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Full Length Research Paper

Studies on bioemulsifier production by *Bacillus licheniformis* PTCC 1595

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Surfactants are amphipathic molecules which reduce surface and interfacial tensions and widely used in pharmaceutical, cosmetic, food and petroleum industries. Biosurfactants are the structurally diverse group of surface-active molecules synthesized by microorganisms. There are several advantages for biosurfactants in contrast with chemical surfactants, such as lower toxicity; higher biodegradability; better environmental compatibility; higher foaming; high selectivity and specific activity at extreme temperatures, pH, and the ability to be synthesized from renewable feed-stock. In the present study, the production of bioemulsifer by *Bacillus licheniformis* PTCC 1595 was studied. *B. licheniformis* was grown in the nutrient broth medium and bioemulsifer production was evaluated every 24 h by surface tension and emulsification index (E24). Then *B. licheniformis* PTCC 1595 was grown in nutrient broth with different conditions in order to get maximum production of bioemulsifer. The best culture medium was found to be nutrient broth medium supplemented with starch, Fe^{2+}, Mn^{2+} and olive oil. After growing the bacteria, the microbial biomass was removed from the supernatant by acidic precipitation method. Its amphipathic structure was established by biochemical and spectroscopy methods and it was confirmed to be a lipopeptide structure.

Key words: *Bacillus licheniformis*, bioemulsifer, surface tension, emulsification index.

INTRODUCTION

Microbial-derived surfactants are amphipatic molecules produced by a wide variety of bacteria, yeasts and filamentous fungi. Increasing environmental concern had led to consider biological surfactants as alternative to chemical manufactured compounds. The most important advantage of biosurfactants when compared to synthetic surfactants is their ecological acceptance, owing to their low toxicity and biodegradable nature (Karanth et al., 1999). Another advantage of biosurfactants is that they can be modified by biotransformation to generate new products for specific requirements (Deleu et al., 2004). Microbial surfactants are complex molecules, comprising a wide variety of chemical structures, such as glycolipids, lipopeptides, fatty acids, polysaccharide–protein complexes, peptides, phospholipids and neutral lipids (Banat et al., 2000). 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 et al., 2002). In the environmental sector, microbial surfactants show promising applications in bioremediation and waste treatment to remove hazardous materials (Mulligan, 2005).

Bacillus licheniformis produces a lipopeptide called lichenysin. Lichenysin is a cyclic lipopeptide and belongs to the most effective biosurfactant discovered so far (Yakimov et al., 1995). The lipopeptide were found to be a mixture of four closely related compounds. The lipophilic part consisting of ¡- , ⁿ- C14 or ¡- , a_{i-} C15 ß-OH fatty acids was linked to the hydrophilic peptide moiety, which contained seven amino acids (Glu, Asp,Val, three Leu and Ile) by a lactone linkage (Jenny et al., 1991).

The lipopeptide showed activity against a variety of yeast strains. Growth of the gram negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* was inhibited by a minimal concentration of 1 mg/ml. Bioemulsifer pro-

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duced by *B. licheniformis* JF-2 exhibits a critical micelle concentration of 10 mg/l and reduces the interfacial tension against decane to 6×10^{-3} dyne cm^{-1}, which is one of the lowest interfacial tensions ever reported for a microbial surfactant (Lin et al., 1994). 15 mg l^{-1} of the purified lipopeptide product decreased the surface tension of water from 72 to 27 mN m^{-1} (Konz et al., 1999).

The strain *B. licheniformis* D-13 produces three hydrophobic peptides (amoebicins d13-A, d13-B, and d13-C) that elicit antiamoebic activity against human-pathogenic and nonpathogenic species of *Naegleria* and have a broad spectrum of antibacterial activity. Amoebicin d13-B causes lysis of amoebae through disorganization of the cell membrane. It also induces permeability to 86Rb and membrane disruption in asolectin vesicles (Glavez et al., 1994).

In this paper, the production of bioemulsifer by *B. licheniformis* PTCC 1595 and some of its properties were determined by using physicochemical methods.

MATERIALS AND METHODS

Test organism

The *B. licheniformis* PTCC 1595 was obtained from the Persian Culture Type Collection, Tehran, Iran. The strain was streaked on the surface of nutrient agar plates (HiMedia Laboratories Limited, Mumbai, India). After incubation at 37°C, distinct colonies were isolated (Konz et al., 1999).

Hemolytic activity

Isolated strains were screened on blood agar plates (Merck) containing 5% (v/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected as the presence of a definite clear zone around a colony (Cameotra et al. 2004).

Surface activity measurement

Surface tension and critical micelle dilution (CMD^{-1} and CMD^{-2}) were determined with a duNouy Tensiometer (Tensiometer K100, KRUSS). All measurements were made on supernatant. CMD^{-1} and CMD^{-2} measurements were performed by measuring the surface tension of 10-times and 100-times diluted supernatant. Negative control consisted of sterile culture medium plus *B. licheniformis* PTCC 1595 (an inoculum), at zero time (Carrillo et al., 1996).

Emulsification test

For estimation of the emulsification index, 5 ml of liquid paraffin was added to 5 ml of supernatant in a graduated tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h. The E_{24} was calculated by measuring the emulsion layer formed (Cooper et al., 1987; Markkar et al., 1997; Patel et al., 1997b; Desai et al., 1994).

Optimization of Growth conditions

B. licheniformis PTCC 1595 was initially grown in 500 ml Erlenmeyer flasks, each containing 100 ml nutrient broth medium. The flasks were incubated at 37°C in a shaker incubator (F.F-81, Pars Azma CO.) at 250 rpm. In some experiments, *B. licheniformis* was grown in nutrient broth with different conditions (aeration rates, temperatures and times of incubation) and additives such as paraffin oil, castor oil, almond oil, olive oil, starch and trace metal cations [$MnSO_4$, $FeSO_4$, $MgSO_4$, $CaCl_2$ respectively] (0.3, 0.001, 0.025 and 0.01% w/v) to the nutrient broth medium in order to get maximum production of bioemulsifer. Samples were withdrawn every 24 h (five cultures for each time) to analyze the surface activity, emulsification index and therefore to select the best conditions and additives for bioemulsifer production (Jenny et al., 1991; Cooper et al., 1987; Lin et al., 1994; Lin et al., 1998).

Isolation of bioemulsier and partial purification

After the bacterial cells were removed from the liquid culture by centrifugation (13000 g, 15 min) in a HEPTICH centrifuge mod., the crude bioemulsifer was isolated by adding concentrated HCl to the supernatant. A flocculated precipitate was formed at pH 2.0 that could be collected by centrifugation (20000 g, 20 min). The precipitate was dried under vacuum in dessicator and kept overnight at 4°C. The crude product was resuspended in dichloromethane. After stirring for one night, the suspension filtered through Whatman No. 1 filter paper to remove the coarse impurities. The filtrate was extracted twice with equal volumes of distilled water (pH 8.0) while stirring for 20 min. After this period, it was left 3 h in a separating funnel to allow the two phases to separate. The aqueous phases containing the bioemulsifer were collected and then were lyophilized overnight (Cameotra et al., 2004; Patel et al., 1997a; patel et al., 1997b; Sim et al., 1997).

Circular dicroism (CD) spectroscopy

CD spectrum was measured at 25°C using an AVIV model 62A DS spectroscopic measurement was carried out in a 1 mm path-length cuvette (Dehghan-noudeh et al., 2005).

Infrared analysis

Infrared (IR-470, Shimadzu Corporation, Japan) spectroscopy was used to confirm exact structure o the bioemulsifer obtained from *B. licheniformis* PTCC 1595. IR spectera were collected between 400 and 4000 wave numbers (cm^{-1}). (Jenny et al., 1991; Yakimov et al., 1995).

UV spectroscopy

The sample was dissolved in dichloromethane and UV spectra were obtained between 200 to 450 nm, using a UV-Visible Spectrophotometer (UV-2100, Shimadzu Corporation, Japan) (Dehghan-noudeh et al., 2005).

Identification of fatty acid

The bioemulsifer was hydrolysed 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 (Jenny et al., 1991).

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

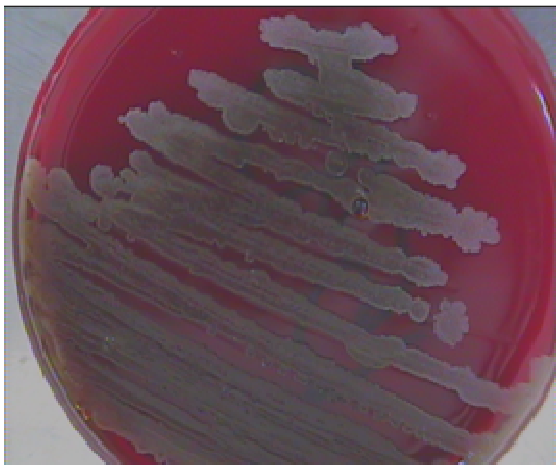

Figure 1. Colonies of *B. licheniformis* PTCC 1595 on blood agar.

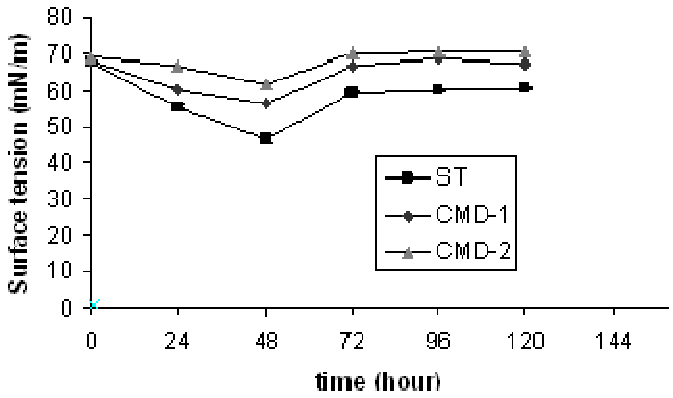

Figure 2. Surface activity profile for supernatant of *B. licheniformis* PTCC 1595. (37°C, 250 rpm).

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 (Yakimov et al., 1995; Dehghan-noudeh et al., 2005).

RESULTS AND DISCUSSION

Bioemulsifer production

The screening of bioemulsifer–producing micro-organism organisms is generally carried out using monitoring parameters that estimate surface activity, emulsifying property and hemolytic capacity. In the present study, these parameters were evaluated as potential predictors of surfactant-producing bacteria. The hemolytic effect was observed for *B. licheniformis* PTCC 1595 (Figure 1). Bioemul-sifer-producing capacity in liquid medium is associated with reduction of surface tension (Youssef et al., 2004). *B. licheniformis* PTCC 1595 was cultured in nutrient broth and bioemulsifer production, as evident from surface tension lowering (Table 1), started from first day and continued until 48 h of growth. CMD^{-1} and CMD^{-2} values (Table 1) followed a similar pattern as surface tension lowering. CMD^{-1} and CMD^{-2} measurements were performed by measuring the surface tension of 10-times and 100-times diluted cell-free broth (Carrillo at al., 1996).

Table 1. Surface tension studies, (critical micelle dilution)$^{-1}$; CMD^{-1}; and (critical micelle dilution)$^{-2}$; CMD^{-2}; results for supernatant of *B. licheniformis* PTCC 1595, grown in nutrient broth medium (37°C, 250 rpm).

Time (h)	Surface Tention (mNm^{-1}) ± SD	CMD^{-1} (mNm^{-1}) ± SD	CMD^{-2} (mNm^{-1}) ± SD
0	67.43±0.0223	68.37±0.0234	69.21±0.0561
24	55.52±0.0339	60.35±0.0190	66.68±0.0122
48	46.51±0.1504	56.48± 0.1213	61.82±0.1999
72	59.46±0.1110	66.47±0.1232	70.32±0.0115
96	60.17±0.2132	68.79±0.0333	70.58±0.0277
120	60.54± 0.0314	67.33±0.0212	70.38±0.0121

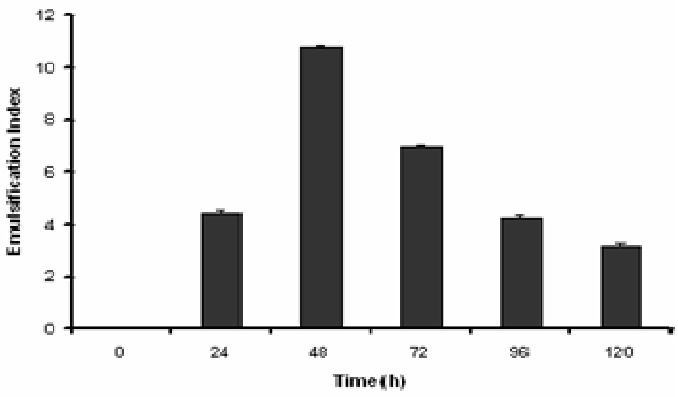

Figure 3. Emulsification index; E_{24}, graph of *B. licheniformis* PTCC 1595 with different times of incubation (37°C, 250 rpm).

Maximum of bioemulsifer production was achieved in 48 h of incubation and CMD values (Figure 2) were minimal at this point. Emulsification index values followed a similar pattern as surface tension lowering (Table 2 and Figure 3). According to these data, 37°C and 300 rpm were selected as best conditions (Figures 4 and 5) (Jenny et al., 1991).

The production yield of bioemulsifer was improved by addition of $FeSO_4$, $MnSO_4$ and starch while $CaCl_2$ and $MgSO_4$ decreased it (Figure 6). The addition of hydrocarbons, such as almond, castor oil and olive oil to the culture medium increased the bioemulsifer production while maximum of yield was achieved with olive oil (Figure 7) (Lin et al., 1994; Jenny et al., 1991).

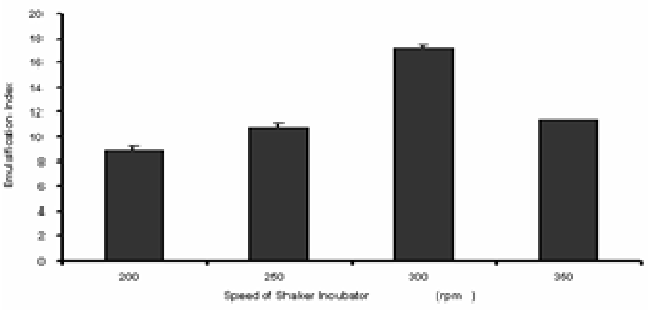

Figure 4. Emulsification index; E_{24}, graph of *B. licheniformis* PTCC 1595 with different aeration rates (37°C, 48 h).

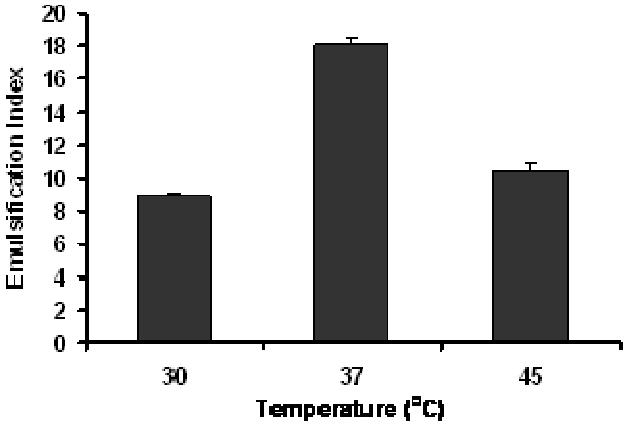

Figure 5. Emulsification index; E_{24}, graph of *B. licheniformis* PTCC 1595 with different temperatures (300 rpm, 48 h).

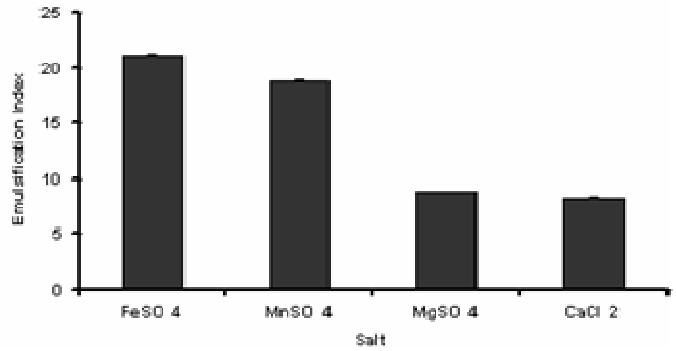

Figure 6. Emulsification index; E_{24}, graph of *B. licheniformis* PTCC 1595 with different salts (300 rpm, 48 h, 37°C).

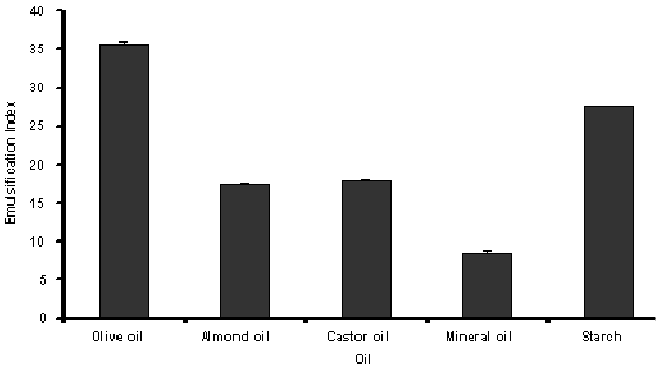

Figure 7. Emulsification index; E_{24}, graph of *B. licheniformis* PTCC 1595 with different carbon sources (300 rpm, 48 h, 37°C).

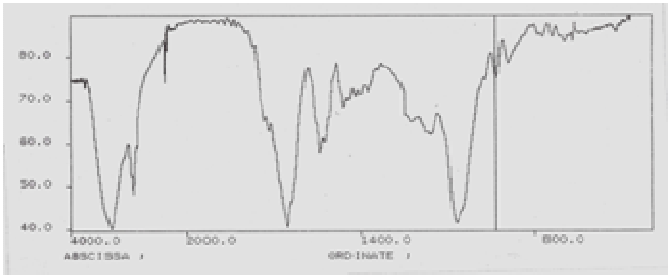

Figure 8. IR spectrum of bioemulsifer.

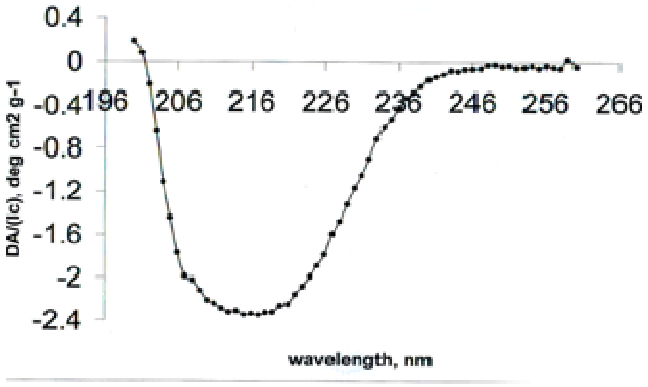

Figure 9. Far UV CD spectrum of bioemulsifier at 25°C.

Characterization of bioemulsifer

IR (KBr, cm^{-1}): 3312 (stretching mode of N-H), 1647 (stretching mode of the C=O) 1539 (deformation mode (combined C-N stretching mode) of the NH bond), 2966, 1456, and 1371 (aliphatic chains (-CH3, -CH2-), 1740 – 1680 (lactone carbonyl), (Figure 8). A far UV CD spectrum of bioemulsifer showed behavior β-sheet form in phosphate buffer solution (Figure 9). The ease of micelle formation may reflect the ease of piling of bioemulsifer 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 behavior of bioemulsifer 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 range 239 nm that indicated the product was lipopeptide which has confirmed previously. These results indicate that the product contains aliphatic hydrocarbons as well as a peptide-like moiety. Bromine water reaction was negative; indicating which the fatty acid chain was saturation. Ninhydrin reaction was negative, indicating the peptide has a blocked N-terminal. Biuret reaction was positive indicating for polypeptide. The results indicate that the bioemulsifer has lipopeptide structure. Hemolytic measurement showed that as same as synthetic surfactants it was able to rupture erythrocyte and therefore could be used as absorption enhancer.

Conclusion

The presence of a lactone ring in bioemulsifer was detected by IR spectrum. The IR spectrum indicated the presence of long chain fatty acids (Figure 8). UV spectrum indicated the presence of peptide groups in the product. A far UV CD spectrum of bioemulsifer showed β-sheet form behavior (Figure 9). These results indicate that the product has lipopeptide structure. Therefore, this bioemulsifer with various activities and properties can be used as an interesting compound in the pharmaceutical, industry as well as energy and environmental fields. Due to its low toxicity it could be considered a suitable surfactant in drug formulations.

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