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PRODUCTION OF BIOSURFACTANTS FROM TWO *BACILLUS* SPECIES

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ABSTRACT

When oil is spilled in marine environment, the lighter hydrocarbon components volatilize while the polar hydrocarbon components dissolve in water. However, because of low solubility of oil, most of the oil components will remain on the water surface. Surfactants enhance degradation by dispersing and emulsifying hydrocarbons

The production of biosurfactant from two bacterial isolates, *Bacillus licheniformis* (B5) and *B. subtilis* (B6) was investigated. The B5 isolate was an efficient producer. Maximum production of biosurfactants from both strains was obtained when using glucose and glutamic acid as carbon and nitrogen sources respectively. The two isolates were unable to grow on water immiscible sunflower oil; a lower growth was shown on crude oil as sole carbon source. The biosurfactant produced by B6 had a high emulsification activity with sunflower oil and the product of B5 had a high activity with corn oil, it also had a good emulsification activity with sunflower oil, crude oil and waste oil. Results showed that Mg^{++} concentration had an effect on the emulsification activity of the two isolates product.

INTRODUCTION

Biosurfactants are microbial surface-active compounds. They acquired their importance from their use in several industrial fields such as pharmaceutical petroleum and food industries (Deziel *et al.,* 1999). Biosurfactants have many properties such as soaping, emulsifying, foaming, dispersing…etc. They are widely used in industrial and environmental fields such as microbially enhanced oil recovery (MEOR), oil tanks cleaning and bioremediation of oil polluted water and soil (Patel and Desai, 1997).

Most of the surfactants used today are chemically synthesized. Many recent studies are focused on the use of microorganisms in the production of biosurfactants (bioemulsifier). Biosurfactants are more active and less toxic than chemical surfactants which are difficult to remove or degrade from the environment (Patel and Desai, 1997; Desai and Banat, 1997). Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil (Kosaric, 2001).

Several *Bacillus* species produce a lipopetide biosurfactant; the most important one is surfactin which is produced from *bacillus subtilis* (Nakano and Zuber.1989; Nitschke and Pastore, 2004)**.** Surfactin is not ribosomally synthesized; it is synthesised by a multi functional enzyme system as that involved in the synthesis of the peptide antibiotics released from bacilli bacteria (Nakano and Zuber, 1989). Moreover *B. licheniformis* has the ability to produce many surface active lipopeptides (Jenny, 1990; Yakimov *et al.,* 1995).

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This work aims at the production of biosurfactants from two species of *Bacillus* and the study of the emulsification activity of the produced biosurfactant.

MATERIAL AND METHODS

Bacterial isolates: *B. subtilis* (B6) and *B. licheniformis* (B5) were isolated from soil and identified at the Department of Biotechnology, University of Baghdad, Iraq. These two isolates have the ability to produce antimicrobial agents (Ouled-Haddar *et al.* 2004). The isolates were maintained at 4°C on nutrient agar slants, brain heart infusion was used for the activation of the isolate.

Production media and culture conditions: Production of the emulsifier was carried out in 250 ml Erlenmeyer flasks containing 50 ml of the medium composed of (g/l): KH_2PO_4: 0.5, K_2HPO_4: 1, KCl: 0.1, $MgSO_4.H_2O$: 0.5, $FeSO_4.7H_2O$: 0.008, $CaCl_2$: 0.05, Urea: 6 and 0.05 ml of trace elements solution (Br: 0.026, Cu: 0.05, Mn: 0.05 and Zn: 0.07) (Sifour *et al.,* 2004), carbon source was added at 4% (wt or vol/vol), pH was adjusted to 7.0. The medium was inoculated with 5% of the 18 hours bacterial culture grown on nutrient broth. Incubation was carried out at 37°C in an incubator shaker at 150 rpm for 48 hours.

The effect of carbon and nitrogen sources on the biosurfactants production was studied:

Carbon source: sunflower oil, glucose, glycerol, and crude oil were tested at 4% (wt or vol/vol).

Nitrogen source: urea, glutamic acid and $NaNO_3$ were used at 6g/l.

Biomass determination: A 5 ml sample was centrifuged at 9000 rpm for 15 min, the pellet was dried at 100ºC and weight was determined.

Determination of emulsification activity: Samples (0.5 ml) of cell free supernatant were added to a screw-capped tube containing 7.5 ml of Tris-Mg [20mM Tris HCl (pH 7.0) and 10mM $MgSO_4$] and 0.1 ml of kerosene. After a vigorous vortex, the tubes were allowed to sit for 1 hour. Absorbance was measured at 540 nm (Patel and Desai, 1997). Emulsification activity (E.A) was defined as the measured optical density. Assays were carried out in triplicates.

Emulsification properties of produced compounds: The emulsification activity of the compounds produced from the two isolates B5 and B6 using different hydrocarbons (kerosene, octane, heptadecane, paraffin, olive oil, sunflower oil, corn oil, crude oil and used oil) was tested. Emulsification activity was measured as described above.

Extraction of biosurfactant: 50 ml of the culture medium was precipitated at 9000 rpm for 10 min at 4ºC. pH of the cell free supernatant was adjusted to 2.0 using 1N HCl, and kept to the next day at 4ºC, then precipitated again at 15000 rpm for 15 min at 4ºC. The precipitate was dissolved in 2 ml distilled water and the pH was adjusted to 7.0 with 1N NaOH (Javaheri *et al,* 1985).

Effect of Mg^{++} ion on the emulsification activity: Different concentrations of $MgSO_4$ (5, 10, 15, 20 and 25) mMol were added to Tris buffer used for the determination of the emulsification activity. Emulsification activity was determined by adding 100 µl of crude extract to Tris Mg (20 mMol of Tris HCl and different concentrations of $MgSO_4$) and 100 µl of kerosene. Activity was measured as described above.

RESULT AND DISCUSSION

The two species of *Bacillus* (*B. subtilis* (B6) and *B. licheniformis* (B5)) have the ability to produce biosurfactant. To determine the appropriate carbon source for the production of biosurfactant, four different

carbon sources were used (Figure 1). For B5, glucose was the best source of carbon for growth (1.31g/l) and for the production of the biosurfactant (E.A. was 0.36). Isolate B6 grew preferably on glycerol (1.28g/l) but high production was shown with glucose as the carbon source (E.A. 0.29). Many studies showed that glucose was the best carbon source for the production of surfactin biosurfactant from *B. subtilis*, with a concentration of 4% (Cooper *et al,* 1981; Mulligan *et al,* 1989). A low growth and a decrease in biosurfactant production was shown with crude oil as carbon source from the two isolates. No growth was seen when using sunflower oil as sole carbon source. *B. licheniformis* JF-2 could not utilize oils for growth (Javaheri et al, 1985). Several isolates of *B. subtilis* could produce biosurfactant only when they are grown on water soluble compounds. This emphasizes that the production of biosurfactant from microorganisms is not only to facilitate the uptake of water immiscible substrates (Cooper and Goldenberg, 1987).

Different nitrogen sources were tested to determine the best source for biosurfactant production (Figure 2).Addition of glutamic acid (6g/l) to the production medium enhanced both growth and biosurfactant production from the two isolates (B5and B6), however $NaNO_3$ was a good substrate for the growth but it gave a low productivity. Glutamic acid was found to be the best nitrogen source for the production of bacitracin from the isolate B5 (Ouled-Haddar et al, 2004). It was reported that $NaNO_3$ and yeast extract were used for the production of biosurfactants from different *Bacillus sp.* (Javaheri *et al,* 1985; Cooper and Goldenberg, 1987).

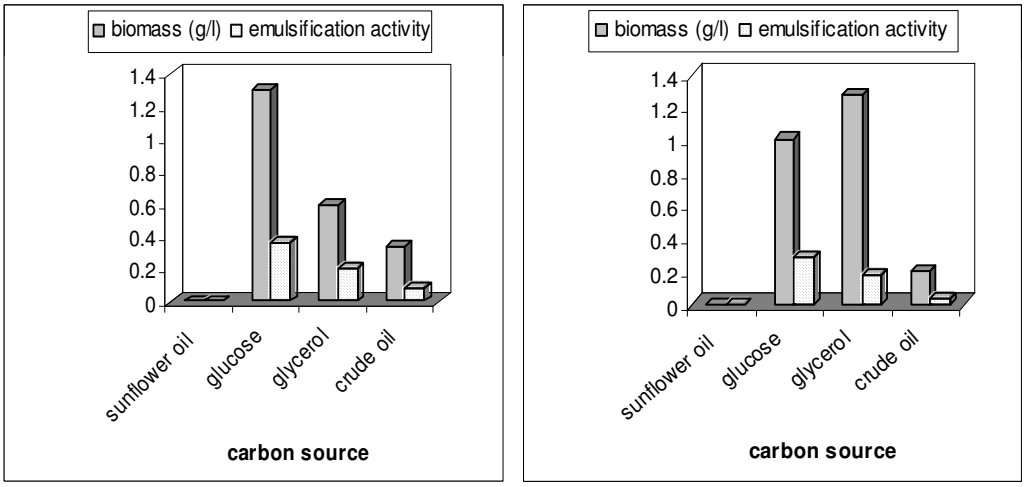
Emulsification activity of the produced biosurfactants using different hydrocarbons

The emulsification activity of the produced biosurfactants was tested with different hydrocarbons. Table 1 showed that the highest E.A. of the produced compounds from isolate B5 was obtained when using corn oil in the reaction mixture while the compounds produced from the isolate B6 showed a high E.A. with kerosene and sunflower oil. It was seen that compounds produced from isolate B5 had a high E.A. when compared with those from isolate B6. Furthermore, compounds from isolate B5 showed good emulsification with sunflower oil, crude oil and used oil, which gave it an importance in bioremediation of oil pollution. It was reported that several biosurfactants had the ability to emulsify crude oil and vegetable oils (Patel and Desai, 1997; Navon-venesia *et al.,* 1995).

Effect of Mg^{++} ions on the emulsification activity

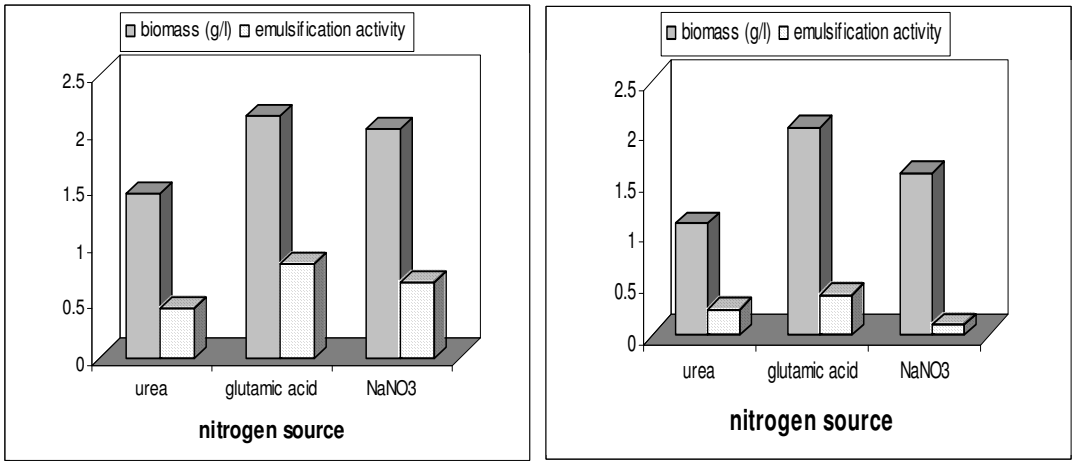
After the extraction of the produced biosurfactant, the precipitate was dissolved in 2 ml distilled water. The crude extract was used to study the influence of magnesium ions on the emulsification activity. Figure 1 showed that Mg^{++} ions had a significant effect on the emulsification activity. A remarkable increase in emulsification activity was obtained with the biosurfactant produced from isolate B5 with the increase of Mg^{++} ions concentration; it reached 1.1 in the presence of 25 mMol $MgSO_4$. On the other hand, the E.A. of the compounds produced from B6 was slightly affected by $MgSO_4$ concentrations and maximum activity was showed when 10 mMol $MgSO_4$ was added. These results showed that crude extract from isolate B5 was more active than that produced from isolate B6. It was reported that Mg^{++} ions and Ca^{++} had an important effect on the emulsification activity of emulcyan (biosurfactant) from *Phormidium* J-1 (Fattom and Shilo, 1985).

In conclusion these two *Bacillus* strains produced efficient biosurfactants especilly from isolate B5 that are able to emulsify crude and used oils. Therefore, they could be utilized for the bioremediation of oil pollution.



(A) (B)

Figure 1: Biosurfactant production from *B. licheniformis* B5 (A) and *Bacillus subtilis* B6 (B) using different carbon sources. Biomass (g/l) was determined after 48h incubation at 37ºC. Emulsification activity was determined as optical density at 540 nm.



A B

Figure 2: Biosurfactant production from *B. licheniformis* B5 (A) and *Bacillus subtilis* B 6 (B) using different nitrogen sources. Biomass (g/l) was determined after 48h incubation at 37ºC. Emulsification activity was determined as optical density at 540 nm.

Table 1: Emulsification activity of biosurfactants from *B. subtilis* B6 and *B. licheniformis* B5 using different hydrocarbons.

Hydrocarbons	Emulsification activity at 540 nm	
	B5	B6
Kerosene	0.7	**0.30**
Paraffin	0.09	0.04
Crude oil	**1.05**	0.15
Used oil	**0.92**	0.15
Heptadecan	0.42	0.15
Octane	0.17	0.14
Corn oil	**1.46**	0.20
Sunflower oil	**1.05**	**0.25**
Olive oil	0.24	0.07

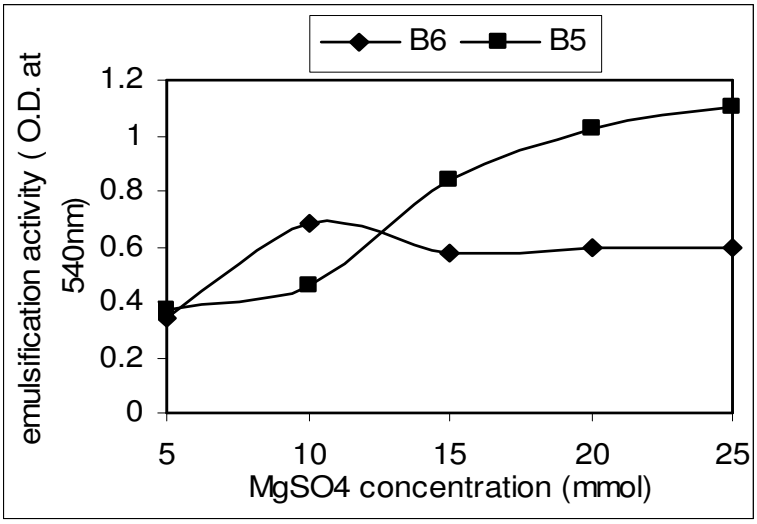


Figure 3: Effect of different concentrations of $MgSO_4$ on the emulsification ctivity of produced biosurfactant from B5 and B6.

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