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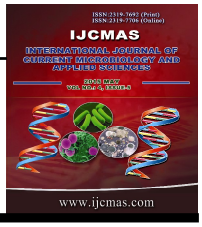

Original Research Article

Screening of Bioemulsifier and Biosurfactant Producing *Streptomyces* from Different Soil Samples and Testing its Heavy metal Resistance Activity

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K e y w o r d s

Surface active compounds, Bioemulsifier, Biosurfactant, Heavy metal Resistance, Streptomyces sp., Actinobacteria

A B S T R A C T

Out of eight strains of actinobacteria isolated from the diesel, petrol and kerosene contaminated soil and from the garden soil, five showed good emulsification activity and produced biosurfactant. These strains were designated as DKMRD1, DKMRD2, DKMRD3, DKMRD4 and DKMRD5. Haemolytic activity, lipolytic assay, drop collapse test, oil displacement method and emulsification activity were employed for screening. All the strains showed positive result for these tests. The strains were also screened for heavy metal resistance activity by using agar diffusion method and tube method. All the strains were resistant to copper sulphate, mercuric chloride, manganese chloride and magnesium sulphate was ranged from 10mM, 50mM, 100mM, 500mM, 1000mM, 5M and 10M. The strains were confirmed as *Streptomyces* sp. by various phenotypic characteristics and biochemical tests. These strains of *Streptomyces* sp. can be improved with genetic engineering approach and can be produced in large scale for the bioremediation of hydrocarbons in heavy metal contaminated soil.

Introduction

Surface active compounds produced by microorganisms are of two main types, those that reduce surface tension at the air water interface (Biosurfactant) and those that reduce the interfacial tension between two immiscible liquids or at the solid liquid interface (Bioemulsifier). Biosurfactants vary in their molecular size and chemical properties (Fiechter, 1992). It occurs as natural chemical entities such as glycolipid, phospholipid and lipopeptide. Effluents from tanneries and industries with heavy metals such as lead, cadmium, copper, zinc, mercury, arsenic and chromium are released in to the ecosystem. Release of these cause foremost serious health hazard to human and animal (Joseph,*et al.*, 2009; Volesky,*et al.*, 1995). An exposure to heavy metals causes cancer, kidney damage and even death. In some cases, exposure to high concentration of heavy metals leads to the development of autoimmunity too (Brooks,*et al.*, 2010). Pollution of soil with heavy metals is an important environmental hazard. Increased level of heavy metals not only decreases the soil microbial activity, Long term exposure

of metals leads to the resistance of organism to metal. Bioremediation techniques have been used in accordance with environmental sustainability concepts. Metals provide distinctive challenge for remediation because they cannot be degraded into innocuous products (Wiatrowski and Barkay, 2005).

Different surface active compounds synthesized by many prokaryotic and eukaryotic microorganisms have been exploited for environmental remediation techniques (Franzetti, *et al*., 2009).Only a limited number of reports published on biosurfactant producing and heavy metal resistance actinobacteria. For the last decade, *Streptomyces* caught a lot of attention for its abundance among soil bacteria and for being biologically active bacteria.

The aim of this study is to isolate and screen the Actinobacteria from diesel, petrol and kerosene contaminated soil and from the garden soil for the production of Biosurfactant, Bioemulsifier and testing its heavy metal resistance activity.

Materials and Methods

Collection of soil samples

Seven different soil samples were collected from different places in Vellore district, Tamilnadu. Soil samples were collected in sterile polythene covers, transported to the laboratory aseptically and refrigerated until further use.

Isolation of Actinomycetes

Soil samples were serially diluted (upto 10^{-7}) and 0.1ml from 10^{-3}, 10^{-4} and 10^{-5} dilutions were spreaded on starch casein agar (SCA) using L-rod. Nystatin and actidione (5mg $100mL^{-1}$) were added to reduce the fungal contamination. The plates were incubated at $28^{o}C$ for 7-14 days.

After incubation the colonies which showed morphological differences were selected, eight pure strains of actinomycetes have been isolated by streak plate method and maintained on SCA slant for further studies.

Screening of bioemulsifier and biosurfactant production

Haemolyticactivity

Haemolysis test was carried out using blood agar plates. The purified cultures were inoculated and the blood agar plates were incubated at $28^{o}C$ for 7 days. The plates were then examined for the zone of clearance around the colonies (Volesky and Holan, 1995).

Lipolytic assay

Luria Bertani (LB) agar supplemented with an olive oil emulsion (1%) plates were made and spot inoculated with fresh culture of actinobacteria and incubated at $30^{o}C$ for 7 days. After incubation the plates were observed for the zone of hydrolysis around the colony (Cappuccino and Sherman, 1992).

Drop collapse test

Biosurfactant production was screened using qualitative drop collapse test (Youssef, *et al.*, 2004). Mineral oil (2µl) was added to 96 well microtitre plates.

The plate was equilibrated for 1 hour at $37^{o}C$ and 5µl of culture supernatant was added to the surface of the oil in the well. The shape of drop on the oil surface was observed after one minute. Distilled water was used as negative control.

Oil displacement method

Oil displacement method was used to screen the activity of biosurfactant. The broth cultures were centrifuged at 6000 rpm for 20 minutes. 40ml of distilled water was added to the petridish followed by the addition of 100µlof diesel oil to the surface of the water. 10 µl of cell free culture broth was added onto the center of the oil film. The diameter of the clear zone on the oil surface was measured and compared with 10µl of distilled water as negative control (Youssef, *et al*., 2004).

Emulsification index

The emulsification capacity was determined by adding 2ml of kerosene to the same amount of cell free culture broth, mixed for 2 minutes on vortex mixer and allowed to stand for 24 hour. E24 index is defined as the percentage of height of emulsified layer divided by the total height of liquid column (Cooper and Goldenberg, 1987).

Emulsification activity

The isolates positive for lipase and haemolysis were grown for 7 days in maltose yeast extract broth (MYE) (Kokare, *et al.,* 2007). Microbial cells were separated by centrifugation at 8000rpm for 15 minutes at 30^{o}C. Cell free supernatant (3 ml) was vortexed vigorously with 0.5ml test oil/hydrocarbon for 2 minutes and incubated at 30^{o}C for one hour for phase separation. Aqueous phase was removed carefully and its absorbance recorded at 400 nm. The blank was prepared similarly by replacing the cell free supernatant by sterile medium. An absorbance of 0.010 units at 400nm multiplied by dilution factor, if any, was considered as one unit of emulsification activity per ml (EU/ml) (Patil and Chopade, *et al.,* 2001).

Screening of heavy metal resistance

Preparation of heavy metals

Screening of heavy metal resistance was carried out using heavy metal salt solutions (Copper sulphate, Mercuric chloride, Manganese chloride and Magnesium sulphate). The concentration of heavy metal salt solutions was ranged from 10mM, 50mM, 100mM, 500mM, 1000mM, 5M and 10M. The salt solutions were prepared with phosphate buffer saline, PBS (pH 6.8). The standard and salt solutions were sterilized separately for 15 minutes at 110^{o}C.

Agar diffusion method

Lawn culture of isolates, grown for 7days in ISP-1 (International Streptomyces Project) broth was prepared. Using sterile well borer, wells were made on the surface of SCA seeded with the *Streptomyces* isolates. To each well 100µl of the standard metal salt solutions were added and incubated at 28^{o}C for 7 days. The area of inhibition (mm) was measured as the distance from the edge of growing colonies to the edge of the well (Hassen, *et al.,* 1998).

Tube method

Tryptone yeast extract broth (ISP-1) was prepared and dispensed in test tubes and sterilized for 15 minutes at 121^{o}C. To each of the labeled tubes, 100µl of the appropriate metal standard solutions and 100µl of culture was added and incubated at 28^{o}C for 7 days (Konopka and Zakharova, 1999).

The tubes were qualitatively measured for turbidity. The tubes with growth were considered as resistant and without growth as sensitive to the particular metal salt solutions.

Identification of isolates

Phenotypic and Biochemical characteristics

Phenotypic characterizations of the selected strains were done based on cell morphology, growth on microbiological media, aerial spore mass color and soluble pigment production. The potential isolates were inoculated on Oat meal agar (ISP-3) for the study of aerial spore mass color. Melanin pigment production was studied on the Peptone yeast extract iron agar (ISP-6).Hydrolysis of starch, casein, tyrosine, esculin, gelatin and urea were tested.

Results and Discussion

Eight strains of *Streptomyces* spp. were isolated from a total of seven soil samples. Out of eight, five showed best results were selected for further study. These strains were designated as DKMRD1, DKMRD2, DKMRD3, DKMRD4 and DKMRD5 respectively. The selected strains were screened for the production of bioemulsifier and biosurfactant. Inoculation of these isolates on blood agar produced clear zone around the colonies indicates that the strains were able to produce surface active compounds. Inoculation on LB agar form clear zone around the colony indicates lipase is the enzyme produced by the *Streptomyces* spp. that acts at oil-water interface and is linked with emulsifier and surfactant production. In the drop collapse test, the drops collapsed because interfacial tension between the liquid and the drop and the hydrophobic surface was reduced. In the oil displacement method the strains displace the oil showing a zone of displacement. The organisms which produce biosurfactant can only displace the oil. In the emulsification index test all five strains emulsify kerosene. In the emulsification activity, all showed good emulsification activity with sunflower oil from 107 EU/ml to 300 EU/ml. All these results were shown in Table 1.

The isolates were also tested for its heavy metal resistance activity. In the agar diffusion method, the plates showed growth around the wells indicates resistance of organisms to that heavy metal at desired concentration. The strains DKMRD1, DKMRD2, DKMRD3, DKMRD4 and DKMRD5 produced no zone for copper sulphate, mercuric chloride, manganese chloride and magnesium sulphate. Also in the tube method the strains produced visible turbidity for these heavy metals. Hence the isolates were found to be resistance to copper sulphate, mercuric chloride, manganese chloride and magnesium sulphate.

The strains can be identified as *Streptomyces* spp. under Actinobacteria by phenotypic and biochemical characteristics were tabulated in Table 2.

During the last few decades Actinobacteria have been extremely fascinating organism with unexhausted reserve of bioactive compounds. Surface active compounds play an important role in emulsifying hydrocarbon. Bioemulsifiers and biosurfactants are very suitable alternatives to chemical surfactants due to their properties (Shete *et al.,* 2006).

In this study six different screening methods were assessed for selecting BE/BS producing actinobacteria. Haemolytic activity is a commonly preferred method to screen surface active compounds. Lipase is an enzyme that acts at oil-water interface and is linked with bioemulsifier and surfactant production (Anu Appaiah and Karanth, 1995). Inoculation on Tributyrin agar plate showed clear zone which

indicates the production of enzyme lipase by *Streptomyces* spp. VITDDK3 (Deepika Lakshmipathy *et al.,* 2010).

In drop collapse test a flat drop was observed around the colonies, which indicates a biosurfactant activity. Bodour *et al* reported that the drop collapse test used to detect biosurfactant producing microorganism in natural environment. Oil displacement method was good to quantify the biosurfactant.

Biosurfactant production may also be tested by using emulsification index. Surface activity and emulsification activity have direct correlation (Surachi *et al.*, 2007).

Loganathan Karthik reported that the *Streptomyces* strains LK-2 and LK-3 showed good emulsification activity. Emulsifying activities determine productivity of bioemulsifier (Bonnila *et al.*, 2005). Javed P Maniyar reported that *Streptomyces* sp. S22 showing highest emulsification activity of 320 EUml. Bioemulsifier production from *Streptomyces* sp.S22 was induced by sunflower oil and their bioemulsifier activity increased from 145 EU/ml to 320 EU/ml (Javed Maniyar *et al.*, 2011). Ellaiah *et al* screened 68 bacterial isolates from soil and found only 6% of isolates with good emulsification activity of up to 61%. This observation is important to suggest that potent biosurfactant producing cultures can be detected through such assays (Ellaiah *et al.*, 2002).

Bioemulsifier and biosurfactant producing strains were also tested for its ability to resist heavy metals. *Streptomyces* VITDDK3 was more resistant to sodium arsenate and least resistant to potassium dichromate (Deepika Lakshmipathy *et al.*, 2010). Abbas and Edward reported that *Streptomyces coelicolor* tolerated copper of up to 0.047mM (Abbas and Edward, 1989). Several bacteria associated with a marine sponge *Fasciospongia cavernosa*, *Streptomyces* sp. (MS101), *Salinobacter* sp. (MS106), *Roseobacter* sp. (MS109), *Pseudomonas* sp. (MS1016), *Vibrio* sp. (MS123), *Micromonaspora* sp. (MS128), *Saccharomonaspora* sp. (MS136) and *Alteromonas* sp. (MS142) showed resistance to heavy metals such as copper, lead, cadmium, cobalt, mercury and nickel (Joseph *et al.*, 2009).All the five strains were resistant to copper sulphate, mercuric chloride, manganese chloride and magnesium sulphate among the metal salt solution used in this study up to 10M. All these results have shown same pattern of results obtained by previous research done many workers.

Table.1 Screening methods for biosurfactant and bioemulsifier production

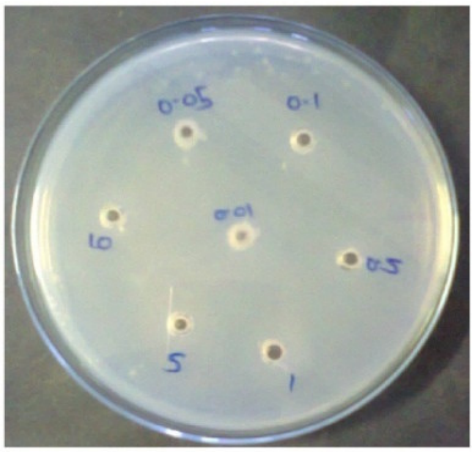
S. No	Name of the Strain	Oil Displacement Method (Diameter in mm)	Emulsification Index (%)	Emulsification Activity (EU/ml)
1	DKMRD1	10	63.63	229
2	DKMRD2	11	44.44	107
3	DKMRD3	12	55.55	300
4	DKMRD4	5	35.48	199
5	DKMRD5	15	31.25	182

Table.2 Phenotypic and biochemical characteristics of bioemulsifier and biosurfactant producing heavy metal resistant actinobacteria (DKMRD1, DKMRD2, DKMRD3, DKMRD4 and DKMRD5)

S.No	Phenotypic and biochemical characteristics	Actinobacteria				
		DKMRD1	**DKMRD2**	**DKMRD3**	**DKMRD4**	**DKMRD5**
1.	Gram's staining	+	+	+	+	+
2.	Colony morphology on SCA	Medium size, irregular, rough and white colored	Small, round, rough and white colored	Small, round, rough and orange colored	Small, round, rough and pink colored	Small, round, powdery and cream colored
3.	Aerial spore mass color	White	White	White	White	Yellow
4.	Melanin pigmentation	Dark brown pigment	No pigment	Dark brown pigment	Dark brown pigment	No pigment
5.	Starch hydrolysis	+	+	+	+	+
6.	Tyrosine degradation	+	-	+	+	-
7.	Esculin hydrolysis	-	+	+	-	+
8.	Gelatin liquefaction	+	+	+	+	+
9.	Urea hydrolysis	+	-	+	+	+

+: positive, -: negative

Figure.1 Heavy metal resistance activity
Growth of organisms around the wells



Hence the isolates DKMRD1-6 were identified as potential strains and was identified as *Streptomyces* spp. based on their phenotypic and biochemical characterization when compared with Bergy's manual of determinative bacteriology.

Day by day the demand for hydrocarbon is increasing in different fields such as industries automobiles, households etc. It is also one of the major environment pollutants. Contamination of soil environment with heavy metals is very hazardous for human and other organisms in the ecosystem. Ecofriendly technologies must be used to clean environment such as degradation by microorganisms. Bioremediation has been accepted method for the treatment of hydrocarbon pollution. The bioemulsifier and biosurfactant produced by heavy metal resistance actinomycetes culture can be used for the bioremediation of hydrocarbon pollutants. Heavy metal resistant actinomycetes can be improved with genetic engineering approach and produced in large scale for the bioremediation of oil and heavy metal contaminated soil.

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