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Isolation, Characterization, and Investigation of Surface and Hemolytic Activities of a Lipopeptide Biosurfactant Produced by *Bacillus subtilis* ATCC 6633

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***Bacillus subtilis* ATCC 6633 was grown in BHIB medium supplemented with Mn^{2+} for 96 h at 37°C in a shaker incubator. After removing the microbial biomass, a lipopeptide biosurfactant was extracted from the supernatant. Its structure was established by chemical and spectroscopy methods. The structure was confirmed by physical properties, such as Hydrophile-Lipophile Balance (HLB), surface activity and erythrocyte hemolytic capacity. The critical micelle concentration (cmc) and erythrocyte hemolytic capacity of the biosurfactant were compared to those of surfactants such as SDS, BC (benzalkonium chloride), TTAB (tetradecyltrimethylammonium bromide) and HTAB (hexadecyltrimethylammonium bromide). The maximum hemolytic effect for all surfactants mentioned was observed at concentrations above cmc. The maximum hemolytic effect of synthetic surfactants was more than that of the biosurfactant produced by *B. subtilis* ATCC 6633. Therefore, biosurfactant would be considered a suitable surface-active agent due to low toxicity to the membrane.**

***Key words*:** *Bacillus subtilis*, biosurfactant, critical micelle concentration, hemolytic activity, surface tension.

In the past few decades, biosurfactants (surfactants of microbial origin) have gained attention because of their biodegradability, low toxicity, ecological acceptability, and capacity to be manufactured from renewable and cheaper substrates (Desai and Banat, 1997; Ishigami, 1997; Makkar and Cameotra, 1999). Several structurally diverse surface-active molecules are producible by a wide spectrum of microorganisms (bacteria, fungi, and yeasts). There are six major types of biosurfactants: hydroxylated and cross linked fatty acids (mycolic acids), glycolipids, lipopolysaccharides, lipoproteins-lipopeptides, phospholipids, and the complete cell itself (Desai and Banat, 1997). Biosurfactants have been tested in environmental applications, such as bioremediation and dispersion of oil spills, enhanced oil recovery, and transfer of crude oil, and have been considered for surfactants of the future, especially in the food, cosmetic, and in agricultural chemicals (Banat *et al*., 2000). *Bacillus subtilis* produces a lipopeptide, called surfactin, with exceptional surface activity (Heerklotz *et al*., 2001, Peypoux *et al*., 1999). The compound has been characterized as a cyclic lipopeptide containing a carboxylic acid (3-hydroxy-13-methyl tetradecanoic acid) and seven amino acids. Structural characteristics show the presence of a heptapeptide with an LLDLLDL chiral sequence linked to a β-hydroxy fatty acid, via a lactone bond, (Peypoux *et al*., 1999). Investigations of its other physiological or biochemical actions have shown that it is also an antibacterial, antitumoral compound against Ehrilich ascites carcinoma cells, and a hypocholesterolemic agent that inhibits cAMP phosphodiesterase and has anti-HIV properties (Peypoux *et al*., 1999). It has only been since 1980 that surfactin has drawn the attention of a number of researchers, as an attractive supplement to chemical surfactants that have detrimental effects on the environment. If economic problems were not such a hindrance, the petroleum industry would certainly find new uses in agriculture and in environmental applications for this compound (Peypoux *et al*., 1999). Recently, diverse new activities have been demonstrated, including emulsification (Razafindralambo *et al*., 1998), foaming (Razafindralambo *et al*., 1998), antiviral, and antimycoplasmic activities (Nissen *et al*., 1997). On top of such properties, surfactin can differentiate viral cells from Mycoplasma cells; proposals have been made to employ surfactin in ensuring the safety of pharmaceutical products (Nissen *et al*., 1997). In this work, we isolated surfactin (SU) from culture of *B. subtilis* ATCC 6633 and some of its properties were determined by using physicochemical methods. The critical micelle concentration and erythrocyte

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hemolytic capacity of the produced biosurfactant were compared to those of synthetic surfactants, including SDS, BC, TTAB, and HTAB.

Materials and Methods

Test organism

B. subtilis ATCC 6633 was obtained from the Persian Culture Type Collection, Theran, Iran.

Cultivation of organism

Brain-Heart Infusion Broth (BHIB) medium supplemented with Mn^{2+} at 1.3×10^{-3} M was used. *B. subtilis* ATCC 6633 was grown in 500 ml Erlenmeyer flasks, each containing 100 ml medium. The flasks were incubated at 37°C on a shaker incubator (ISH-3000, Akhtarian) at 220 rpm for 96 h (Randhir, 1997).

Isolation and purification of biosurfactant

After the bacterial cells were removed from the liquid culture by centrifuging at 15000 × g for 25 min at 10°C in a Beckman centrifuge with the use of a mod. JA. 25.50 rotor, crude biosurfactant was isolated by adding 6 M HCl to the supernatant. A flocculated precipitate formed at pH 2.0, and was collected by centrifuging (15000 × g for 20 min, 10°C). The precipitate was dried under vacuum in a dessicator and kept overnight at 4°C. The crude product was resuspended in dichloromethane and stirred overnight. The suspension was then filtered through Whatman No. 1 filter paper to remove any coarse impurities. The filtrate was extracted twice with equal volumes of distilled water (pH 8.0) after stirring for 20 min. After this period, the mixture was transferred into a separating funnel and left for 3 h to allow for the separation of the two phases. The aqueous phases containing the biosurfactant were withdrawn and acidified to pH 2.0 through the addition of 6 M HCl. The biosurfactant, which was precipitated in the form of white to yellowish crystals, was recovered by centrifuging at 15000 × g for 15 min, dried under vacuum, and weighed to evaluate the yield (Cooper *et al.*, 1981; Feignier *et al.*, 1995; Carrillo *et al.*, 1996; Desai and Banat 1997; Randhir, 1997).

Circular dicroism (CD) spectroscopy

CD spectrum was measured at 25°C using an AVIV model 62A DS spectropolarimeter (AVIV, USA). The CD spectroscopic measurement was carried out in a 1 mm path-length cuvette (Provencher and Glochner, 1981).

Infrared analysis

Infrared (Perkin Elmer paragon 1000) spectroscopy was used to confirm the exact structure of the biosurfactant obtained from the *B. subtilis* ATCC 6633. IR spectra were collected between 400 and 4000 wave numbers (cm^{-1}) (Cooper *et al.*, 1981).

UV spectroscopy

The sample was dissolved in dichloromethane and UV spectra were obtained between 200 to 450 nm, using a UV-Visible Spectrophotometer (UV-160A, Shimadzu Corporation, Japan) (Arima *et al.*, 1968).

Identification of fatty acid

The biosurfactant was hydrolyzed with 6 M HCl at 110°C for 20 h and the lipid moiety was subsequently separated by extraction with chloroform. Several drops of bromine water were then added to the extract (Feignier *et al.*, 1995; Peypoux *et al.*, 1994).

Identification of amino acids

Ninhydrin and biuret reactions were used in order to identify the amino acids. The ninhydrin reagent was added to the sample. A deep blue color developed after the heating of amino acid or peptide with ninhydrin. The biuret reagent was then added to the sample. A positive result was then indicated by a violet or pink ring, due to the reaction of peptide bond proteins or short-chain polypeptides, respectively. Such a result would not occur in the presence of free amino acids (Feignier *et al.*, 1995).

Determination of Hydrophile-Lipophile Balance (HLB)

Two emulsifiers (an oil-soluble, Span 80, and a water-soluble, Tween 80) were first selected; one with a low HLB value, emulsion A, and the other with a high HLB value, emulsion B, (each containing 0.5% of surfactant). A series of emulsions were prepared which covered both stock emulsions, A and B, by mixing them in varying proportions. Therefore, an emulsion (water, oil) was prepared with mixture of biosurfactant and the other surfactant with known HLB, which was added to the graduated tube. The tubes were centrifuged at 1500 rpm. The amount of aqueous phase separated was measured in milliliters using the graduations on the tubes at two minute intervals. Four readings were taken for each emulsion and compared with other emulsions in the series. The net HLB value of the biosurfactant emulsion was equal to the HLB of emulsion with the same value of separation phase. The HLB value of biosurfactant was then calculated from biosurfactant emulsion Required HLB (RHLB) (Orafidiya *et al.*, 2002).

Red blood cell suspension

Approximately 10 ml of blood from a healthy volunteer was taken and added to a heparinized tube and placed in ice. The plasma and buffy coat were removed by centrifuging at 2200 g for 10 min. The erythrocytes were washed three times, in at least five times their volume of McIlvaine's buffer, at pH 7.0. The erythrocytes were adjusted to approximately 12% hematocrit by resuspending them in buffer to 3.33 times their original weight. The

erythrocyte suspension was stored on ice at 4°C and was used within 48 h of collection (Gould *et al.*, 2000).

Erythrocyte hemolytic measurement

A suspension of red blood cells (0.2 ml) was then incubated for the required time with an equal volume of the test sample, prepared in McIlvaine's buffer, at 37°C. To assay for the amount of haemoglobin released after incubation, the mixtures were spun in a microcentrifuge (Biofuge Pico, Germany) for 15 sec, and 0.2 ml of the resulting supernatant was added to 3 ml Drabkin's reagent. Positive controls consisted of 0.2 ml samples, taken from uncentrifuged mixtures of erythrocyte suspensions (0.2 ml) and buffer (0.2 ml), which were added to 3 ml Drabkin's reagent to obtain a value for 100% haemolysis. A negative control, included to assess the levels of spontaneous hemolytic, comprised 0.2 ml buffer mixed with 0.2 ml erythrocytes. After centrifugation for 15 sec, 0.2 ml sample of supernatant was added to 3 ml Drabkin's reagent. The absorbance (540 nm) of the samples was determined spectrophotometrically and the values were expressed as a percentage of the maximum hemolysis (Gould *et al.*, 2000).

Critical micelle concentration determination

Different aqueous concentrations of surfactants were prepared. Surface tensions were measured with a tensiometer using the duNouy procedure (model-703, Sigma) with a platinum ring at 23°C. The surface tension-concentration plots were used to determine critical micelle concentrations (cmc) (Randhir, 1997).

Results and Discussion

Characterization of biosurfactant

The FTIR spectrum of the sample (Fig. 1); showed strong absorption bands of peptides at 3312 cm^{-1}, 1647 cm^{-1}, and 1539 cm^{-1}. These bands resulted from the stretching mode of N-H, stretching mode of the C=O bond, and the deformation mode (combined C-N stretching mode) of the N-H bond, respectively. The bands 2966 cm^{-1}, 1456 cm^{-1}, and 1371 cm^{-1} reflect aliphatic chains (-CH_3, -CH_2-) of the sample. The absorption region at 1740 - 1680 cm^{-1} was due to lactone carbonyl absorption, a result which is comparable with another report (Ferre *et al*, 1997). A far UV CD spectrum of biosurfactant showed the behavior of β-sheet form in phosphate buffer solution (Fig. 2). The ease of micelle formation may reflect the ease of the piling of biosurfactant molecules organized by β-sheet formation. The exposure of a large number of carboxylic groups on the surface due to β-sheet organization may contribute to the special behaviors of biosurfactant such as the ease of surface β-sheet micelles and the ease of surface adsorption (Ishigami *et al*., 1994). On the other hand, presence of peptide bonds was clearly demonstrable from UV spectrum at a range of 237 nm, which indicated the product

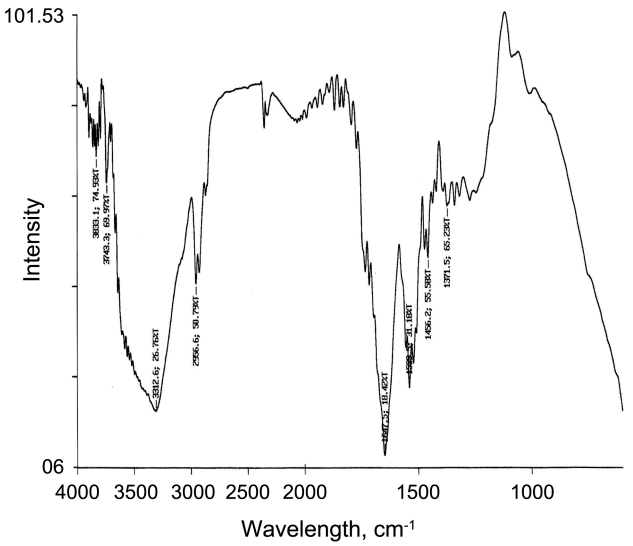

Fig. 1. IR spectrum of biosurfactant.

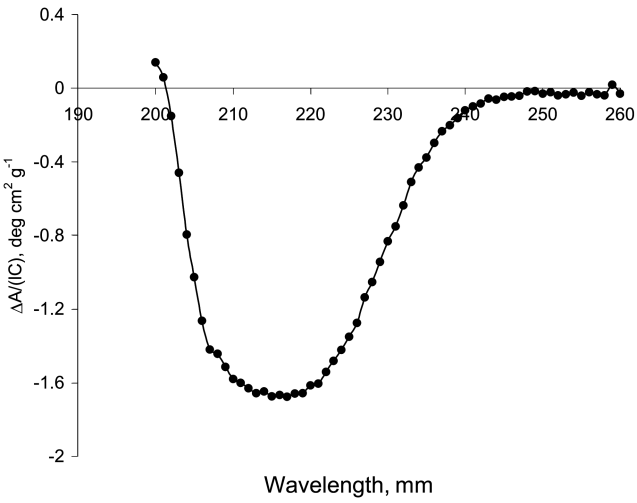

Fig. 2. Far UV CD spectrum of biosurfactant at 25°C.

was lipopeptide, as previously has confirmed by Arima *et al*. These results indicate that the product contains aliphatic hydrocarbons as well as a peptide-like moiety. Bromine water reaction was negative; indicating that the fatty acid chain was saturated. Ninhydrin reaction was negative, indicating that the peptide has a blocked N-terminal. Biuret reaction was positive indication for polypeptides, a finding which is verified by other reports (Arima *et al*., 1968; Cooper *et al*., 1981; Ferre *et al*., 1997 and Ishigami *et al*., 1994). The results indicate that the biosurfactant has a lipopeptide structure.

HLB property

The HLB of the biosurfactant was 21.27. According to Griffin's HLB scale (Kayes, 1989), it has high HLB and therefore is an o/w emulsifier, detergent, and solubilizing agent.

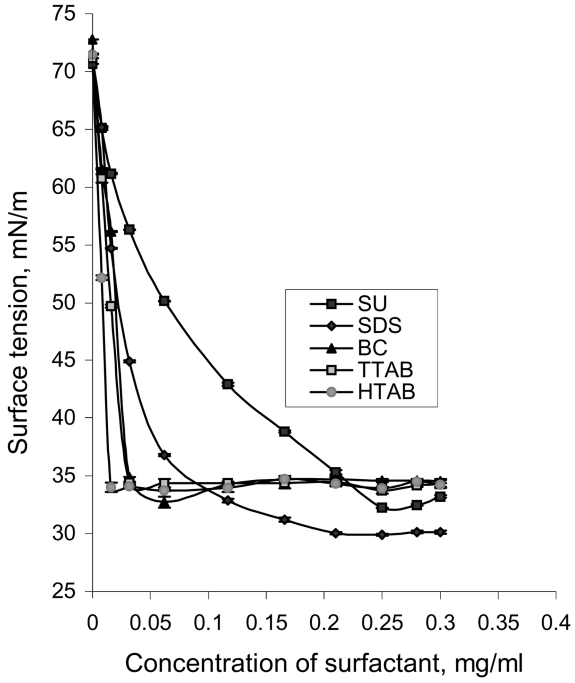

Fig. 3. Surface tension activity of various surfactants, including SU, SDS, BC, TTAB, and HTAB.

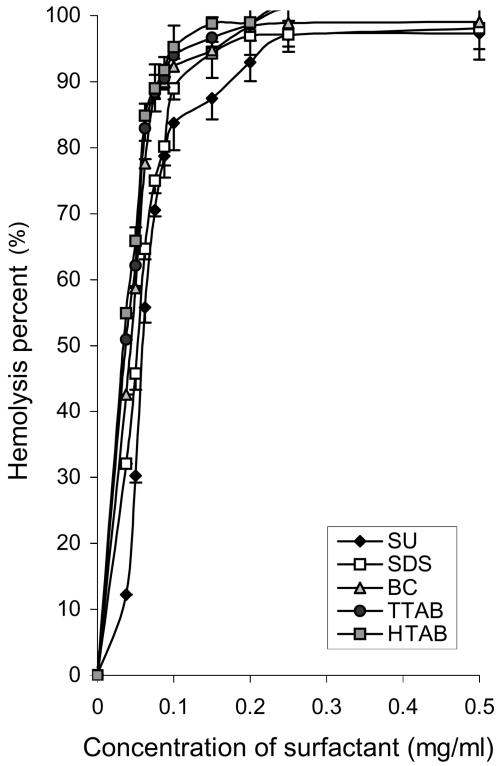

Fig. 4. Hemolytic effect of various surfactants, including SU, SDS, BC, TTAB, and HTAB, on human erythrocytes at 37°C.

Surface activity and hemolytic capacity

According to the data obtained in this study, the average surface tension of pure water at 23°C was 72.54 ± 0.74 mN/m, and SDS showed the maximum lowering surface tension activity (Fig. 3). Fig. 4 shows the hemolytic capacity of SDS, BC, TTAB, HTAB, and the produced biosurfactant. The results indicate that among surfactants tested, HTAB had the maximum efficacy for disruption of red blood cell membranes. As Fig. 4, indicates, hemolytic capacity of surfactants increases with concentration, and the definite concentration of their hemolytic capacity (~ 100%) was above the relevant critical micelle concentration. The increase in membrane permeability and subsequent osmotic lysis of the cell is thought to be due to the formation of mixed micelles in the lipid bilayer (Vinardell, 1996). According to the data presented in this study, the hemolytic activity of chemical surfactants (SDS, BC, TTAB, and HTAB) was more than that of the biosurfactant produced by *B. subtilis* ATCC 6633. The cationic surfactants (TTAB and HTAB) showed high hemolytic efficacy compared to anionic surfactants (SDS and biosurfactant). Similar to the case of synthetic surfactants, erythrocyte hemolytic measurement showed that the product was able to rupture erythrocytes, and therefore could be used as an absorption enhancer. Surface tension study of the biosurfactant showed that cmc was above minimal hemolytic concentration (MHC).

In conclusion the results indicate that the product has lipopeptide structure. Biosurfactant produced by *B. subtilis* ATCC 6633 with various activities, such as detergent, solubilizing, and absorption enhancer properties, can be used as an interesting compound in the pharmaceutical industry, as well as energy-related and environmental fields. Due to its low toxicity to the membranes, it would be considered a suitable surfactant in drug formulations.

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