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# Biosurfactant Production in Batch Culture by a *Bacillus licheniformis* Isolated from The Persian Gulf

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**Abstract:** Biosurfactants are amphipathic molecules that have received increasing attention in recent years because of their role in the growth of microorganisms on water-insoluble hydrophobic materials such as hydrocarbons and because of their commercial potential in the cosmetics, food, oil, mining and agricultural industries. In this study, we used easy methods such as hemolytic test, oil spreading technique and surface tension measurement to confirm biosurfactant production by a halophilic strain isolated from the Persian Gulf. Also, the effects of carbon, nitrogen, NaCl, $FeSO_4$ sources on biosurfactant production were studied. Optimal cell growth and biosurfactant production occurred when the strain was grown in cultures with 0.5% crude oil, glycerol, glucose and sucrose as carbon source and 0.2 g urea per g carbon source as nitrogen source. Two percent NaCl and minimal concentration (1 mg $L^{-1}$) of $FeSO_4$ were suitable.

**Key words:** Biosurfactant, oil spreading, surface tension

## INTRODUCTION

Surfactants are amphiphilic compounds that reduce the free energy of a system by replacing the bulk molecules of high energy at the interface (Mulligan and Gibbs, 1993). Some surfactants, known as biosurfactants, are biologically produced by yeast or bacteria from various substrates such as sugars, oils, alkanes and wastes (Lin, 1996). The reason why these microorganisms produce surfactant is not always so obvious. In some bacteria, biosurfactants facilitate microbial oil up take and degradation by emulsifying the substrate hydrocarbon (Koch *et al*., 1991). Biosurfactants are classified into glycolipids, fatty acids, lipopeptids, polymeric and particular types, based on the structure of their hydrophobic part (Kitamoto, 2001; Banat *et al*., 2000). Currently, biosurfactants are used in enhanced oil recovery, hydro carbon bioremediation, agriculture, cosmetics, pharmaceuticals, detergents, personal care products, food processing, metal treatment and processing, pulp and paper producing and paint industries (Mulligan *et al*., 2001; Ron and Rosenberg, 2001; Banat *et al*., 2000). In this study, we used sensitive and rapid methods to detect biosurfactant production by microorganism and investigated the effect of cultural conditions on its cell growth and biosurfactant production.

## MATERIALS AND METHODS

All data reported in this study are from triplicate measurement.

**Growth study:** The organism used in this study was *Bacillus* sp. which was isolated from Persian Gulf by G. Ebrahimipoor, Department of Biology, Shahid Beheshti University, Tehran, Iran. The organism was maintained on a slant of M1 medium at 4°C and subcultured every month (M1(per liter):1.25 g of yeast extract; 0.75 g peptone; 15 g of agar). The Mineral Salt Medium (MSM) was a mixture of solution A and solution B. Solution A contained (per liter): 1.95 g $g^{-1}$ oil of $NH_4Cl$; 0.24 g $g^{-1}$ oil of $Na_2HPO_4$ (Gibbas, 1975); 0.01 g of $FeSO_4.7H_2O$;14 g of $MgSO_4.7H_2O$; 32 g of NaCl; 0.05 g of KCl; 0.01 g of $CaCl_2.2H_2O$. Solution B contained (per liter): 70 mg of $ZnCl_2$; 100 mg of $MnCl_2$; 200 mg $CoCl_2$; 100 mg NiCl; 20 mg $CuCl_2$; 50 mg $Na_2MoO_4.H_2O$; 26 mg $Na_2SeO_4$; 10 mg $NaVO_3$; 30 mg $Na_2WO_4.2H_2O$; 1 mL 25% HCl. One milliliter of solution B was added to 1000 mL of solution A to form the MSM. The pH of the medium was adjusted to 8.0. Cultivation was performed on a rotatory shaker with an agitation speed of 100 rpm at 35°C.

**Analytical methods:** Starter culture. One loop of bacterial isolated from a 24 h streaked cultured was inoculated into 50 mL of M1 broth in a 100 mL flask and incubated with

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shaking at 100 rpm at 35°C for 24 h. Inoculum with $OD_{620}$ = 0.1($10^7$ bacteria $mL^{-1}$) (10%) was transferred into the MSM 100 mL in a 500 mL flask and incubated at 100 rpm for 5 days in an incubator shaker.

**Cell growth measurement:** Culture broth was collected and diluted with medium broth to obtain optimum dilution. The absorbance was measured at 620 nm ($OD_{620}$) by a spectrophotometer UV-VIS Double Beam PC, Labomed INC (Kim *et al*., 1999).

**Cell dry weight determination:** Samples were centrifuged (10000 x g) for 15 min at 4°C. The pellet was suspended in distilled water and recentrifuged. Biomass dry weight was determined by drying at 105°C for 24 h (Cooper and Goldenberg, 1987).

**Protein concentration:** Protein concentration of samples was determined by the Bradford (1976) method using bovine serum albumin as the standard.

**Hemolytic test:** Hemolytic activity bacterial strain was determined by streaking onto sheep blood agar plates and incubating at 35°C for 48-72 h (Mulligan *et al*., 1984; Banat, 1993).

**Oil displacement test:** Twenty microliter of crude oil was put onto the surface of 50 mL of distilled water in a petri dish (25 cm diameter). A thin membrane of oil formed immediately. Then 10 μL of supernatant was gently dropped on the center of the oil membrane. A clear halo was visible. The area of this circle was measured and calculated for Oil Displacement Area (ODA) using the following equation (Morikawa *et al*., 2000): ODA = 22/7 $(radius)^2$ $cm^2$.

**Emulsification activity:** To measure emulsification activity, 0.5 mL of crude oil was added to 2 mL of supernatant in test tube, vortexes at high speed for 1 min and stood for 24 h. The emulsification index is the height of the emulsion layer, divided by the total height, multiplied by 100 (Cooper and Goldenberg, 1987).

**Solubility test:** Two milligram of the acetone precipitated biosurfactant was tested its solubility in solvent systems (2 mL) such as distilled water, alkaline water, methanol, ethanol, acetone, ethyl acetate, chloroform and hexane. The mixture was allowed to stand for 24 h and the solubility was observed (Kim *et al*., 1997).

**Surface tension measurement:** Surface tension was measured using a Du Nouy tensiometer. Measurements were made on supernatant samples after centrifugation.

**Biosurfactant Extraction:** After cultivation, a crude biosurfactant was obtained by centrifugation (10000 x g, 15 min, 4°C) to remove the cells. The supernatant was precipitated with an equal volume of chilled acetone and standing overnight at 4°C. The precipitate was collected by centrifugation and used for further purification by Thin-layer Chromatography (TLC) (Kim *et al*., 2000).

**Analysis the component of partially purified biosurfactant by TLC:** The crude biosurfactant was separated by TLC using aluminum sheets silica gel 60 $F_{254}$ plates with various solvent systems including: chloroform/methanol/acetic acid at 65:15:2; chloroform/methanol at 80:20; chloroform/methanol/water at 65:25:4; chloroform/methanol/acetic acid/water at 50:5:4:1; acetone/water at 90:10. The components were observed under UV light. The best separated solvent system was selected for separation of biosurfactant by TLC.

## RESULTS

Initial characterization of bacterium. Bacterial strain is a facultatively anaerobic, gram-positive, motile, rod-shaped bacterium. The spores were oval and subterminally positioned. The strain hydrolyzed gelatin; used citrate and propionate; and reduced nitrate, but did not produce indole or hydrogen sulfide. The strain is catalase and oxidase positive and grows in medium with a range of 0 to 17% (wt/vol) NaCl, at temperatures of 25 to 45°C and at pH of 5 to 10. According to Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986), the strain is characterized as Bacillus licheniformis. More characterization is needed by fingerprinting and sequencing of 16s RNA.

**Effect of type and concentration of carbon source:** Biosurfactant production depends both on the type and amounts of carbon source present in the medium (Adamczak and Bednarsk, 2000; Davila *et al*., 1997; Davis *et al*., 1999). In this study, water-soluble carbon sources such as glycerol, glucose, sucrose and molasses and hydrocarbons such as n-hexane, n-decane, tetradecane in addition to crude oil were used as carbon source. The cell growth, biomass and surface tension of supernatant were measured after 5 days incubation of flask containing MSM medium and 1% concentration of each carbon source in shaking incubator at 100 rpm at 35°C (Makkar and Cameotra, 1997). Based on the results, molasses, hexane, crude oil, glycerol were the best for cell growth. But with crude oil, glucose, sucrose, tetradecane and glycerol, the reduction of surface tension was the

Table 1: The study of various parameters following different carbon sources

| | Carbon sources | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameters | Crude oil | Molasses | Glycerol | Hexan | Decane | Tetradecane | Glucose | Sucrose |
| Biomass (g $g^{-1}$ carbon source) | 0.520 | 0.680 | 0.520 | 0.600 | 0.500 | 0.520 | 0.40 | 0.440 |
| Surface tension (mN $m^{-1}$) | 0.035 | 0.052 | 0.051 | 0.055 | 0.056 | 0.049 | 0.046 | 0.047 |
| OD 620 nm | 0.697 | 1.162 | 0.589 | 1.091 | 0.963 | 1.256 | 0.598 | 0.610 |
| Protein (mg $g^{-1}$ carbon source) | 25.17 | 28.59 | 24.51 | 23.75 | 25.22 | 25.46 | 24.80 | 25.46 |

Table 2: The study of various parameters following different nitrogen sources

| Parameters | Urea | $KNO_3$ | Yeast extract | $NH_4Cl$ |
|---|---|---|---|---|
| Biomass (g $g^{-1}$ carbon source) | 0.880 | 1.200 | 2.700 | 1.300 |
| Surface tension (mN $m^{-1}$) | 0.035 | 0.051 | 0.056 | 0.053 |
| OD 620 nm | 0.687 | 0.630 | 0.860 | 0.648 |
| Protein (mg $g^{-1}$ carbon source) | 26.50 | 25.17 | 65.84 | 25.15 |

Table 3: The study of various parameters following (1.5% NaCl added to MSM medium

| Parameters | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Biomass (g $g^{-1}$ carbon source) | 0.26 | 0.34 | 0.46 | 0.3 | 80.34 | 0.32 |
| Surface tension (mN $m^{-1}$) | 0.061 | 0.057 | 0.049 | 0.055 | 0.061 | 0.062 |
| OD 620 nm | 0.385 | 0.671 | 0.768 | 0.648 | 0.487 | 0.373 |
| Protein (mg $g^{-1}$) carbon source | 28.16 | 30.2 | 34.65 | 32.14 | 27.89 | 26.49 |

Table 4: Study of different parameters following different Fe concentrations (1, 10, 50 and 100 mg $L^{-1}$) were added to the MSM

| Parameters $FeSO_4$ (mg $L^{-1}$) | 1 | 10 | 50 | 100 |
|---|---|---|---|---|
| Biomass (g $g^{-1}$ carbon source) | 0.44 | 0.32 | 0.26 | 0.24 |
| Surface tension (mN $m^{-1}$) | 0.037 | 0.043 | 0.052 | 0.053 |
| OD 620 nm | 1.15 | 0.98 | 0.418 | 0.37 |
| Protein (mg $g^{-1}$ carbon source) | 35.41 | 29.53 | 26.50 | 25.15 |

best. Also, the crude oil concentration varying from 0.5 to 3% was added to the MSM medium as the suitable carbon source. Optimal cell growth and biosurfactant production occurred at 0.5% concentration (Table 1).

**Effect of type and concentration of nitrogen source** The nitrogen source can be an important key to the regulation of biosurfactant synthesis. Medium MSM with a crude oil concentration at 0.5% served as the basic medium for the optimization of nitrogen sources. In this study, organic and inorganic nitrogen sources such as $KNO_3$, $NH_4Cl$, urea and yeast extract were studied (Mercade *et al*.,1993). In the presence of yeast extract, biomass concentration was high but biosurfactant production was poor. The best result was obtained with urea and nitrate as indicated by the lower surface tension values of the culture broth. Also, the best result of lowering surface tension was at 0.2 g urea per g carbon source and above this concentration, biosurfactant production decreased. Guerra-Santos *et al*. (1984) showed that the use of complex medium additives such as yeast extract should be avoided. *Arthrobacter paraffineus* ATCC19558 preferred ammonium and urea to nitrate as nitrogen source (Duvnjak *et al*., 1983). Also, Syldatk *et al*. (1985) showed that nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant produced (Table 2).

**Effect of concentration of NaCl:** The bacterial strain grew in a medium with a range of 0 to 15% (wt/vol) NaCl. Optimal growth occurred in M1 medium with 4% NaCl. So, NaCl concentration in medium culture affected cell growth and biosurfactant production. In this study, concentrations (0-5%) of NaCl were added to the MSM medium and incubated at 35°C in shaking incubator at 100 rpm for 5 days. The cell growth was slightly affected by different concentrations and optimal cell growth was at 2% NaCl. Surface tension reduced in 0-5% concentrations of NaCl but the lowest surface tension was at 2% concentration. This result is comparable with the results obtained by Lin *et al*. (1993). They showed that the addition of more than 2% NaCl in medium culture of *Bacillus licheniformis* JF-2 resulted in a decrease in biosurfactant production. Also, Yakimov *et al*. (1995) isolated *B. licheniformis* BAS50 which optimal growth and surfactant production occurred at 13% NaCl concentration (Table 3).

**Effect of concentration of $FeSO_4$:** Of the mineral elements, Fe had an important influence on *P. aeruginosa* biosurfactant production (Guerra-Santos *et al*., 1984). At high Fe concentrations (2 mg $g^{-1}$ of glucose and above) formation of biosurfactant did not occur. In this study, the Fe concentration was varied from 1 to 100 mg of $FeSO_4.7H_2O$ $L^{-1}$ (1, 10, 50, 100 mg $L^{-1}$). With decreasing Fe concentration, cell growth and biosurfactant production increased. Guerra-Santos *et al*. (1984) showed that a minimal Fe concentration was suitable for biosurfactant production by *P. aeruginosa* (Table 4).

## DISCUSSION

The *Bacillus* strain used in this study showed hemolytic activity by clearing zones around the colonies on blood agar medium. So, the hemolytic assay allows a rapid assessment of biosurfactant in culture and no special equipment or expensive reagents are needed (Moran *et al*., 2002). Also, the area of clearly formed oil displacement circle was measured as the activity of

surfactants. It was a circle of 7 cm diameter, with an ODA equals 38.5 $cm^2$. Although the mechanisms of the oil displacement by surfactants have not yet been clarified on the molecular level, this method provided us with a sensitive and easy system of surfactants. By measuring the height of the emulsion layer of supernatant against crude oil, the strain showed 55% emulsification activity. According the solubility the acetone precipitated biosurfactant dissolved well in distilled water but its solubility in other solvents wasn't stable after 24 h. The culture supernatant reduced surface tension from 72 to 35 mN $m^{-1}$. This reduction is considered moderately active. The best-known lipopeptide biosurfactant is surfactin which lowers the surface tension to 27 mN $m^{-1}$ (Morikawa *et al*., 1993). *Arthrobacter* species produce extracellular glygolipids (Li *et al*., 1984) which lowers the surface tension of water to 30 mN $m^{-1}$. Some *Clostridium* and *Desulfovibrio* strains produce extracellular compounds that lower the surface tension to about 50 mN/m (Cooper *et al*., 1980; Javaheri *et al*., 1985). The surface-active compounds were isolated from the culture supernatant. The best separated solvent systems for TLC were chloroform/methanol/acetic acid at 65:15:2 and chloroform/methanol at 2:1. The compounds were observed under UV light as pink spots.

## CONCLUSIONS

In this study, biosurfactant production of microorganisms was assayed by qualitative and quantitative methods. Both hemolytic activity and oil displacement showed biosurfactant production and surface tension reduction confirmed the results. Optimal cell growth and biosurfactant production occurred when the bacterial strain was grown in cultures with 0.5% crude oil, glycerol, glucose and sucrose as carbon source and 0.2 g urea per g carbon source as nitrogen source. In this way, we showed that 2% NaCl and minimal concentration of $FeS_4O$ were suitable for biosurfactant production.

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