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# Potential role of microbial surfactants in environment control recovered from oil contaminated and non-contaminated sites

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**Abstract**
A total of 20 samples were collected from contaminated (oil contaminated) as well as non-contaminated (agricultural) sites. A total of 10 bacterial isolates were recovered from these samples out of which 6 were recovered from non contaminated sites and 4 were recovered from contaminated sites gave emulsification index ranged from 44% to 73%. Different carbon sources viz. maltose, starch, sucrose, mannitol and nitrogen sources viz. urea, peptone, potassium nitrate and ammonium nitrate were screened to obtain optimum emulsification activity by KMSS09 and KIWS11. In this study mannitol and peptone was evaluated as best carbon and nitrogen source for the production of bioemulsifier. Further these potential isolates were evaluated for some environmental applications viz. Microbial Enhanced Oil Recovery and Bacterial Adhesion to Hydrocarbon assay having important role in bioremediation. The percentage oil recovered by KMSS09, KIWS11 and *P. aeruginosa* MTCC 2297 was 51.67%, 71.67% and 85.0% respectively. In BATH assay, percentage of bacterial adherence by KMSS09, KIWS11 and *P. aeruginosa* MTCC 2297 was 80.4%, 86.3% and 93.2% respectively showing wide applicability in bioremediation for pollution remediation of metal and hydrocarbon contaminated field.

**Keywords:** Bioemulsifier, Bioremediation, MEOR, BATH Assay

## INTRODUCTION

An emulsifier is a substance that stabilizes an emulsion by increasing its kinetic stability. The word "emulsion" comes from the Latin word for "to milk", milk being (among other things) an emulsion of milk fat and water. The term Emulsion is used when both the dispersed and the continuous phase are liquid (Mason *et al.,* 2006). Emulsifiers are class of surfactants that are amphipathic in nature posses both hydrophobic and hydrophilic domain and stabilize dispersions (Ron and Rosenberg, 2001). Emulsion can be formed by two immiscible liquids which may be described as an unstable dispersion system of two liquids by the action of an emulsifier (Horozov and Binks, 2004).

The synthetic emulsifier and surfactant production are often petrochemical dependent which have lower biodegradability, toxic to environment and may accumulate in the ecosystem. The emulsifiers and surfactants of microbial origin have drawn increasing interest because of their biodegradability, non toxic and potential commercial applications in various sector viz. foods, agriculture, pharmaceuticals and medicine, cosmetics, oil recovery, bioremediation etc. (Banat *et al.,* 2010; Farahbakhsh *et al.,* 2010). Many microorganisms can produce emulsifying agents which are extracellular compounds that allow microorganisms to assimilate water insoluble nutrients that are poorly miscible in water and use them as carbon source for cellular metabolisms (Banat *et al.,* 2000; Shete *et al.,* 2006). These emulsifiers derived from microorganisms collectively called microbial emulsifier or bioemulsifiers. Besides their biodegradable nature the bioemulsifiers are more effective over a wide range of pH, temperature, salinity and allow organic synthesis under eco friendly condition (Banat *et al.,* 2000; Nurbas and Acikel, 2010). Bioemulsifiers are produced by a variety of micro-organisms including bacteria, yeast and filamentous fungi (Abouseoud *et al.,* 2008; Mulligan, 2005). The bacterial genera associated with bioemulsifiers production include *Bacillus, Rhodococcus, Corynebacterium, Mycobacterium* and *Pseudomonas, Thiobacillus thiooxidans, Acinetobacter* species (Mousa *et al*., 2006) play important role in bioremediation.

## MATERIALS AND METHODS

**Chemicals and glasswares:** All the regents and solutions were prepared by using chemicals from Hi-media, Loba-chemicals. All the glasswares like test tubes, beakers, Petri plates, Erlenmeyer's flasks etc. were of Borosil grade.

**Sample collection:** A total of 20 samples were collected from the contaminated and non-contaminated sites. Out of which two soil samples were from Bhaskar Auto Spare and Service Station, Bilaspur, H.P., two from Tata Service Station, Bilaspur, H.P., and seven from Cedar Wood Oil Industry, Ratti, Sundernagar, District Mandi, H.P. Along with these contaminated samples four rhizospheric soil samples were collected from sugarcane, wheat, maize and mustard fields of Tangori village. Two water and two soil samples were also collected from hot water spring of Manikaran Sahib, District Kullu, H.P. The untreated effluent sample from Winson Textile Industry, Baddi, H.P. All these samples were collected aseptically in sterile polythene bags with the help of sterile spatula and brought to the microbiology laboratory of Shaheed Udham Singh College of Research and Technology, Tangori and kept at 4˚C till further processing. Standard strain of *P. aeruginosa* MTCC 2297 was procured from IMTECH, Chandigarh.

**Medium enrichment for isolation of Bioemulsifier producer:** One gram of soil samples and five ml of water samples were inoculated in 100 ml minimal salt medium supplemented with 2% glucose and 5%

petrol in 250 ml Erlenmeyer's flask. The flask was then incubated at 35±2˚C on a shaker incubator at 170 rev m¯□ for 72 hours for enrichment of bioemulsifier producer (Bodour *et al.,* 2004). Samples were sub-cultured into freshly prepared 100 ml minimal salt medium supplemented with 5% petrol. After sub-culturing 0.5 ml samples were streaked on nutrient agar plate and were incubated at 37˚C for 24 hrs. Then the morphologically different colonies were selected for the bioemulsifier activity

**Screening of isolates for bioemulsifier activity:** Screening for the bioemulsifier activity was carried out by using oil spreading method (Rodrigues *et al.,* 2006) and emulsification index (Anyanwu and Chukwudi, 2010).

**Oil spreading test:** For oil spreading test 50 ml distilled water was taken in large Petri plate and 20µl crude oil was added to the surface of water. Then 10µl cell free supernatant was added to the surface of water. The positive test was indicated by formation of clear zone over the oil water surface.

**Emulsification index:** An aliquot of bacterial growth (72 hrs) was taken and centrifuged. 2 ml cell free supernatant and equal volume of kerosene oil was taken in test tubes and vortexed vigorously for one minute. The test tubes were allowed to stand for 24 hrs and emulsification index was measured.

$$(E24\ index)\% = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100$$

**Effect of medium composition on bioemulsifier production:** The effect of medium composition on production of bioemulsifier was studied with effect of different carbon and nitrogen sources on bioemulsifier production as method described by (Makkar and Cameotra, 2002). Different carbon sources such as maltose, starch, sucrose, mannitol and nitrogen sources such as urea, peptone, potassium nitrate and ammonium nitrate were used to test the productivity of bioemulsifier in terms of emulsification activity.

**Effect of carbon sources on bioemulsifier production:**The effect of different carbon sources was studied on growth and bioemulsifier production by enriching the minimal salt media with maltose, starch, sucrose and mannitol. The minimal salt media was supplemented with concentration of carbon sources 2% w/v.

**Effect of nitrogen sources on bioemulsifier production:** The effect of different nitrogen sources was studied on growth and bioemulsifier production. The minimal salt medium was enriched with urea, peptone, potassium nitrate and ammonium nitrate in concentration of 2% w/v. Bioemulsifier producing isolates KIWS-11, KMSS-09 and 2297 were tested for the following applications:

**MEOR (Microbial Enhanced Oil Recovery):** To check oil recovery / mobilization potential of selected isolates from the complex natural matrix experiments were designed to check the ability of culture filtrate to extract oil using packed sand column as described below

**Preparation of sand column:** Prior to filling in the column sand sieved through a sieve and washed with dilute HCl and then with distilled water 2-3 times to remove traces of acid, air dried and packed in the glass column up to the length of 50 cm.

**Preparation of sample for oil mobilization:** Culture was grown in minimal salt medium supplemented with 2% glucose, 1% peptone and pinch of yeast extract at 37˚C for 72 hours. After sufficient growth, cells were separated by centrifugation at 8,000 rpm for 10 minutes and supernatant containing the bioemulsifier was used for oil recovery experiments.

**Running of column:** Before running the column, it was equilibrated with minimal salt media. After equilibration, 6 ml of crude oil was added along with equal volume of supernatant and allowed to percolate through the sand filled column for 24 hours. After 24 hours amount of mobile oil recovered was calculated. A control column (containing oil and uninoculated media) was also run along with samples for comparison.

$$\%\ \text{age of oil recovery} = \frac{\text{Volume of oil recovered}}{\text{Total volume of oil used}} \times 100$$

**BATH (Bacterial Adhesion to Hydrocarbons):** Cultures were taken in centrifuge tubes at centrifuged at 5,000 rpm for 15 minutes. Cells obtained from centrifuged culture broth were washed twice and suspended in buffer salt solution (g/L 16.9 $K_2HPO_4$ and 7.3 $KH_2PO_4$) to give an OD of 0.5 at 600 nm. Cell suspension (2 ml) with 100 µl crude oil was vortex shaken for 3 minutes in centrifuge tubes. After shaking, crude oil and aqueous phases were allowed to separate for 1 hour. OD of aqueous phase was then measured at 600 nm in a spectrophotometer. For each culture, 3 independent determinations were made and mean values were calculated (Thavasi *et al.,* 2011).

%age of bacterial adherence = [1— (OD shaken with oil — OD original)] x 100

## RESULTS AND DISCUSSIONS

A total of 20 samples were collected from contaminated (oil contaminated) as well as non-contaminated (agricultural) sites. Morphologically distinct bacterial colonies were selected. A total of 10 bacterial isolates were recovered from these samples out of which 6 were recovered from non contaminated sites and 4 were recovered from contaminated sites (Table: 1). These isolates were purified in nutrient agar plate to obtain pure culture and stored in nutrient agar slant and kept at 4° C in refrigerators for further characterization.

Table 1. Bacterial isolates with their site of isolation

| Sample collection site | Isolate ID |
|---|---|
| **Non-Contaminated Sites** | |
| Sugarcane rhizospheric soil | KSRS-89 |
| Wheat rhizospheric soil | KWSS-14 |
| Mustard rhizospheric soil | KMSS-09 |
| Maize rhizospheric soil | KMZS-11 |

| | |
|---|---|
| Manikaran hot spring water | KMKW-01 |
| Manikaran soil | KMKS-02 |
| **Contaminated Sites** | |
| Winson textile effluent | KIWS-11, KIWS-09 |
| Bhaskar Auto Spare and Service Station soil | KBSS-03 |
| Tata Service Station untreated effluent | KTSS-04 |

**Screening of isolates for Bioemulsifier activity**

Ten isolates from diverse sites were screened for bioemulsifier activity by means of oil spreading test and emulsification index.

**Oil spreading test**

All the isolates were subjected to the oil spreading test, out of ten isolates, nine isolates shows positive result. The positive test was indicated by the formation of clear zone over the oil-water interface. The formation of clear zone confirm that the supernatant of all the nine isolates contain the bioemulsifier activity. Similar results has been reported by the, Morikawa *et al.* (2000) while characterizing the isolates for biosurfactant activity and used oil spreading test as screening method. Youssef *et al.* (2004) have been also reported that bacterial strain SCMU106 form diameter of zone of clearance on the oil-water surface showing bioemulsifier activity. It was also acknowledged that oil spreading test is easy, requires less space, highly sensitive and rapid test for the screening of biosurfactant/bioemulsifier producer (Plaza *et al.,* 2006).

**Emulsification index**

The entire nine isolates positive for oil spreading test were further screened for emulsification index. Emulsification index of the potential isolates was measured with cell free supernatant and kerosene oil. $EMI_{24}$ of bioemulsifier producing isolates were ranged from 44-73%. The stability of the emulsion formed by different isolated were varied from a week to month. Bacterial isolates KMSS-09 and KIWS-11 were forming stable emulsion and are stable for a month while rest seven isolates were form emulsion and are stable for a week (Table: 2). Bioemulsifier is an amphipathic molecule containing both hydrophobic and hydrophilic moieties due to which they form emulsion with oil and water inter-phase for the stabilization of oil and water inter-phase. Similar, result have been reported by Rahman *et al.* (2002) that emulsification of various hydrocarbons using *Pseudomonas* strain DS10-129 quantitatively in term of height of emulsified layer formed after mixing of oil and cell free culture broth. Another, similar result reported with bacterial strain SCMU106 and the $E_{24}$ was ranged from 7.8-63.3 EA% (Techaoei *et al.,* 2007). These results showed that the bioemulsifier synthesis are very necessary for the bacterial community growing in oil contaminated sites where the main carbon source is the hydrocarbon and easily not available for growth and metabolism. Therefore, bioemulsifier directly interact with the hydrocarbon substrate and make them available for the microorganism for their cellular activity (Amiriyan, 2004).

Table 2. Emulsification Index (%$E_{24}$) for bacterial isolates after 72hrs of incubation

| Isolate ID | $EMI_{24}$ (%) | Stability |
|---|---|---|
| KSRS-89 | 58 | 1 week |
| KWSS-14 | 57 | 1 week |
| KMSS-09 | **71** | **2.5 months** |
| KMZS-11 | 60 | 1week |
| KMKW-01 | 61 | 1 week |
| KMKS-02 | 56 | 1 week |
| KIWS-11 | **72** | **2.5 months** |
| KBSS-03 | 44 | Not stable |
| KTSS-04 | 48 | Not stable |
| *P. aeruginosa* MTCC 2297 | **73** | **2.5 months** |

**Effect of medium composition on bioemulsifier production**

The maximum emulsion producers viz. KMSS-09 and KIWS-11 along with reference strain *P. aeruginosa* MTCC 2297 were screened for effect of different carbon and nitrogen source on their emulsification activity.

**Effect of carbon sources on bioemulsifier production**

To check the effect of different carbon sources on bioemulsifier production by, KMSS-09 and KIWS-11, four carbon sources viz. maltose, starch, sucrose, and mannitol were screened. The effect of different carbon sources on bioemulsifier production mentioned above are shown in Fig. 1. From above study it can be depicted that starch and mannitol was the good carbon source for the production of bioemulsifier. The emulsification activity was ranged from 59-73% in starch and 56-74% in mannitol respectively. It is well known that carbon source is required for the cellular metabolism and energy source for the diverse microorganisms. Subasioglu and Cansunar, (2008) has also reported that the mannitol was the best carbon source studied for maximum rhamnolipid production by *P. aeruginosa*. Similarly, it have been reported that the bacterial strain B160 showed the highest productivity of bioemulsifier in the medium supplemented with monosaccharide (glucose or fructose) in comparison with other carbohydrates (disaccharide and polysaccharide) tested (Youssef *et al.,* 2004). *Pseudomonas putida* 21BN grew on soluble substrates, such as glucose or on poorly soluble substrates, such as hexadecane. When grown on hexadecane as the sole carbon source the biosurfactant lowered the surface tension of the medium to 29

mN/m and formed stable and compact emulsions with emulsifying activity of 69% (Tulena *et al.,* 2001). Thompson, (2000) has also used potato effluent waste as the best carbon source for the production of surfactin by *Bacillus subtilis*. It is also known that potato is a good source of starch and can be support microbial growth and serve as carbon source.

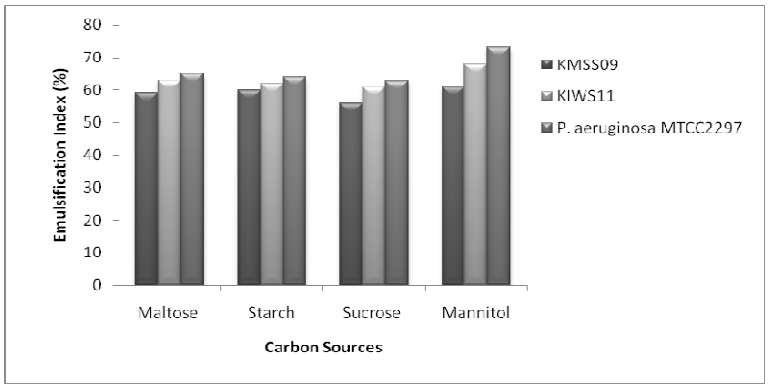


Fig 1. Effect of Carbon sources on bioemulsifier production in terms of emulsification index

**Effect of nitrogen sources on bioemulsifier production**

Nitrogen is main constituent of proteins which are very essential for growth of microbial world. Nitrogen is also essential for enzyme production, as the product of microbial metabolism, which helps in fermentation process. In this study minimal salt medium was supplemented with diverse nitrogen sources to confirm their effect on bioemulsifier production. Different nitrogen sources used were urea, peptone, potassium nitrate and ammonium nitrate. Their effect on bioemulsifier production was checked in terms of EMI (emulsification index) and compared with that of *P. aeruginosa* MTCC 2297. Peptone was observed to be the best nitrogen source for KMSS-09 and KIWS-11 (Fig. 2). It was revealed that supplementation of media with ammonium nitrate lead to maximum production of biosurfactant giving maximum EMI (56%) and minimal surface tension (31 dyne/cm) (Abouseoud *et al.,* 2008).

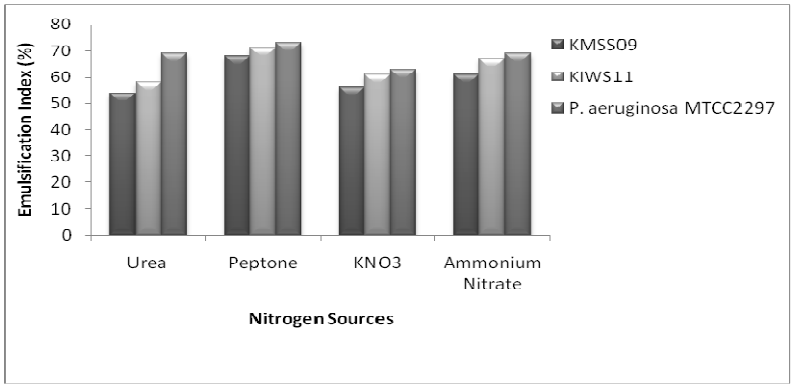


Fig 2. Effect of Nitrogen sources on bioemulsifier production in terms of emulsification index

Bioemulsifier producing isolates were used for some environmental applications viz. (Microbial Enhanced Oil Recovery and Bacterial Adhesion To Hydrocarbon assay).

**MEOR (Microbial Enhanced Oil Recovery):** Bioemulsifier producing isolates were used to study MEOR by using sand pack column and compared with *P. aeruginosa* MTCC 2297 strain. It was observed that KIWS-11, KMSS-09 and *P.aeruginosa* MTCC 2297 recoverd 72%, 52% and 85% respectively (Table: 3). Results were supported by the study of bioemulsifier produced by *Bacillus licheniformis* K125 which gave 43±3.3 enhanced oil recovery (Suthar *et al.,* 2008). In MEOR methods metabolites of micro organisms such as biosurfactants, biopolymers, acids, biomass etc are used to recover oil from the sites where assessment of oil is very difficult (Sen, 2008). Increase in the environmental pollution resulted the use of biosurfactants and bioemulsifiers as an alternative and eco friendly method to clean up the environment (Pacwa-Plociniczak, 2011).

Table 3. MEOR by Bioemulsifier producing isolates

| Isolates | Volume of oil used | Volume of oil recovered | %age of oil recovery |
|---|---|---|---|
| KMSS-09 | 6 ml | 3.1 ml | 51.67 |
| **KIWS-11** | **6ml** | **4.3 ml** | **71.67** |
| *P. aeruginosa* MTCC 2297 | 6 ml | 5.1 | 85.0 |

**BATH (Bacterial Adhesion to Hydrocarbons) assay:** Very important property of bioemulsifiers is degradation of hydrocarbons by attaching with them on their surfaces. In this work Bioemulsifier producing isolates were used for BATH assay and were compared with the standard strain of bioemulsifier producing *Pseudomonas aeruginosa* MTCC 2297. It was observed that KIWS-11, KMSS-09 and 2297 showed 86.3%, 80.4% and 100% bacterial adherence to hydrocarbons respectively (Table: 4). Results were supported by the study of *Lactobacillus delbrueckii* (Franzetti *et al.,* 2009) and *P. aeruginosa* which showed 93.2±1.2% cell adherence (Thavasi *et al.,* 2011).

Table 4. Percentage of bacterial adherence by different isolates

| Isolates | Original OD(at 600 nm) | Mean of OD shaken with oil (at 600nm) | %age of bacterial adherence |
|---|---|---|---|
| KMSS-09 | 0.555 | 0.751 | 80.4% |
| KIWS-11 | 0.528 | 0.665 | 86.3% |
| *P. aeruginosa* MTCC 2297 | 0.553 | 0.553 | 100% |

## CONCLUSION

Bioemulsifiers are a class of surfactants that are amphipathic in nature possess both hydrophobic and hydrophilic domain and stabilize dispersions. In the present study an attempt was made to isolate potential bioemulsifier producing bacteria and optimize best medium and condition for the production of bioemulsifier, emphasis also focused on some potential applications viz. MEOR and BATH assay. The percentage oil recovered by KMSS09, KIWS11 and *P. aeruginosa* MTCC 2297 was 51.67%, 71.67% and 85.0% respectively. In BATH assay, percentage of bacterial adherence by KMSS09, KIWS11 and *P. aeruginosa* MTCC 2297 was 80.4%, 86.3% and 93.2% respectively showing that these isolates can be exploit for the purpose of bioremediation.

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