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Research Article

ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF BIOSURFACTANT FROM SOIL SAMPLE

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ABSTRACT

The present study aimed to isolate and characterize bio surfactants bacteria. Biosurfactants are surface active compound like chemical surfactants but synthesized by microbes like bacteria, fungi and yeast. Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are usually non-toxic and biodegradable. Bio surfactant showed diversity, flexibility in operation, and more ecofriendly than chemical surfactant. Bio surfactants are thermotolerant, halo bacteria and oil degrading in nature, which help in various industrial applications in near future. Biosurfactants are produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition and their nature and the amount depend on the type of microbes producing a particular biosurfactant. And in resulting wide variety of biosurfactant can be produced accordingly to the demand and uses. The advantage of biosurfactants reported by several scientists from worldwide and compared their efficiency with chemically synthesized pesticides and drugs aimed to screen effective bio surfactant producing bacteria. Biochemical characterization of biosurfactant contributes for the social benefits. Biosurfactants are biodegradability, generally low toxicity biocompatibility and digestibility which allow their application in cosmetics, pharmaceuticals and as functional food additives. Other applications include detergents, health care, pulp and paper, coal, textiles, ceramic processing and food industries excluding herbicides and pesticides formulations. This project is eco-friendly and is used in cleaning the environment with the help of these microbes

Keywords: Agar well diffusion, Bacillus, Biosurfactants, Isolation.

INTRODUCTION

Bio surfactants are amphiphilic compounds produced on living surfaces, mostly microbial cells excreted extracellular substances which are active molecules at the surface and interface. The bio surfactant has one of the following structures: mycolic acid, glycolipids, polysaccharide–lipid complex, lipoprotein or lipopeptide, phospholipid, etc. Few bio-surfactants have been reported as bio pesticide (Lipopeptides and Rhamnolipid) for controlling stored product insect pest. By properties like biodegradability, substrate specificity, chemical and functional diversity, and rapid/controlled inactivation; bio surfactants are gaining importance in various industries like agriculture, food, textiles, petrochemicals, etc. The economic growth of India largely depends on the agriculture-based industry. Lot of chemicals based pesticides are applied for insect pest management to increase the yield. Unfortunately, they are accumulated in soil and affecting the plant growth and product yield. Past two decades ago, several scientists reported that the toxins from certain strains of bacteria, like *Bacillus thuringenesis* and B. *sphaericus* are shown to be highly effective against mosquito larvae at very low dosage and safe to non-target organisms (Lacey and Undeen, 1986; Mulla, 1990; Walton and Mulla,1992). However, the bio-larvicide formulation from Bs strain is reported to be less effective against *Anopheles culicifacies* and hardly effective against *Aedes aegypti* (Mittal, 2003).

Several alternative methods are available other than biosurfacants. Due to the beneficial role of bio-surfactants, it needs to be improved. Survey of the literature reveals that application of bio surfactants in the field of pesticides is still in its infancy. In India, several laboratories have initiated studies on bio surfactants. Increasing interest

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shown possible replacement of synthetic surfactants with the bio surfactants in the pesticide formulation is effective in eco-friendly clean-up.

Oil pollution and remediation technology has become a global phenomenon of increasing importance. Most of the hydrocarbons are insoluble in water and their degradation using microorganisms have a key role in combating environmental pollution. Hydrocarbon degrading microorganisms produce biosurfactants of different chemical nature and molecular size which are surface active compounds which increases the surface tension of the hydrophobic water-insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation (Pekdemir *et al.*, 1999).

Almost all surfactants currently produced are chemically derived from petroleum. These synthetic surfactants are usually toxic themselves and are hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic emulsifiers have, in recent years, drawn much attention to the microbial production of surfactants or biosurfactants (Urum and Pekdemir, 2004).

Surfactants are surface active compound that reduce the interfacial tension between two liquids or that between a liquid and a solid. Surfactants are organic compound that contain both hydrophobic (head part of the surfactant) and hydrophilic (tail part of the surfactant) moieties. Thus surfactant contains both water insoluble i.e. water repellent group as well as water soluble i.e. water loving group. Biosurfactants are also surface-active compound like chemical surfactants but unlike the chemical surfactant, biosurfactant are synthesized by microbes like bacteria, fungi and yeast. Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are usually non-toxic and biodegradable.

Recently interest in biosurfactant has increased because of its diversity, flexibility in operation, and more ecofriendly then chemical surfactant (Saharan *et al.,* 2011 and Eduardo *et al.,* 2011). Furthermore, possibility of their production on large scale, selectivity, performance under intense conditions and their future applications in environmental fortification also these have been increasingly attracting the attention of the scientific and industrial community. These molecules have a potential to be used in a variety of industries like cosmetics, pharmaceuticals, humectants, food preservatives and detergents (Saharan *et al.,* 2011). But the production of biosurfactant on industry level is still challenge because of using high costly synthetic media for microbial growth. Biosurfactants are classified based on diversity in their structure and their microbial origin. They contain a hydrophilic group, that contain an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic group of unsaturated or saturated hydrocarbon chains or fatty acids. Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition and their nature and the amount depend on the type of microbes producing a biosurfactant.

Biosurfactants derived from living organisms, mainly microorganisms have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, and lower CMC. These properties have led to several biosurfactant applications in the food, cosmetic and pharmaceutical industries (Thanomsub *et al.*, 2004).

The most commonly isolated biosurfactants are glycolipids and lipopeptides. They include rhamnolipids released by *Pseudomonas aeruginosa* (Nitschke *et al.,* 2005), sophorolipids from *Candida* sp. (Daverey and Pakshirajan, 2009), as well as surfactin and iturin produced by *Bacillus subtilis* strains (Ahimou *et al.*, 2000).

Thus, based on their chemical composition, the biosurfactants produced by microorganisms are of many types such as glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides (Franzetti *et al.,* 2010; Banat *et al.,* 2010). Biosurfactants has received considerable attention in the field of environmental remediation processes such as bioremediation, soil washing, and soil flushing.

Biosurfactants influence these processes because of their efficacy as dispersion and remediation agents and their environmentally friendly characteristics such as low toxicity and high biodegradability. Although biosurfactants exhibit such important advantages, they have not yet been employed extensively in industry because of relatively high production costs (Sivapathasekaran *et al.,* 2010; Kiran *et al.,* 2010; Satpute *et al.,* 2010).

Biosurfactant or surface-active compounds are a heterogeneous group of surface active molecules produced by microorganisms, which either adhere to cell surface or are excreted extra cellulary in the growth medium (Fietcher 1992; Zajic and Stiffens, 1994; Makker and Cameotra, 1998). These molecules reduce surface tension and Critical Micelle Dilution (CMD) in both aqueous solutions and hydrocarbon mixtures. These properties create microemulsion in which micelle formations occur where hydrocarbons can solubilize in water or water in hydrocarbons (Banat, 1995). Several types of biosurfactant have been isolated and characterized, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids, lipopolysaccharides and other fully characterized. The majority of known biosurfactants are synthesized by microorganisms grown on water immiscible hydrocarbons, but some have been produced on such water- soluble substrates as glucose, glycerol and ethanol (Abu-Ruwaida *et al.,* 1991).

Biosurfactants are a unique class of compounds that have been shown to have a variety of potential applications in the remediation of organic and metal-contaminated sites, in the enhanced transport of bacteria, in enhanced oil recovery, as cosmetic additives and in biological control.

However, little is known about the distribution of biosurfactant-producing bacteria in the environment. The common culturable surfactant-producing bacteria are in undisturbed and contaminated sites. A series of 20 contaminated (i.e., with metals and/or hydrocarbons) and undisturbed soils were collected and plated on R A agar. The 1,305 colonies obtained were screened for biosurfactant production in mineral salts medium containing 2% glucose. Forty-five of the isolates were positive for biosurfactant production, representing most of the soils tested. The 45 isolates were grouped by using repetitive extragenic palindromic (REP)-PCR analysis, which yielded 16 unique isolates. Phylogenetic relationships were determined by comparing the 16S rRNA gene sequence of each unique isolate with known sequences, revealing one new biosurfactant-producing microbe, a Flavobacterium sp. Sequencing results indicated only 10 unique isolates (in comparison to the REP analysis, which indicated 16 unique isolates). Surface tension results demonstrated that isolates that were similar according to sequence analysis but unique according to REP analysis in fact produced different surfactant mixtures under identical growth conditions. The 16S rRNA gene database commonly used for determining phylogenetic relationships may miss diversity in microbial products (e.g., biosurfactants and antibiotics) that are made by closely related isolates. In summary, biosurfactant-producing microorganisms were found in most soils even by using a relatively limited screening assay. Distribution was dependent on soil conditions, with gram-positive biosurfactant-producing isolates tending to be from heavy metal-contaminated or uncontaminated soils and gram-negative isolates tending to be from hydrocarbon-contaminated or co-contaminated soils (Adria *et al.,* 2003).

Despite this work, our understanding of biosurfactants as a class of molecules remains limited. This is partially because the present body of knowledge has been developed around a relatively small number of well-characterized biosurfactants. Contributing to this is the lack of a concerted effort to perform a comprehensive screening for biosurfactants and the microorganisms that produce them. Such an effort is hampered by the fact that common genes or regulatory pathways do not exist among the different types of biosurfactant producers. Thus, molecular approaches alone are not useful in screening for biosurfactant producers; instead, screening must be done by using an activity measurement such as surface tension analysis. Surface tension is a parameter that is commonly used to describe the effectiveness of a surfactant (Adria *et al.,* 2003).

MATERIALS AND METHODS

Isolation of bacterial strains

Bacterial strains were isolated from soil samples using the standard protocol. Soil samples were suspended and serially diluted in sterile saline solution (0.89% w/v^{-1} NaCl). Tubes containing 0.1 ml of appropriately diluted solution were plated on nutrient agar (Himedia, Mumbai, India) plates and were incubated at 30°C for 24 hrs. Morphologically distinct single colonies were subculture on nutrient agar plates and screened for antibacterial activity.

Detection of antibacterial strains

The antibacterial activity of the isolates was determined by a deferred-antagonism plating assay (Tagg *et al.* 1976). Nutrient agar plates were streaked with test organisms and incubated at 30°C for 24 hr and then the plates were overlaid with soft agar (0.75% agar) containing 10^6 CFU ml^{-1} of the stationary-phase culture of indicator strains. The plates were incubated at 30°C for 24-48 hr and examined for the zone of inhibition.

Agar well diffusion assay

The strain which was selected as potential bacteriocin producers were grown in luria broth at 37ºC for 24 hrs. Cells were separated by centrifugation at 10,000 rpm for 10 min at room temperature. Around 6mm diameter wells were made on preinoculated agar media and each well was 100 µl of culture supernatant added (Toba *et al.* 1991). Inhibitory activity was performed against *Staphylococcus aureus*. Inhibition zones around the wells were measured and recorded.

Gram staining

Gram staining is used to detect the fundamental difference in the cell wall composition of bacteria. A clean, greese free glass slide was taken and wiped with alcohol. A thin smear of the organisms was made on the slide and it was fixed by air drying. The smear was flooded with crystal violet (primary stain) for 60 second and then washed with distilled water. Few drops of Gram's Iodine were added on the smear and kept for 60 seconds followed by washing with distilled water. Decolorization of the smear was done by using alcohol. Then the smear was stain by counter stain safranin and kept for 20 seconds followed by washing with distilled water. The slides was finally washed with distilled water, air dried and viewed under the oil immersion objective of the light microscope to obtain colony morphology.

Biochemical characterization of biosurfactant

The microbial colonies were identified by using the following biochemical tests. In case of fungal isolates, biochemical characterization was not followed.

Take 1.3 g of Luria broth add 100 ml of H_2O then inoculate the culture with the help of sterile loop in the Laminar flow to prevent contamination. Cultures were grown at 37°C for 24 hrs. After culture grown, culture can be added to Biochemical kit. Biochemical test were done as follows.

Indole Test

1-2 drops of Kovac's reagent (R008) were added. Development of reddish pink color within 10 seconds

indicated the positive reaction. Reagent remained pale colored if the test was negative.

Methyl Red Test

1-2 drops of Methyl Red reagent (1007) were added. Reagent remained red in color if the test was positive. Reagent decolorized and become yellow if the test was negative.

Voges Proskaeur's Test

1-2 drops of Baritt reagent A (Ro29) and 1-2 drops of Baritt reagent B (R030) were added. Pinkish red color development within 5-10 minutes indicated a positive test. No change in color or a slight copper color (due to reaction of Baritt reagent B) denoted a negative reaction.

RESULTS

Single colony was isolated by serial dilution (Figure 1). Agar well diffusion experiment was performed for the activity (Figure 2). Morphological studies by staining were performed. Biochemical characterization (Table 1 and Figure 4) were performed and the results were showing biosurfactant activity, which was checked by agar well diffusion and gram staining (Figure 4).All isolates were characterized through Bergey"s manual these cultures were characterized. This work was eco-friendly and was used in cleaning the environment with the help of these microbes.

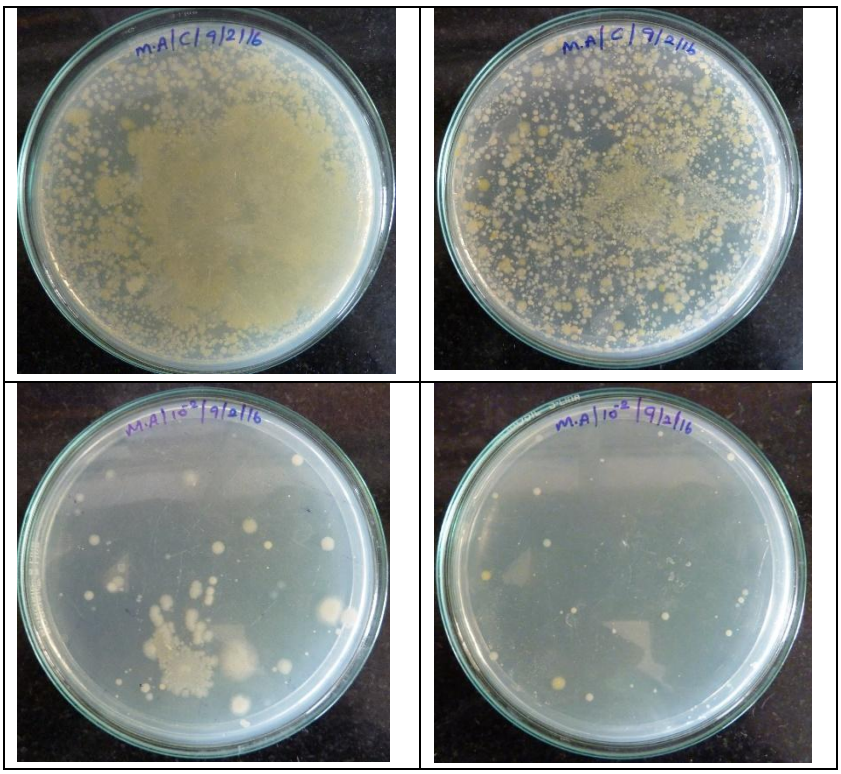

Figure 1. Isolation of bacterial strains.

Figure 2. Agar well diffusion assay.

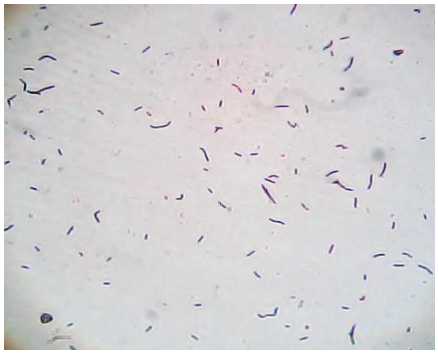

Figure 3. Gram staining.

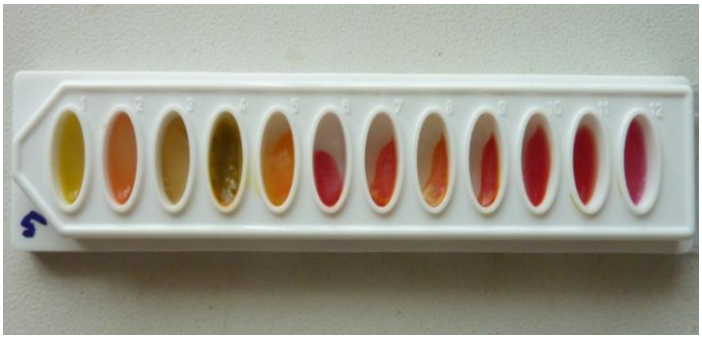

Figure 4. Biochemical characterization.

Table 1. Biochemical tests.

Indole tests	Negative
Methyl red tests	Negative
VP tests	Negative
Citrate utilization tests	Positive
Oxidase tests	Positive
Nitrate tests	Positive
Catalase tests	Positive
Gelatin	Positive
Casein hydrolysis	Positive
Urease	Positive
Lipase	Positive
Starch hydrolysis	Negative

DISCUSSION

Bacterial isolates about were isolated from oil contaminated soil samples by plate and dilution techniques. They were further screened for biosurfactant activities by hemolytic test, blue agar test, drop collapsing and emulsification index, as reported by Satpute *et al*. (2008) that more than one screening methods should be included in the primary screening as to identify potential biosurfactant producers. Potent bio-surfactant producing bacterial species from natural habitats like oil reservoir have been reported; however, the diversity of these in various habitats has not been studied. So in the present study, we aimed to screen, identify and characterize the novel bio-surfactant producing bacteria from washing powder contaminated soil in hotel outlet and its application in insect pest management.

Conventional arthropod control strategy involves application of broad-spectrum of chemicals and pesticides, which often produce undesirable effects. Moreover, growing public disquiet about the environmental and human risk associated with chemical pesticides, emergence of pesticide resistant insect populations as well as rising prices of new chemical pesticide have stimulated the search for new eco-friendly vector control tools (Mittal, 2003).

The results on blood agar media were similar to the work done by Mulligan *et al*. (1984) and Mulligan *et al*. (1989), who have isolated biosurfactant overproducer mutants with blood agar method. The flat drop appearance in micro titer plate confirmed the positive result for drop collapse test as suggested by Jain *et al*. (1991), proving the use of drop collapse method as a sensitive and easy method to test for biosurfactant production. Dark blue halo zone in the methylene blue agar plate supplemented with CTAB confirmed the presence of anionic biosurfactant. An alternative approach previously developed for the detection of extracellular rhamnolipids and other anionic glycolipids (Siegmund and Wagner, 1991) were employed in this study for the screening of rhamnolipid biosurfactants production by *P. aeruginosa* MM1011 and mutants. The assay was developed based on the property that the concentration of anionic surfactants in aqueous solutions can be determined by the formation of insoluble ion pairs with various cationic substances.

The formation of insoluble ion pair precipitates in the agar plate containing methylene blue exhibited dark blue color against the light blue background. The diameter of the dark blue region previously has been shown to be semi-quantitatively proportional to the concentration of the rhamnolipid biosurfactants (Siegmund and Wagner, 1991). Only 10 isolates showed positive results for all the 4 screening methods viz., hemolytic test, blue agar test, drop collapsing and emulsification index. The 10 isolates were further tested for maximum biosurfactant production by inoculating into the MSM medium. Among the selected isolates, PB3A showed maximum biosurfactant producing ability. The best isolate PB3A was identified as *P. aeruginosa* based on microscopic and biochemical analysis according to Bergey's manual of determinative bacteriology.

All strains were tested for haemolytic activity, which is regarded by some authors as indicative of biosurfactant production and used as a rapid method for bacterial screening (Banat, 1995). After haemolysis test, stabilization of an oil and water emulsion is commonly used as a surface activity indicator. Several studies focused on high emulsifying abilities (Francy *et al.,* 1991; Bicca *et al.,* 1999; Bodour *et al.,* 2004).

Identification of biosurfactant producing bacteria can be further confirmed by measurement of surface tension. Reduction of surface tension measurements by isolated bacteria from Iranian crude oil reservoirs indicates the production of surface-active compounds. Similar results obtained by Banat *et al.* (2000). They isolated several bacteria which showed the ability to reduce culture-broth surface tension to values below 40 mN/m. Salt concentration also affected biosurfactant production depending on its Effect of cellular activity which is very near to the results obtained by Yakimov *et al.* (1995).

Petroleum contaminated soil samples were collected and the organisms were isolated then the preliminary tests to identify the organisms (Thavasi *et al*., 2009). In this study, *Pseudomonas aeruginosa* showed the highest production of biosurfactant in both cell free culture and pellets. For the emulsification of the present study among the cell free cultures, *Pseudomonas aeruginosa* showed the highest production of biosurfactant in both cell free culture and pellets. For the emulsification of the present study among the cell free cultures, *Pseudomonas aeruginosa* showed the highest production (0.113%) of biosurfactant and also in the pellets *Pseudomonas aeruginosa* showed the highest production of biosurfactant (0.044). These results showed that highest extracellular biosurfactant production, compared to intracellular biosurfactants by isolates (Padmapriya *et al*., 2012).

Bioremediation is a process in which the environment can cleanup through microbes. There are so many types of microbes which used to eliminate contamination in sea water. The contamination can be in the form of oil or some chemicals. This type of contamination may lead to adverse effect on the organisms, animals as well as plants mainly found in sea water.

Bioaugmentation is the introduction of a group of natural microbial strains or a genetically engineered variant to treat contaminated soil or water. Bioaugmentation is commonly used in municipal wastewater treatment to restart activated sludge bioreactors. Most cultures available contain a research based consortium of Microbial cultures, containing all necessary microorganisms. Phytoremediation describes the treatment of environmental problems bioremediation through the use of plants that mitigate the environmental problem without the need to excavate the contaminant material and dispose of it elsewhere.

Phytoremediation is energy efficient with low to moderate levels of contamination and it can be used in conjunction with other more traditional remedial methods as a finishing step to the remedial process (Pandey *et al*., 2014).Oil degrading and total heterotrophic bacteria isolated from the sea water sample were 1.05x10 and 2.13x10 CFU/ml, respectively. Percentage calculation showed that oil degrading bacteria contributed 0.5% of the total heterotrophic bacterial (99.5%) population in the water sample. All the 105-oil degrading bacterial strains isolated in Bushnell Haas agar were subjected to screening for their biosurfactant production. Results on identification of 105 bacterial strains revealed that out of 105 isolates, 34 strains belong to gram positive and 71 strains to gram negative group represented by 18 bacterial species from 11 genera. Scientific names of the isolated bacterial species and number of isolates from each species n which Pseudomonas aeruginosa was found as the dominant species (Thavasi and Jayalakshmi, 2003). Dominant existence and biosurfactant producing Pseudomonas strains in hydrocarbon polluted environment was reported by many researchers (Yateem *et al*., 2002; Bodour *et al*., 2003; Das and Mukherjee, 2005) supporting the results obtained in this study.Biosurfactant produced by *L. delbrueckii* using peanut oil cake in this study showed its potential to be used in bioremediation process. Unlike medicinal applications, environmental application of biosurfactants needs comparatively less purity and high activity. In this study peanut oil cake was used as the carbon source for

biosurfactant production. Even though the biosurfactant was not purified to its purest form and structurally not well characterized but the results on emulsification and biodegradation experiments revealed the potential use of this biosurfactant in bioremediation of hydrocarbon pollution. Which emphasize that for environmental applications the biosurfactants need not be pure and could be synthesized from a mixed cheaper carbon source like peanut oil cake used in this study. All these approaches will make the bioremediation process an economically and environmentally viable mitigation technology (Thavasi *et al*., 2011).

CONCLUSION

From the result above Single Bacillus Gram's positive colony were isolated by serial dilution. Agar well diffusion studied were performed for the activity. Morphological studies by staining were performed. Biochemical characterization showed biosurfactant activities are able to produce lipopeptides type biosurfactant was checked by agar well diffusion method. All isolates were characterized through Bergey's manual these cultures were characterized. This project is eco-friendly and is used in cleaning the environment with the help of these microbes

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