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STUDIES ON BIOSURFACTANT PRODUCTION BY *ACINETOBACTER CALCOACETICUS* (PTCC 1318)

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Abstract

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 biosurfactant by *Acinetobacter calcoaceticus* has been studied. *A. calcoaceticus* PTCC 1318 was grown in the nutrient broth medium and biosurfactant production was evaluated every 24 h by surface tension and emulsification index. Then *A. calcoaceticus* PTCC 1318 was grown in nutrient broth with different conditions in order to get maximum production of biosurfactant. The best culture medium was found to be nutrient broth medium supplemented with NaCl and almond oil.

After growing the bacteria, the microbial biomass was removed from the supernatant by acidic precipitation method. Its amphipathic structure was established by some biochemical methods and it was confirmed as lipopolysaccharide-protein structure.

Keywords:

Acinetobacter calcoaceticus, Biosurfactant, Surface tension, Emulsification index.

Introduction

Microbial-derived surfactants are amphipathic 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 (1). Another advantage of biosurfactants is that they can be modified by biotransformation to generate new products for specific requirements (2). 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 (3).

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Potential applications of biosurfactants include emulsification, phase separation, wetting, foaming and surface activity that can be exploited in food, oil, cosmetic and pharmaceutical industries (4). In the environmental sector, microbial surfactants show promising applications in bioremediation and waste treatment to remove hazardous materials (5). *Acinetobacter calcoaceticus* produces a potent polyanionic amphipathic heteropolysaccharide biosurfactant called emulsan. The heteropolysaccharide backbone contains a repeating trisaccharide of *N*-acetyl-D-galactosamine, *N*-acetylgalactosamine uronic acid, and an unidentified *N*-acetyl amino sugar. Fatty acids are covalently linked to the polysaccharide through *o*-ester linkages. Emulsan is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001 to 0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (6). Emulsan is easily isolated from culture broth and can be produced using hydrophobic and hydrophilic substrates such as carbohydrates, hydrocarbons, vegetable oils or wastes from food industry (7). In this study, the production of bioemulsifier by *Acinetobacter calcoaceticus* PTCC1318 and some of its properties were determined by using some physicochemical methods.

Materials and methods

Test organism

The *A. calcoaceticus* PTCC 1318 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 30°C for 24 h, distinct colonies were isolated (Fig. 1) (8).

Surface activity measurement

The biomass was gathered by centrifugation (HEPTICH centrifuge mod.) at 11000 g for 20 min and the supernatant was obtained for further tests as follows. Surface tension and critical micelle dilution (CMD^{-1} & 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 *A. calcoaceticus* PTCC 1318 (an inoculum), at zero time (8).

Fig. 1: Colonies of *A. calcoaceticus* PTCC 1318 on blood agar.

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 (6,8,9).

Optimization of Growth conditions

A. calcoaceticus PTCC 1318 was initially grown in 500-mL Erlenmeyer flasks, each containing 100 mL nutrient broth medium. The flasks were incubated at 35^{o}C in a shaker incubator (F.F-81, Pars Azma CO.) at 350 rpm. In some experiments, *A. calcoaceticus* PTCC 1318 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 and olive oil, and trace metal cations [NaCl, $CaCl_2$, $MgCl_2$, respectively] (10 g L^{-1}, 10 g L^{-1}, 10 g L^{-1}) were added to the nutrient broth medium in order to get maximum production of biosurfactant. Samples were withdrawn every 24 h (three cultures for each time) to analyze the surface activity and emulsification index and therefore to select the best conditions and additives for biosurfactant production. The supernatants were tested for studying surface activity and emulsification parameter (10 – 13).

Isolation of biosurfactant and partial purification

After the bacterial cells were removed from the liquid culture by centrifugation (11000 g, 20 min) in a HEPTICH centrifuge mod., the crude biosurfactant was isolated by adding three volumes of chilled acetone to the supernatant and incubation at 4˚C for 15 h. A flocculated precipitate was collected by centrifugation (17000 g, 30 min) and dissolved in minimum volume of sterile water (pH 7.0). This solution was then lyophilized (10).

Infrared analysis

Infrared (Perkin Elmer paragon 1000) spectroscopy was used to confirm structure of the biosurfactant obtained from *A. calcoaceticus* PTCC 1318. IR spectra were collected between 400 and 4000 wave numbers (cm^{-1}).

Identification of lipid moiety

The biosurfactant was hydrolyzed with 6 M HCl 110°C for 20 h and subsequently the lipid moiety was separated by extraction with chloroform. Then several drops of bromine water were added to the extract (14).

Identification of sugar moiety

Two drops of 20% L-napthol solution (in ethanol) was added and mixed to 2 ml of a 0.1% solution of the sample. 2 ml of concentrated H_2SO_4 was poured to the side of the tube (molish test) (10).

Biuret reaction

The Biuret reagent is made of sodium hydroxide and copper (II) sulfate. The blue reagent turns violet in the presence of proteins, and changes to pink when combined with short-chain polypeptides. The sodium hydroxide does not participate in the reaction at all, but is merely there to provide an alkaline medium so that the reaction can take place. The principle can be demonstrated with the chemical compound biuret which, just as proteins, is able to complexate copper (II) ions (10).

Results and Discussion

Biosurfactant production

Reduction of surface tension is a selection criterion for biosurfactant-producing capacity of microorganisms in liquid medium (8,15,16). Therefore, *A. calcoaceticus* PTCC 1318 was cultured in nutrient broth and biosurfactant 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 (8,15). Maximum of biosurfactant production was achieved in 48 h of incubation and CMD values (Fig. 2) were minimum at this point. Emulsification index values followed a similar pattern as surface tension lowering (Table 1 and Fig. 3).

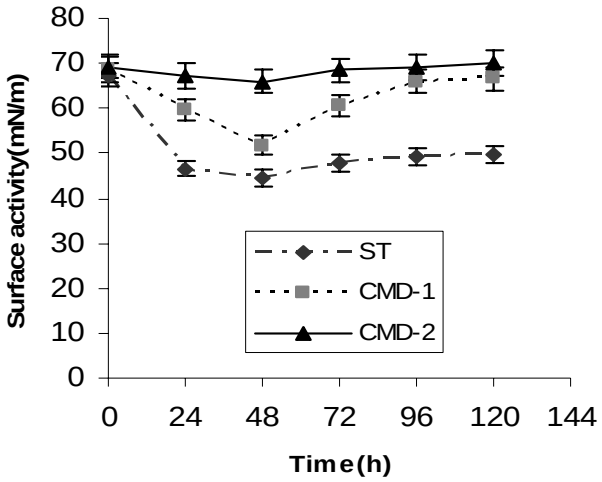

Fig. 2: Surface activity profile of *A. calcoaceticus* PTCC 1318 (30˚C, 250 rpm).

Table 1: Surface tension studies, $(Critical\ micelle\ dilution)^{-1}$; CMD^{-1}; $(Critical\ micelle\ dilution)^{-2}$; CMD^{-2}; and Emulsification index; E_{24}; results for supernatant of *A. calcoaceticus* PTCC 1318, grown in nutrient broth medium (30˚C, 250 rpm)

E_{24} ±SD	CMD^{-2} (mN/m) ±SD	CMD^{-1} (mN/m) ±SD	Surface Tention (mN/m)±SD	Time (h)
0±0.00	69.33±0.05615	68.66±0.02345	67.4±0.02236	0
4.25±0.13	67.19±0.012232	59.73±0.019010	46.6±0.03393	24
10.86±0.19	65.92±0.1999	51.72± 0.1213	44.46±0. 15047	48
6.66±0.12	68.5±0.011571	60.59±0.1232	47.96±0.11108	72
4.34±0.24	69.02±0.0277	65.93±0.03333	49.32±0.2132	96
3.377±0.17	70.08±0.01213	66.54±0.02122	49.72± 0.03142	120

When *A. calcoaceticus* PTCC 1318 was grown in the nutrient broth medium, the production of the biosurfactant was relatively poor. According to these data, 30˚C and 350 rpm were selected as best conditions (Figs. 4 and 5) (8,11).

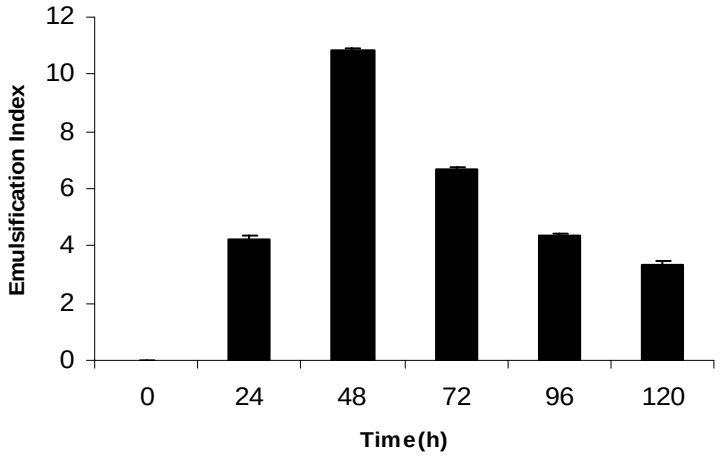

Fig. 3: Emulsification index; $E_{24,}$ graph of *A. calcoaceticus* PTCC 1318 at different times of incubation (30°C, 250 rpm).

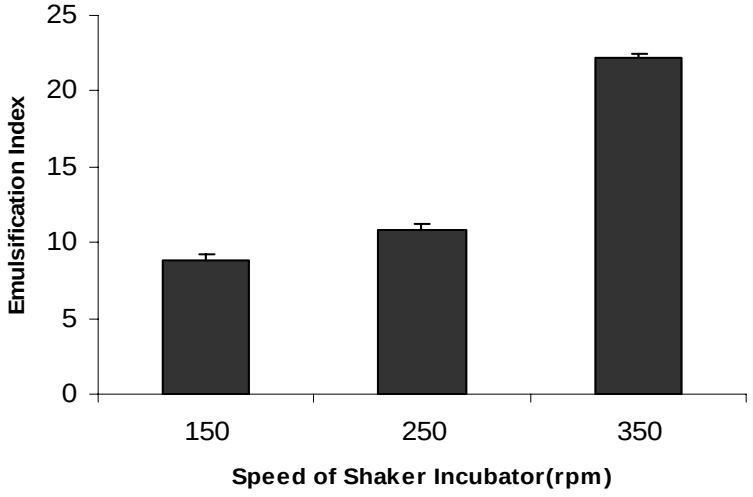

Fig. 4: Emulsification index; $E_{24,}$ graph of *A. calcoaceticus* PTCC 1318 at different aeration rates (30°C, 48 h).

The production yield was improved by addition of sodium chloride while calcium and magnesium chloride decreased it (Fig. 6). Also, the yield of biosurfactant was improved by addition of oils, such as liquid paraffin, almond and olive oils to the culture medium (nutrient broth). All of the oils caused enhancement of yield while this increase was the most in case of almond oil (Fig. 7) (11).

Therefore, from emulsification index studies, it can be concluded that when sodium chloride and almond oil was added to nutrient borth medium, the best yield of biosurfactant was obtained. The difference between observed data of surfactants was significant ($p<0.01$).

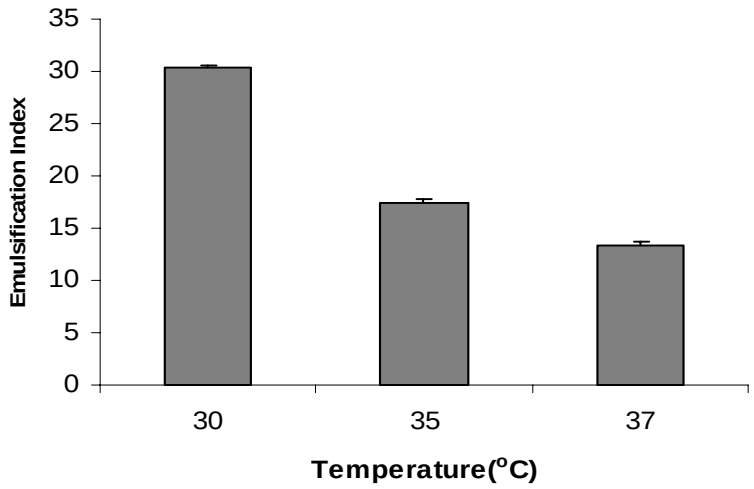

Fig. 5: Emulsification index; $E_{24,}$ graph of *A. calcoaceticus* PTCC 1318 at different temperatures (350 rpm, 48 h).

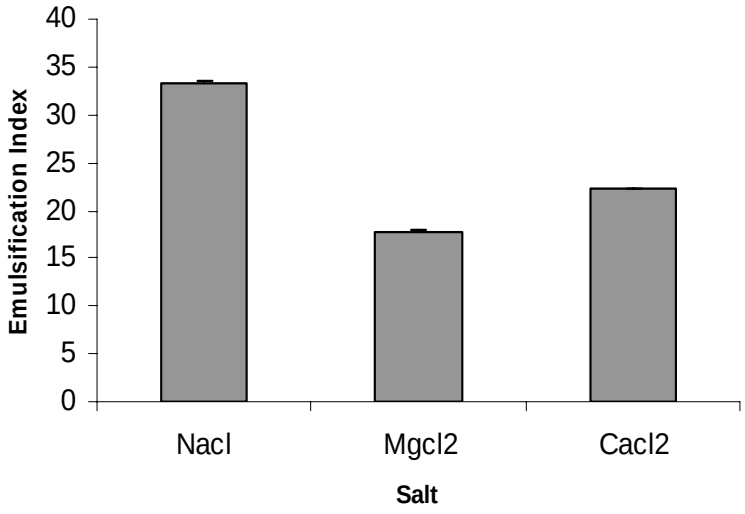

Fig. 6: Emulsification index; $E_{24,}$ graph of *A. calcoaceticus* PTCC 1318 with different salts (350 rpm, 48 h, 30°C).

IR (KBr, Cm^{-1}): 3743 (NH) 3312 (OH), 2956, 1456 and 1398 (CH_3, CH_2), 1647 (CO, amide) , 1072 & 1274 (CO , ether), (Fig. 8). Molisch's test is a chemical test for the presence of carbohydrates, based on the dehydration of the carbohydrate by sulfuric acid to produce an aldehyde. All carbohydrates, monosaccharides, disaccharides, and polysaccharides should give a positive reaction. In this study molisch's test was positive; indicating for carbohydrate. Biuret reaction was positive indicating for polypeptides and proteins. Bromine water reaction was positive indicating that the fatty acid chain was unsaturated. In conclusion, the results indicate that the product has lipopolysaccharid-protein structure and these data which are comparable with other reports. Biosurfactant produced by *A. calcoaceticus* PTCC 1318 with various activities, and can be used as an interesting compound in pharmaceutical industry, as well as environmental fields. Due to its low toxicity it could be considered as a suitable surfactant in drug formulations.

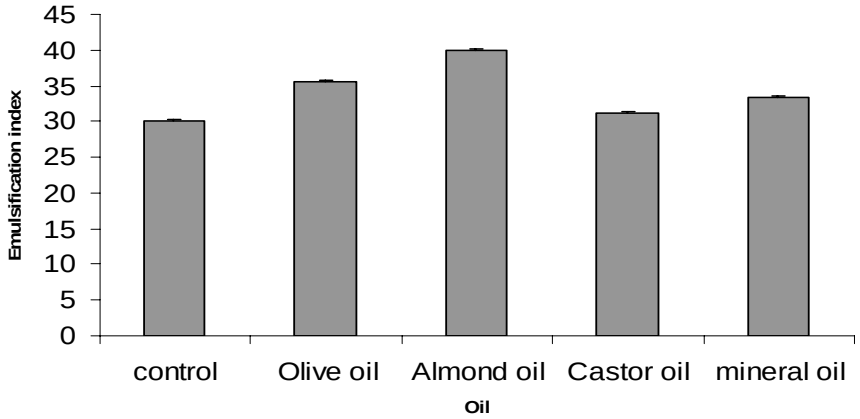

Fig.7: Emulsification index; E_{24}, graph of *A. calcoaceticus* PTCC 1318 with different hydrocarbons (350 rpm, 48 h, 30°).

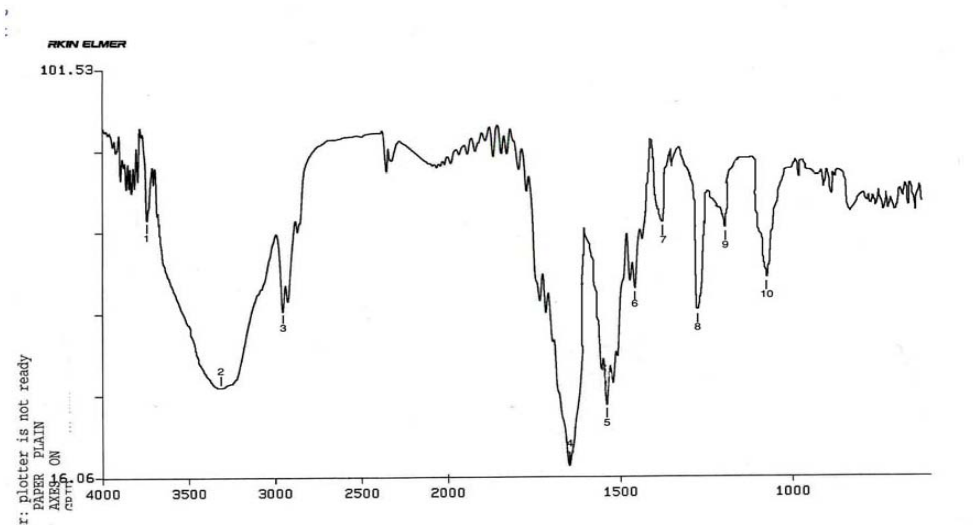

Fig.8: IR spectrum of biosurfactant.

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