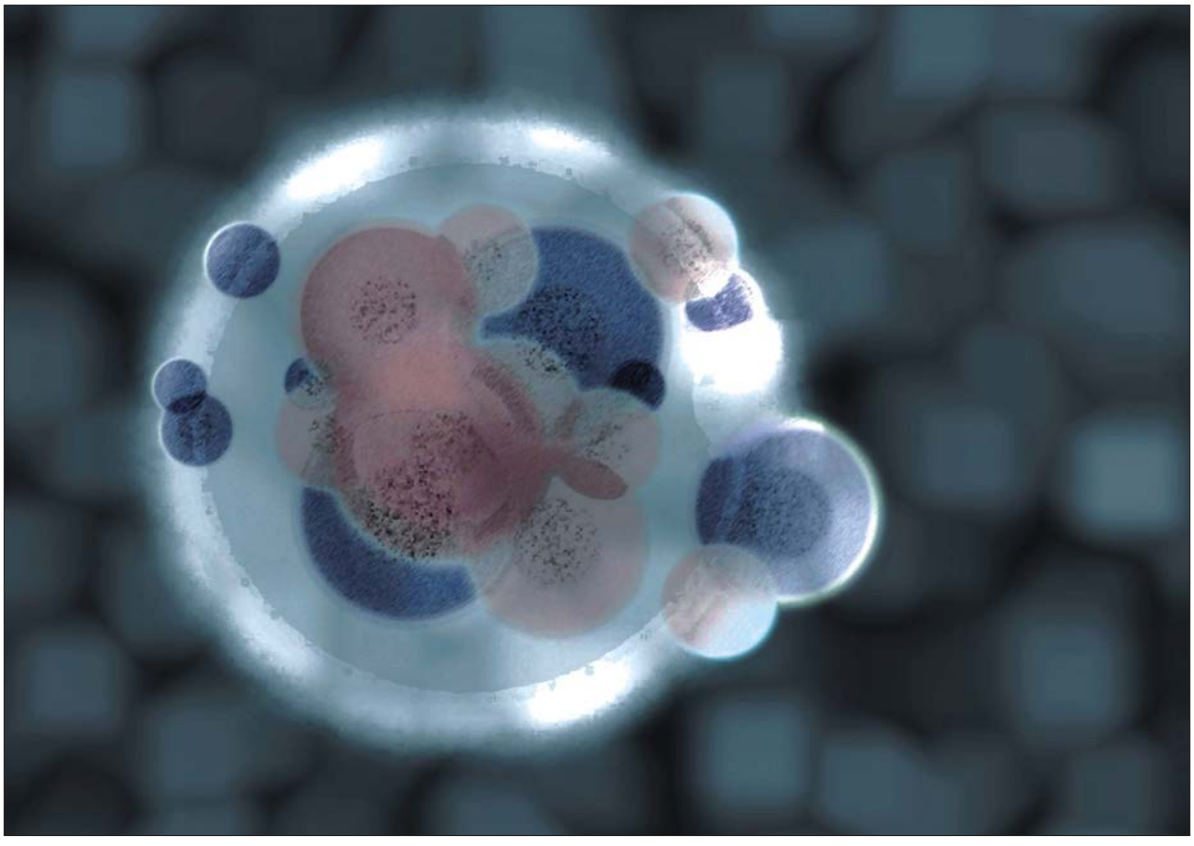

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 7 (7): 1266-1269, 2007
ISSN 1727-3048

Biosurfactant Production by the Strain Isolated from Contaminated Soil

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Abstract: A bacterial strain was isolated from vegetable oil contaminated soil, identified and named as *Brevibacterium* sp. strain S-34. Hemolysis of erythrocytes and emulsification of the oil in culture media indicated surface active agents production by the strain. Thin layer chromatography studies revealed that the secreted biosurfactants were glycolipids. The potential production of biosurfactants was assessed using different carbon sources (glucose, glycerol, molasses, gasoline, Canola oil and waste oil). Optical density, pH and surface tension were employed to determine the biosurfactant activity of strain S-34 in mineral salts medium with each carbon source. The microorganism was able to grow well on glycerol (50 g L^{-1}) and reducing the surface tension of medium from 69 mN m^{-1} to 30 mN m^{-1} by production of 2.4 g L^{-1} total biosurfactant after 72 h.

Key words: Biosurfactant, surface tension, oil contaminated soil

INTRODUCTION

Surfactants, which constitute an important class of industrial chemicals widely used in almost every sector of modern industry, are surface active compounds capable of reducing surface and interfacial tension between liquids, solids and gases (Desai and Banat, 1997). Most of the surface active compounds currently in use are chemically synthesized, however increasing environmental awareness has led to serious consideration of biological surfactants as possible alternatives to existing products (Kim *et al*., 1999). Microbially-derived surfactants are amphipatic molecules produced by a wide variety of bacteria, yeasts and filamentous fungi (Christofi and Ivshina, 2002). Having both polar and nonpolar domains, biosurfactants are able to partition at water-oil or water-air interfaces and thus reduce the interfacial or surface tension (Banat *et al*., 2002; Banat, 1995). The most important advantage of biosurfactant when compared to synthetic surfactants is their ecological acceptance, owing to their low toxicity, biodegradable nature, production from renewable resources, greater ability to complex heavy metals and the potential for in-soil production by native species (Ron and Rosenberg, 2001; Christofi and Ivshina, 2002; Karanth *et al*., 1999). Another advantage of biosurfactants is that they can be modified by biotransformation to generate new products for specific requirements (Deleu and Paquet, 2004). Potential applications of biosurfactants include emulsification, phase separation, wetting, foaming and surface activity that can be exploited in food, oil, cosmetic and pharmaceutical industries (Makkar and Cameotra, 2002). In the environmental sector, microbial surfactants show promising applications in bioremediation and waste treatment to remove hazardous materials (Mulligan, 2005). Microbial surfactants are complex molecules, comprising a wide variety of chemical structures such as glycolipids, lipopeptids, fatty acids, polysacharid-protein complexes, peptides, phospholipids and natural lipids. Some species of *Pseudomonas*, *Burkholderia*, *Arthrobacter*, *Rhodococcus*, *Mycobacterium* and *Torulopsis* were recently shown to produce a variety of glycolipid biosurfactants (Häussler *et al*., 1998, 2003; Rosenberg and Ron, 1999; Tuleva *et al*., 2002; Gunther *et al*., 2005). Strains S-34 which was isolated from contaminated soil showed the ability to grow on different hydrocarbons and

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produce surface active compounds. So the aim of present study was to determine the influence of different carbon sources on biosurfactant production by the strain S-34.

MATERIALS AND METHODS

Microorganism: Samples of oil contaminated soil were aseptically collected from a vegetable oil industry in Babol, north of Iran and sent to the Microbiology Laboratory of School of Pharmacy, Medical Sciences/University of Tehran, where they were immediately analyzed. Ten grams of each oil contaminated soil was suspended in 100 mL of NaCl 0.9% and then 100 μL aliquots of each suspension were spread on the surface of agar plates containing 5% sheep blood. After incubation of the plates at 35°C for 48 h, colonies with clear zone of hemolysis were selected and tested for biosurfactant activity. An inoculum was prepared by transferring single colonies into 100 mL of Tryptic Soy broth (TSB, Merck) incubated at 30°C for 18 h. A 10% inoculum volume of TSB culture was used to inoculate 500 mL Erlenmeyer flask containing 100 mL liquid mineral salt medium (g L^{-1}): $NaNO_3$ 15, NaCl 1.1, KCl 1.1, KH_2PO_4 4.3, K_2HPO_4 3.4, $MgSO_4.7H_2O$ 0.5, yeast extract 0.5; 5 mL trace element stock solution composed of (g L^{-1}): $FeSO_4.7H_2O$ 0.00028, $CaCl_2.4H_2O$ 0.24, $ZnSO_4.7H_2O$ 0.29,$CuSO_4.5H_2O$ 0.25, $MnSO_4.H_2O$ 0.17 (Linhardt *et al*., 1989) supplemented with 5% Canola oil and incubated on a rotary shaker at 30°C and 200 rpm for 3 days. After centrifugation of the culture medium at 8000 x g for 20 min, the supernatant was collected and the surface tension was measured using a Du Nouy ring tensiometer (McInerney *et al*., 1990). The isolates were maintained on Tryptic Soy agar (TSA, Merck) slants at 4°C and subcultures were made every 2 weeks. The strain with highest biosurfactant activity was chosen for identification and optimization of the culture medium using different carbon sources.

Morphological and physiological characterization of the isolate: The isolate was characterized using standard microbiological techniques, including Gram-staining, spore staining, motility, oxidase, catalase, oxidation, fermentation, gas production, indole, utilization of urea, hydrolysis of casein and gelatin (Gerhardt *et al*., 1994) and identified according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Biosurfactant extraction and determination: The biosurfactant was extracted from culture medium after cell removal by centrifugation at 8000 x g for 20 min. The pH of the culture supernatant was adjusted to 2.0 with 1 N HCl and an equal volume of chloroform: methanol (2:1) was added. The mixture was shaken well for 1 min and allowed to stand until phase separation. The organic phase was separated and extraction was repeated twice more. The pooled extract was concentrated in a rotary evaporator at 40°C to obtain the total biosurfactant which was then dried to constant weight at 50°C under vacuum. The concentrate was analyzed by thin-layer chromatography (TLC) on silica gel (K6F 60 A, Whatman, Inc.) with a solvent system of chloroform, methanol and acetic acid (65:15:2 v/v/v). Biosurfactant spots were developed by α-naphtol-H_2SO_4 reagent (Makkar and Cameotra, 1997).

Effect of different carbon sources on growth and biosurfactant production: To investigate the effect of different carbon sources on biosurfactant production, bacterial cultures were inoculated into the basal mineral salt medium with 5% of each carbon substrate of glucose, glycerol, molasses, gasoline, canola oil and waste oil.

The flasks were incubated on a rotary shaker at 30°C and 200 rpm for 7 days. Surface tension was measured after 72 h of cultivation and growth was assayed by measuring pH and optical density at 600 nm (OD_{600}) each day.

RESULTS AND DISCUSSION

Isolation of biosurfactant producing strains: After incubation of blood agar plates, sixty nine colonies with hemolytic activity were selected and subcultured on TSA to obtain pure cultures. The single colonies were transferred to the mineral salt medium for determination of biosurfactant activity. Twenty two isolates were able to produce surface active compounds when grown in medium containing 5% Canola oil and reduced culture broth surface tension to lower than 46 mN m^{-1} in comparison to the control (69 mN m^{-1}).

The best among these isolates, designated as strain S-34, which showed the lowest surface tension (37 mN m^{-1}) was selected for further studies.

Strain identification: Colonies of strain S-34 appeared white, gray on Muller-Hinton agar medium. In microscopic examination Gram-positive cocobacilli was observed. The strain was halophilic, grew aerobically and was catalase positive and oxidase negative. Some characteristics of the isolate are summarized in Table 1. It did not utilize galactose, maltose, lactose, rhamnose, arabinose, rafinose. Comparison of morphological, physiological and biochemical properties of the strain with Bergy's manual of Determinative Bacteriology (Holt *et al*., 1994) showed that the majority of features of tested strain were close to species of *Brevibacterium*.

Table 1: Morphological and biochemical characteristics of strain S-34

Test	Result
Cell morphology	Irregular coccobacilli
Strictly aerobic	+
Facultative anaerobic or microaerophilic	–
Endospore	–
Oxidase test	–
Catalase test	+
Motility	–
Gelatin and casein hydrolysis	+
Urease	–
DNase	+
Utilization of carbon sources:	
Galactose, maltose, fructose	–
Esculin, xylose, mannose	–
Rhamnose, arabinose, rafinose	–
Sucrose, lactose	±

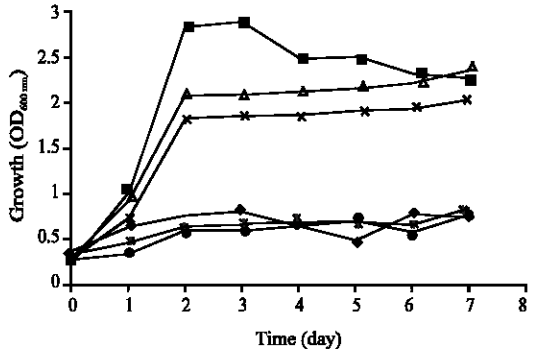

Fig 1: Effect of carbon source on growth ($OD_{600\ nm}$) of strain S-34 on molasses (◆), Canola oil (■), glycerol (△), glucose (×),gasoline (✱) and waste oil (●)

Detection of biosurfactants: The extracted biosurfactants in the organic phase were examined by thin layer chromatography (TLC) and visualized with different specific reagents. No spots were visualized after staining with ninhydrin. Purple spots detected after spraying with α-naphtol solution in acidified ethanol and heating at 110°C for 5 min, indicated the spots were presumptively glycolipid biosurfactants.

Effect of carbon sources on growth and biosurfactant production: As shown in Fig. 1, the strain S-34 showed satisfactory growth when cultivated on glucose, glycerol and Canola oil while growth was lower on gasoline, waste oil and molasses. Maximum cell growth was attained after 2 days incubation which was in proportion to pH variations. The pH of cultures containing molasses, Canola oil and glycerol increased to about 8, 6.9 and 6.5, respectively while the increase of pH was very little on waste oil and gasoline after 2 days incubation (Fig. 2). The pH of culture reduced to about 4.5 when glucose used as carbon source.

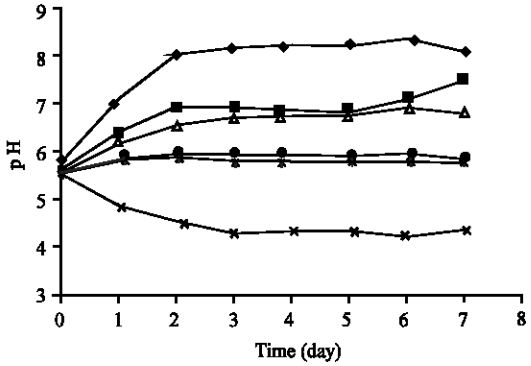

Fig. 2: Effect of carbon source on pH of strain S-34 on molasses (◆), Canola oil (■), glycerol (△), glucose (×), gasoline (✱) and waste oil (●)

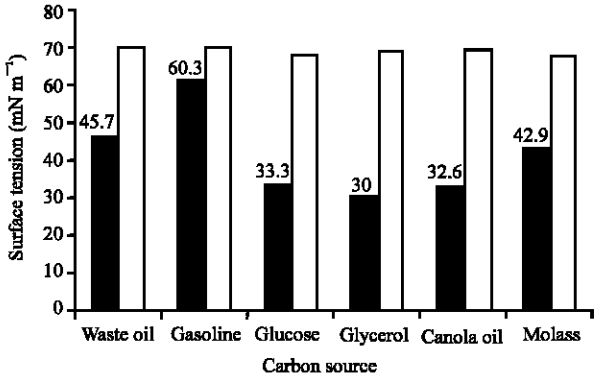

Fig. 3: Effect of carbon source on surface tension activity of strain S-34. Surface tension of inoculated culture medium after 72 h incubation (■), sterile culture medium (□)

The lowest surface tension values were obtained with glycerol, 30 mN m^{-1} and then for Canola oil, 32.6 mN m^{-1} and glucose, 33.3 mN m^{-1}. The surface tension values for other carbon substrates were more than 40 mN m^{-1}: molasses, 42.9 mN m^{-1}; waste oil, 45.7 mN m^{-1} and gasoline, 60.3 mN m^{-1} (Fig. 3). The maximum glycolipid concentration of 2.4 g L^{-1} was attained with glycerol.

The lower surface tension obtained with glycerol than Canola oil may be due to more water solubility and availability of the substrate to the microorganism. The ester linkage in vegetable oil should be hydrolyzed to release glycerol and fatty acids for biosurfactant production. It is also reported that alkenes are not metabolizable under anaerobic conditions and molecular oxygen is required for initial oxidation of alkenes to alcohol (Chayabutra *et al*., 2000). So glycerol which is an oxidized vegetable oil component seems to be a suitable intermediate in biosurfactant production.

It is known that most of bacteria optimize uptake of insoluble hydrocarbons by producing biosurfactants that promote substrate emulsification and/or solubilization and/or enhance cell hydrophobicity thus allowing the cells to get into direct contact with the oil phase (Rosenberg and Ron, 1999). Glucose is a water-immiscible hydrocarbon which might have lower induction on biosurfactant synthesis than hydrophobic substrates. Higher surface tensions obtained with molasses, waste oil and gasoline are probably due to their lower availability to microorganism and/or containing toxic substances.

In conclusion we report on biosurfactant production by *Brevibacterium* sp. isolated from oil contaminated soil in different carbon sources for the first time. *Brevibacterium* S-34 was able to grow on carbon sources of glucose, glycerol, molasses, gasoline, Canola oil and waste oil, but glycolipid-type biosurfactants were produced on glucose, glycerol and Canola oil. The lowest surface tension was observed with glycerol, reducing the surface tension of medium from 69 to 30 mN m^{-1} by production of 2.4 g L^{-1} total biosurfactant after 72 h.

ACKNOWLEDGMENT

This study was fully supported by a research grant from the Pharmaceutical Sciences Research Center, Medical Sciences/University of Tehran for which the authors are thankful.

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