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# Biosurfactant production trait of *Bacillus cereus* isolated from Mercury contaminated soil

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**ABSTRACT**

*Release of heavy metals into the environment could be hazardous to the biota in soil. Microorganisms prevalent in heavy metal contaminated soil could be useful in decontaminating the soil. Biosurfactants are used in bioremediation of metal – contaminated soil. The application of biosurfactant producing bacteria for bioremediation of metal contaminated soil could be a promising approach .With this view, the present study was designed to tap the biosurfactant production potential of bacteria harbouring the metal contaminated soil. Bacteria were isolated from the mercury contaminated soil and their biosurfactant production potential was evaluated. Isolate No.3, 6 (500 mg/ L) and 8, 11, 17 and 19 (400 mg/ L and 500 mg/ L) were resistant to mercury at higher concentrations. Among the bacteria, Bacillus cereus was identified to produce biosurfactant.*

**Key words:** Mercury, soil, bacteria, biosurfactant

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## INTRODUCTION

Although methods like excavation and land filling are practiced for cleaning up metal contaminated soil, Biosurfactant and biosurfactant producing microorganisms could be a sustainable and eco - friendly option. Biosurfactants are amphiphilic, biodegradable, non- hazardous, active at extreme temperature, pH and salinity [1-5]. In heavy metal polluted soils, biosurfactants form complexes with metals at the soil interface, which is followed by desorption of the metal and removal from the soil surface leading to metal ions concentration and their bioavailibilty in the soil solution [6].Heavy metals are not biodegradable; they can only be transferred from one chemical state to another, which changes their mobility and toxicity. Microorganisms can influence metals in several ways. Some forms of metals can also be accumulated by microorganisms by metabolism – dependent (passive) or by intracellular, metabolism – dependent (active) uptake. Microorganisms can influence mobility indirectly by affecting pH or by producing or releasing substances which change mobility of the metals [7, 8]. In this study, we have isolated mercury resistant bacteria from mercury contaminated soil and evaluated their potential to produce biosurfactant.

## MATERIALS AND METHODS

Ten different soil samples of sugarcane field were collected from different localities in BHEL, Kailasapuram, Trichy area. Soil samples at a depth of approximately 20 cm were taken in sterilised polyethylene bags using sterilised spatula and stored at 4 °C until analysis. Mercury content of the soil was determined by AAS (Atomic absorption spectroscopy). Hg content of the soil was 0.1 mg $L^{-1}$ , which was found to be above the permissible limit of

TNPCB (0.01mg $L^{-1}$). 1 g of soil was dissolved in 100 ml of sterile distilled water to make soil suspension. 100 µl of serially diluted ($10^{-6}$ dilution) soil suspension was poured on the nutrient agar plates. After incubation for 24 hours at 37 °C, colonies were formed, which were selected for testing their biosurfactant production potential. The isolates which were able to produce biosurfactant were identified by the methods mentioned in Bergey's manual of systematic bacteriology [9].

**Minimum Inhibitory Concentration (MIC)**

**Preparation of mercury standards:**

Stock solution of different Hg for 1000 mg $L^{-1}$ was prepared by dissolving the calculated amount of required $HgCl_2$ salts in distilled water and the volume was made to 100 ml using standard flask. The stock solution was further made into different concentrations of 100, 200, 300, 400 and 500 mg $L^{-1}$. This was used for Minimum Inhibitory Concentration tests. For the determination of Minimum Inhibitory Concentration, the pure cultures isolated were used for this test. Using sterile swabs cultures were lawn cultured on sterile nutrient agar medium and wells were made using sterile cork borer. 20 µl of Hg solution of different concentrations were pipetted into different wells using micropipette and were incubated at 30 $^{o}$C for 24 hours. After incubation, the plates were observed for zone of clearance [10].

**Antibacterial susceptibility assay:**

Antibacterial susceptibility assay was carried out by Disc diffusion method wherein sterile nutrient agar plates were prepared and spread with test bacterial cultures. Thereafter, the antibiotic discs (Tetracycline, Kanamycin, Streptomycin, Amphicilin and Chloramphenicol) were placed onto the inoculated plates. The plates were then incubated at 37 ºC overnight. The antibacterial activity of each antibiotic was expressed in terms of mean of diameter of Zone of Inhibition (in mm) produced at the end of incubation period.

**Detection of biosurfactant activity of bacteria:**

**Blue agar plate method:**

Methylene blue agar plates containing cetyl trimethylammonium bromide (CTAB) (0.2 mg / ml; Himedia, India) and methylene blue (5 µg / ml) were used to detect extracellular anionic glycolipid production [11]. Biosurfactants were observed by the formation of dark blue halo zone around the colonies.

**Blood agar haemolysis :**

Blood agar haemolysis method is used to screen biosurfactant producing strain. This method is based on the fact that biosufactants are able to haemolyse the red blood cell present in blood [12]. Cultures of selected isolates were spot inoculated on blood agar plates. These plates were incubated for 24 - 72 hours at 37 ºC. After incubation, the plates were observed for zone of haemolysis. This zone of haemolysis indicates production of biosurfactant.

**Drop collapse test:**

Drop collapse test was performed by following the procedure described by Jain *et al.,*

[13] and modified by Bodour and Miller-Maier [14]. 2 µl of crude oil was applied to the well regions delimited on the covers of 96-well micro-plate lids and allowed to equilibrate for 24 hours. Five microliters of the cell free culture broth was transferred to the oil coated regions and the drop size was observed 1 minute later with the aid of a magnifying glass. A result was considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by de-ionised water (negative control).

**Oil spreading assay:**

Oil spreading experiment was performed using the method described by Morikawa *et al*., [15]. 20 ml of distilled water was added to a plastic petri dish followed by addition of 20 µl of crude oil to the surface of the water to form a thin oil layer. 10 µl of cell free culture supernatant was then gently placed on the centre of the oil layer. The presence of biosurfactant would displace the oil and a clear zone would form. The diameter of the clearing zone on the oil surface would be visualised under visible light and measured after 30 seconds, which correlates to the surfactant activity, also known as oil displacement activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed and Triton X-100 was used as the positive control.

**Emulsification index** (EI) (E24):
The emulsification index (E24) was measured using the method described by Cooper and Goldenberg [16]. This method was used to check the stability of the BS (Biosurfactant) extracted. BS activity was measured by adding 6 ml of kerosene to 4 ml of cell-free supernatant. The mixture was vortexed at high speed for 2 minutes. The height of emulsion was measured by taking the layer formed in between aqueous and hydrocarbon layer. Measurement was taken after 24 hours. Emulsions formed by the isolates were compared to those formed by distilled water as control. E 24 was calculated at 25 °C. The emulsification index was determined using the following formula.

Emulsification index (E 24) (%) = (Height of Emulsion layer) ×100/ (Total height)

**Screening of potential bacterial isolates**
The confirmed isolates were inoculated into BH (Bushnell – Hass broth) liquid media and incubated at 25 °C for 7 days. 3 separate BH agar plates were prepared and spread with 100 µl of oil (crude oil), then the prepared wells (8 mm) were loaded with 50 µl of broth culture and plates were incubated at 37 °C for 48 hours. The diameter of zone of clearance around the well was measured [17, 18].

**Secondary screening by gravimetric analysis:**
To obtain more potent strains for crude oil degradation, secondary screening was performed with potential isolates. About 100 ml of Bushnell Hass broth was prepared in 3 different conical flasks and 1g of crude oil was added. Oil degrading isolates were added as an inoculum and the flask was incubated at 30 ºC for 7 days in a rotary shaker at 120 rpm. After incubation, diethyl ether was added to the flask and mixed well. The complete mixed broth was transferred to the separating flask, which was left for 20 minutes for oil and broth separation. The broth was separated in the lowest portion. Diethyl ether was added to remove complete oil from separating flask. Oil along with solvent was collected in a pre - weighed petri plate.

After the complete evaporation of the solvent the plate was weighed. The estimation of residual oil left after degradation was made by the amount of oil in a pre - weighed plate [19]. The percentage of oil degradation was calculated as {1- (Xo-X1)/ Xo}100% (%), where Xo- initial amount of crude oil, X1- amount of crude oil after degradation [ 20, 21].

**Confirmatory method Phenol: sulfuric acid method**
Biosurfactant producing strains selected from above screening methods were inoculated in MSM broth and incubated at 37 ºC on rotary shaker for 4 - 5 days. After incubation, the broth was centrifuged at 10,000 rpm for 15 minutes and supernatant was collected while the pellet was discarded. 1 ml of collected supernatant was mixed with 1ml of 5 % phenol and 5 ml of concentrated $H_2SO_4$ was added in drop wise manner. Presence of biosurfactant in the supernatant was confirmed by change in yellow colour to orange colour [22].

## RESULTS AND DISCUSSION

Out of 20 bacterial isolates from the Hg contaminated soil, 7 bacterial isolates exhibited resistance against Hg (Isolates 3, 6, 8, 11, 17, 19, 20) (table 1). Further, MIC for mercury was registered by bacterial isolate No. 3 and 6 at 500 mg $L^{-1}$ (3 mm, and 2 mm, respectively). Bacterial isolate No. 8 registered MIC for Hg at 400 mg $L^{-1}$ (2 mm) and 500 mg $L^{-1}$ (4 mm). Bacterial isolate No. 11 at 300 mg $L^{-1}$ (2 mm), 400 mg $L^{-1}$ (3 mm) and 500 mg $L^{-1}$ (5 mm).Isolate 17 also registered MIC for Hg at 300 mg $L^{-1}$ (2 mm), 400 mg $L^{-1}$ (3 mm) and 500 mg $L^{-1}$ (6 mm). Similarly, bacterial isolate 19 also registered MIC for Hg at 300 mg $L^{-1}$ (3 mm), 400 mg $L^{-1}$ (5 mm) and 500 mg $L^{-1}$ (7 mm) (table 2, fig 1). Antibiotic sensitivity test exhibited by Hg resistant bacteria is represented in table 3.Bacterial isolate 3, 17 and 19 were resistant to 10 µg of tetracycline. Bacterial isolate No. 3, 8, 11, 17 and 19 were resistant to 10 µg of kanamycin. Bacterial isolate No. 8,11,17 and 19 exhibited resistance to amphicillin (30 µg). Streptomycin (30 µg) resistance was exhibited by bacterial isolate No. 11, 17 and 19. Chloramphenicol (30 µg ) resistant was exhibited by bacterial isolate 17 ,19 and 20.

**Table – 1 Isolation of Mercury resistant bacteria from mercury contaminated soil**

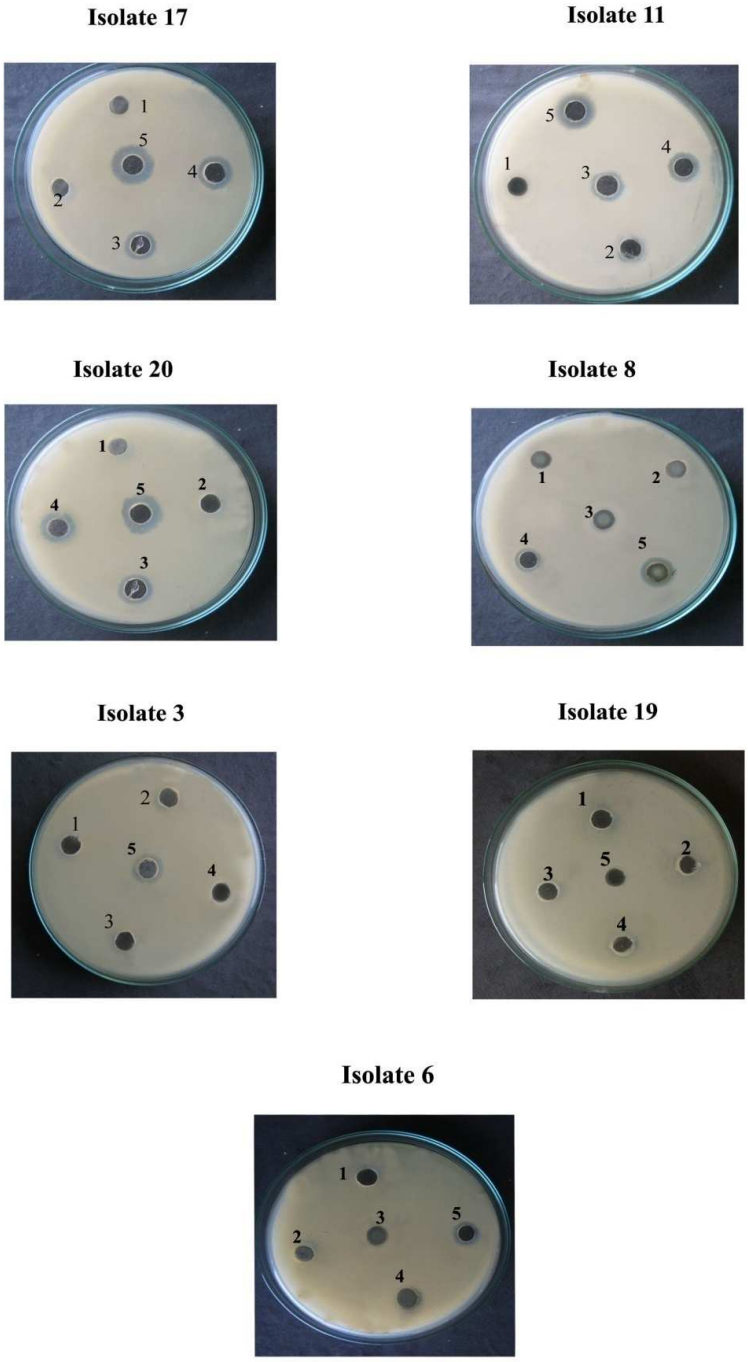
| S. No | Bacterial isolates | Mercury resistant |
|---|---|---|
| 1. | Isolate 1 | - |
| 2. | Isolate 2 | - |
| 3. | Isolate 3 | + |
| 4. | Isolate 4 | - |
| 5. | Isolate 5 | - |
| 6. | Isolate 6 | + |
| 7. | Isolate 7 | - |
| 8. | Isolate 8 | + |
| 9. | Isolate 9 | - |
| 10. | Isolate 10 | - |
| 11. | Isolate 11 | + |
| 12. | Isolate 12 | - |
| 13. | Isolate 13 | - |
| 14. | Isolate 14 | - |
| 15. | Isolate 15 | - |
| 16. | Isolate 16 | - |
| 17. | Isolate 17 | + |
| 18. | Isolate 18 | - |
| 19. | Isolate 19 | + |
| 20. | Isolate 20 | + |

*+ resistant to mercury , – not resistant to mercury*

**Table 2 Minimum inhibitory Concentration of mercury against soil bacteria**

| Bacterial isolates | Concentration of mercury | | | | |
|---|---|---|---|---|---|
| | 100 mg/L | 200 mg/L | 300 mg/L | 400 mg/L | 500 mg/L |
| Isolate 3 | - | - | - | - | 3 mm |
| Isolate 6 | - | - | - | - | 2 mm |
| Isolate 8 | - | - | - | 2 mm | 4 mm |
| Isolate 11 | - | - | 2 mm | 3 mm | 5 mm |
| Isolate 17 | - | - | 2 mm | 3 mm | 6 mm |
| Isolate 19 | - | - | 3 mm | 5mm | 7 mm |
| Isolate 20 | - | - | - | - | - |

**Fig1 Minimum inhibitory concentration of Mercury against soil bacteria**




100 mg/ L, 2 - 200 mg/ L, 3- 300 mg/ L, 4- 400 mg/ L, 5- 500 mg/ L

**Table – 3 Isolation of antibiotic resistance bacterial isolates**

| S.No | Isolates | Antibiotic resistance | | | | |
|---|---|---|---|---|---|---|
| | | T(10µg) | K(10µg) | A(30µg) | St(30µg) | C(30µg) |
| 1. | Isolate 3 | R | R | S | S | S |
| 2. | Isolate 6 | S | S | S | S | S |
| 3. | Isolate 8 | S | R | R | S | S |
| 4. | Isolate 11 | S | R | R | R | R |
| 5. | Isolate 17 | R | R | R | R | R |
| 6. | Isolate 19 | R | R | R | R | R |
| 7. | Isolate 20 | S | S | S | S | R |

*T- Tetracycline, K-Kanamycin, St-Streptomycin, A-Amphicilin, C-Chloramphenicol*
*S-Sensitive, R- Resistance*

**Antibiotic resistsnce of Mercury resistance isolates**

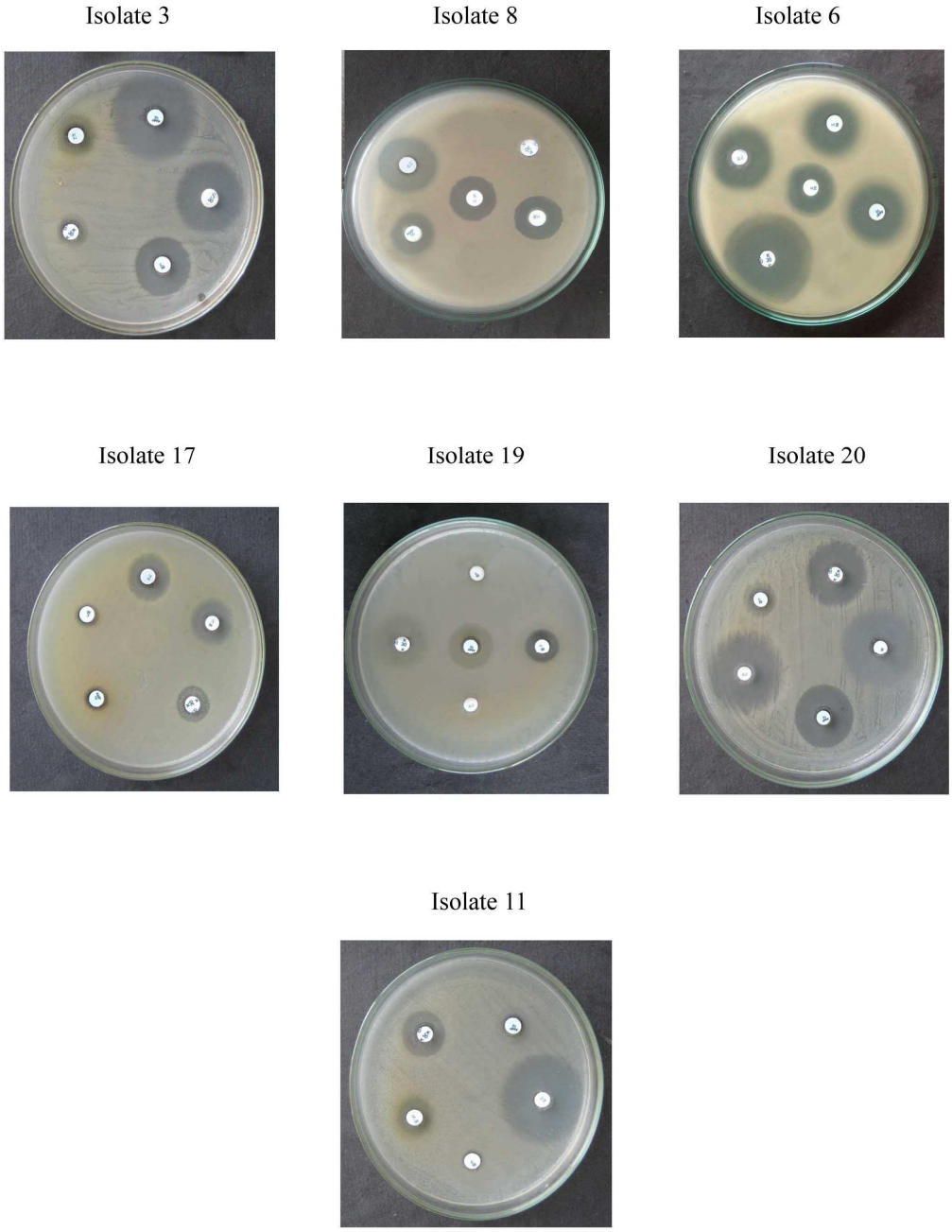



**Fig 2 .Antibiotic resistance of mercury resistant bacteria**

**Table – 4 Screening of biosurfactant producing bacterial isolates**

| S. No | Isolates name | Blue agar | Blood hemolysis | Drop collapse test | Oil spreading test | Emulsification test |
|---|---|---|---|---|---|---|
| 1. | Isolate 3 | + | + | + | ++ | **20 %** |
| 2. | Isolate 6 | + | - | + | + | - |
| 3. | Isolate 8 | - | + | + | + | - |
| 4. | Isolate 11 | + | + | + | ++ | **28 %** |
| 5. | Isolate 17 | + | + | + | - | - |
| 6. | Isolate 19 | + | + | + | + | **36 %** |
| 7. | Isolate 20 | + | + | - | - | - |

*+ = Positive ++ = Highly Positive*

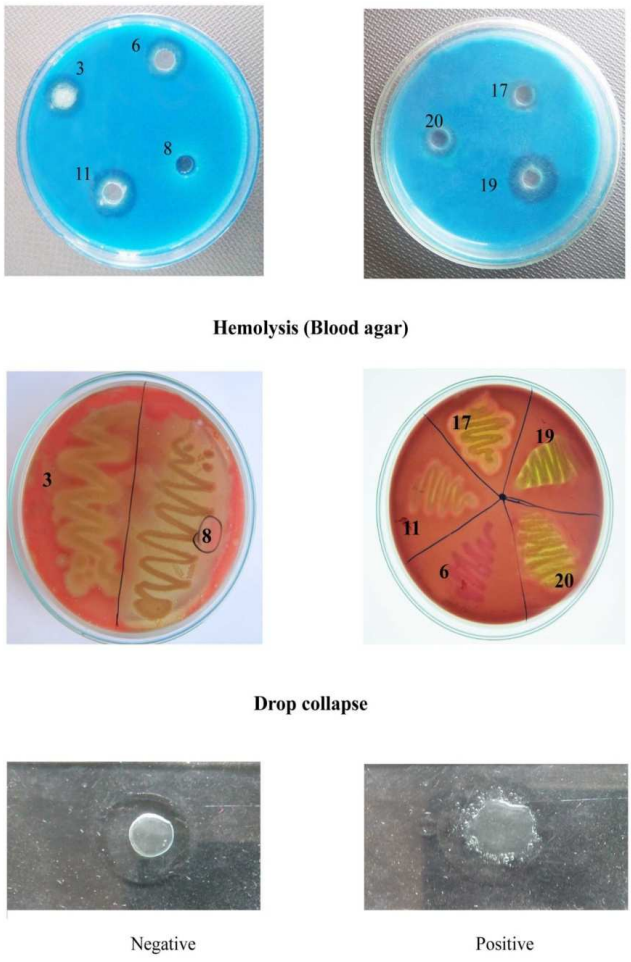


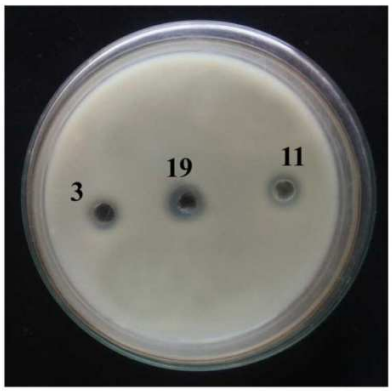

**Fig 3. Screening of biosurfactant producing bacteria**

## Blue agar method

**Table – 5 Screening of biosurfactant production potential of bacterial isolates**

| S.No | Bacteria solates | Agar well method (mm) | Gravimetric method (mg) |
|---|---|---|---|
| 1 | Isolate 3 | 1 | 0.601 |
| 2 | Isolate 6 | - | - |
| 3 | Isolate 8 | - | - |
| 4 | Isolate 11 | 2 | 0.664 |
| 5 | Isolate 17 | - | - |
| 6 | Isolate 19 | 3 | 0.795 |
| 7 | Isolate 20 | - | - |

**Fig.4. Screening of potential isolates by agar well method**




**Confirmation of potential isolates by Phenol: sulfuric acid method**

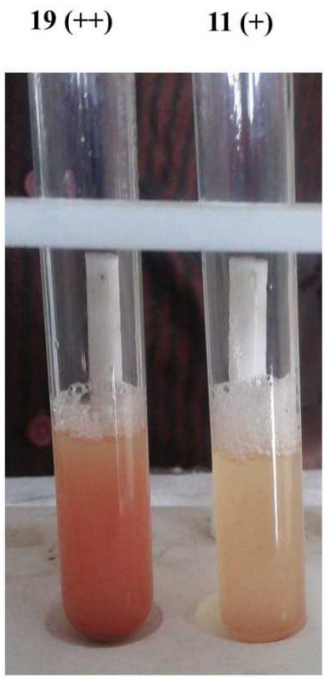



**Table – 6 Confirmation of biosurfactant production potential of bacterial isolates method Phenol: sulfuric acid method**

| S.No | Isolates | Result |
|---|---|---|
| 1. | Isolate 11 | + |
| 2. | Isolate 19 | ++ |

**Table 7 Biochemical characterisation biosurfactant producing bacterial isolate No. 19**

| S. No | Biochemical tests | Results |
|---|---|---|
| 1 | Gram stain | Gram -Positive |
| 2 | Shape | Rod |
| 3 | Spore staining | + |
| 4 | Motility | + |
| 5 | Catalase | + |
| 6 | Oxidase | - |
| 7 | Citrate | + |
| 8 | Indole | - |
| 9 | Glucose | Acid production |
| 10 | Lactose | Gas production |
| 11 | Sucrose | Acid production |
| 12 | Mannitol | - |
| | **Identified organism** | ***Bacillus cereus*** |

Dark blue halo zone in the methylene blue agar plates supplemented with CTAB confirmed the presence of anionic biosurfactant (Isolate 3, 6, 11,17 ,19 and 20). Isolate 3, 8, 11, 17, 19 and 20 showed positive results for haemolytic activity indicated by formation of clear zone around the colonies (Table 4). The supernatant of the six strains were added to the plates containing oil. The strain No. 3, 6, 8, 11 and 19 displaced oil thus showing a zone of displacement (table 4). By drop collapse test, it is evident that the bacterial isolates 3, 6, 8, 11,17 and 19 produced biosurfactant.

Isolates 3, 11 and 19 showed positive results which indicates their ability to emulsify crude oil (20 %, 28 % and 36 % , respectively). Further, the biosurfactant producing potential of bacterial isolates 3, 11 and 19 were confirmed by agar well method. Highest zone of clearance was elicited by bacterial isolate 19 (3mm) followed by bacterial isolate 11 (2mm) and 3 (1mm). Isolate No. 1, 11 and 19 were known to degrade oil (0.601 mg, 0.664 mg and 0.795 mg, respectively). Comparatively, bacterial isolate 19 exhibited highly positive result by phenol sulphuric acid method. These results confirm that bacterial isolate 19 could be used in the bioremediation of Hg contaminated soil observed in this study. Further, bacterial isolate 19 was identified as *Bacillus cereus* (table 7).

As evidenced in this study, Arun karnwal and Vaishali Bharadwaj [23] have isolated ten bacterial isolates from metal contaminated area in and around Baddi industrial areas and have identified these isolates as gram - negative bacteria and morphologically rod shaped. Furthermore , they have found that all the isolates belonged to the genus *Bacillus*. In addition, they have found that the bacterial strains vb4 was potent producer of biosurfactant as well as have efficient removal ability of zinc and chromium. The biosurfactant potential was determined by greater zone of hydrolysis by vb4 on blood agar plates and produced highest rhamnolipid by **TAB** method. The present observation agrees with that of Vijayanand and Divyashree [24] who have isolated bacteria from effluent water and have reported that all the six isolated bacteria exhibited oil displacement and have attributed it to the production of biosurfactant. Further, these strains also displayed haemolytic activity, which indicates their ability to produce biosurfactant on hydrophilic media [25]. Further, they have attributed it to the lysis of the RBCs present in the medium. Blood agar is an enriched and selective medium which allow only haemolytic organisms to grow by

utilising blood and hence the production of biosurfactants cause the lysis of cells which is an indication of production of biosurfactant by these organisms. The organism showed complete zone of haemolysis. The ability of isolate 3, 11, and 19 to emulsify crude oil exhibited in this study coincides with that of Vijayanand and Divyashree [24] who have also evinced all the six bacterial isolates displayed emulsifying activity with olive oil after 24 hours of inocubation and have ascribed it to the ability of these bacteria to degrade hydrocarbons by producing biosurfactant, which is the property of the biosurfactant producing organisms. Dark halo zone observed in methylene blue agar plate supplemented with CTAB confirmed the presence of anionic biosurfactant. This results agrees with that of Vijayanand and Divyashree [24].

The present observation is in good accord with Pradyut Saikia *et al* ., [26] who have also observed that *Klebsiella sp., Staphylococcus sp. , and Bacillus sp*. exhibited resistance to lead (600 µg/ ml, 400 µg/ml and 200 µg/ml, respectively), zinc (600 µg/ml, 400 µg/ml and 400 µg/ml, respectively) and copper (400 µg/ ml, 600 µg/ ml and 400 µg/ ml, respectively). Simultaneously, these bacteria also elicited resistance to amphicillin *(Klebsiella sp*; 120 µg/ ml; *Staphylococcus sp:* 40 µg/ ml; *Bacillus sp*: 60 µg/ ml) and cefotaxime (*Klebsiella sp:* 140 µg /ml; *Staphylococcus sp*: 80 µg/ ml; *Bacillus sp*: 90 µg/ ml).They have concluded that some of the organisms adapt themselves to protect or fight against high concentration of toxic metals and this property can be explored in the field of bioremediation.

Kavya *Bai et al.,* [10] have reported that bacterial isolates from marine beach shore , Triplicane beach shore, Kasimedu beach shore, interior sea water of Kasimedu beach shore were not resistant at lower concentrations (100 and 200 mg $L^{-1}$) of heavy metals (Co, Hg, Ag, Cu, Pb and Cr).On the other hand, they have observed that few bacteria exhibited slight zone of inhibition , whereas , most of the bacteria did not show inhibitory zone. Further, most of the bacteria did not show any significant zone of inhibition in case of heavy metals like Co, Cu and Cr even at 500 mg/ L. Comparatively, the inhibition was more for Co, Ag, Cu and Cr at 500 mg/ L. These observations are inconsistent with the present findings. Since *Bacillus cereus* produces biosurfactant, it could be used to bioremediate Hg contaminated soil.

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