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Ability of indigenous *Bacillus licheniformis* and *Bacillus subtilis* in microbial enhanced oil recovery

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ABSTARCT: Microbially produced lipopeptide have been isolated and studied for microbial enhanced oil recovery. About 60 gram positive bacteria isolated from soil contaminated with crude oil, near the crude oil storage tank in Tehran Refinery, Tehran, Iran. However, most of these studies have produced lipopeptide by one of the pure-culture microbes isolated in a laboratory. Among the isolates, heamolytic tests revealed two biosurfactant producers. The isolated strains were designated as C2, E1. By using morphological, biochemical and molecular biology tests (16 SrRNA), the strains identified as *Bacillus licheniformis* and *Bacillus subtitlis*, respectively. Emulsification activity and measurement of surface tension indicated that, the isolates were high producers of biosurfactant. The product of C2 and E1 is mainly lipopeptide. This product reduce surface tension from 65 to 30 mN/m. Emulsified activity of crude oil was 92% for C2 and 90 % in case of E1. This is the first report of indigenous *Bacillus licheniformis* and *Bacillus subtilis* from a soil contaminated with oil in an Iranian refinery with ability to produce biosurfactant.

Key words: *Surface tension, emulsification activity, lipopeptide, crude oil, Bacilli*

INTRODUCTION

Microbial enhanced oil recovery (MEOR) is an important tertiary recovery technology utilizing microorganisms and/or their metabolic products for recovery of residual oil. It is generally accepted that approximately 30 % of the oil present in a reservoir can be recovered using current enhanced oil recovery (EOR) technology (Abtani and Reestaazad, 2001; Khire and Khan, 1994; Premuzic, 1992). However, techniques involving the use of chemical or physical processes such as pressurization, water flooding of steaming is generally inapplicable to most oil reservoirs (Vanbyke, 1991). The use of chemical surfactants for cleaning up oil reservoirs in an unfavorable practice that is hazardous and costly and will leave undesirable residues which are difficult to dispose without adversely affecting the environment (Turkovskaya, 1999). Poor oil recovery in existing producing wells may be due to several factors. The main factor is the low permeability of some reservoirs or the high viscosity of the oil which results in poor mobility. High interfacial tensions between the water and oil may also result in high capillary forces retaining the oil in the reservoir rock (Banat, 1995a). Biosurfactants are a diverse group of surface active chemical compounds that are produced by a wide variety of microorganisms such as *Bacillus sp*. (Cooper and Goldenbergy, 1999; Sung and Kuo, 1998). Having both polar and non-polar domains, biosurfactants are able to partition at the water/air or water/oil interfaces and thus lower the interfacial of surface tension (Youssef *et al.*, 2004). Such properties make them good candidates for EOR (Bannat, 1995b; Bannat *et al*., 2000). This biological surface active compounds have several advantages over the chemical surfactant such as lower toxicity, higher degradability and better environmental compatability, higher foaming, higher selectivity and specific activity at extreme pH, temperature and salinity (Fietcher, 1992; Sullivan, 1998; Gautham and Taygi, 2006; Bodour and Miller, 2002; Gerson, 1998). In the present studies, isolation and identification of lipopeptide producing bacilli and extraction of biosurfactant were assessed.

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The bio-products excreted by these strains cause a series of very desirable changes in the properties of the crude oil (Mahmudieh Lab, 2007).

MATERIALS AND METHODS

Soil samples were obtained from contaminated area close to the storage and distribution center of oil products in Tehran refinery. All samples were collected in triplicates. Processing on soil samples began immediately upon arrival in the laboratory. 1 mL of each soil sample was added to 9 ml of 0.9 % sodium chloride solution. The mixture was placed on a reciprocal shaker for 1 h. to produce a well dispersed suspension (Francy and Thomas, 1991; Adria *et al.*, 2003). By inoculating sheep blood agar, heamolytic test was performed (Banat, 1995). Lipopeptides are metabolites which are capable to destroy the membrane of erythrocytes. Surface tension was determined using a DU-NOUY test (Akhavan Sepahy *et al.*, 2005). *Bacillus Sp.* was isolated by pour plate technique on plate count agar. Individual culture was preliminary identified by morphological, biochemical and molecular biology techniques. Using the taxonomic scheme of Bergey's manual of determinative bacteriology and 16S rRNA using fD_1 and rD_1 primers. 16S rRNA method has several steps as below:

- DNA extraction by Amersham kits
- electrophoresis in agarose gel
- PCR with special primers that contains nucleotides as below:

fD_1 5'-AGAGTTTGATCCTGGCTCAG-3'
rD_1 5'-AAGGAGGTGATCCAGCC-3'

Samples had been sent to SQ lab Co. (Germany) for further molecular biology studies. By receiving the result, other strain was compared and matched strains were detected. The graphs had been drawn by special software recognized as the unknown nucleotides. Eventually, the results were compared with gene bank sequences (Blackwood and Turenne, 2004). Nutrient broth (Merck) was used for preparation of the inoculums. The cultures were grown in this broth for 8-12 at 35 °C (OD_{450} nm 0.8-0.9). For biosurfactant synthesis a mineral salts medium (MSM) with the following composition was used: Na_2HPO_4 2 g/L, KH_2PO_4 2g/L, $MgSO_4.7H_2O$ 0/01 g/L, $NaNO_3$ 2.5 g/L, NaCl 0.8 g/L, $CaCl_2$ 0/2 g/L, KCl 0/8 g/L, $FeSO_4.7H_2O$ 0/001 g/L and 1 mL of trace element solution containing (mg/L) $ZnSO_4.7H_2O$ 525 mg/L , $MnSO_4.4H_2O$ 200 mg/L, $CuSO_4.5H_2O$ 705 mg/L, $Na_2MnO_4.2H_2O$ 15 mg/L, $COCl_2.6H_2O$ 200 mg/L, H_3-BO_3 15 mg/L, $NiSO_4.6H_2O$ 27 mg/L (Francy and Thomas, 1991). The carbohydrate (glucose, sucrose, fructose and mannose) were added to a final concentration of 2 % (V/V). The hydrocarbon substrates (crude oil) were added separately at 2% (V/V) concentration. Different nitrogen sources such as sodium nitrate, peptone and yeast extract were added (3%) to the culture medium. Growth studies were done in flasks containing 200 mL medium at 30-50 °C with shaking at 200 rpm. Biosurfactant production at different NaCl concentration, pH, the NaCl was determined. pH of the medium were adjusted accordingly. Experiments were done in duplicate and the results reported are averages of there independent experiments (Tulra *et al.*, 2002). Emulsification activity was measured using the method described by Cooper and Goldenbergy (1999) and Tabatabaee *et al.* (2005). The method used for lipopeptide extraction was modified from the previous work of Youssef *et al.* (2004). In a liter flask, 200 mL culture was grown in medium (MSM) with 2% crude oil. After about 48 h., aliquot were checked for biosurfactant production by using the surface tension test. Cells were then removed by centrifugation at 10.000 rpm for 15 min. at 4 ºC. The culture broth were acidified to pH 2.0 by 1% HCl and extracted with ethyl ether (1:2 V/V). The ether phase was evaporated under vacuum at 35 °C. The residual lipid extract was then redisolved in dichloromethane and fractionated on a activated silica-gel 60 plates (20 × 20 × 0/25 cm, MERK, F.R.G) using chloroform-methanol-acetic acid-water mixture (25:15:4:2) as a solvent system. The lipid components were detected as brown spots on the plates after spraying with potassium dichromate in sulfuric acid and heating at 150 °C for 15 min. Amino-acid components were detected as red spots after the plates were sprayed with nine hydrine solution and heated for 10 min at 110 °C. These components were identified by comparison with published reports and against known standards (Abu Ruwaida and Banat, 1991; Tulra *et al.*, 2002; Kim *et al.*, 2000).

RESULTS AND DISCUSSION

The production of surface active compounds or lipopeptide by microorganisms has been a subject of increasing interest in recent years, especially due to their potential applications in enhanced oil recovery. Because of the diversity of microorganisms in such a environment and the heterogeneity of many

environments at a microbial level, a large number of different methods have been developed to detect and/or identify as large a proportion of the microorganisms present as possible. Anyhow, due to the diversity of microorganisms and the different niches in which they inhabit and no single method is applicable for the extraction, detection or identification of all the microorganisms in soil contaminated with oil sample. Hence, this study tried with specific medium for bacilli species. About 60 strains of Bacillus isolated from contaminated area close to the storage and distribution center of oil products in Tehran refinery. These strains were selected for further studies. Since hemolytic activity is one of the tools to characterize bacteria, hemolytic activity of bacterial strains was determined by inoculating sheep blood agar. Lipopeptides are also metabolites which are capable to destroy the membrane of erythrocytes. Depicted results in Fig. 1 is enough evidence for both the strains ability to produce biosurfactant. The isolated strains were identified by morphological, biochemical and molecular biology technique using the taxonomic scheme of Bergy's manual of determinative bacteriology and 16 SrRNA gene. PCR products were first obtained using *Taq* DNA polymerase and genomic DNA as a template and f D_1 and r D_1 as primers following 10 min. of initial denaturation at 94 °C; 30 cycles of 45 s at 94 °C, 45 s at 56 °C and 60 s at 72 °C and a final elongation step of 10 min. at 72 °C. Products were visualized with ethidium bromide after running on a 1% agarose gel. DNA was extracted from the gels. PCR products were diluted 1:50 and used as a template for incorporation under the same PCR conditions, using DIG PCR labeling mixture (Roche). the obtained results of 16 SrRNA, determined with special software comparison of the sequence with the sequences available in the database revealed 99 % identity with *Bacillus licheniformis* and *Bacillus subtilis*. A standard emulsion assay was used to determine the emulsifying activities of pure isolated bacilli strains. Units of emulsifying activity are defined in Materials and Methods. The specific emulsifying activities of the *Bacillus licheniformis* were 92 % and *Bacillus subtilis* 90 %, respectively. However, identified species had a capability to reduce surface tension in their culture broth to values 30 and 29 mN/m. Some effective biosurfactants are able to reduce the surface tension of water from 65 mN/m to 29 mN/m. The obtained results are comparable with the results

Fig. 1: Heamolaysis test on blood agar

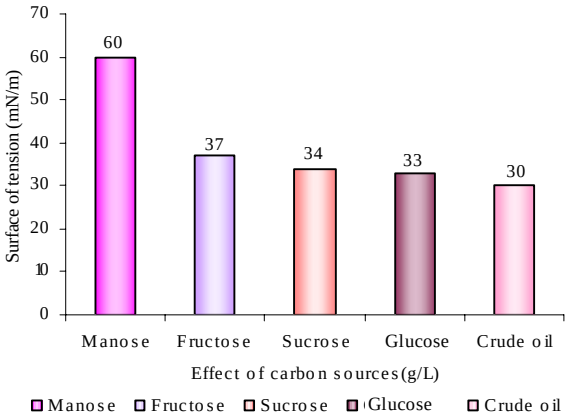

Fig. 2: Effect of carbon sources on surface tension of *Bacillus licheniformis* biosurfactant.

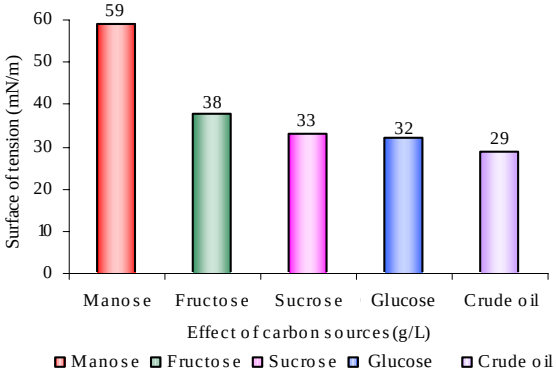

Fig. 3: Effect of carbon sources on surface tension of *Bacillus subtilis* biosurfactant

obtained by Banat (1995a). The ability of these strains to grow on crude oil, glucose, sucrose, manose, lactose as carbon sources is shown in Figs. 2 and 3. the depicted results revealed that (in Figs. 2 and 3) crude oil was the best source of carbon and energy for biosurfactant

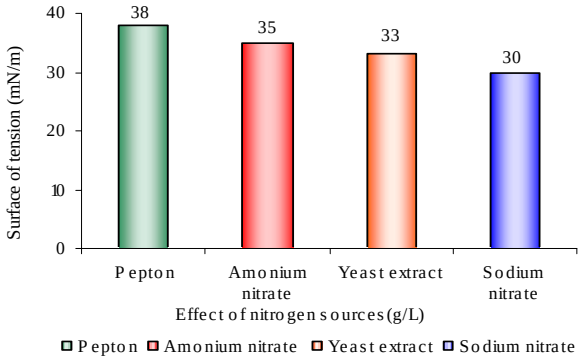

Fig. 4: Effect of nitrogen sources on surface tension of *Bacillus licheniformis* on production lipopeptide

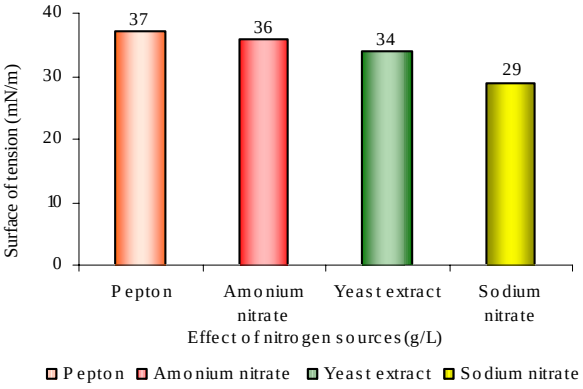

Fig. 5: Effect of nitrogen sources on surface tension of *Bacillus subtilis* lipopeptide

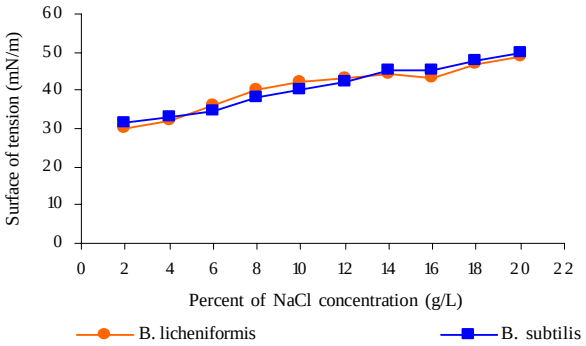

Fig. 6: Effect of NaCl concentration on surface tension using *Bacillus licheniformis* and *Bacillus subtilis*

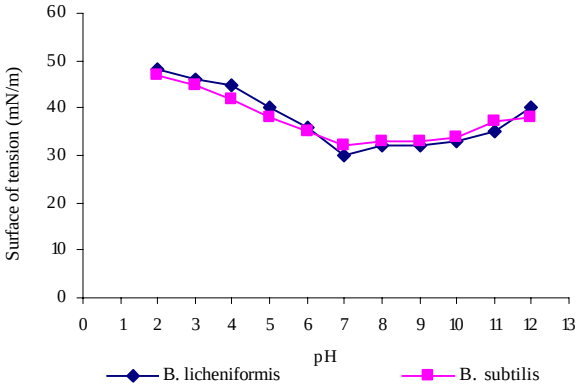

Fig. 7: Effect of pH on surface tension of *Bacillus licheniformis* and *Bacillus subtilis*

production by the isolated bacteria. In present study, the conditions were standardized for the maximum lipopeptide production by *Bacillus licheniformis* and *Bacillus subtilis* at mesophilic growth conditions. The results of variation in nitrogen sources such as Yeast extract, ammonium nitrate, peptone and sodium nitrate are shown in Figs. 4 and 5. The effects of these nitrogen sources on surface tension by *Bacillus licheniformis* and *Bacillus subtilis* showed 35 mN/m and 36 mN/m reduction, respetively. The depicted results in Figs. 4 and 5 showed that sodium nitrate is applicable for lipopeptide production using locally isolated strains. A number of studies have indicated that the type of medium and growth conditions can influence the type and yield of biosurfactant. In this regard, this study examines the effect of carbon, nitrogen sources, pH and NaCl concentrations. The isolated *Bacillus* strains in this investigation were able to utilize different carbon sources in order to produce lipopeptide in mesophilic condition. Although there are reports on biosurfactant production in mesophilic condition, but Makkar and Cameotra (1998) isolated a thermophilic *Bacillus* strain on a hydrocarbon containing medium. Biosynthesis of lipopetide was reduced when above 6 % NaCl were used. Different NaCl concentrations (2-20 %) had effects on lipopeptide biosynthesis. As it is shown in Fig. 6, sodium chloride addition to the medium affected reduction of surface tension. Higher NaCl concentration in the medium did not affect the ability of the isolated bacteria in the reduction of surface tension considerably. The isolated strains behaved like the studies of Yakimov and Amro (1997). They reported that isolation of strain of *Bacillus licheniformis* from a petroleum reservoir was able to produce surfactant oprimally at 6 % NaCl concentration (Banat, 1993). Fig. 7 shows the effect of pH on the surface tension of *Bacillus licheniformis* and *Bacillus subtilis* biosurfactant, respectively. These strains were grown at pH values between 6 to 9 but the optimum pH for both the indigenous bacilli were 6.8. When these strains were grown at pH values of 4, there is no considerable lipopeptide production. Preliminary the analysis of the biosurfactant produced

Table 1: Experimental Rf data for surface active lipid components isolated from *Bacillus licheniformis* culture

Extract fraction	Rf	Presumed component
A	0.06-0.12	Monoglyceride
B	0.20-0.24	Diglyceride
C	0.50-0.60	Neutral lipids
D	0.8	Amino-acid

Table 2: Experimental Rf data for surface-active lipid components isolated from *Bacillus subtilis* culture

Extract fraction	Rf	Presumed component
A	0.05-0.14	Monoglyceride
B	0.20-0.26	Diglyceride
C	0.40-0.80	Neutral lipids
D	0.9	Amino-acid

by *Bacillus subtilis* and *Bacillus licheniformis* culture indicated that the presence of different lipid compounds can be identified on the basis of their Rf-values (Tables 1 and 2) as monoglycerides, diglycerides and natural lipids. A potassium dichromate in sulfuric acid fraction was also detected, indicating that the lipid extract contains a peptide substance. The present findings are similar to those reported in the literature for surface active lipids isolated from *Bacillus licheniformis* BA550 and *Bacillus licheniformis* jf-2 (Mc Inerney, Javaheri, 1990; Yakimov and Timmis, 1995; Jenny, 1992; Youssef *et al.*, 2004). The potential of these strains to produce biosurfactant and their use in the oil industries, especially for MEOR, is apparent.

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