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RESEARCH ARTICLE

ISOLATION AND SCREENING OF MARINE BACTERIAL STRAINS FOR BIOSURFACTANT PRODUCTION

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ABSTRACT

Surfactants are the amphiphilic compounds which reduce surface tension between molecules. In the past decades, such compounds were reported to be produced on the surface of living organisms, especially microorganisms and are termed biosurfactants. These compounds are used for a variety of industrial purposes because of their varied advantages such as biodegradability, renewability, functionality under extreme conditions etc. The main areas of usage include oil and petroleum industries, pollutant site remediation, detergent formulations, cosmetics, food additives, pharmaceutical industry etc. Though these are promising compounds, the large-scale industrial production is difficult due to material costs, processing costs and manufacturing output. The work is done to identify and isolate microorganisms from marine system with a view to carry out in depth studies on the activity of the isolated strains and ways by which these strains can be used as economically viable sources for biosurfactants. Twelve strains were isolated from the marine water sample. Eight strains showed growth in oil supplemented medium. Four of these strains showed slightly higher growth than the rest of isolates. Emulsification index was calculated for these strains and two strains, Tb1 and Tb3 showed maximum emulsion index in both kerosene and diesel. But none of the tested strains showed glycolipid production.

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INTRODUCTION

Biosurfactants are surface active compounds, produced extracellularly by bacteria, fungi and yeast, (Sen, 2010). These are complex molecules which include lipopeptides, glycolipids, polysaccharide protein complexes, fatty acids and phospholipids (Desai and Banat, 1997). These compounds have shown to have numerous applications in industries such as additives in food (Maria *et al*., 2009), cosmetics, detergent formulations, waste water treatment (Banat *et al*., 2010 and Damasceno *et al*., 2012), lubrication, softening, making emulsions, biomedical and pharmaceutical industries etc. In the recent time, biosurfactants have gained importance because of their unique properties such as biodegradability, low toxicity, ecological acceptability and ability to be taken from renewable and cheaper substrates, (Mohan *et al*, 2006) and their functionality under extreme conditions. Hence they find their use in crude oil recovery and pollution site remediation. Enhanced oil recovery, crude oil drilling, lubricants, and bioremediation of environmental pollutants, health care, food processing, medical applications as adjuvants and as antimicrobial biocontrol agents are some other applications of biosurfactants. But currently the industrial production of this category of compounds is difficult due to the associated raw material cost, high processing cost and low manufacturing output, (Henkel *et al*, 2012). Therefore, identification of new and better sources of biosuractants proves to be of importance. Studies have shown that biosurfactants are found on the cell surface of a variety of microorganisms. These compounds help the microbial cells by increasing the bioavaiability of poorly soluble PAHs and resins, (Olivera, *et al*., 2003). Microorganisms, particularly marine have shown to have numerous potentials in terms of production of enzymes and bioactive metabolites (Lekshmi and Ayona, 2014). This is because of the fact that marine microorganisms have developed unique metabolic and physiological capabilities to thrive in extreme habitats and produce novel metabolites which are not often present in microbes of terrestrial origin (Fenical, 1993). Marine microbes act as are potent source of various industrially important bioactive compounds include enzymes, exopolysaccharides (EPS), biosurfactants, antibacterial, antiviral, anticancer compounds etc. This study was conducted

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with an intention of identifying and isolating biosurfactant producing microorganisms of marine origin and assessing their potential.

MATERIALS AND METHODS

Sample collection

Marine water samples for the isolation of bacterial strains were collected from Sangumugham, Vizhinjam and Veli coast, Thiruvananthapuram, Kerala. The samples were collected in sterile bottles and were brought to laboratory maintaining standard conditions and refrigerated.

Enrichment and Isolation of bacterial strains

Microorganisms from marine samples were enriched in marine Zobell broth and incubated at 30°C for 2 days in shaker at 200 rpm. Isolation of bacteria was done by the serial dilution and pour plate technique. A loopful of inoculum from the marine Zobell broth was streaked onto the marine zobell agar and incubated at 30°C for 2 days. Single, separated colonies were selected and the subcultures were maintained on marine zobell slants at 4°C until further use.

Screening of biosurfactant producing bacterial strains

Primary screening of biosurfactant producing bacterial strains were done by inoculating the isolated bacterial strains in 100 ml of Marine Zobell Broth containing 2 to 3 drops of mixtures of oils (petrol, diesel, kerosene (P+K+D)) in equal amounts, and incubated with continuous shaking (200 rpm) one week at room temperature using a shaker. After that the growth of the bacterial strains were observed by measuring absorbance of the culture at 600 nm. The strains possessing biosurfactant producing activity was identified, as evidenced by emulsification of oil and maximum growth and were chosen for further experimentation (Dhail 2012).

Emulsification Index

Several colonies of pure culture were suspended in test tubes containing 2 mL of mineral salt medium. After 48 h of incubation, 2 mL hydrocarbon (oil) was added to each tube. Then, the mixture was vortexed at high speed for 1 min and allowed to stand for 24 h. The emulsion index was calculated. It (E24) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100 (Bodour *et al*., 2004).

Emulsification index (E24) = (Height of the emulsion layer/ Total height) X100

Blue Agar Plate Method

Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic biosurfactant (Satpute *et al*., 2008). Thirty microlitre of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm). The plate was then incubated at 37°C for 48-72 h. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production.

Emulsification Assay (EA)

Microorganisms were cultured overnight in 10 ml ZMB in 100 ml Erlenmeyer flasks at 28°C and 200 rpm. Cultures were centrifuged at 10,000 rpm at room temperature for 15 min. Three millilitres of supernatant was mixed with 0.5 ml of petrol. This mixture was mixed vigorously for 2 minutes. This mixture was left undisturbed for one hour at 28°C to separate aqueous and oil phases. Aqueous phase was removed with the help of 1 ml micropipette and absorption was measured. Uninoculated broth was taken as blank. Absorbance of aqueous phase was measured by using a spectrophotometer at wavelength of 400 nm. Emulsification activity per ml (EU/ml) was calculated by using the formula, 1 Emulsification unit = 0.01 x Dilution factor (Satpute *et al*., 2008)

RESULTS AND DISCUSSION

Screening of Biosurfactant producing bacterial strains

Primary screening of biosurfactant producing bacterial strains was done by inoculating all the isolated strains in Zobell marine broth added with mixture of oils such as petrol, kerosene and diesel. The growth of the strains after incubation was measured in terms of cell OD taken at 600 nm and the results are shown in Table 1.

Table 1. Primary screening of Biosurfactant producing bacterial strains

Bacterial strains	Growth in terms of cell density (OD at 600 nm)
Ab1	Nil
Ab2	Nil
Ab3	0.004
Ab4	0.114
Bb1	Nil
Bb2	Nil
Bb3	0.028
Bb4	0.124
Tb1	0.208
Tb2	0.005
Tb3	0.126
Tb4	0.004

The results revealed that out of the 12 strains 8 (Ab3, Ab4, Bb1, Bb3, Bb4, Tb1, Tb2, Tb3 and Tb4) strains showed growth in the medium added with oil. Strains Ab1, Ab2, Bb1 and Bb2 did not show any growth in the oil containing medium. Out of the 8 strains, 4 strains: Ab4, Bb4, Tb1 and Tb3 showed slightly higher growth when compared to other strains (0.114, 0.124, 0.208 and 0.126 respectively). Other strains showed only a moderate growth rate which is negligible. So the bacterial strains which showed considerable growth rate were further selected for the screening tests for biosurfactant production.

Emulsification Index

The bacterial strains which showed maximum growth in the hydrocarbon added media were used for finding the

biosurfactant activity by calculating the emulsification index using kerosene and diesel and the results are shown in Table: 2

Table 2. Emulsification index shown by the bacterial strains

Bacterial strains	Emulsification index (E24) in kerosene (%)	Emulsification index (E24) in Diesel (%)
Ab4	26	22
Bb4	29	23
Tb1	59	62
Tb3	48	51

The results revealed that among the strains, Tb1 and Tb3 showed maximum emulsion index in both kerosene and diesel where as the strains Ab4 and Bb4 showed less emulsion index which revealed that both the strains are least efficient in biosurfactant production.

Blue Agar Plate Method

Those strains which showed maximum growth in the primary screening were selected to test their ability to produce glycolipids and the results are shown in Plate 1. None of the tested strains showed glycolipid production.

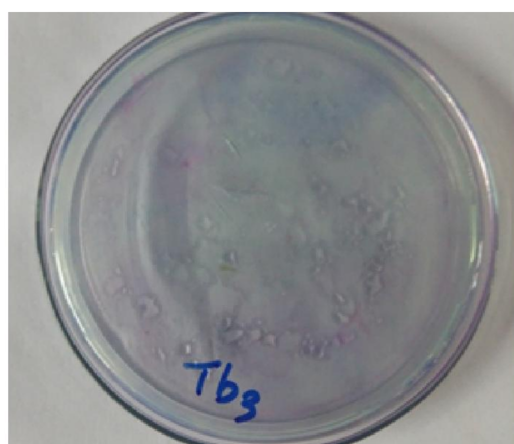

Plate 1. Blue agar plate method for biosurfactant production

Emulsification Assay

The bacterial strains which showed maximum growth in the hydrocarbon added media were used for the estimation of biosurfactant activity by calculating the emulsification assay using petrol and the results are shown in Table: 3. The results highlights that strain Tb1 and Tb3 showed maximum emulsification assay when compared to other strains.

Table 3. Emulsification assay shown by the bacterial strains

Bacterial strains	Emulsification assay (EA) in petrol
Ab4	10.08
Bb4	18.11
Tb1	75.04
Tb3	69.36

1 Emulsification Unit=0.01O.D multiplied by dilution factor of absorbance at 400 nm

There are so many microorganisms thriving in different condition which produce biosurfactants. These are used in biodegradation of various harmful chemicals in oils. Kiran *et al*. (2010) suggested that the single screening method is not suitable for identifying all types of biosurfactants. He recommended that several screening methods should be included during primary screening to identify potential biosurfactant producers. In the present study an attempt was made to screen biosurfactant producing bacterial strains through screening methods by using petrol, kerosene and diesel and Emulsification Index was calculated. Emulsification activity determines the productivity of bioemulsifier. The culture showing > 30 % emulsification activity can be considered as positive for biosurfactant production (Satpute *et al.,* 2008). In current study strain Tb1 and Tb3 showed > 30% emulsification activity both when added with diesel and kerosene (Table 2). Emulsification Assay is one of the important and effective methods for screening the biosurfactant producers. Chopade and Patil, (2012) introduced emulsification assay based on emulsification units of the tested oils. By examining the emulsification units it is possible to select a potent biosurfactant producer. In this study, strains Tb1 and Tb3 showed high emulsification assay than the other two strains (Table: 3). Hence, these strains are identified positive for the production of biosurfactants. Confirmation of biosurfactant production through other screening methods is essential to test their potential for production.

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