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Screening of Biosurfactant Production in Bacteria Isolated from Oil and Pesticide Contaminated Soil of Ranchi District

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***Abstract:*Biosurfactants, the surface-active compounds are produced by a few microorganisms.As such biosurfactantsare superior to synthetic surfactants in terms of cost of production and industrial application.Present study deals with the isolation, characterization, screening, and extraction of biosurfactant-producing bacteria frommotor oil dumped and pesticide contaminated agricultural fields of Ranchi District, Jharkhand, India. Mineral salt medium (MSM) supplemented with hydrocarbon was used for the enrichment of putative biosurfactant producers. Out of ten isolates, five isolates (F1 to F5) showed growth on hydrocarbon supplemented plate suggesting its use as main carbon source. Three isolates namely F1, F4 and F5 were from the waste oil contaminated soil and remaining two (F2 and F3) from pesticide contaminated site. Based on traditional microbiological methods, characterization and identification were made which showed that F1, F4 and F5 isolates belong to *Staphylococcus aureus* and F2 and F3 to*Bacillus subtilis* species. Biosurfactant production was tested by hemolysis,emulsification index (E24) and drop collapsing tests, results showed positive test for all the assay suggesting the potential of biosurfactant production by all the five isolates. Among all the five isolates, F1 showed maximum emulsification index (44.44%) followed by F4 (35%) both belonging to strains of*Staphylococcus aureus*.The remaining isolates F2, F3 and F5 also showed appreciable level of E24(24-28%). Biosurfactants produced by all the five isolates wereextracted using solvents, the dry weight showed close correlation with E24. Further work is needed to confirm the identity of all the isolates using 16S rRNA sequencing and chemical characteristics of biosurfactants employing standard analytical techniques.**

***Index Terms:*Biosurfactant, Sta*phylococcus aureus, Bacillus subtilis*, Emulsification, Selective Media**

I. INTRODUCTION

Surfactants are surface-active materials which tend to reduce the surface tension of a liquid in which it dissolves. These can be potentially used in various chemical industries like detergents, paints, paper products, pharmaceuticals, cosmetics, and petroleum (Elazzazy*et al*., 2015). Chemical based surfactants in cosmetic formulations are one of the most challenging problems because it causes allergy and irritation in skin (Sil*et al*., 2017; Vecino*et al*., 2017). Biosurfactants are generally produced by microorganisms and classified according to their chemical structure and microbial origins (Marchant & Banat, 2012).They are amphiphilic compounds associated with both hydrophobic and lyophobic characteristics (Fracchia*et al*., 2012; Hu *et al*., 2017; Liu *et al.*, 2017). Biosurfactants, the surface-active molecules, show properties of lower toxicity and higher biodegradability(Cameoptra&Makker2004). Due to above unique properties, biosurfactants have gained much attention by several industries including cosmetics and pharmaceutical for their use in the production of conditioners, soaps, creams, moisturizers, cleansers, skin, and healthcare products (Boruah&Gogoi., 2013; Chakraborty *et al*., 2015). In this context, green synthesis of cosmetics and other related products using biosurfactants may bring revolution in industries(Ferreira *et al.*, 2017).Although large scale production of biosurfactants is costly, still several industries have initiated its production for uses in beverages, food, metallurgy, petrochemicals, bioremediation, and biological control (Perfumo*et al*., 2010; Vedaraman&Venkatesh., 2011).As such, majority of the biosurfactants-producingmicroorganismsarefound in soils, their detailed screening is lacking. However, soil types are one of the important factors in the production of biosurfactants by any

bacteria. It has been reported that Gram-positive biosurfactant-producing bacteria aremostly found in heavy metal-contaminated soilswhereasGram-negative bacteria are found inhydrocarbon contaminated soils(Adria *et al*.2003;Bodour*et al*., 2003).Nevertheless, uncontaminated soils also show growth of both Gram-positive and negative bacteria with potential to produce biosurfactants.Considering the above distribution pattern of biosuractants-producing bacteria, we became interested to isolate and screen biosurfactant-producing isolates from soils contaminated with pesticides and motor oil wastes.).

II. MATERIALS AND METHODS

A. Collections of soil samples

Ten soil samples contaminated with pesticides and motor oil (kerosene, petrol, and diesel) were collected from different sites/agricultural fields of Ranchi district. Accordingly, ten grams of soil samples from each site (four from pesticide and 6 from oil contaminated sites) were collected and placed in a sterilized polythene bag and brought to the laboratory for the isolation of bacteria. The choice of above sites for sample collection was based on the fact that soil contaminated with petro-chemicals and pesticides may be a good source of hydrocarbons and may favour growth of biosurfactant-producing microorganisms. These samples were tentatively designated as F1 to F10.

B. Isolation and cultivation of bacteria

One gram soil from each sample was taken in a culture tube and mixed with 10 ml sterilized double distilled water. After thorough mixing and vortexing, the tubes were kept at room temperature for 30 min for settling of soil particles. Serial dilutions down to 10-5 of the suspension were prepared from each sample. For the isolation of bacteria, two media namely nutrient agar (NA- contained 0.5 % peptone, 0.3 % beef extract, 0.5 % NaCl and 5.0 % agar-agar)for general isolation and mineral salt medium (MSM) forselective isolation of biosurfactant-producing bacteria were used. MSM contained the following salts (g/l); $NaNO_3$-2.0, NaCl-0.8, KCl-0.8, $CaCl_2.2H_2O$- 0.10,KH_2PO_4-2.0, $Na_2HPO_4.12\ H_2O$-2.0, $MgSO_4$-0.20, $FeSO_4.7H_2O$-0.001, agar-agar-10.0 and 2 ml standard trace elements. Glucose was replaced with the addition of 3% crude oil (v/v) as sole source of carbon. 50 µl of inoculums from 10-4 dilution of each sample were streaked on MSM solid agar-agar plate and incubated at 30ºC in a bacteriological incubator. After 72 h of incubation, appearance of colonies was examined in each plate. Colonies appearing on plates were picked and restreaked on fresh MSM agar-agar plates. Well-separated colonies from each sample were selected for further purification in NA solid medium.

C. Characterization of bacteria

Morphological and biochemical tests were done for characterization and tentative identification of bacteria following Bergey's manual of systematic bacteriology. The colony characteristics (shape, size, and color) were determined by observing the NA plates visually and under light microscope. Biochemical tests such as Gram's staining, catalase, citrateutilization, methyl red, indole, gelatin and starch hydrolysis, nitrate reduction and sugar fermentation were performed (Eddouaoudae*t al*., 2011).

D. Test of growth of bacterial isolates in selective media

Two selective media namely Tryptic soy agar (TSA) (Faddin and Jean 1985) and Bacillus differentiation agar were used and tested for the growth of specific genera in these media. For the preparation of TSA medium, 40 g of dehydrated medium was dissolved in one litre of double distilled water and autoclaved at 15 lbs (121ºC) for15 min. After cooling of medium, 7% sterilized sheep blood was added to the sterile molted medium and immediately poured in the plates. Colonies appearing on MSM were streaked separately on Petri plates in triplicate and incubated at 30ºC for 72 h. Beta haemolysis was checked for the confirmation of putative species. *Bacillus* differentiation agar medium was prepared by dissolving 22.0 g of the medium (obtained from HiMedia, Mumbai) in 1000 ml double distilled water and autoclaved as described above. After cooling the medium to 40-45ºC, medium was poured in different Petri plates. 50 µl inoculums of all the isolates growing on MSM were streaked on agar plates and incubated at 30ºC for 72 h. Colour of the medium (agar medium) was checked for tentative identification of species.

E. Screening of biosurfactant production

Screening of biosurfactant-producing strains was done by the following methods:

Hemolysis test

For the screening of biosurfactant-producing bacteria method developed by Mulligan *et al*. (1984) was used. This method is based on the hemolysis of red blood cells by the enzyme produced by test organism. Accordingly, one colony of each isolate was streaked on sheep blood agar plates and incubated for 48 h at 37ºC. Changes in colour of the medium surrounding the colony were observed to note the types of hemolysis. The colony surrounded by darkening zone was considered as alpha-hemolysis, appearance of clear white or halo zone as beta-hemolysis and no change in the medium as gamma- hemolysis.

Emulsification test (E_{24})

Emulsification test was done following the method of Cooper and Goldenberg (1987). 5 ml overnight grown culture in nutrient broth medium was centrifuged at 8000rpm for 10 min and the supernatant was collected in a sterilized tube. Equal volume of kerosene oil was added to the supernatant and vortexed vigorously for 2 min.The tube was kept at room temperature for 24 h. Subsequently, the emulsion index was measured by the formula mentioned below.

E_{24} = Height of visible emulsion layer/total height (sample plus emulsion layer) x 100

Drop collapsing test

Drop collapsing test was performed as per the methods described earlier (Jain *et al*., 1991; Bodour& Maier, 1998). 2 ml of pure engine oil (2T, Indian Oil Co. Ltd) was put in a 96 wells micro titer plate and left for 24 h at room temperature. 72 h grown cultures in MSM of test isolates were centrifuged at 8000 rpm for 10 min and the pellet was discarded. The clear supernatant (200 µl) was gently added in each well containing 2T and after 3 min the shape of the drop was observed visually with the use of a magnifying glass.

Blue agar plate (BAP) test

Mineral salt agar medium, supplemented with glucose (2%), 0.5 mg/ml cetyltrimethyl ammonium bromide (CTAB), and 0.2 mg/ml of methylene blue (MB) was prepared for the detection of anionic biosurfactant (Satpute*et al.,* 2010). In CTAB-MB agar plates, 30 µl of actively grown culture was streaked and the plates were incubated at 37°C for 48 to 72 h. Appearance of a dark blue halo zone around the colonies was considered positive to produce anionic type of biosurfactant.

F. Extraction and partial purification of biosurfactant

Bacterial dry weight (g/ml) was measured spectrophotometrically based on Beer-Lambert's law. Dry weight was calculated by using cell weight formula:

Dry weight of total cell (g/ml) $=W_3-W_2/W_1$

where

W_1= the volume of the culture

W_2=Weight of the test tube

W_3 =final weight

All the isolates were grown in MSM at pH 7 and 37^{o}C for seven days. Bacterial cells were harvested by centrifugation and the supernatant was filtered through a 0.22µm Millipore membrane filter. Supernatant was acidified by 2 N HCL to attain pH of 2.The acidified solution was kept at 4^{o}C overnight for complete precipitation and thereafter centrifuged at 10,000 for 20 min. Precipitate obtained was dried and used as crude biosurfactant. The dried precipitate was extracted twice with chloroform-methanol (4:1 v/v), the resulting extract was poured in a large size Petri plate. Organic solvent from the plate was evaporated using rotary evaporator or hair drier. The residue obtained served as partially purified biosurfactant. Residue was completely dried by placing the plate in a hot air oven at 100^{o}C for 30 min. Weight of the plate with residue was taken and subtracted with the weight of the same plate taken before the addition of the extract. The weight of residue was treated as the quantity of biosurfactant.

III. RESULTS AND DISCUSSION

Inoculums prepared from all the ten samples when streaked on MSM agar plates showed appearance of colonies and growth by five samples comprising three from motor oil waste dumped sites (F1, F4 and F5) and two from pesticides contaminated sites (F2 and F3). However, inoculums of all the samples showed growth of bacteria on solid NA agar non-selective medium.Tentative characterization of all the five isolates was made by performing Gram's staining and biochemical tests. Results showed that all the five isolates belong to Gram positive group and are either rod or coccus shaped. Colonies of all the five isolates appeared as white clustered on nutrient agar plate. Morphologically isolates F1, F4, and F5 showed cocci shape and F2 and F3 as bacilli. These five isolates were characterized in detail employing biochemical tests (Table I).

Table I: Biochemical tests of all the five isolates (F1- F5)

Biochemical tests	***Bacillus* species (asreference)**	***Staphylococcus* (as reference)**	**Isolates (F1, F4 & F5)**	**Isolates (F2 & F3)**
Gram-staining	+	+	+	+
Catalase Test	+	+	+	+
Citrate Test	+	+	+	+
Methyl Red Test	–	–	+	–
VP (VogsProsekauer)	+	+	+	+
Indole	–	–	–	–
Gelatin	+	+	+	+
Starch hydrolysis	+	+	+	+
Nitrate reduction	–	+	+	+
Sugar fermentation (sucrose/lactose)	+	+	+	+

It is evident from the data of biochemical tests that these isolates share most of the characteristics of the genera *Staphylococcus* and *Bacillus*as per the parameters listed in Bergey's manual of systematic bacteriology. Further analysis ofresults showed that F1, F4 and F5 isolates may belong to *Staphylococcus* and F2 and F3 to *Bacillus* species (TableI).

With a view to identify both the above genera at species level, selective media were used. Accordingly, F1, F4 and F5 isolates were grown in tryptic soy agar supplemented with sheep blood. All the three isolates showed growth on TSA agar plate as well asbeta haemolysis (Fig. 1). Thesecharacteristics resembled to those exhibited by *Staphylococcus aureus*. Henceforth, based on data of morphological characters, biochemical tests, and growth in selective medium, it is concluded that F1, F4 and F5 belong to *Staphylococcus aureus* species. This conclusion is based on the report of researchers (Faddin&Jean.,1985)who used TSA as a selective medium for the isolation of *S. aureus*. Similarly, isolates F2 and F3 were grown on *Bacillus* differentiation agar medium (selective medium) which is mainly used to differentiate *Bacillus cereus* and *Bacillus subtilis*based on mannitol fermentation (Turnbull, 1996; Madigan &Martinko, 2005). It was noted that the color of the medium changed to

yellow upon growth which is a typical characteristic of *B. subtilis*. Our findings clearly suggest that isolates F2 and F3 may be assigned as the species of *B. subtilis*. Altogether, our findings suggest that the species of *S. aureus*and*B. subtilis* are capable to grow in motor oil dumped sites and pesticides contaminated filed soil respectively. It is pertinent to mention that the identification of all the five isolates is based on traditional microbiological techniques, exact identification employing 16S rRNA sequencing is needed for the confirmation of bacterial species reported in this study (Muyzer*et al.*, 1993; Osborn*et al.*, 2000;Madigan &Martinko., 2005). However, to our knowledge our study is the first documenting the isolation of bacterial species from the motor oil dumped sites of Ranchi.

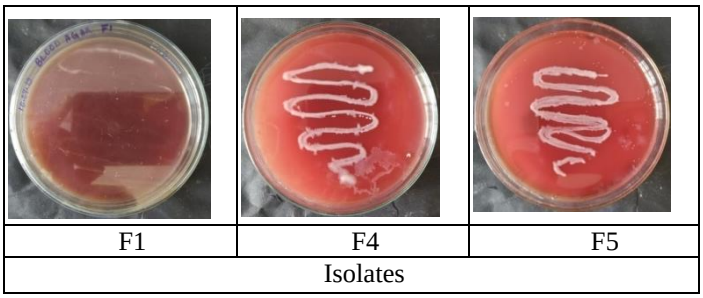

Fig. 1. Photograph showing growth of three isolates (F1, F4 and F5) on selective medium (MSM) used for the isolation of *Staphylococcus* species

Knowing that bacteria isolated from hydrocarbon contaminated sites are efficient biosurfactant producers, our interest aroused to test the same in all the five isolates of this study. Accordingly, emulsification capacities of all the isolates based on emulsification index(in %)was measured and. The highest value (44.44 %) was found in F1 followed by 35 % in F4 (Fig.2). F2 had the lowest value (24 %). As such isolates from motor oil dumped sites had higher emulsification index values in comparison to isolates of pesticides contaminated sites (Fig.2).Data of emulsification index showed close correlation with the dry weight of biosurfactants produced by all the five isolates upon growth under identical condition. As expected, the highest dry weight of surfactant was attained by F1 followed by F4, the lowest value being in F2 (Fig. 3). Emulsification attribute of all the five isolates is also evident from the photograph (Fig. 4). Similar to our findings, Jain *et al*. (1991) have also reported differences in emulsifying potential between *Pseudomonas aeruginosa* UG2 and *Acinetobactercalcoaceticus* ATCC 31012 especially during changes in carbon source used as substrate for growth.

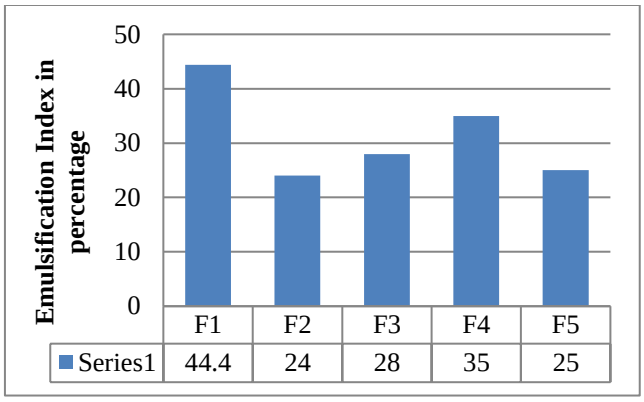

Fig. 2. Emulsification index (E_{24}) in percent of all the five isolates. Data are based on three experiments performed under identical conditions. All the experiments were performed in triplicate.

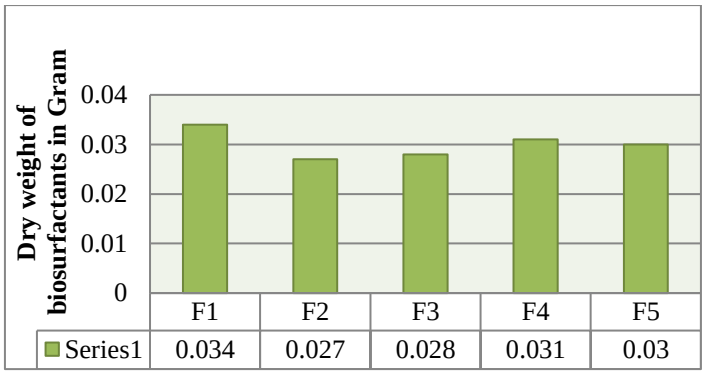

Fig 3. Dry weight (in g) of biosurfactant from different isolates (F1, F4 and F5) and *Bacillus* species (F2 and F3)

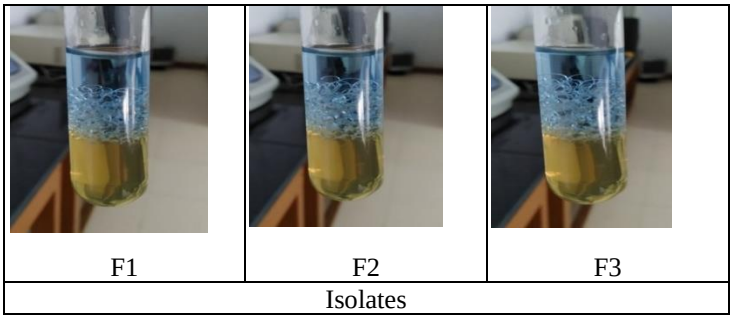

Fig.4. Photograph showing emulsification properties of F1, F3 and F5

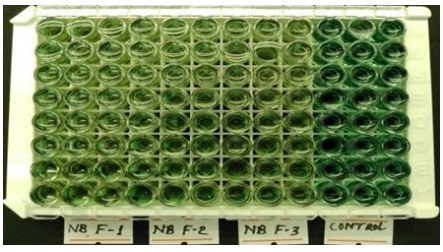

Fig 5. Photograph showing drop collapsing test for selected isolates

With a view to confirm the biosurfactant production potential, drop collapsingand blue agar plate tests for all the five test isolates were performed. In drop assay test, the drop appeared as a flat structure after the addition of supernatant in the plate suggesting the production of biosurfactant by the test isolates (Fig.5). Our findings are consistent with several other reports where drop collapsing test was found rapid and reliable method for thescreeningof biosurfactant production in several bacteria (Jain *et al*., 1991; Schulz *et al.,* 1991;Bodour& Maier, 1998; Bodour*et al*., 2003).Additionally, blue agar plate assay also showed the formation of dark blue halo zone surrounding the colonies of all the test isolates. Results of all the tests conducted for the screening of biosurfactant production are represented in (TableII). Based on the results of all the tests, it may be safely concluded that all the isolates are endowed with metabolic machinery to produce biosurfactants. Our findings support the report of earlier study conducted by a few researchers wherein majority of the bacteria isolated from oil or hydrocarbon contaminated sites were found biosurfactant producers (Schulz *et al.,* 1991;Bodour*et al.,* 2003; Vedaraman&Venkatesh., 2011).We have provided sufficient evidences in support of biosurfactant production by all the five isolates but could not analyze its chemical structure and properties. It would be worthwhile to characterize the biosurfactant reported in this study by using mass spectrometry, NMR and MALDI-TOF MS (Ahmad *et al.,* 2012).

Table II. Screening of biosurfactant-producing bacterial isolates based on different tests

Isolates	Hemolysis test (types)	Emulsification index (E_{24} value)[a]	Drop collapsing[b]	BAP test[c]
F1	β	44.44	Flat	+ve
F2	β	24	Flat	+ve
F3	β	28	Flat	-ve
F4	γ	35	Flat	-ve
F5	γ	25	Flat	+ve

a -value in %, b- shape of drop, and c-halo zone formation

CONCLUSION

Biosurfactants are proving more importantthansynthetic surfactants mainly because of their varied structural types, biodegradable potential, low cost of production using waste materials and may be used in industrial processes requiring high pH, temperature, salinity etc. They are now widely used in industries involved in the production of cosmetics, pharmaceuticals, and food preservatives. However, extensive search for biosurfactant-producing bacteria has not been made especially from harsh habitats till date. Herein, we report the isolation of five isolates from sites contaminated with motor oil wastes and pesticides of Ranchi district which were found capable to produce biosurfactant. Among five isolates, three were tentatively identified as the species of *Staphylococcus aureus* and two as *Bacillus subtilis*. Confirmation of biosurfactant production was made by using several standard assays reported by several researchers. *S. aureus*showed higher amount of biosurfactant production as compared to B.subtilis. It is anticipated that these isolates may be used at industrial level for small scale production of biosurfactants.Hopefully, extensive survey and screening of polluted sites of Ranchi district for bacteria may lead to the isolation of novel and hitherto unknown biosurfactant- producing isolates.

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