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Studies on the bioemulsifier production by a bacterial isolate

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Bioemulsifiers have gained a lot of importance in biodegradation, bioremediation, medical, pharmaceutical and food industries due to their non-toxicity. In the present study bacteria producing bioemulsifier were isolated from mangroves and saltpans on mineral salt medium (MSS) containing crude oil as sole carbon source. Amongst the 19 isolates picked up to screen for bioemulsification activity, 9 isolates showed potential bioemulsification activities which were in the range of 2.0-45.5 % for pellets while 1.7-56.5% for supernatants. Two isolates MS-3 and MS-18 that showed the highest Emulsification index with supernatant were selected and identified based on biochemical characteristics as *Aeromonas* (MS-3) and *Vibrio* (MS-18) species. The isolate MS-3 was selected for detailed studies. The bioemulsifier was isolated, purified and characterised using TLC, FTIR and found to be glycolipid in nature. The critical micelle concentration (CMC) of the present emulsifier was found to be 12 mg/ ml with xylene as the hydrocarbon.

Introduction

Bioemulsifiers are low molecular weight amphiphilic compounds. They show broad chemical diversity and can be glycolipids, phospholipids, lipopeptides, polysaccharide protein complexes, lipopolysaccharides, neutral lipids, etc [1, 2]. They may/ may not lower surface or interfacial tension but aid in emulsification by micelle formation when grown in aqueous media with hydrocarbon in it [3- 5]. Many microorganisms such as *Acinetobacter*, *Pseudomonas, Bacillus,Micrococcus* etc. are known to produce bioemulsifying polymers [6 10]. Bioemulsifiers produced by microorganisms have distinct advantage over their commercial counterparts as they are less toxic, biodegradable and effective at wide ranges of pH, salinity and temperature [11, 12]. Thus they have gained immense importance in recent years and become indispensable in food, cosmetic, petroleum, pharmaceutical industries, environmental bioremediation, agriculture and bioprocessing [7, 13-18]. Studies on bioemulsifiers have shown large number of organisms capable of producing such compounds, however ecosystems from where such bacteria are isolated are also important in view of novel resources. In the present study, it was envisaged to isolate bacteria from mangrove swamps and saltpans to assess their capacity to produce bioemulsifier in the presence of hydrocarbons. This paper describes the isolation of such bacteria and purification and characterisation of the potential bioemulsifier produced by the promising isolate, *Aeromonas* sp.

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Materials and methods

Isolation of bacterial isolates and screening for potential bioemulsifier producing isolates

Bacterial cultures were isolated from water and soil samples from mangroves [15] and saltpans on Salt nutrient agar (SNA), 15% NaCl- tryptone- yeast extract agar (NTYE) and 25% NTYE .The medium SNA contained 500ml of mineral salt solution (MSS) agar and 500 ml of nutrient agar (HiMedia). The mineral salt solution (MSS) agar contained per 1000 ml KH_2PO_4 20 g, K_2HPO_4 5.0 g, $(NH_4)_2$ SO_4 30 g, NaCl 0.1 g, $FeSO_4$ $7H_2O$ 0.01 g, $MgSO_4$ $7H_2O$ 0.2 g, $CaCl_2$ $2H_2O$ 0.01 g, $MnSO_4$ $7H_2O$ 0.002 g, Yeast Extract 0.03% , Glucose 0.03% and agar 20 g, with pH 7.2 [18]. 15% NTYE and 25% NTYE agar medias contained per 100 ml $MgSO_4$ $7H_2O$ 2 g, KCl 0.5 g, $CaCl_2$ $2H_2O$ 0.02 g, Yeast extract 0.3 g, Tryptone 0.5 g and Crude salt 15/25 g, with pH 7.0. Potential bioemulsifier producing bacteria were selected by growing the isolates on 10 ml of Mineral salt solution (MSS) with 0.2 ml of crude oil as carbon source under shaking conditions (REMI, Orbital Shaking Incubator, CIS 24 BL) at 28°C. Growth was monitored every 24 h for 14 days (2 weeks) visually.

Response of the selected isolates to varying concentrations of NaCl in presence of hydrocarbon

The selected isolates were grown on liquid MSS broth with 2% crude oil, MSS agar with 15% NaCl and 25% NaCl with 0.5% crude oil, as the sole source of carbon. Isolates were also plated on SNA agar with 0.5% crude oil. The tubes and plates were incubated at 28°C for 5 days and the growth was inspected visually.

Studies on emulsification

The isolates were grown in 10 ml Salt Nutrient Broth (SNB contained 500ml of mineral salt solution (MSS) and 500 ml of nutrient broth (HiMedia) with pH 7.2) for 48 h at 28°C and centrifuged (Eppendorf centrifuge 5804R) at 9000 rpm for 10 minutes, and the emulsification activities of the supernatant and pellet were determined.

a) Determination of the Emulsification Activity (EA)/ Emulsification Index (EI)/ E24

Two ml of supernatant and pellet of each isolate was added with 2 ml of xylene (hydrocarbon) to each tube and vortexed for 2 minutes and allowed to settle for 1 h [19]. The pellet was suspended in sterile MSS medium and 2 ml of this suspension was added to the suspension tube followed by xylene as hydrocarbon [7, 20-22]. The tube was then vortexed using cyclomixer (REMI CM 101 cyclomixer) and allowed to stand for 10 mins. The height of the emulsified layer and the height of the liquid column were measured.

EA was calculated as

EA = Height of the emulsified layer in cm x 100
Total height of the liquid column in cm

b) Determination of type of emulsification

The supernatant and pellet were tested for type of emulsification. Set 1 had 2 ml of supernatant or pellet in sterile MSS medium with 2 ml of xylene and 1 drop of oil-o-red indicator while set 2 had the indicator crystal violet. The mixture was vortexed at high speed and allowed to settle for 1 h. The distribution of dye in the two phases was observed.

Identification and biochemical characterization of selected isolates:

The selected isolates MS-3 and MS-18 were tested for cultural morphological and biochemical tests and identified upto the generic level with reference to Bergey's Manual of Systematic Bacteriology[23]. The promising isolate, MS-3 was selected for further studies.

Relation between growth and emulsification index of MS-3

The isolate, MS-3 was grown on 200 ml of SNB Medium at 28°C under shaker conditions (REMI, Orbital Shaking Incubator, CIS 24 BL) at 200 rpm. Ten ml of sample was withdrawn every 2 h for 48 h and increase in the absorbance at 600 nm of the culture suspension was monitored using a UV-VIS spectrophotometer (Shimadzu). In order to measure the emulsification index the culture suspension was centrifuged (Eppendorf centrifuge 5804R) at 9000 rpm for 10 minutes and the initial turbidity of the supernatant was determined at 450 nm. The emulsification index (EI) of the supernatant was determined as above.

Extraction and purification of bioemulsifier

The culture was grown on 1000ml SNB medium in a 2000ml flask at 28°C for 48 h under shaking (REMI, Orbital Shaking Incubator, CIS 24 BL) at 200 rpm and centrifuged (Eppendorf centrifuge 5804R) at 9000 rpm for 10 minutes to obtain supernatant and pellet. 250 ml of ethyl acetate was added to the supernatant twice to extract bioemulsifier. The solvent layer was pooled and concentrated till the ethyl acetate evaporated leaving behind residue of solid crude bioemulsifier [19].

Characterisation of bioemulsifier

TLC and FTIR

TLC of the crude bioemulsifier was carried out by using the solvent system chloroform: methanol: acetic acid: distilled water (25:15:4:2). The spots were visualised using iodine vapours as the visualising agents for lipids [18, 27] and 1-naphthol and sulphuric acid reagent for sugars. The Rf values of the spots were recorded. The solid bioemulsifier was mixed with KBr and analysed using Fourier′s transform infrared spectroscopy (IR- Prestige by Shimadzu)[17, 21, 28].

Studies on critical miscelle concentration (CMC) of crude bioemulsifier

A known quantity of the crude bioemulsifier was dissolved in 10 ml distilled water and the CMC was calculated as the amount that gives the maximum emulsification with a known quantity of the hydrocarbon [4, 25, 26, 29]. Varying concentrations of the crude bioemulsifier were prepared using distilled water. Emulsification was checked and turbidity of aqueous layer was determined at 450 nm using the spectrophotometer.

Results and Discussion

Isolation of bacteria and screening for potential bioemulsifier producing isolates

Microbial isolates from mangroves and saltpans showed varied colony counts (Table: 1). It was noted that 15% NTYE supported the highest number of bacteria as compared to 25% NTYE and SNA agar.All isolated bacterial cultures were tested for their ability to grow in the presence of hydrocarbon. Out of 19 isolates, 17 isolates showed turbidity and visible reduction in hydrocarbon layer in 14 days. It was interesting to note that 15 from 17 isolates showed good emulsification with reduction in the crude oil layer and cell growth as determined by visual turbidity. However, 4 cultures showed no or poor growth both in terms of decrease in crude oil layer as well as turbidity in spite of incubating it for 10 days (240 h.). Such isolates have been reported earlier from similar hydrocarbon containing ecosystems [13, 16, 24]. Amongst the 15 isolates 9 isolates were selected based on their ability to exhibit good turbidity and visible reduction in the hydrocarbon layer within 48 -72 h.

Response of selected isolates to varying concentrations of NaCl in presence of hydrocarbon

The 9 selected isolates were screened for their sodium chloride tolerance at different concentrations. It was observed that all 9 cultures showed growth when the isolates were grown on MSS broth with 2% crude oil. However, only 6 isolates showed growth on MSS agar with 15% NaCl and 0.5% crude oil, while none of the isolates showed growth on MSS agar with 25% NaCl and 0.5% crude oil.

Studies on emulsification

Emulsification index and type of Emulsification of the selected isolates

The type of emulsification of the 9 isolates was determined and 3 isolates showed good turbidity, however no change was observed in distribution of oil-o-red dye between the aqueous and the organic layer. Hence all isolates showed oil-in-water emulsification. Interestingly, 8 isolates decolourised crystal violet instead of distribution. It is therefore recorded that the emulsification occurs between the interphase layer of aqueous and organic layer. Such emulsification activity can be assayed by using the central layer height which increases with the increase in emulsifier concentration. Eight isolates showed good turbidity for both the pellet and supernatant and 7 decolourised crystal violet. Two isolates showed poor turbidity and retained the colour of crystal violet. Earlier studies also reported similar results where dyes were used to know the nature of emulsification [32, 33].Out of 9 isolates tested for emulsification, 2 isolates viz; MS-3 and MS-18, exhibited the highest emulsification index of 56.6% and 10.4% respectively with the supernatant. These were selected for further studies. These isolates also showed the oil-in-water type of emulsification and decolourisation of crystal violet dye [22, 26, 31].

Table 1—Total viable counts of bacteria from mangroves and saltpans

SAMPLE	COUNTS cfu/ml		
MANGROVES	15% NTYE AGAR	25% NTYE AGAR	SNA AGAR
SEDIMENT	1.324×10^6	0.4×10^3	87.36×10^3
WATER	2.9913×10^6	0.2×10^3	9.77×10^3
SALTPANS			
SEDIMENT	5.4×10^3	1.3×10^3	76.711×10^3
WATER	3.348×10^6	0.1×10^3	2.942×10^3

Identification and biochemical characterisation of bioemulsifier producing isolates

The 2 cultures selected for further studies based on their emulsification activity i.e. isolates MS-3 and MS-18 were subjected to biochemical and physiological characteristics and tentatively identified up to generic level based on Bergey's Manual of Systematic Bacteriology. The isolate MS-3 was tentatively identified as *Aeromonas* and isolate MS-18 as *Vibrio*. Earlier studies have shown hydrocarbon degrading *Aeromonas* and *Vibrio* isolated from the marine environment and soil sediments.[34- 36]. Thus the isolates in the present study differ from the earlier reports due to their ecosystem.Further studies were carried out using MS-3 (*Aeromonas*) due to its high emulsification index and decolourisation activity.

Growth curve of MS-3

A typical bacterial growth curve pattern of the isolate MS-3 was seen (Fig: 1a). The lag phase was absent owing to inoculation with actively growing culture. The exponential phase ended at 16 h where the stationary phase began and continued up to 32 h and thereafter there was a steady decline indicating that the culture has entered the death phase. The graph of time v/s emulsification index in Fig: 1b showed

that the emulsification index remained steady. Fig: 1c indicated the reduction in turbidity of supernatant with growth and it was seen that the turbidity gradually decreased in 48 h. The decrease at first was slow and the pace then increased after 16 h [37- 40]. It was interesting to note that the turbidity of supernatant reduced from 0.3 to 0.15 during growth as the emulsification index increased of the supernatant. This indicates that the bioemulsifier is produced after 10 h after which turbidity of supernatant starts decreasing.

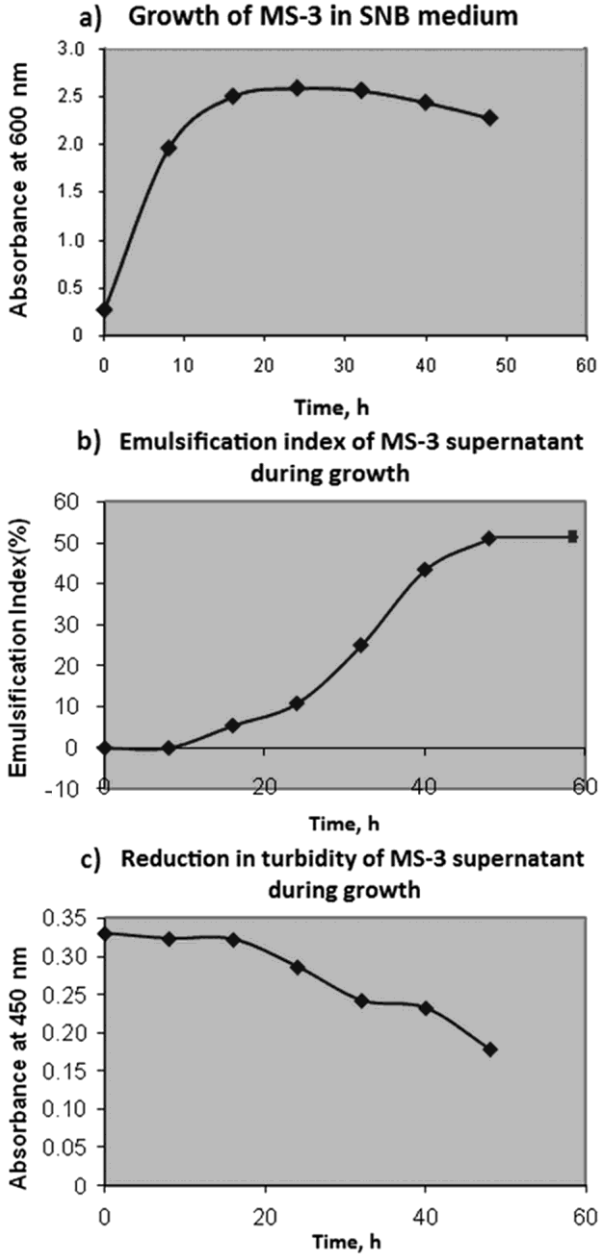

Fig. 1—Relationship between growth, emulsification index and reduction in turbidity of the supernatant of MS-3

Studies on critical miscelle concentration (CMC) of crude bioemulsifier

CMC is an important criteria in understanding efficiency of bioemulsifier [4, 25, 41, 42]. It is reported that a low concentration giving a CMC is more potential for application in environmental bioremediation studies and processes [41, 42]. In the present study CMC of the crude bioemulsifier (Fig: 2) was determined. It was interesting to note that CMC of both was at a concentration of 12 mg/ ml of the bioemulsifier with absorbance at 450 nm.

Characterisation of bioemulsifier

Specific groups of bioemulsifier can be identified using chromatographic and spectroscopic techniques [5, 17, 18, 21, 28, 43]. In the present study the extracted bioemulsifier was subjected to TLC and FTIR. TLC of crude bioemulsifier for lipids showed a spot at Rf of 0.6 and a spot at 0.25 for sugars correlating this with reported data [18]. Studies on bioemulsifier from Iranian soils have been reported and the bioemulsifier was characterised as glycolipid based on TLC. The FTIR profile of the crude bioemulsifier showed a broad peak between wavelengths of 3400-2700 cms^{-1} indicating –OH group.Sharp peaks were obtained between 2900-2800 cms^{-1} showing C-H stretching and between 1700-1600 cms^{-1} indicating the presence of C=O stretching. It is therefore indicated that the bioemulsifier is a complex acid having a carbohydrate moiety which confirms the results of TLC. Bioemulsifiers are generally categorised by their chemical composition and microbial origin. Major classes of low molecular mass bioemulsifiers are glycolipids, lipopeptides and phospholipids. These efficiently lower surface and interfacial tension. The high molecular mass bioemulsifiers include polymeric and particulate surfactants and are more effective as emulsion stabilising agents. Most bioemulsifiers are

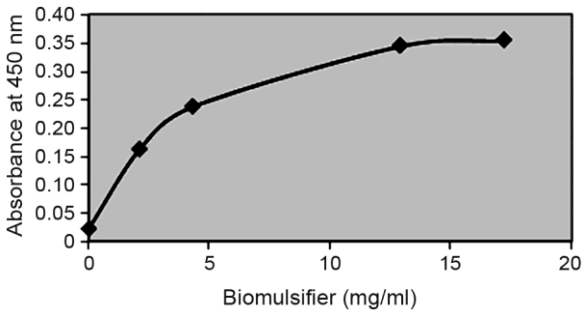

Fig. 2—CMC of crude bioemulsifier isolated from supernatant of 48 h old culture MS-3

either anionic or neutral (amphiphilic) with the hydrophobic moiety based on long chain fatty acids or fatty acid derivatives and the hydrophilic portion being a carbohydrate, amino acid, phosphate or cyclic peptide. In the present study we found that the bioemulsifier was a glycolipid. A number of glycolipids are reported such as rhamnolipids of *Pseudomonas* sp, trehalolipids of *Mycobacterium* sp *Nocardia* sp *Corynebacterium* sp, etc. Such glycolipids find potential applications in clinical biology as antimicrobial and antiviral agents, bioremediation of hydrophobic contaminants like poly aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), removal of heavy metals from contaminated soils and genetic engineering [44, 45].

Conclusion

Microorganisms are known to produce bioemulsifiers in presence of complex carbon sources such as hydrocarbons. These organisms adapt and survive in stressful environments and their occurrence in mangroves and saltpans was investigated. The study showed that emulsification index increased as the culture entered the stationary phase. The CMC of crude bioemulsifier was found to be 12 mg/ml. The TLC for detecting lipids and sugars showed brown and reddish-purple spots respectively. The TLC and FTIR suggest that the bioemulsifier is a glycolipid as it contained both sugar and lipid moieties. The isolates from the present studies can be used for environmental bioremediation of oil spill and in estuarine, river and mangrove areas.

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