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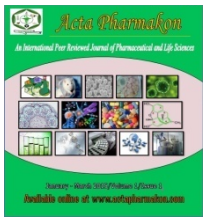

Research Article

Isolation and Screening of Bio-Surfactant Producing Microorganisms from Oil Contaminated Soil

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ABSTRACT

Bio-surfactants are surface-active compounds produced by microorganisms. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures. In this study, we collected contaminated soil from garages and screened for bio-surfactant-producing microorganisms. Twenty seven bacterial strains were isolated and cultured by enriching carbon and nitrogen sources. Each culture medium was sampled to confirm the ability of bio-surfactant production. Bio-surfactant ability was conducted using emulsification activity determination (EA), oil spreading technique and parafilm M method. The results reveal that twelve strains of bacteria from garage sites presented positive activity. Among these, the emulsifying capacity evaluated by the E24 emulsification index range from 7.8-63.3% EA. In addition, the oil displacement area (ODA) was displayed at 9.62-66.50 cm^2 and the collapse of droplets on parafilm M method was showed with the average of 5-8 mm. Interestingly, the bacterial isolate (KYNG106) selected from garage site gave the highest values in emulsification activity, oil spreading and parafilm M determination. Phylogenic relationships of the KYNG106 was determined comparing the 16Sr DNA gene sequences, revealing them as isolates of *Bacillus cereus* that can be used in pilot scale for industrial production of new bio-surfactant/bio-emulsifier.

1. INTRODUCTION

Naturally occurring surface-active compounds derived from micro organisms are called bio-surfactants. Bio-surfactants are amphiphilic biological compounds produced extracellular or as part of the cell membrane by a variety of

yeast, bacteria and filamentous fungi.[1] The ability to reduce surface tension is a major characteristic of surfactant. Surfactants are key ingredients used in detergents, shampoos, toothpaste, oil additives, and a number of other consumer and industrial products. The total surfactant production has exceeded 2.5 million tons in 202 for many purposes such as polymers, lubricants and solvents. The growth rate is related to the world demand in detergents since this sector uses over 50% of surfactant production.[2] The bio-surfactants are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycol-lipids, antibiotics, lipo-peptides, etc.

Bio-surfactants, lead to an increasing interest on these microbial products as alternatives to chemical surfactants.[3] There are numbers of reports on the synthesis of various types of bio-surfactants by microorganisms using water-soluble compounds such as glucose, sucrose, ethanol or glycerol as substrates.[4] Petroleum related industry was found to be one of the industries that have a greater potential to produce bio-surfactants producing microorganism. In the present study, the bio-surfactant producing microorganism isolated from oil contaminated soil was screened and characterized for the production of bio-surfactants.

2. MATERIALS AND METHODS

Samples were collected from oil contaminated soil from garage, Kalyan. Microorganisms from the soil samples were isolated from liquid enrichment cultures containing 0.1% soy bean oil as a carbon source. One gram of soil sample was incubated into 100 mL of culture medium. The Mckeen medium (20 gL^{-1} glucose, 5.0 gL^{-1} glutamic acid, 1.0 gL^{-1} K_2HPO_4, 1.02 $gL^{-1}MgSO_4$, 0.5 gL^{-1} KCl) supplemented with 1 mL of trace elements solution (0.5 gL^{-1} $MnSO_4$,.7H2O, 0.16 gL^{-1} $CuSO_4$,.$5H_2O$ and 0.015 $gL^{-1}FeSO_4$,.$7H_2O$) adjusting to pH 7.0 was used as cultural medium. The cultures were incubated on rotary shaker (150 rpm) for 3 days at 45 °C (for the hot spring soil samples) and 30°C (for garage sites and culture collection strains). The culture suspension was screened for biosurfactant production by oil spreading test. The bacterial suspension was counted on decimal dilution plate. The bio-surfactant producing bacteria were purified using the Mckeen medium containing soy bean as a carbon and energy source. The isolates were then maintained on nutrient agar.[5]

2.1 Oil Spreading Test

The selected strains were compared by measuring of the diameter of the clear zones occurred when a drop of a bio-surfactant-containing solution is placed on an oil-water surface. The 50 ml of distilled water was added to a large Petri dish (15 cm diameter) followed by the addition of 20 µl of crude oil to the surface of water, 10 µl of supernatant of culture broth. The diameters of clear zones of triplicate assays from the same sample were determined.[6]

Figure 1: The spreading of extracellular-bio-surfactant on oil surface layer of bacterial isolate KYNG106

2.2 Emulsification Index (E24)

The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture samples was determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, vortexed at high speed for 2 min and allowed to stand for 24h. The E24 index is given as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column(cm). The percentage of emulsification index calculated by using the following equation. [7,8]

$$\mathbf{E24} = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100$$

Table 1: The clear zone of microorganisms on oil surface layer

Bacterial strains	Clear zone of bio-surfactant (cm)	Oil displacement area (cm^2)
KYNG10	7.2	40.73
KYNG08	7.0	38.50
KYNG106	9.2	66.50

2.3 Parafilm M Test

The 25 µl of bacterial supernatants when mixed with 1% xylenecyanol were added to the hydrophobic surface of parafilm M. The shape of the drop on the surface was inspected after 1min. The diameters of droplets were evaluated. The sodium lauryl sulfate and phosphate buffer (pH 7.0) were used as a positive and negative control, respectively.[9,10]

3. RESULTS AND DISCUSSION

Twenty seven bacterial strains were isolated from the garage soil. Twelve of twenty-seven bacterial strains from garage samples presented oil spreading activity. It was suggested that this site has a variety of hydrocarbon substrates. The Bacterial strain KYNG110 showed the highest clear zone and oil displacement area at 66.5 cm^2 (Table 1 and Figure 1). While, bacterial isolates KYNG10 and KYNG08 showed lower surfaces activities as lower diameters were found at 40.73 and 38.50 cm^2 respectively. This method is better predicted bio-surfactant production than the drop collapses method because it is very

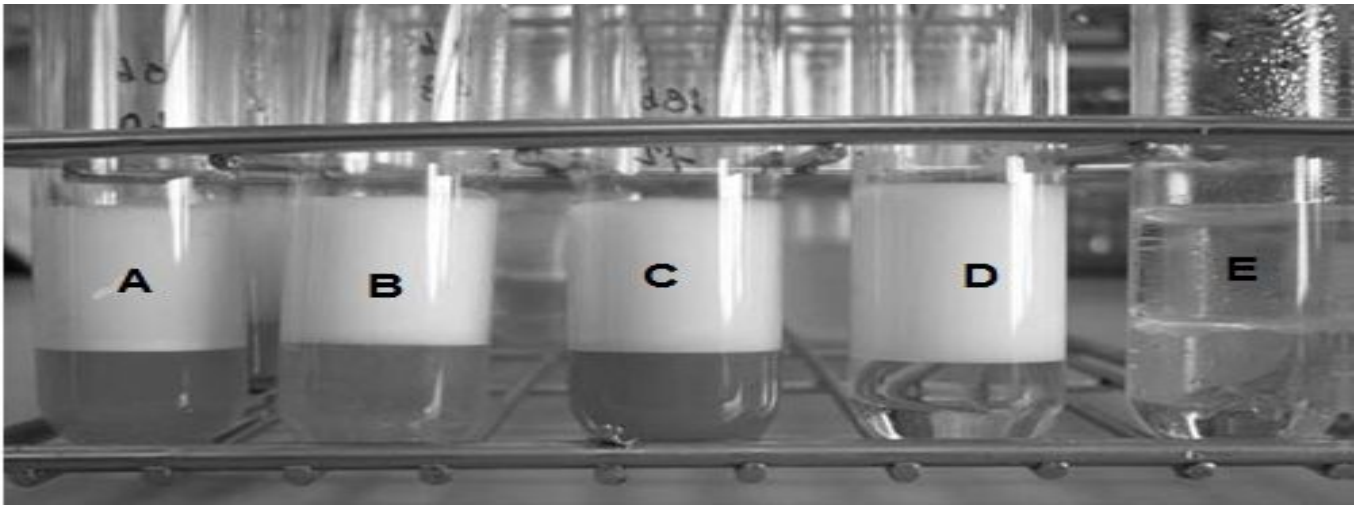

Figure 2: The emulsion form of isolate KYNG106 after various incubation periods (A) emulsion formed at 36 h incubation, (B) emulsion formed at 48 h incubation, (C) emulsion formedat 60 h incubation, (D) 1% Sodium lauryl sulphate (E) Phosphate buffer pH 7

sensitive for detection [11] and it has several advantages in requiring a small volume of samples. They are rapid and easy to be carried out, and do not require specialized equipment. [12] Thus, the bacterial strain KYNG110 was selected for emulsification index and parafilm M tested.

Emulsification activity (E24) of the bio-surfactant from KYNG106 was measured with kerosene and culture-free broth. E24 ranged from 7.8-63.3 EA%. The emulsification of bacterial isolate KYNG106 was detected from the first day of incubation period and showed the highest of emulsion formed at 60 hrs. The degree of emulsification and the stability of the emulsions formed is presented in the Figure 2. The emulsion will expose their stability when it is stored at room temperature. Emulsion layer have been maintaining their form although the duration is more than a week.

The bacterial isolate KYNG106 showed the highest amounts of extra cellular bio-surfactant when compared to another isolates (Figure 3). This methodology proved to be cheap and effective for the screening, maintenance and qualification of bio-surfactant-producing bacteria.[13] In addition, it was correlated with surface tension. [14]

It is interesting that isolate KYNG106 showed different and distinct emulsifying response when exposed to each of hydrocarbon sources. Following complementary screening, a potential bio-surfactant producing strain was isolated and was further characterized. Sequencing of 16S rDNA produced by polymerase chain reaction of bacterial DNA using universal primers revealed that better bio-surfactant-producing isolates were related to *Bacillus cereus*. Determination of the morphological and biochemical traits of these isolates confirmed the results of phylogenetic studies.

4. CONCLUSIONS

The soil sample from garages was found to be a good source for screening of bio-surfactant-producing bacteria. The bacterial isolate KYNG106 displayed the highest activity after detection with oil spreading test, emulsification index and parafilm M method. KYNG106 identified as *Bacillus cereus* with bio-surfactant-producing ability and emulsion capacity were isolated from petroleum-contaminated soil. Its ability to reduce surface tension and emulsion capacity makes them new potential candidates for bio-surfactant and bio-emulsion production. Further studies have been initiated to identify their properties and consequently determine the potential of their different industrial applications in particular enhanced oil recovery application.

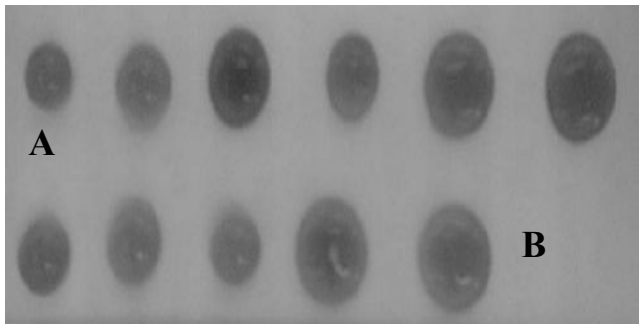

Figure 3: The activity of surface tension of cell-free broth on parafilm M surface (A) Fresh medium (B) Cell-free broth of bacterial isolate KYNG106

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