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Isolation, production and characterization of bioemulsifiers of marine bacteria of coastal Tamil Nadu

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Present study consists 18 morphologically different marine bacterial species isolated from coastal Tamil Nadu, examined for production of bioemulsifiers. All the 18 isolate produces bioemulsifiers and exhibit emulsification index (E24) of 85-95% with hydrocarbons containing fatty acids and <75% with other hydrocarbons. Resultant emulsion found stable till day 100 without any phase separation. Polymeric protein nature of the bioemulsifiers produced by the marine bacterial species could be the reason for the stability. Hypothesis involved in the biosynthesis of bioemulsifiers and the formation of phenomenal stable emulsions are also discussed in the present study.

[Keywords: marine bacteria, bioemulsifiers, stable emulsion, emulsification index]

Introduction

Coastal areas in general, are the potential source for the microbes. Microbes are well known for their varied bioactive properties, which include production of secondary metabolites, highly thermostable enzymes and bioactive compounds[1]. Human beings in their day today life use emulsifiers in different forms and most of the materials of human use contain emulsifiers. Though the potency of the synthetic emulsifier is well known their non-degradable nature necessitates the high need for eco-friendly and environmental benign bioemulsifiers. Bioemulsifiers are generally amphipathic molecules, grouped as (i) low molecular weight compounds such as glycolipids, fatty acids, phospholipids, and lipopeptides, which lower the interfacial tension between hydrophobic liquids and water and reduce the energy required for emulsions formation and (ii) polymeric bioemulsifiers, which stabilize the emulsions[2-4].

Mostly, bioemulsifiers are insoluble in water reduce the interfacial tension and surface tension equivalent to synthetic surfactants and also play a major role in hydrocarbon degradation. According to Iguchi[5], Rapp[6] and Itoh and Inoue[7] bioemulsifiers emulsify hydrocarbons to sub-micron droplets, which are then utilized by the microorganisms. Some of the microorganisms produce emulsifiers that make the cell surfaces hydrophobic which enhances hydrocarbon uptake[8].

With regard to bioemulsifiers production, few reports are available for the production without hydrocarbons using terrestrial bacterial species and only scanty information on yeast[9]; *Planococcus* sp. (marine origin[10]) and *Pseudomonas*[11]. However, there are few reports on marine microbial isolates, produces bioemulsifiers without hydrocarbons. Attempts had been made in the present study to screen marine bacterial isolates for the production of bioemulsifiers without supplementing hydrocarbons and further examined the composition, emulsification property and stability of emulsion with hydrocarbons (aliphatic and aromatic). It also consist explanation on biosynthesis of bioemulsifiers in the absence of hydrocarbons and the reasons for stability of emulsion.

Materials and Methods

Marine bacterial species isolated from sea sediments of coastal region of Tamil Nadu, Chennai, India using zobell marine agar (Hi media, Mumbai, India), as per the standard procedure (serial dilution method) employed for the culturing of pure organisms. About eighteen isolates designated as ETW– 1; ETW–2, ETW-3, ETW- 4P, ETW–5P, ETW–6, ESS-1, ESS-2g, ETS-1, ETS- ZA, ETS- 13, ETS-14, ETS-OR, ESW–NA12S, ESW–NA, KSW–ZA, KSW-5g, KSW-10; (ETW - corresponds to samples collected from Ennore coastal area; ESS-corresponds to samples of

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Mamallapuram coastal area; ETS-corresponds to Cuddalore coastal area; ESW-corresponds to Mandapam coastal area; KSW-Kalpakkam coastal area) were screened and stored at 4°C until use.

Bioemulsifiers production carried out in 1000 ml Erlenmeyer flask containing 200 ml of zobell marine media (Hi media, Mumbai, India). The medium was inoculated with 10^7-10^9 cells/ml of pre-grown chosen isolates in zobell marine broth and incubated at 37°C under shaking condition at 180 rpm for 48 h. Cells were removed after incubation by centrifugation at 10,000 rpm for 10 min at 4°C. To cell free broth equal volume of ice-cold ethanol was added for precipitation. Resultant precipitate was separated and dialyzed against water. The dialysate was considered as partially purified bioemulsifiers and used for the following studies.

Interfacial tension of cell free broth and partially purified bioemulsifiers assessed using GBX-3S tensiometer (DM) at room temperature (modified method of Anita *et al*[10]) by dipping the platinum-iridium ring into the aqueous sample (2 mg/ml), layering an equal volume of hydrocarbon (5 ml) on the surface. Hexane (petroleum fraction, density 0.665; (analytical grade)) was used as hydrocarbon. Results on Interfacial tension measurements were expressed as milli Newton per meter (mN/m) in SI units.

Assessment of bioemulsification activity, Emulsification index (E_{24}) and stability of emulsion

To 1.0 ml of hydrocarbon (heptadecane), added 10 mg of partially purified bioemulsifiers obtained from the chosen isolates and vortex for 30 min. Turbidity of the resultant solution measured at 600 nm after 30s using UV-visible spectrophotometer (Shimadzu, Japan). One unit of emulsifying activity defined as an increase in absorbance of 0.1 with respect to the quantity of the bioemulsifiers added.

To assess the emulsification index, to 1.0 ml of cell free broth taken in the test tube containing 1.0 ml of various hydrocarbons (oils of sesame, groundnut, sunflower, soybean, rice bran), benzene, toluene and kerosene in individual tubes, vortex for 30 min and kept at room temperature. Emulsification index (E24) calculated after 24 h, by measuring the height of emulsion layer with respect to original volume (modified method of Cooper *et al*[12]).

For the assessment of stability of emulsion, emulsions obtained from above experiments kept at room temperature for the period of 0-100 d at room temperature. Visual observations made for any phase separation such as, flocculation, creamy and coalescence. Samples exhibiting nil phase separation were considered as high stable emulsion.

Characterization of water-soluble bioemulsifiers

To assess the composition of bioemulsifiers obtained, samples are subjected to both qualitative and quantitative estimation of carbohydrates proteins and phospholipids according to the standard procedures followed.

Results and Discussion

A wide variety of microorganisms; viz., bacteria, yeast and fungi produce bioemulsifiers and the majority share goes to bacterial species[13]. Microbial emulsifiers are important secondary metabolites produced by both marine and non-marine microbes. Out of marine microorganisms, *Acinetobacter* sp. is well studied for its bioemulsifiers production. However emulsifiers produced by a large number of terrestrial organisms are in reports[14]. In the present study, about 18 morphologically distinct bacterial strains isolated from sea-sediments collected at various places of Tamil Nadu for their bioemulsifiers production. Of these, 6 were isolated from the Ennore coastal area (ETW samples), 2 from Mamallapuram coastal area (ESS samples), 5 from Cuddalore coastal area (ETS samples), 2 from Mandapam coastal area (ESW samples) and the remaining 3 from Kalpakkam coastal area (KSW samples). All 18 bacterial strains grew well on zobell marine agar media, compared to nutrient agar (prepared in sea water) and classified as moderately halophilic, based on Larsen[15] report. With reference to gram staining, 16 isolates are Gram-positive (*Bacillus* genera) and the rest two are Gram-negative (*Pseudomonas* genera). Bicca *et al*[16] reported, most bacteria isolated from the sites with a history of contamination by oil or its byproducts are gram-negative and this may be the characteristic that contributes to the survival of these populations in such harsh environments. Yim *et al*[17] employed zobell media for the production of exopolysaccharide with emulsifying ability using marine *Alteromonas sp*. strain 00SS11568. Anita *et al*[10] reported bioemulsifiers production by marine isolates grown in zobell media. In the present study, we observed bioemulsifiers production by the chosen marine isolates in heterotrophic bacterial media (zobell marine media).

With regard to bioemulsifiers production, most of the reports suggests, necessity of hydrocarbon supplementation to induce the production. Phetrong *et al*[18] reported *Acinetobacter calcoaceticus* RAG-1 produced bioemulsifiers when grown in *n*-hexadecane and ethanol-containing medium. Zinjarde and Pant[19] reported Yarrowia lipolytica NCIM 3589 produced bioemulsifiers in the presence of *n*-hexadecane or crude oil. Sarubbo *et al*[20] reported *Candida glabrata UCP 1002* produced an extracellular emulsifier in the presence of cottonseed oil. In the present study, bioemulsifiers production observed without hydrocarbon supplementation. Similar observations made by Sarubbo *et al*[9] with *Candida lipolytica,* which produces bioemulsifiers with water-soluble substrate as the carbon source.

Regard to recovery of bioemulsifiers, acetone[21], ammonium sulfate[22], methanol and ethanol precipitation[23] are in use. In the present study we used ethanol because of the appreciable bioemulsifiers yield irrespective of the species. Table 1 demonstrates yield of bioemulsifiers and the bioemulsifying activity of 18 marine bacterial species. Of the 18 isolates only six isolates exhibit appreciable bioemulsifying activity as well as the yield. The maximum yield of 25.0 g/l (wet weight) exhibited by the isolate ETW-5P isolated from coastal areas of Ennore, without any hydrocarbon supplementation. Shepherd *et al*[24] reported bioemulsifiers yield of 0.26 to 0.93 g/l by yeast *Candida utilis*, while the yield of extracellular emulsifying agent from *Curvularia lunata* reported as 2.6 g/l[25].

Results on carbohydrate, protein and phospholipid analyses of water-soluble emulsifiers reveals, the obtained emulsifiers contain maximum percentage of oligopeptides of (-leu-isoleu-ala-met-) followed by repeating units of deoxymannose with phosphate moiety. No evidence for phospholipids existence in the product. Thus, the product obtained was grouped as carbohydrate polymer with oligopeptides linked with phosphate group. Based on these results, the structural elucidation of bioemulsifiers made and illustrated in Figure 1.

Table 1 summarizes the interfacial tension analysis of bioemulsifiers produced by the entire 18 marine bacterial isolates. Similar to bioemulsifiers production only six isolates reduces the interfacial tension (22-32.5 mN/m). Reduction in interfacial tension of 10-45.5 mN/m reported by bioemulsifiers of terrestrial hydrocarbon degrading organisms, *Rhodococcus erythropolis*[25]; *Mycobacterium* sp.[12]; *Pseudomonas fluorescens*[6]; *Alcaligenes faecalis* respectively. In addition, reduction of interfacial tension to 1.8 mN/m observed by Abu-Ruwaida *et al*[3] with *Rhodococcus* sp. With regard to marine microbial sources, bioemulsifiers of *Planococcus maitriensis* reduces the interfacial tension of hexane from 45.5 to 20.9 mN/m[10].

Examination of emulsification activity, emulsification index and stability of emulsion reveals that emulsion formation and stability of emulsion was high irrespective of the bioemulsifiers of the bacterial species. Figure 1(a-g) illustrates the emulsification index of six potent bioemulsifiers of carbohydrate-protein polymer. The emulsification index of six bioemulsifiers for vegetable oils determined as 85-95%. However, with kerosene, the percentage of emulsification index observed was only < 75%.

With regard to stability of emulsions, we observed, emulsion formed with vegetable oils are more stable (more than 90 d) than emulsion formed with kerosene (only up to 30 d). Anita *et al*[10] reported stability of emulsion formation with cedar wood oil using crude exopolymer emulsifier upto 45 d. However Haba *et al*[26] observed stable emulsion with kerosene for bioemulsifiers of *Pseudomonas aeruginosa* 47T2 for 90 d. Figure 2 depicts stability of emulsion (without emulsifiers; with synthetic emulsifiers and with bioemulsifiers of ETW-5P) after 90 d of incubation at room temperature. We observed, stability of the emulsion with bioemulsifiers is on par with synthetic surfactants without any phase separation. In general, stability of emulsion depends on various physical and biochemical factors: physical factors include; (i) size (ii) shape (iii) distribution of emulsion (iv) external force (agitation/vortex) (v) pH (vi) ionic strength (vii) water hardness (viii) volume of the hydrocarbons (ix) concentration of bioemulsifiers and finally the (x) metal ions. With regard to biochemical factors, (i) nature (ii) structure and (iii) molecular weight of the emulsifier; (vi) electrostatic and (vii) steric forces (viii) exo-cellular components (including proteins and enzymes) (ix) unspent nutrients and finally the (x) temperature, etc.

In the present study, the high stability in emulsion observed upto more than 90 d in all the hydrocarbons containing fatty acids, could be due to one of the factors explained above. Further, it is not very clear,

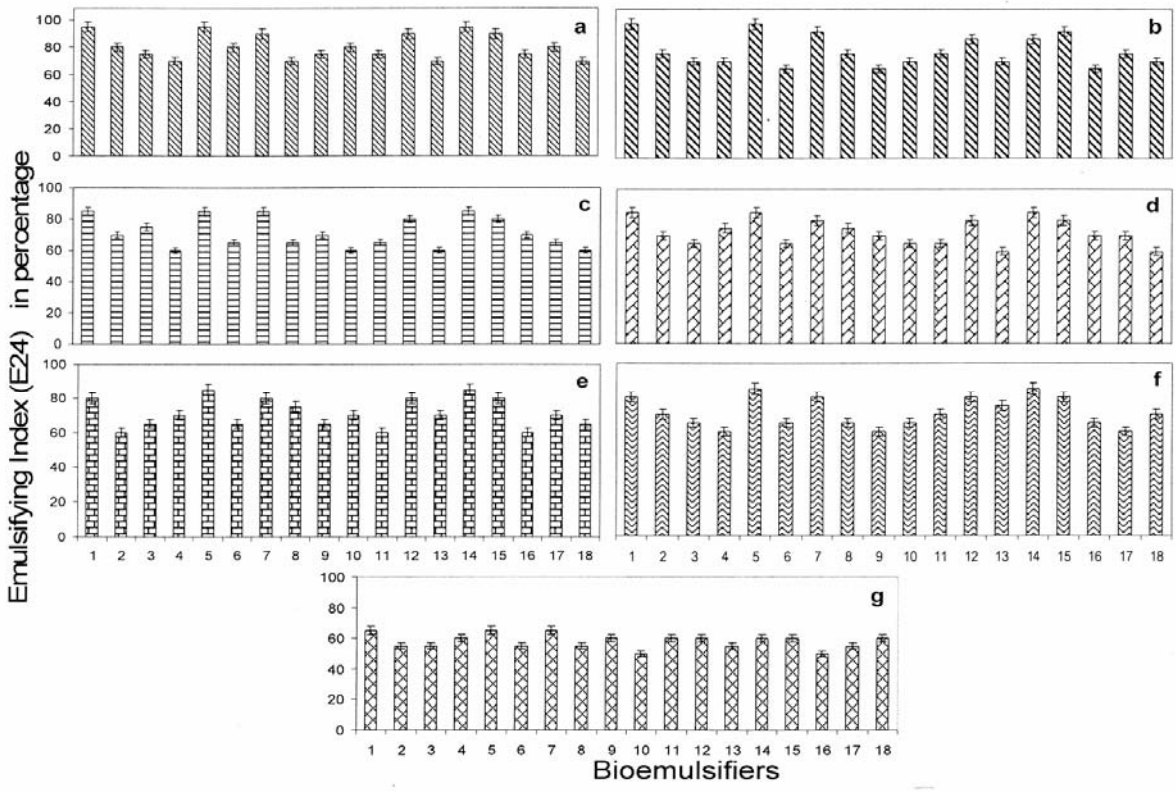

Figure 1—Emulsifying index of the bioemulsifiers of marine microorganisms with various hydrocarbons. a-soybean oil; b-sunflower oil; c-olive oil; d-peanut oil; e- sesame oil; f- rice bran oil; g-kerosene

Table 1—Yield, bioemulsifying activity and interfacial tension of bioemulsifiers of isolated 18 marine bacterial species

Sample code	Name of isolates	Yield (g/l)*	Bioemulsifying activity (U/ml)*	Interfacial tension (mN/m)*
BS-1	ETW-1	20 ± 1	13.5 ± 1.4	25 ± 2
BS-2	ETW-2	10 ± 2	4.52 ±0.8	38 ± 2
BS-3	ETW-3	10 ± 2	4.61 ±1.2	39 ± 2
BS-4	ETW-4P	12 ± 2	6.65 ±1.0	35 ± 4
BS-5	ETW-5P	25 ± 2	15.0 ±1.2	22 ± 2
BS-6	ETW-6	10 ± 2	4.59 ±1.2	38 ± 2
BS-7	ESS-1	22 ± 1	14.3 ±1.6	24.5 ± 4
BS-8	ESS-2g	12 ± 1	5.71 ±1.8	33 ± 2
BS-9	ETS-1	10 ± 1	4.34 ± 1.4	40 ± 1
BS-10	ETS-ZA	10 ± 2	4.57 ± 1.4	39 ± 2
BS-11	ETS-13	10 ± 1	4.48 ± 1.2	40 ± 2
BS-12	ETS-14	18 ± 1	12.06 ± 1.2	31 ± 1
BS-13	ETS-OR	8 ± 2	3.98 ± 1.4	42 ± 1
BS-14	ESW-NA12S	20 ± 1	9.34 ± 1.4	32.5 ± 2
BS-15	ESW-NA	20 ± 1	9.66 ± 1.4	32.1 ± 2
BS-16	KSW-ZA	10 ± 2	4.14 ± 1.6	41 ± 2
BS-17	KSW-5g	10 ± 2	4.82 ±1.2	37 ± 2
BS-18	KSW-10	10 ± 1	3.95 ± 1.4	42 ± 2
Standard	Hexane-water	-	-	46 ± 2

* - mean ± SD of triplicates

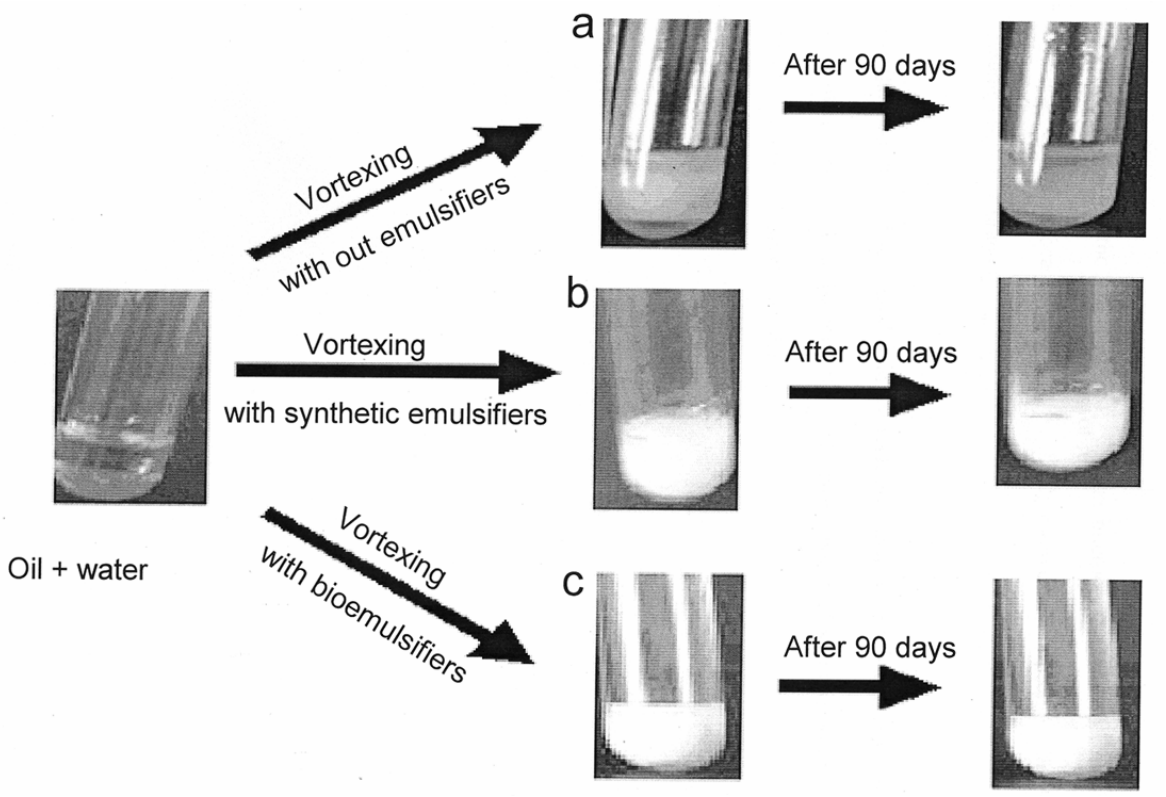

Figure 2—Comparison of emulsion stability of (a) without emulsifier; (b) with synthetic emulsifiers and (c) with bioemulsifiers of marine bacterial species, observed before and after 90 days of incubation

which factors play the major role and nevertheless, water-soluble nature of the bioemulsifiers of six isolates may contribute to the stability. Characterization of bioemulsifiers produced by the chosen isolates reveals, soluble nature of the emulsifier might be due to polymeric nature of protein carbohydrate complex, ie., presence of mannose and rhamnose as backbone with leu-isoleu-ala-met- peptide linkage. Though both carbohydrate backbone and the amino acids link are grouped under hydrophobic, however, exhibit hydrophilic activity and thus makes the bioemulsifiers water-soluble. In addition, the reduced level of interfacial tension compared to bioemulsifiers of other bacterial species may also be taken for consideration. Nevertheless, still more elucidation is required to confirm the structure and property of the bioemulsifiers produced by the chosen marine isolate.

With regard to water-soluble bioemulsifiers production by the chosen marine isolates in the absence of hydrocarbons, the question on why and how these isolates produce bioemulsifiers without any inducers. Since, marine sources are more vulnerable to oil spills and waste dumping, and have the opportunity to thrive in the hydrocarbon enriched environments. When these isolates brought and cultured under laboratory conditions, their growth and metabolism is non-dependent on the external nutrients. Further, during microbial growth, in general, organisms synthesize (de novo synthesis) carbohydrates, lipids, proteins and enzymes (nothing but biocatalysts) with the help of nutrients supplemented in growth media. According to Desai *et al*[27], biosynthesis of bioemulsifiers mediated by (i) induction; (ii) repression (iii) nitrogen and multivalent ions. Though these statements are coincidence with the reported findings, however, in our study, bioemulsifiers production is not induced or repressed and the produced bioemulsifiers is soluble in water. Explanation on biosynthesis of water soluble bioemulsifier is summarized by four different sequential processes, viz., (i) extrusion of cell wall, results with micelle formation followed by separation of micelle in to hydrophobic and hydrophilic groups by catabolic mechanisms; (ii) de novo synthesis of intracellular bioemulsifiers and expelled out by exocytosis; (iii) grouping of separated hydrophilic and hydrophobic (fatty acids) components

of (i) and (iv), carbohydrates, proteins, metal ions, biocatalysts, etc, (iv) synthesis of fatty acids by carbohydrate metabolism (lipogenesis) results with the extracellular bioemulsifiers production. These explanations suggest the high possibility of extracellular bioemulsifiers existence without any hydrocarbon induction during the growth of marine bacterial isolates.

Conclusion

Emulsifying activity of marine bacteria assessed in the present study implies isolates of Tamil Nadu coastal region able to exhibit bioemulsifier production without hydrocarbon induction. Moreover, the produced bioemulsifiers are water-soluble and exhibit high emulsification index with aliphatic hydrocarbons. High percentage of emulsion index might be due to the presence of fatty acids and the high stability could be due to the presence of carbohydrate backbone with polymeric protein linkage in the structure of the bioemulsifiers. The yield and potency on emulsification property of the bioemulsifiers of marine bacteria suggests the possibility of exploitation of marine bacteria in the production of bioactive compounds.

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