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Research Article

AGRO-INDUSTRIAL WASTES: A COST-EFFECTIVE SUBSTRATE FOR MICROBIAL SURFACTANT PRODUCTION

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ABSTRACT
Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties produced mainly by the hydrocarbons degrading microorganisms. These have potential applications in agriculture, food, cosmetics, pharmaceutics and bioremediation. In the present study it was aimed to isolate potential bacterial isolates, studying their surfactant efficiency along with the screening of inexpensive agro-industrial wastes for the production of biosurfactants to minimize the production cost. Total 15 samples were collected from various contaminated and non-contaminated sites and 7 different bacterial isolates were recovered. All the bacterial isolates were positive for oil spreading test and emulsification index. The range of emulsification index was ranged from 32% to 72% in Nutrient broth and 34% to 78% in Bushnell Hass broth respectively. The maximum and the stable emulsion former viz. AG-1 and HC-3 were tested for the effect of different inexpensive carbon sources viz. molasses (1-5% v/v), whey and orange peel fruit waste on emulsification capacity. After 72 hrs. of incubation, the isolates AG-1, HC-3 and *P. aeruginosa* MTCC 2297 showed maximum EMI of 53%, 68 % and 78% at 5% molasses concentrations while for whey, it was 57%, 67% and 69% and for fruit waste it was 55%, 57% and 60% respectively. Further, the potential biosurfactant producing isolates were characterized on the basis of cultural, morphological and biochemical characteristics and the result were compared with Bergey's manual of systematic bacteriology and partially identified as the member of genera Pseudomonas and Bacillus. The isolates were used for potential application i.e MEOR. The percentage oil recovered by AG-1, HC-3 and *P. aeruginosa* MTCC 2297 was 44%, 64% and 82% respectively.

Keywords: *Agro-Industrial Waste, Cost Effective, Microbial Surfactant, MEOR*

INTRODUCTION
Microbial surfactants or Biosurfacatants are surface active metabolites produced by microorganisms when grown on water miscible or oily substrates: they either adherent to microbial cell surfaces or are secreted in the culture broth. They posses the characteristic property of reducing the surface and interfacial tensions using the same mechanisms as chemical surfactants. Research in the area of biosurfactant has expanded quite in the recent years due to its potential use in different areas such as the food industry, agriculture, pharmaceuticals, the oil industry, petrochemistry and the paper and pulp industry amongst others (Abouseoud *et al.,* 2007). Biosurfactants reduce surface tension, Critical Micelle Concentration (CMC) and interfacial tension in broth aqueous solutions and hydrocarbon mixtures due to its hydrophilic and hydrophobic moiety (Banat *et al.*, 2010). A variety of microorganisms, including bacteria, fungi, yeasts, have been reported to produce biosurfactants. Several of these biosurfactants are well described chemically and catagorized into high and low molecular mass compounds. The low molecular mass biosurfactants include glycolipids and lipopeptides, such as rhamnolipids and surfactin. The high molecular mass compounds include proteins and lipoproteins, or complex mixtures of these polymers (De-souza *et al.*, 2003). Biosurfactants are used in the remediation of organic and metal contaminated site, enhanced oil recovery and as cosmetic additives (Bodour *et al*., 2003). They are powerful natural emulsifiers capable of reducing the surface tension of water roughly 76 mN/m to 25-30 mN/m. The biosurfactant activity makes them excellent candidates for assisting in the breakdown and removal of oil

spills. Biosurfactants also demonstrate for its antibacterial and antifungal activites, suggesting possible roles in the medical and agricultural fields (Gunther *et al*., 2005). Nevertheless, from economic standpoint, biosurfactants are not yet competitive with the synthetics. Biosurfactants can only replace synthetic surfactant if the cost of the raw material and the process is minimal. So far, several renewable substrates from various sources, especially from industrial waste have been intensively studied for microorganism cultivation and surfactant production at an experimental scale (Maneerat, 2005). The principle aim of the present investigation are the detection the bacterial isolates which produced surfactant and can mitigate the requirement and the use of inexpensive wastes as substrate for the biosurfactant production to reduce the cost of production along with waste management.

MATERIALS AND METHODS

The research work was carried out in the Microbiology Research laboratory of SUS College of Research and Technology, Tangori, Mohali (India).

Chemicals and Glassware

All the chemicals used for preparation of reagents and solutions were procured from Hi-media, SD fine chemicals, Loba chemicals and were of AR grade. All the glassware were of Borosil grade.

Procurement of biological materials

The standard culture of *Pseudomonas aeruginosa* MTCC 2297 was procured from the Institute of Microbial Technology (IMTECH), Chandigarh. The strain was used as a reference strain in the present study.

Collection of Samples

A total of 15 different samples were collected from various contaminated and non contaminated sites. Non contaminated samples were collected from mustard, wheat, sugarcane and maize rhizopheric soils. Contaminated water samples were collected from Setia textiles Muktsar (Punjab), soil contaminated with hydrocarbon such as petrol filling stations and motor servicing station. All these samples were collected aseptically in sterile polythene bags and brought to the Microbiology laboratory of Shaheed Udham Singh College of Research and Technology, Tangori and kept at 4°C till further processing.

Pre-enrichment of the biosurfactant producer

For the pre enrichment of the biosurfactant producing bacteria Bhushnell Haas broth (BH) liquid media which is used as enrichment media for the biosurfactant producer. The media was supplemented with 2% glucose (w/v) and 5ml of kerosene oil as a sole carbon source. Pinch of yeast extract is also added. Samples were inoculated in the different Erlenmeyer flasks and kept for incubation in the rotor shaker for 72 hours at 37°C at 180 rev m^{-1}. The flasks with positive growth were re-cultured in the same medium for confirmation for biosurfactant production. After the enrichment, growth was observed in the flask. Nutrient agar plates were then prepared. The growth seen in different flasks, the aliquots from these flasks were spread over the nutrient agar plates. The plate was aerobically incubated at 35 ± 2°C. Morphologically distinct colonies were further screened for biosurfactant activity.

Preservation and maintenance of isolates

The colonies which came out for biosurfactant production were selected and streaked on nutrient agar slants and kept at 4°C in refrigerator for further screening. For the long term preservation of these isolates they are kept in the glycerol broth at -70°C.

Screening of the isolates producing biosurfactants

Two tests were performed for further screening of distinct colonies for biosurfactant production oil spreading method and emulsification index.

Oil spreading method

In this test 50ml of the distilled water was taken in the petriplates. 20µl of kerosene oil was added on the surface of water and then 10µl of cell free broth culture was added to it. The formation of clear zone on the oil-water surface shows the positive test. The diameter of this clear zone will indicate the presence of biosurfactant in the supernatant.

Emulsification index

Emulsification index is used to measure the emulsification ability of the biosurfactant. Emulsification index (E24) is used to characterize the biosurfactant emulsifying, generally, the hydrophobic phase in hydrophilic phase. Firstly 2 ml of the sample was added to the tube reaction. Then same amount of the kerosene oil was added as the amount of sample taken. Then the solution was mixed with a vortex for 2 min. this solution is then kept for 24 hours undisturbed. The E24 is given as the percentage of the height of the emulsified layer (mm) divided by the Total height of the liquid column (mm).

$$EMI_{24}\ (\%) = \frac{\text{Total height of the emulsified layer x100}}{\text{Total height of the liquid layer}}$$

Screening of inexpensive carbon sources for biosurfactant production

For the reduction in cost of the biosurfactant production inexpensive carbon sources which are usually waste are used in the present study. The carbon sources such as molasses, whey and fruit waste are used in this study.

Effect of molasses concentrations

Growth media consists of distilled water with different concentrations of molasses (1%, 2%, 3%, 4%, and 5% V/V). The pH must be adjusted to 7.0. The media was then autoclaved and was used as the fermentation media. The media was then inoculated with high and stable emulsion forming bacterial isolates and then incubated for three days in ratatory shaker at 37°C ± 1°C at 180 rev m^{-1} and EMI_{24} was calculated.

Effect of whey

Whey is the end product of cheese industry. This is used as the carbon source for the biosurfactant production in terms of emulsification activity as per the method described by (Lee *et al.*, 2008) Whey was boiled for 10 min, cooled to 4 °C, and solid particles were removed by filtration through the cotton. Then it is centrifuged at 4000 rpm for 10 min. The final pH was maintained to 4.0-4.5 with 1N H_2SO_4. Whey was again boiled for 10 min, cooled and pH was maintained at 7.0 with 1N NaOH. The product was filtered and autoclaved and is used as a medium for the biosurfactant production. Then the media was inoculated with emulsion forming high isolates of bacteria and incubated for three days in rotator shaker at 37°C ± 1°C at 180 rev^{-1}. During the course of fermentation sample was withdrawn in every 24 hours and the EMI_{24} was calculated.

Effect of fruit waste

The citrus fruit peels are taken and dried in the sunlight for 3-4 days. Then these peels were grinded. The grinded peels were then placed in water and centrifuged at 10,000 rpm for 10 min. The pellet was discarded and supernatant was taken in the 250 ml flask and was then inoculated with high emulsion forming isolates of biosurfactant producing bacteria.

Characterization of potential biosurfactant producing isolates

Morphological characterization: Isolates were characterized morphologically by Gram's staining to determine Gram's reaction pattern.

***Cultural Characterization*:** The cultures with high emulsification activity were tested for their cultural characterization by streaking the isolates in nutrient agar plates. The colonies formed were then observed for cultural characteristics like margin, shape, elevation, density and pigmentation.

Biochemical characterization: Series of biochemical tests were performed for characterization and identification of high and stable biosurfactant producing bacterial isolates. Isolates were subjected to various biochemical test viz. IMViC, Catalase, Urease, Fermentation of carbohydrates, Triple sugar iron etc.

MEOR (Microbial Enhanced Oil Recovery):

To check oil recovery / mobilization potential of selected strains 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.

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

RESULTS AND DISCUSSION

Enrichment and recovery of the biosurfactant producing bacteria

Total of 15 samples were collected from contaminated and non-contaminated sites. Morphologically 07 distinct bacterial colonies were selected, out of these 7 bacterial isolates, 4 isolates were recovered from non contaminated sites and 3 isolates were recovered from oil contaminated sites. These isolates were purified in nutrient agar plates to obtain pure culture and stored in nutrient agar slant and kept at 4 °C in refrigerators for further characterizations.

Screening of isolates for biosurfactant activity

7 isolates from diverse sites were further screened for biosurfactant activity by means of oil spreading test and emulsification index for the confirmation.

Oil spreading test

All the isolates subjected to the oil spreading test showed the positive result. The positive test indicated by the formation of clear zone over the oil-water interface. The formation of clear zone confirmed that the supernatant of all the 7 isolates contains the biosurfactant activity. The surfactants present in the supernatant forming and emulsion with the oil and forming a clear zone. Similarly, the Morikawa *et al. (*2000) reported, while characterizing the isolates for biosurfactant activity and used oil spreading test as screening method. (Plaza *et al*., 2006; Youssef *et al*., 2004) demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms.

Table 1: Showing the Oil Spreading Test for different bacterial isolates.

Isolate ID	Oil Spreading test
AG-1	++++
AG-2	++
AG-3	++
HC-1	++
HC-2	++
HC-3	++++
HC-4	+
P. aeroginosa MTCC 2297	++++

+: Low activity; ++: Medium activity; ++++: High activity

Emulsification index

The entire 7 isolates positive for oil spreading test were further screened for emulsification index. Emulsification index of the potential isolates were measured with cell free supernatant and kerosene oil.

EMI_{24} of the bioemulsifier producing isolates were ranged from 32% to 72% in Nutrient Broth and 34% to 78% in Bushnell Hass Broth. It was also seen that isolates AG-1, HC-3 and *P. aeruginosa* MTCC-2297 produced maximum yield of emulsification index. Their E_{24} was ranged from 71% - 78% in Bushnell Hass broth and 70% - 74% in Nutrient broth and were stable for about 35-42 days (Table 4.3). While other isolates AG-2, HC-4 and HC-1 formed emulsion that broke in 2 – 5 days and were not stable even for a week. Similarly, two bacterial strains identified as *Ralstonia picketti* (BP 20) and *Alcaligenes piechaudii* (CZOR L-1B) were isolated from petroleum hydrocarbon-contaminated soil following bioremediation treatment with emulsification index (EI24) was almost 100% for all tested compounds except diesel oil (Plaza *et al.,* 2005).

Table 2: Emulsification Activity of Different Bacterial Isolates

ISOLATES	EMI %		Stability of emulsion
	NB	BHB	
AG-1	**72%**	**74%**	**38 days**
AG-2	52%	53%	3 days
AG-3	55%	57%	4 days
HC-1	59%	62%	5 days
HC-2	32%	34%	2 days
HC-3	**70%**	**71%**	**35 days**
HC-4	51%	53%	5 days
***P. aeruginosa* MTCC-2297**	**74%**	**78%**	**42 days**

Screening of Inexpensive Carbon Sources for Biosurfactant Production

Effect of Molasses Concentration

Molasses with different concentration (1.0% - 5.0%) were inoculated with potential biosurfactant producer viz. AG-1, HC-3 and *P. aeruginosa* MTCC 2297 strain to find out the optimal molasses concentration for maximal emulsification activity. The different isolates were inoculated in various concentrations of molasses and their results are shown in fig 1. From the above experiments it can be depicted that emulsification activity of biosurfactant producer were increased with increase in molasses concentration up to 5% (v/v). The maximum emulsification activity of AG-1, HC-3 and *P. aeruginosa* MTCC 2297 was 53%, 68 % and 78% at 5% molasses concentration. In a similar study it has been reported that the biosurfactant production reached maximum when 5% (v/v) of molasses concentration was with Maximal surfactant production (0.25 g L^{-1}) occurred after 96 hrs of incubation, when cell reached the stationary phase of growth (Patel and Desai, 1997). Similar, reports have been reported with molasses as a carbon sources after dilution without any additional supplements (Joshi *et al.,* 2008). Molasses medium supplemented with soya-okara can be suitable medium for biosurfactant production. *P. aeruginosa* MTCC 2297 growing in a fermentation medium having molasses (4%) and soya-okra (0.15%) with pH 6.7 – 7.0 incubated at 35 - 37°C yielded maximum emulsification index (70%) after 120 hrs of incubation period (Panesar *et al.,*2011).

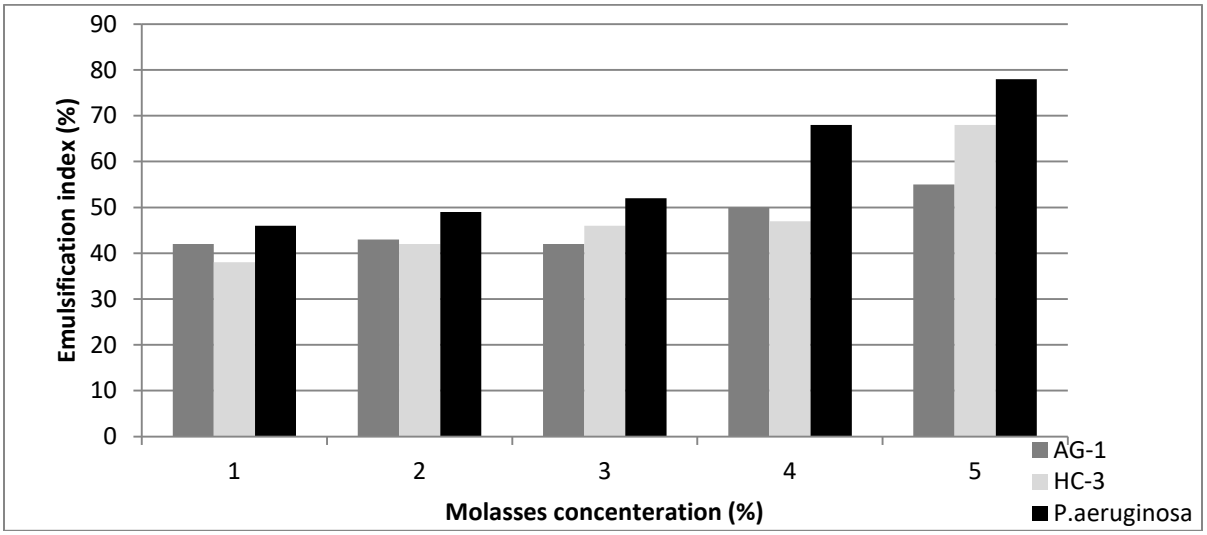

Figure 1: Effect of molasses concentration on Biosurfactant production in terms of emulsification activity after 72 hrs of incubation.

Effect of Whey

Processed whey was inoculated with two high and stable emulsion forming screened bacterial isolates i.e., AG-1 and HC-3 along with the standard strain of *P. aeruginosa* MTCC 2297 and incubated in the rotatory incubator at 37 ± 1°C at 180 rev m^{-1} for the time period of 3 days. Samples were drawn at every 24 hrs of incubation and emulsification index was calculated for each isolates. It has been observed that AG-1, HC-3 and *P. aeruginosa* showed maximum of 57%, 67% and 69% of emulsification index at the end of 72 hrs. It have been suggested that dairy waste liquor supports good microbial growth and was used as cheap raw material for biosurfactant production Dubey and Juwarkar, (2004). *P. aeruginosa* BS2 cultivated on whey waste produced 0.92 g L^{-1} biosurfactant as the secondary metabolites and its maximal production occurred after the onset of the nitrogen limiting conditions. The isolated biosurfactants possessed the potent surface-active properties, as it effectively reduced the surface tension of water from 72 to 27 Nm m^{-1} and formed 100% emulsion in variety of water insoluble compounds (Dubey and Juwarkar, 2001). Similarly, *Lactobacillus pentosus* that was growing on whey at 31°C effectively lowered the surface tension of medium from 54 mN m^{-1} to 45 mN m^{-1} (Rodrigues *et al.*, 2006a).

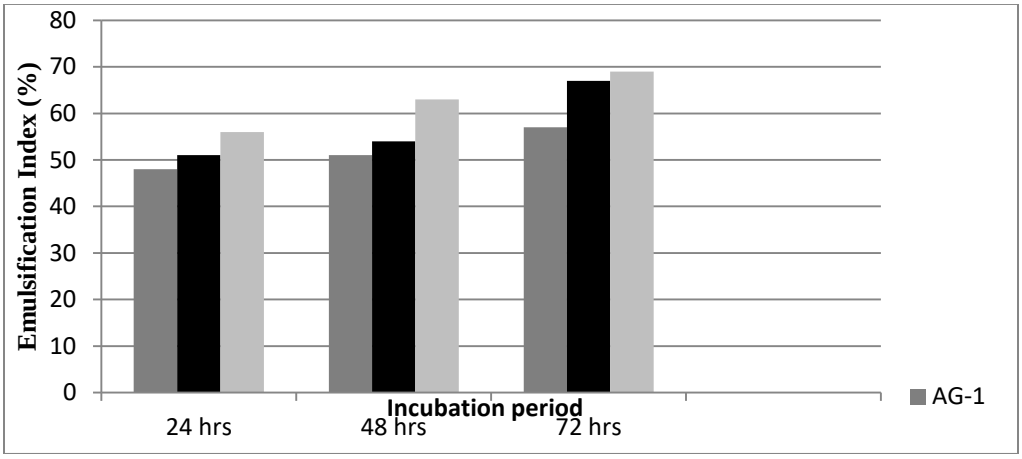

Figure 2: Effect of Whey on biosurfactant activity of isolates at incubation time of 24, 48 and 72 hrs.

Effect of fruit waste

Similarly, as in whey the two stable emulsion producing isolates along with the reference strain were inoculated in the orange peel fruit waste effluent and was incubated in the incubator shaker at 37 ± 1°C at 180 rev $m^{-1}$for three days. The results are depicted in fig. 4. Fruit wastes are used as the carbon source for the production of biosurfactants. The isolates AG-1, HC-3 and *Pseudomonas aeruginosa* MTCC 2297 produced a maximum emulsification activity of 55%, 57% and 60% respectively at the end of 72hrs. George and Jayachandran (2009) found that *Pseudomonas aeruginosa* MTCC 2297 growing on various cost effective waste materials such as orange peelings, carrot peel waste, lime peelings, coconut oil cake and banana wastes produce a surface-active compound rhamnolipid biosurfactant by submerged fermentation. The orange peel was found to be the best substrate generating 9.18 g/l of rhamnolipid with a surface tension reduction up to 31.3 mN/m. It has been reported that cassava flour processing effluents used as a substrate for biosurfactant production by *Bacillus subtilus* ATCC 21332. *B. subtilus* LB5areduced the surface tension of the medium from 49.5 mN m^{-1} to 26.6 mN m^{-1} and produced crude biosurfactant concentration of 3.0g L^{-1} (Pekin *et al.*, 2005).

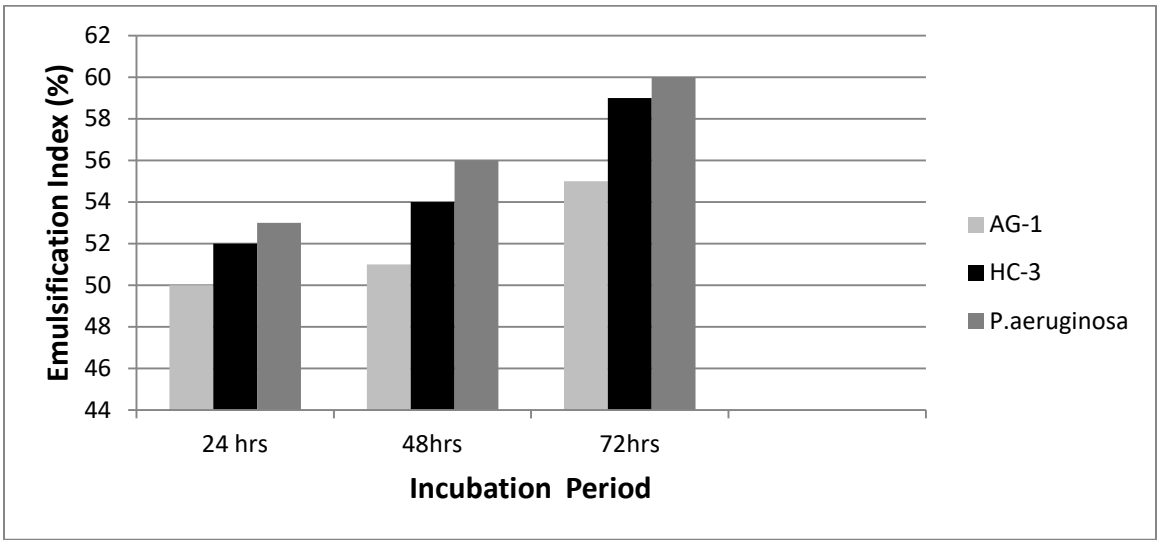

Figure 3: Effect of fruit waste on biosurfactant activity of isolates at incubation time of 24, 48 and 72 hrs.

Partial identification of the isolates

In this study bacteria were isolated from the soil and water from different contaminated sites and from rhizopheric soil. All the bacterial isolates were characterized in terms of morphological and biochemical reactions and they were then identified on the basis of the specific characteristic compared with the use of Bergey's manual of systemic bacteriology. Isolate AG-1 found to be small rod, gram positive, while HC-3 was rod gram negative.

Identification of isolates was done by the biochemical test based on the criteria described by Bergey's manual of determinative bacteriology. For Indole, MR, VP and urease all the isolates showed negative result. The Gelatin liquification, Nitrate and Oxidase, both the isolates showed positive results. Catalase and citrate test were positive for HC-3 and *P. aeruginosa* MTCC 2297 and were negative for AG-1. In case of triple iron test (TSI), HC-3 and *P. aeruginosa* MTCC 2297 showed K/A (Alkali/Acid), while for AG-1, H_2S production was observed. On the basis of these morphological, cultural and biochemical reactions and above characteristics were compared with Bergey's manual of systematic bacteriology, HC-3 was partially identified as *Pseudomonas* sp. and AG-1 was tentatively assigned as *Bacillus* sp.

Microbial Enhanced Oil Recovery

During oil recovery from packed sand columns, all the three potential isolates showed more than 44% of oil recovery, when incubated with equal volume of mobile oil and supernatant containing bioemulsifier. It was observed that AG-1, HC-3 and *P. aeruginosa* MTCC 2297 showed maximum oil recovery 44%, 64% and 82% respectively. The results are dipcted in table 4.6 and fig. 4. Similar results were supported by the study of bioemulsifier produced by *B. licheniformis* K125 which gave 43 ± 3.3 enhanced oil recoveries (Suthar *et al.,* 2008). In MEOR methods, metabolites of microorganisms such as biosurfactants, biopolymers, acids, biomass etc are used to recover oil from the sites where assessment of oil is very difficult (Sen, 2008). Similarly, biosurfactants produced by *B. subtilus PT2* and *P. aeruginosa* recovered 62% and 57% oil respectively (Pacwa-Plociniczak, 2011). It has been reported by various workers that the produced bioemulsifier decrease the capillary force between the oil and rock surface by decreasing surface tension and increase the mobility of oil resulting in its easy recovery. Increase in the environmental pollution results the use of biosurfactants and bioemulsifiers as an alternative and eco-friendly method to clean-up the environment (Pacwa – Plociniczak, 2011).

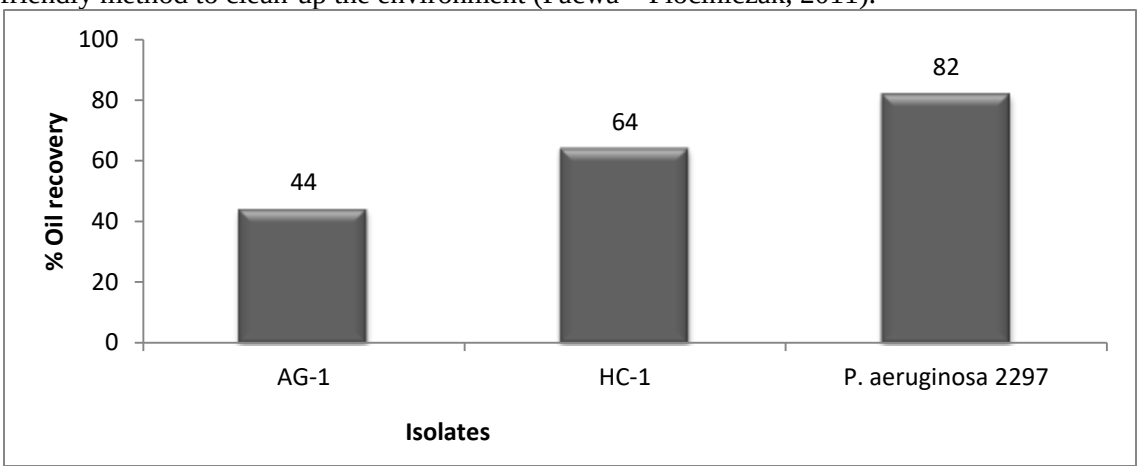

Figure 4. Microbial enhanced oil recovery

CONCLUSION

From the above studies it was concluded that the biosurfactant producing bacteria can be isolated from both the agricultural as well as the oil contaminated sites. The rate of the production can be reduced by using the agro-industrial waste as the substrate for biosurfactant production. In the present study it has been observed that both the isolates along with reference strain produced maximum emulsification activity i.e. bisurfactant with 5% molasses concentration at 37 °C ± 1 °C for 72 hours of incubation period. It was also observed that whey and citrus fruit waste also support the microbial growth and in future can be exploit as substrate for the commercial production of biosurfactant to reduce its production cost along with waste management. These bacterial isolates also showing good oil recovery activity therefore can be used for the recovery of oil in petrochemical industry or can be useful as environment cleaning agents in bioremediation. With the emphasis on the building of a sustainable society in harmony with the environment, the introduction of green technology in all the fields of the industries is one of the most important challenges. Considering the technological and ethical background, utilization of biosurfactants, which are eco-friendly and highly functional, have become more and more important future aspects.

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