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Analysis and Characterization of Bioemulsifer Produced by Surfactant Degraders

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Out of the 5 surfactant degraders obtained from lake shore sample were grown in Mineral salt medium with different oils as substrates. The emulsification index was calculated in which *Enterobacter* sp. 3EAG2 showed maximum emulsification index and was further studied and partially purified by Acetone precipitation. Maximum bioemulsifier production was obtained during the stationary phase after 72 hours using almond oil as a substrate. The bioemulsifier was found to be stable at 10º C & 37 º C. The bioemulsifier showed the presence of proteins, carbohydrates and also lipids in its backbone. The HPTLC & GC-MS analysis showed the presence of 3- hydroxyl- 2- alkyl fatty acids & saturated fatty acids, n-alkanes and esters of carboxylic acid that formed the sugar moiety respectively. The bioemulsifier showed efficient oil removing capacity and on immobilization of the *Enterobacter* sp. 3EAG2 cells in Sodium alginate beads, excellent detergent degradation was also observed over a period of 5 days.

Key words: Bioemulsifier, chemical emulsifier, Immobilization, HPTLC & GCMS.

Bioemulsifiers have received increasing attention in recent years because of their role in the growth of microorganisms on water-insoluble hydrophobic materials such as hydrocarbons and because of their commercial potential in the cosmetics, food, and agricultural industries.[1] Conventional chemical surfactants have been extensively used and their derivatives are costly and of serious environmental concern; they are potential threats to the environment due to their recalcitrant and persistent nature. Therefore, with current advances in biotechnology, attention has been paid to the alternative environment friendly processes for production of different types of biosurfactants from microorganisms [2].

Emulsifiers typically have a hydrophobic and a hydrophilic end. The emulsifiers will surround oil (or other immiscible molecule) and form a protective layer so that the oil molecules cannot "clump" together. This action helps to keep the dispersed phase in small droplets and preserves the emulsion. As a consequence, bioemulsifiers can partition preferentially at the interface [3].

A large number of bacterial species are capable of producing bioemulsifiers. The majority of the *Acinetobacter* strains, including both

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hospital and environmental isolates produce high molecular weight bioemulsifiers. The best studied are the bioemulsans of *Acinetobacter calcoaceticus* RAG-1 and *A. calcoaceticus* BD4 [4]. Liposan, a bioemulsifier produced by *Candida lipolytica,* is primarily composed of carbohydrates [5]. Toledo *et al* found that *Bacillus subtilis, Alkaligenes faecalis and Enterobacter* sp. isolated from waste crude oil when grown in the presence of hydrocarbons produced bioemulsier [6].

Unlike chemically synthesized surfactants, which are usually classified according to the nature of their polar grouping, bioemulsifiers are generally categorized mainly by their chemical composition and microbial origin. Rosenberg and Ron suggested that bioemulsifiers can be divided into low-molecular-mass molecules, which efficiently lower surface and interfacial tension, and high molecular- mass polymers, which are more effective as emulsion-stabilizing agents. The major classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high-mass surfactants include polymeric and particulate surfactants. Most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long-chain fatty acids or fatty acid derivatives, whereas the hydrophilic portion can be a carbohydrate, amino acid, phosphate or cyclic peptide.[7]

Advantages of a bioemulsifier over a chemical emulsifier include biodegradability, generally low toxicity, biocompatibility and digestibility, availability of raw materials, acceptable production economics, and use in environmental control, specificity, and effectiveness [8].

The effectiveness of the bioemulsifier as biostimulating agent in oil bioremediation processes has been demonstrated by several authors in different experimental assays. For example they have been shown to be really efficient in combination with other products frequently used in oil remediation such as they are inorganic fertilizer (NPK) and oleophilic fertilizer (i.e. S200C). On the other hand, the Bioemulsifiers have shown to be more efficient in the treatment of soil with high percentage of clay. Finally, their efficacy has been proved in other biotechnological processes such as *insitu* treatment and biopiles [9].Other extensive bioremediation studies were successfully carried out on the oil contaminated desert sand in Kuwait, both *in situ* and on site. In all these applications, indigenous microbial populations were utilized by introduction of specific nutrients (N and P) and oxygen to encourage biosurfactant production and hydrocarbon utilization [10].The current study is aimed at characterization of the bioemulsifier produced by surfactant degraders.

MATERIAL AND METHODS

Isolation of surfactant degraders

The surfactant degrading organisms were obtained from a lake shore sample at Borivali, Mumbai after enrichment in a Mineral based media containing surfactant as sole carbon source. The enriched broth was then serially diluted with sterile saline and 0.1 ml of the dilutions were spread on sterile nutrient agar plates. 5 colonies were selected at random and preserved on nutrient agar slants until further use.

Screening for bioemulsifier production

The preculture seed of the 5 isolates was prepared by inoculating a loopful of bacterium into sterile nutrient broth, 50 ml in a 250 ml Erlenmeyer flask that was kept on a rotary shaker at 180 rpm for 24 hours at 30° C until an O.D of 1.0 was reached. The seed inoculum was produced by inoculating the above preculture 1% (v/v) into sterile mineral salts medium (100 ml in 250 ml flask) which consisted of (per litre solution) 0.5 g K_2HPO_4, 1 g NH_4Cl, 2 g Na_2SO_4, 2 g KNO_3, 0.2 g $MgSO_4.7H_2O$, and 0.002 g $FeSO_4.7H_2O$. under the same conditions until an exponential growth was seen [11]. Different oils (almond oil, groundnut oil, sunflower oil, mustard oil and coconut oil) and hydrocarbons (diesel and kerosene) were used as substrates during fermentation. The microbial cells were separated by centrifugation at 8000g for 20 min at 30º C. 2ml of the cell free supernatant and 0.5 ml of the suitable hydrocarbon (kerosene) were vortexed at a high speed (10,000 rpm) for 1 min using a cyclomixer. The emulsification index was calculated after incubation at 30° C for 24 hrs. The maximum bioemulsifier producer is designated as strain K hence forward.

Cell-surface hydrophobicity assay

Cell surface hydrophobcity is an important factor in the adherence of the organisms at liquid- hydrocarbon interface and is a prerequisite for their growth. The absorbance

measures the affinity of the organism to the hydrocarbon. Cell-surface hydrophobicity of the *Enterobacter* sp. 3EAG2 was determined using almond oil. The volume of test hydrocarbon used ranged from 1 to 5 ml. Incubation of the culture and hydrocarbon was carried out at 30°C. Absorbance was recorded at 420nm [12].

Partial purification of bioemulsifier

The organism *Enterobacter* sp. 3EAG2 producing maximum levels of bioemulsifier was grown in the Mineral salt medium with almond oil at 30° C for a period of 3 days. The cell-free culture broth obtained by centrifugation at 8000 g for 20 min was mixed with three volumes of chilled acetone and incubated at 4°C for 15 h. The precipitate was collected by centrifugation at 10,000 g for 30 min and dissolved in minimum volume (1-2 ml) of sterile distilled water (pH 7) [11]. The bioemulsifier was then used for further analysis.

Chemical analysis and characterization of bioemulsifier

A portion of the partially purified bioemulsifier was used for chemical analysis. Protein content was measured using the method described by Lowry *et al.* (1951) with bovine serum albumin as standard. Reducing sugar content was estimated using the dinitro salicylic acid method with glucose as a standard. A portion of the extracted bioemulsifier was tested for presence of lipids using Sudan IV reagent. To determine and characterize the components of crude bioemulsifier extract, various analytical methods like HPTLC and GC-MS techniques were employed [13].

Stability of the emulsion

The bioemulsifer that was obtained was checked for its stability at different temperatures. After 1 h incubation of the vortexed mixture of test oil and emulsifier at 30°C, the absorbance of aqueous layer at 420 nm was recorded. One set was incubated at 37°C and the other at 10°C. Absorbance of aqueous layer was noted every 20 hours for a period of 3 days [11].

Oil Removal Potential

To a piece of clean cotton cloth (1x1 cm^2) ,0.1 ml of engine oil was added. The cloth was dried and after 24h, 5 ml of the bioemulsifier extract was taken into two sterile tubes into one of which the sample cotton cloth was introduced. The contents were vortexed for 2mins at high speed. The cloth was then removed and rinsed with distilled water and dried at room temperature. The results thus obtained were observed and compared with 1% SLS and distilled water [14].

Detergent Degradation and Bioemulsifier Production

The *Enterobacter* sp. 3EAG2 was checked for detergent degradation. Mineral Salt medium was used with detergent. Immobilized sodium alginate entrapped cells were introduced in the medium containing detergent to check the efficiency of the degradation by immobilized organisms. Emulsification assay was performed to confirm bioemulsifier production. Degradation of detergent and hence, reduction in its concentration was estimated on day 1 and day 5 titrimetrically.

RESULTS AND DISCUSSION

The isolates obtained from the lake shore samples which were surfactant degraders grew on various carbon sources such as hydrocarbons, alcohols, fatty acids and triglycerides [14]. Chemical composition of the test oils was also taken into consideration during these studies. All the test oils comprised mainly of three fatty acids, viz. oleic acid, linoleic acid and palmitic acid, in varying proportions. Oleic acid and linoleic acid are unsaturated fatty acids, whereas palmitic acid, stearic acid and arachidic acid are saturated fatty acids [15]. Accordingly, almond oil displayed a higher degree of unsaturation as compared to coconut oil and mustard oil. Still, almond oil was attacked most readily by the test strains, whereas mustard oil, with the least degree of unsaturation, was not easily emulsified. Bioemulsifier production by various organisms is speculated to be due to its ability to grow on various hydrophobic substances prevalent in the environment[11].

Among the 5 strains of surfactant degraders, strain K exhibited maximum bioemulsifier production. In general, the almond oil was emulsified to a greater extent, while coconut oil and mustard oil were least emulsified. These findings suggest that the emulsification activity depends on the microorganism's affinity for the hydrocarbon substrate which involves a direct interaction with the hydrocarbon itself rather than an effect on surface tension of media. The 16s rRNA typing done at Chromus Biotech Pvt. Ltd identified

the Strain K to be most similar to *Enterobacter* sp. 3EAG2 (99% homology): (Gen Bank entry: GQ 383912)

On studying the kinetics of the bioemulsifier production of *Enterobacter* sp. 3EAG2 in almond oil, maximum bioemulsifier

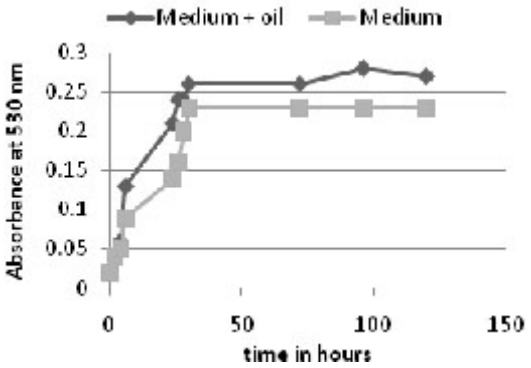

Fig. 1. Growth Kinetics

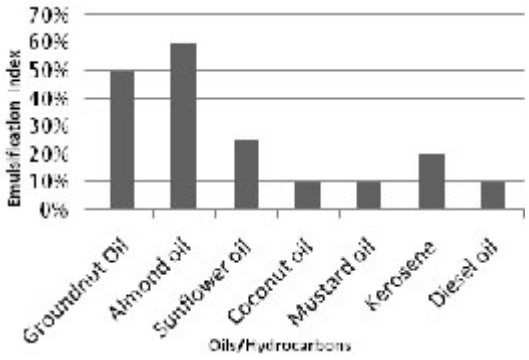

Fig. 2. Effect of different oils/hydrocarbons on the bioemulsification activity

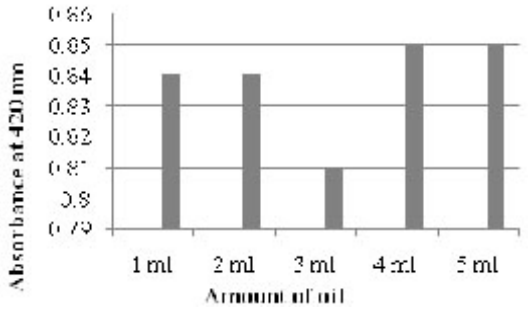

Fig. 3. Cell Hydrophobocity Assay

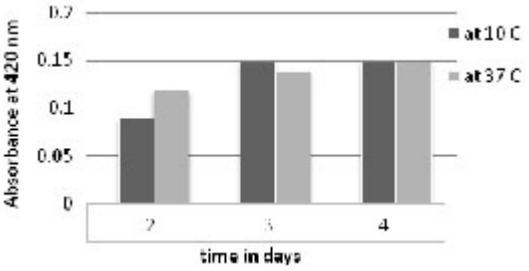

Fig. 4. Stability of bioemulsifier at 10º C & 37º C

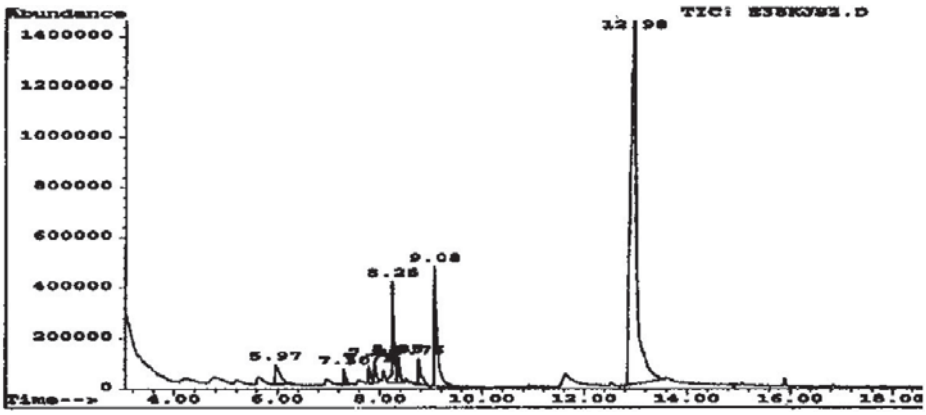

Fig. 5. Chromatogram obtained of emulsan

production was obtained over a period of 5 days (120 hours). Within 24-30 hours of growth, a sharp rise in the bioemulsifier concentration followed by a slow and gradual increase over the next 4 days was observed (Fig. 1). This biphasic growth curve appears to indicate a vigorous utilization of nutrients followed by a slow induction of emulsifier formation. Possibility that induction of capacity to grow on the added oil is also seen here. So the fermentation was carried out until a constant growth was obtained. On comparison with the control, the growth of the microorganisms ceased within 24 hours. Thus the oil present in the test sample acts a carbon source which gets emulsified into small droplets thus increasing the surface area. The oil droplets are utilized by the organisms thus resulting in the emulsification of the medium. Patil & Chopade (2001) and Mujumdar have also separately demonstrated that bioemulsifier from *Acinetobacter* sp. is produced during the stationary phase[5].

The cell hydrophobocity assay (Fig. 3) is the measure of the affinity of the microorganism to the hydrocarbon. This can enable the use of organisms in oil- spills which are very extensive and the concentration of the oil is not known [11].Cell-surface hydrophobicity assay revealed that in spite of the increasing amount of the oil, *Enterobacter* sp. 3EAG2 are able to utilize the almond oil as a carbon source.

The bioemulsifier can be precipitated by various methods like the Ammonium sulphate precipitation method, and/or the ethanol and methanol precipitation method. However, the results obtained by these methods were not satisfactory as compared to the Acetone precipitation[16]. The bioemulsifier was therefore precipitated by Acetone precipitation.

The stability of the emulsion at various temperatures is very important from the point of view of its use in various industries like pharmaceuticals and also in food formulations where temperature plays an important role for preservation[4]. High emulsion stability at room temperature is preferable as it alleviates the need of storage at low temperature for preservation and also extends the shelf life of the product. Moreover, emulsification of almond oil by the partially purified bioemulsifier resulted in considerable reduction in the viscosity, which is a desired property for an emulsifier [11].The emulsion stability at 10°C and 37°C (Fig. 4) showed the bioemulsifier produced by *Enterobacter* sp. 3EAG2 was stable, thus enabling better use even at different temperatures.

Protein concentration was estimated by Folin- Lowry's assay. The bioemulsifer showed a protein content nearly 270µg/ml. The role of each isolate in the release of the emulsan from the cell surface has been well established. The emulsan contains protein which is an important component of the backbone. Various emulsan isolated previously contain protein which is important for the emulsification of water in oil suspension[8]. The bioemulsifier has an appreciable content of proteins in their backbone. This asserts the role of the protein in the emulsification activity.

Along with various proteins, carbohydrates also play an important role in the emulsification of oil in water suspension[8]. The carbohydrates were estimated using DNSA method with Dextrose as the standard. Here the bioemulsifier showed 120 µg/ml of carbohydrates. This suggests that along with protein, the carbohydrate is also an important constituent of the emulsan backbone and may also be responsible for the biodegradation of the bioemulsifier.

The bioemulsifier with no carbohydrate may contain certain amount of lipids, esterase which also contribute to the emulsification of the oil in water suspension[17]. On addition of Sudan IV reagent to the extracted bioemulsifier, it gave red coloured precipitate, thus confirming the presence of lipids in the bioemulsifier.

The HPTLC analysis and the corresponding results were in accordance with the literature data. Unsaturated long chain fatty acids with an alkyl branch in the 2- position and a hydroxyl group in the 3- position, were revealed to be an active part of the partially purified emulsan extract. Violet colour did not develop when the plate was dipped into Ninhydrin reagent, thus ruling out the possibility of phosphatidy-lethanolamines being present. Red colour developed when the plate was sprayed with 2',7'-Dichlorofluorescein indicating the presence of 3-hydroxyl- 2- alkyl fatty acids[18]. With further purification of the extracted bioemulsifier, probably, phosphatidylethanolamines could be shown to be the part of the complex.

The major peaks in the mass spectra

obtained in case of the bioemulsifier on comparing with the standard database revealed the presence of saturated fatty acids, n- alkanes and esters of carboxylic acid that formed the sugar moiety. Depending upon the retention times the polarity of the components as checked, with polar groups taking less time and compounds with non- polar groups emerging later, n- alkanes including Eicosane and butylated hydroxyl toluene were among those which emerged first. Decanoic acids and its derivatives viz. penta- , octa- decanoic acids were detected. Oleic acid accounted for the unsaturation in the emulsan complex. The methyl ester derivatives of the above carboxylic acid moieties were present, as also presented in published data. (Fig. 5)

Bioemulsifiers are potentially capable of removing oil. Here the oil removing ability of bioemulsifier was compared with Sodium Lauryl Sulphate. The bioemulsifier reducing the surface tension, brings about good wetting & subsequent water spreading. The bioemulsifier with hydrocarbon in the surrounding forms stable oil-in-water emulsion. The oil molecules get distributed into small globules and the bioemulsifier surround the globules so that they dont clump together. This action helps to keep the dispersed phase in small droplets and as a consequence the bioemulsifiers get partitioned at the interface[19], in the process removing the oil molecules off the cloth. This acivity exhibited by the bioemulsifier was shown to remove oil from the cotton cloth quite efficiently.

Detergents comprising mostly of linear chain alkylbenzenesulphonates (LAS) are degraded by bioemulsifier producing organisms. In this studies *Enterobacter* sp. 3EAG2 and immobilized cells as well, showed excellent degradation rates, with 50 mg/ 100 ml and 30 mg/ 100 ml of detergent within 5 days respectively. With some modifications in the immobilization methods, probably, the biodegradation could be accomplished even more efficiently at faster rate. The present work has demonstrated good emulsification activity by *Enterobacter* sp. 3EAG2 isolated from lake shore sample.

CONCLUSION

The present study demonstrated the production of glycolipopeptide type bioemulsifier by *Enterobacter* sp. 3EAG2**,** thus providing another source of bioemulsifier producer in the presence of oil as a carbon source. Ultimately, on the basis of their biological and physicochemical properties, biosurfactants can be used as an emulsifier and conservative agent in agrochemical, food industries as well as in bioremediation applications and with some further knowledge regarding the regulatory and molecular mechanisms, pathways for biodegradation etc. and thus the precise manipulations in the same, *Enterobacter* sp. 3EAG2 seemingly would be an ideal one in such industrial applications.

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