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DETERMINATION OF STRESS RESISTANCE IN BIOSURFACTANT-PRODUCING BACTERIAL ISOLATES

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ABSTRACT

Most of the commercially available surfactants are produced from petroleum but concern about the environment has stimulated the search for microbially produced surfactants for the industrial utilization and bioremediation of hydrocarbons. Biosurfactants are more effective, environmentally friendly, and stable than many synthetic surfactants. In previous work, we determined that biosurfactant-producing microorganisms were naturally present at contaminated soil of automobile workshops. In this study, we examine the potential of biosurfactant producers to tolerate other contaminants including heavy metals and antimicrobial compounds for the effective utilization of such microorganisms for the bioremediation of hydrocarbons at heavy metal and antimicrobial contaminated regions. For the present study seven heavy metals ($NiCl_2$, $CuSO_4$, $CdCl_2$, $CoCl_2$, $CrSO_4$, $PbSO_4$, $ZnSO_4$) and four different antimicrobial compounds (Ampicillin, Streptomycin, Erythromycin, Chloremphenicol) were tested. Biosurfactant-producing bacteria were found to constitute a significant proportion (up to 35%) of heavy metal tolerant and up to 25% were found to resist antimicrobial compounds. Isolates were further characterized for colonial and cellular morphologies and analyzed through different screening methods such as oil spreading method, drop collapse method, CTAB agar plate method, BATH assay and emulsification capacity methods. Resistance against heavy metals and antibiotics has made these isolates ideal for bioremediation under stressful environmental conditions.

Keyword: Screening methods, biosurfactant- producing strain, maximum tolerable concentration

INTRODUCTION

Bacteria produce a wide range of extracellular products with many properties and applications among which biosurfactants are unique class of surface active amphiphilic compounds. These compounds reduce surface and interfacial tension by accumulating at the interface of immiscible fluids and thus increase the solubility, bioavailability and subsequent biodegradation of the hydrophobic or insoluble organic compounds (Tuleva *et al*., 2002). There are many types of biosurfactants based on their chemical nature such as glycolipids, lipopolysaccharides, oligosaccharides and lipopeptides that have been reported to be produced by diverse bacterial genera (Banat, 1993; Banat *et al*., 2010). Biosurfactants have received considerable attention in the field of environmental remediation processes because of their efficacy as dispersion and remediation agents and their environmental friendly characteristics such as low toxicity and high biodegradability (Das *et al*., 2009; Kiran *et al*., 2010). There are many reports describing the effects of exogenously added microbial biosurfactants in enhancing the bioremediation of crude oil-polluted soils by indigenous microbes (Abalos *et al*., 2004; Owsianiak *et al*., 2009).

Due to their unique properties and vast array of applications, identification of new biosurfactant producing microbes is in great demand. There are nine different screening methods that have been reported as criteria to screen biosurfactant producing microbes such as hemolytic assay (Volchenko *et al*., 2007), bacterial adhesion to hydrocarbons (BATH) assay (Smyth *et al*., 2010), drop collapse assay (Bodour and Miller, 1998), oil spreading assay (Yonebayashi *et al*., 2000), emulsification assay (Afshar *et al*., 2008), surface tension measurement, titled glass slide test, blue agar plate and hydrocarbon overlay agar assay (Satpute *et al*., 2008). All these tests have variable sensitivity and specificity. Methods like hemolytic assays are not reliable and sensitive, because this method will categorize microbes in two groups as hemolytic and nonhemolytic. Strains that are hemolytic are believed to be biosurfactant producers, but there are other products such as virulence factors that can lyse the blood cells and also biosurfactants with poor diffusion in agar may not be able to lyse the blood cells. Thus, the results from hemolytic assay on blood agar plate are not so sensitive (Youssef *et al*., 2004).

This study was conducted to find heavy metal and antimicrobial resistant strains with positive biosurfactant-producing capability in order to utilize such isolates for future bioremediation activities knowing that most of the hydrocarbon contaminated regions are also contaminated with heavy metals and antimicrobial compounds (Shoeb *et al*., 2012a). Screening criteria used in this study include: BATH assay, drop collapse assay, oil spreading assay and emulsification assay. Eight different bacterial strains were screened for biosurfactant production, characterized for cellular and colonial morphologies and analyzed for the resistance against heavy metals and antimicrobial compounds.

MATERIAL AND METHODS:

Bacterial Strains

A total of eight bacterial strains previously isolated by Shoeb *et al*., (2012b) from the soil of automobile workshop were used for further characterization and screening of biosurfactants.

Media and Chemicals

The selected bacterial strains were subculture and maintained in Luria burtani (LB) broth and agar (OXOID). The antibiotic resistance test was performed in LB agar whereas metal tolerance in tris minimal media because it has low ability of metal binding (Mergeay *et al*., 1985). All the antibiotics used in this study were from Sigma, USA and metal salts from BDH, Germany.

Cellular Morphology

The colonies of all eight strains were gram stained (Duguid, 1989), their shape arrangement and gram reaction was observed under 100 X oil immersion compound microscope.

Colonial Morphlogy

The selected bacterial strains were streaked on solidified LB agar plates and incubated at 37°C for 24-48 hours. The colonies were observed and characterized for form, elevation, margin and opacity.

Antimicrobial Tolerance

In order to determine the maximum tolerable concentration (MTC) against different antimicrobial agents such as Ampicillin, Streptomycin, Erythromycon, and Chloremphenicol. Stock solution of each compound was prepared as described in Sambrook *et al*., (1989). Selective LB agar plates of variable concentrations (25-250 µg/ml) with the difference of 25µg/ml were prepared. The bacterial strains were streaked and incubated at 37°C for 24hrs.

Heavy Metal Tolerence

All the strains were assessed for MTC of heavy metals salts such as $NiCl_2$, $CuSO_4$, $CdCl_2$, $CoCl_2$, $CrSO_4$, $PbSO_4$, $ZnSO_4$. 1M stock solution of each of the metal salt was prepared and selective plates were prepared by adding appropriate dilutions (0.5mM -2.0 mM) with the difference of 0.25 mM. Bacterial strains were streaked from pre-culture on metal supplemented tris minimal media agar plates. The plates were incubated at 37°C and growth was observed after 24-48 hours.

Detection of Biosurfactant Activity

All the strains were screened for their biosurfactant activity; the activity was tested by following methods:

Drop Collapse Method

A modified drop-collapse was performed in 96-well microplate. Firstly plate was rinsed by hot water and ethanol 3 times then each well was thinly coated with crude oil. A 5µl of each sample droplet was added to the centre of a well and observed after 1 min. the droplet was collapsed, depending upon the amount of surfactant in the sample. It was compared it with distilled water as negative control.

Oil Spread Assay

The oil spread assay was adapted from the method described by Morikawa *et al*., (2000). 20ml of distilled water followed by the addition of 10µl of crude oil to the surface of water in a Petri plate and 10 µl of supernatant (from culture broth) was added to the oil surface. The diameter of clear zone formed on the oil surface was measured and compared to 10µl of distilled water as negative control.

Bath Assay

Cell hydrophobicity was measured by Bacterial Adherence to Hydrocarbons (BATH) assay according to a method described by Rosenberg *et al*. (1980). The cell pellets collected in the LB broth were washed twice and suspended in a buffer salt solution (g l-1, 16.9 K2HPO4 and 7.3 KH2PO4) and diluted using the same buffer solution to an optical density (OD) of ~ 0.5 at 610 nm. To the cell suspension (2 ml) in test tubes 100 µl of crude oil was added and vortex-shaken for 3 min. Crude oil and aqueous phases were allowed to separate for 1 h. OD of the aqueous phase was then measured at 610 nm in a spectrophotometer (DU 730 spectrophotometer of Beckman Coulter). OD values indicated percentage of cells attached to crude oil through the formula: $H= A_0\text{-}A/A_0 \times 100$ (A_0 is O.D before adding hydrocarbon whereas A is the O.D after adding hydrocarbon)

Blue Agar Plate Method

Pure bacterial cultures were cultivated on light blue mineral salts agar plate containing the cationic surfactant cetyltrimethylammonium bromide (CTAB) and the basic dye methylene blue as described by Siegmund and Wagner (1991). Anionic biosurfactant-producing colonies on blue agar plate are identified following the formation of dark blue halos around the colonies. To strengthen the visual effect of this method, small wells were made and the cultures were incubated in the wells.

Emulsification Activity

Emulsicfication activity was measured through calculated emulsification index (E24) (Cooper and Goldenberg, 1987). 2 ml samples of cell free supernatant and 2 ml of m-xylene were added to a screw cap tubes and vortex at high speed for 2 min. The mixtures were incubated at room temperature for 24 hours. The emulsification index (E24) was calculated by formula.

$E_{24} = h_{emulsion} / h_{total} \times 100$

RESULTS

Revival of Isolates

All the eight pre-isolated cultures were successfully revived from the slants of LB agar and were characterized and analyzed for biosurfactant producing capability though number of screening tests.

Cellular Morphology

Cellular morphology was observed during Gram staining of each strain. Details for cellular morphology and Gram reaction are given in Table 1.

Colonial Morphology

The colonial morphology of most of the strains was pin-pointed and circular, color was pale yellow, elevation was convex, all colonial margins were entire type; opacity was usually translucent. A detailed result for colonial morphology has been given in Table 2.

Table 1. cellular morphology.

Isolates	shape	Arrangement	Gram reaction
DGEF01	Round	Scattered	Gram negative
DGEF02	Rod	Scattered	Gram negative
DGEF03	Round	Scattered	Gram negative
DGEF04	Rod	Scattered	Gram negative
DGEF05	Rod	Scattered	Gram negative
DGEF06	Round	Chain	Gram negative
DGEF07	Rod	scattered	Gram negative
DGEF08	Round	cluster	Gram negative

Table 2. colonial morphology.

Isolates	Colour	Size	Shapes	Margin	Elevation	Opacity	Surface
DGEF01	Yellow	Pinpoint	Round	Wavy	Convex	Translucent	Shiny & smooth
DGEF02	Yellow	Pinpoint	Round	Entire	Convex	Translucent	Shiny & smooth
DGEF03	Offwhite	Small	Round	Entire	Convex	Opaque	Shiny & smooth
DGEF04	Yellow	Pinpoint	Round	Entire	Convex	Translucent	Shiny & smooth
DGEF05	Yellow	Pinpoint	Round	Entire	Convex	Translucent	Shiny & smooth
DGEF06	Offwhite	Small	Round	Entire	Convex	Opaque	Shiny & smooth
DGEF07	Yellow	Pinpoint	Round	Entire	Convex	Translucent	Shiny & smooth
DGEF08	Yellow	Pinpoint	Round	Entire	Convex	Translucent	Shiny & smooth

Table 3. Results of MTCs for Antimicrobial Compounds.

	Amp(µg/ml)	Sm(µg/ml)	Ery(µg/ml)	Cm(µg/ml)
DGEF01	1000	>2000	375	400
DGEF02	600	>2000	375	400
DGEF03	900	>2000	375	400
DGEF04	900	>2000	375	400
DGEF05	600	>2000	375	400
DGEF06	900	>2000	375	150
DGEF07	600	>2000	250	400
DGEF08	600	>2000	375	400

Isolates which showed MTC of 2000 µg/ml were not checked further for higher concentrations. AMP=Ampicillin; Sm=Streptomycin; Ery=Erythromycin; Cm=Chloremphenicol.

Table 4. Results of MTCs for Heavy Metals.

	Ni (mM)	Pb (mM)	Cr (mM)	Co (mM)	Cd (mM)	Cu (mM)	Zn (mM)
DGEF01	0.5	1.0	-	2.0	3.25	1.5	3.0
DGEF02	-	2.0	-	2.0	3.25	1.75	3.0
DGEF03	0.75	2.0	-	2.0	3.25	1.25	3.0
DGEF04	0.5	1.5	-	2.0	3.25	1.75	2.75
DGEF05	0.75	1.5	-	2.0	4.0	1.75	2.75
DGEF06	0.5	1.5	-	2.0	4.0	2.0	3.0
DGEF07	-	1.5	-	2.0	4.0	1.75	2.75
DGEF08	0.75	1.5	-	2.0	3.25	1.75	2.75

Isolates which could not grow on 0.5 mM concentration of any metal were considered sensitive. Isolates which showed MTC of 4.0 mM were not checked further for higher concentrations. Heavy Metals were Ni=Nickel; Pb=Lead; Cr=Chromium; Co=Cobalt; Cd=Cadmium; Cu=Copper; Zn=Zinc

Maximum Tolerable Concentration (MTC) For Antimicrobial Agents

Isolates showed high MTCs against different antimicrobial agents. Results are given in Table 3.

Maximum Tolerable Concentration (MTC) For Heavy Metals

Most of the isolates were capable of growing at high concentration of different heavy metals except chromium against which all the isolates were sensitive. Detailed results have been given in Table 4.

Screening Methods

Drop Collapse Method

All the isolates showed positive result for drop collapse method. Detailed result is shown in Table 5.

Oil-Spread Assay

All isolates showed positive and rapid result when a drop of supernatant of culture was placed on oil (Fig. 1). Detailed results are given in Table 5.

Bath Assay

BATH assay revealed that all bacterial strains were positive for the BATH assay, which indicated the affinity of the bacterial cells towards hydrophobic substrate. Detailed results of BATH assay are given in Table 5.

Blue Agar Plate Method

All eight isolates showed halo formation through CTAB agar assay (Fig. 2). Results are given in Table 5.

Emulsification Index

In Emulsification assay it was assumed that if the cell free culture broth used in this assay contains biosurfactant then it will emulsify the hydrocarbons present in the test solution (Fig. 3). In this study, xylene was used as the hydrophobic substrate. Detailed results are given in Table 5.

Fig. 1. Oil-spread method.

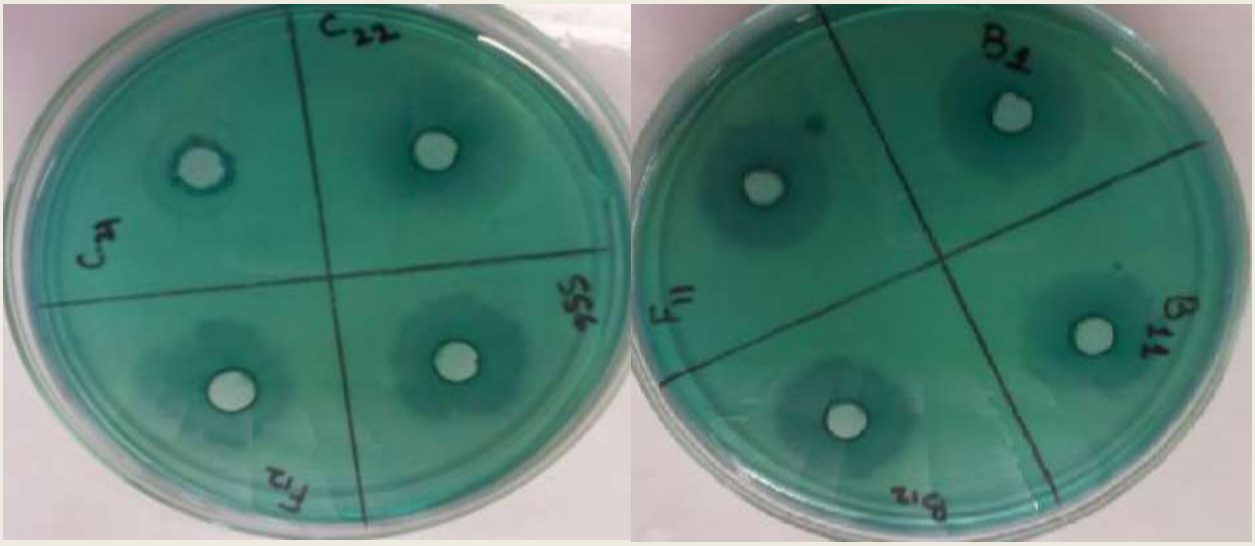

Fig. 2. CTAB Agar Plate Method.

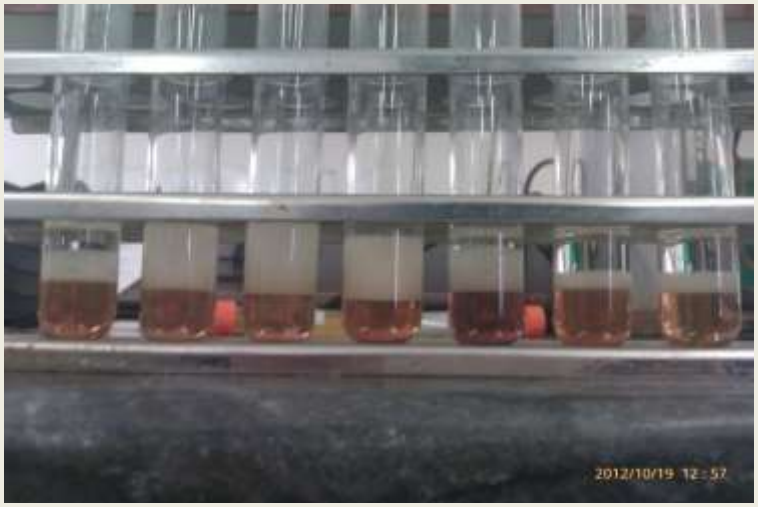

Fig. 3. Emulsification layer.

DISCUSSION

Continual increase in the use of biosurfactants for the environmental and industrial purposes has made these surface-active compounds crucial for investigation (Qiao and Shao, 2010). More and more are being used in the fields of agriculture, cosmetics, pharmaceutics and enhanced oil recovery-EOR (Arutchelvi *et al*., 2008; Franzetti *et al*., 2008). Biosurfactants have versatile properties and pronounced ability to reduce the surface or interface tension of the solvent, and are therefore widely utilized in environmental remediation (Develter and Lauryssen, 2010).

Table 5 Biosurfactant production by bacterial isolates.

Isolates	Drop Collapse	Oil Spread	Bath Assay $\frac{AB-A}{AB} \times 100$	Blue Agar Plate (Size of the Halos in mm.)	Emulsification Index (EI) $E_{24}=H_E/H_T$ X 100
DGEF01	Collapse	Positive	22.95	50	10.7%
DGEF02	Collapse	Positive	13.41	50	**50.0%**
DGEF03	Collapse	Positive	10.8	50	28.6%
DGEF04	Collapse	Positive	47.10	50	14.3%
DGEF05	Collapse	Positive	64.86	50	**46.4%**
DGEF06	Collapse	Positive	59.83	5	**57.1%**
DGEF07	Collapse	Positive	60.61	60	**60.7%**
DGEF08	Collapse	Positive	47.99	40	**42.9%**

Emulsification >30% is indicated in bold to denote high emulsification activity.

In situ bioremediation strategies may involve the stimulation of native bacterial strains or the introduction of nonnative microorganisms (Plante *et al*., 2008). These microbes then transform contaminants into non- or less-hazardous chemicals. Considering such applications we have analyzed number of potent biosurfactant-producing bacterial strain isolated from the soil of automobile workshop earlier (Shoeb etal., 2012b) for heavy metal and antimicrobial resistance considering significance of stress tolerance for the direct application of such isolates in the contaminated environment.

All the tested isolates gave positive results for the screening tests indicated that the isolates are biosurfactant producers. The cause of getting all the strains positive for biosurfactant production is theinitial use of R2A medium for the isolation of these cultures after sampling (Shoeb *et al*., 2012b). R2A medium is a culture medium which tends to promote slow growing bacteria and inhibit faster growing bacteria hence commonly used as a selective medium for the screening of biosurfactant-producing bacteria (Bodour *et al*., 2003; Anandaraj and Thivakaran, 2010).

All the isolates showed positive biosurfactant producing activity through screening tests. Drop collapse is one of the rapid screening tests of biosurfactant-producing bacteria and all the isolates were able to collapse the crude oil layer. Results were compared with negative control which was autoclaved distilled water.

Oil spread method is an authentic method used to detect the biosurfactant activity. All isolates showed positive and rapid results. Test was performed in several replications to confirm the results. BATH assay results revealed that DGEF05 showed highest adherence (64.86%) with crude oil followed by that DGEF07 (60.61%) and that DGEF06 (59.83%). All the isolates showed a positive affinity and drop collapse activity with crude oil. Both the drop collapse and cells adhered to crude oil have several advantages for the future application of these isolates.

The CTAB agar plate method is a semi- quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. It was developed by Siegmund and Wagner (1991). Anionic surfactant secreting isolates when cultivated on the cationic surfactant CTAB blue agar plate, form dark blue halos. All the isolates of our study showed dark blue halo showing production of rhamnolipids, of which DGEF07 showed maximum production while DGEF05 showed minimum.

Emulsification activity is one of the criteria to support the selection of potential biosurfactant producers and it is assumed that if the cell free culture broth used in this assay contains biosurfactant then it will emulsify the hydrocarbons present in the test solution. In this study, m-xylene was used as the hydrophobic substrate. The emulsion was stable for ~72 h at room temperature without any significant change in the emulsification. All the isolates have showed emulsification activity but approximately 62% of isolates with good emulsification activity of up to 60%. During this study, emulsification of the cultures showing >30% emulsification activity were also positive for biosurfactant production in two or three other methods can be selected showing that these isolates have the potential to be used as a source for biosurfactant/bioemulsifier production and can play a key role in emulsifying

hydrocarbons (Satpute *et al*., 2008).In the end it might be concluded that we have successfully screened biosurfactant producing bacteria and anticipated that in future these isolates can be utilized for their biosurfactant production and bioemulsifier activity. Our results indicate that isolates with higher tolerance against toxic contaminants are more suitable for wide-scale implementation of these isolates for the bioremediation of environment.

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