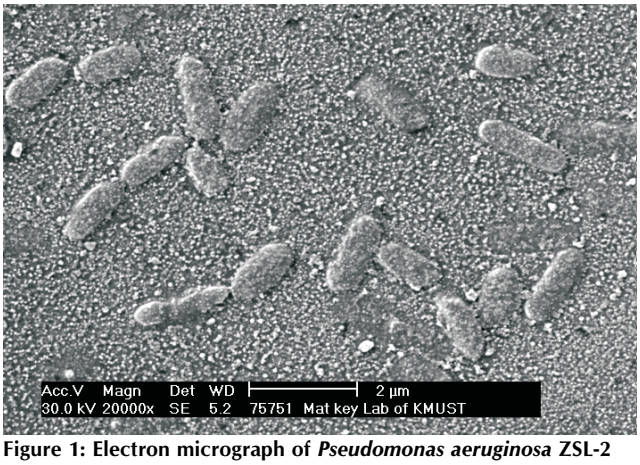

Figure 1: Electron micrograph of *Pseudomonas aeruginosa* ZSL-2

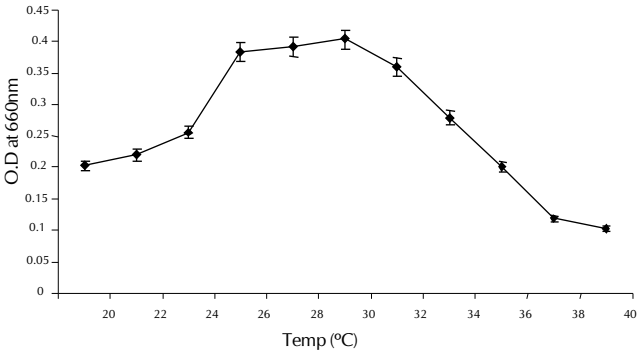

Figure 2: Effect of temperature on growth of *Pseudomonas aeruginosa* ZSL-2

incubation (Fig. 2). Growth was observed in broad range of pH 5 to 11, showed good growth at pH range 7 to 10; however the maximal growth occurred at pH 8. No growth was observed above pH 11 or below pH 5 (Fig. 3). These results indicate that *Pseudomonas aeruginosa* ZSL-2 is alkali-tolerant. The results of growth observed on NaCl concentrations ranging from 0-5%, w/v (Fig. 4) with an optimum around 2%. Increase in

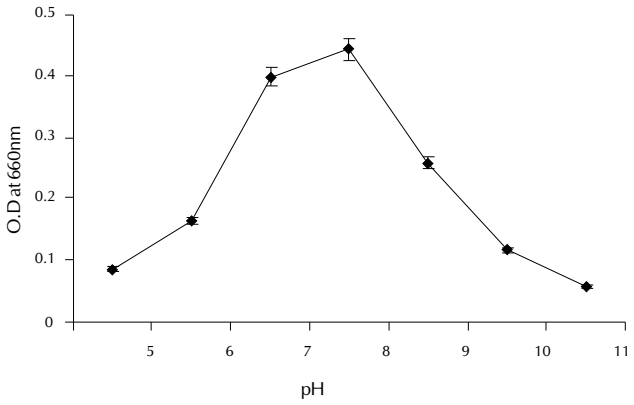

Figure 3: Effect of pH on growth of *Pseudomonas aeruginosa* ZSL-2

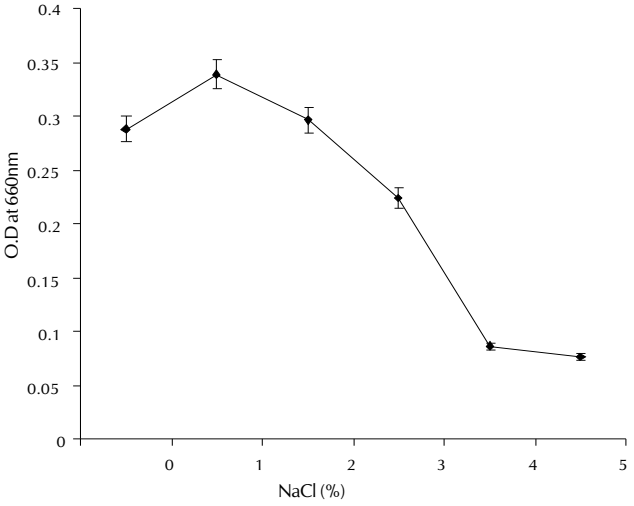

Figure 4: Effect of NaCl concentration on growth of *Pseudomonas aeruginosa* ZSL-2

NaCl concentration beyond 2% decreases the growth rate and very little growth was observed at 5% NaCl concentration revealed that the bacterium grew slightly better when the growth medium supplemented 2% NaCl. It is commonly assumed that marine bacteria, since they live in the sea, are salt tolerant organisms.

When grown on other polysaccharides as a sole source of carbon and energy, the bacterium did not utilized any other polysaccharides except agar and carrageenan as sole source of carbon and energy (Table 3). The *Pseudomonas aeruginosa* ZSL-2 produced extracellular agarase (7.285 U/mL) and carrageenases (6.954 U/mL) when grown on MMS medium supplemented with agar at 30 hrs.

CONCLUSIONS

Newly isolated marine bacterium is identified as *Pseudomonas aeruginosa* ZSL-2 is found to utilize both agar as well as carrageenan and capable of producing extracellular agarase and carrageenases. The *Pseudomonas aeruginosa* ZSL-2 has high industrial potentials for production of agarase and carrageenases.

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