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BIOSURFACTANTS PRODUCED BY A NEWLY ISOLATED *PSEUDOMONAS FLUORESCENS HW-6* DURING GROWTH ON HEXADECANE

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ABSTRACT. The newly isolated from industrial wastewater samples *Pseudomonas fluorescens* strain HW-6 produced biosurfactants during growth on hexadecane as sole source of carbon and energy. Hemolysis of erythrocytes, growth inhibition of *Bacillus subtilis* and thin-layer chromatography revealed that the secreted biosurfactants are rhamnolipids. They decreased the surface tension of water from 72 mN m^{-1} to 32 mN m^{-1} and achieved a critical micelle concentration value of 20 mg l^{-1}. They efficiently emulsified aromatic hydrocarbons, kerosene, *n*-paraffin and mineral oil. The results showed that the newly isolated *Pseudomonas fluorescens* HW-6 and its produced glycolipids with effective surface and emulsifying properties represent a promising potential for application in bioremediation of polluted with hydrocarbons wastewaters.

KEY WORDS. Biosurfactants, Rhamnolipids, Hydrophobicity, *Pseudomonas fluorescens*

INTRODUCTION

Oil pollution is an environmental problem of increasing importance. Microorganisms are the main degraders of petroleum hydrocarbons in contaminated ecosystems (Leahy and Colwell, 1990). Bioremediation is limited by the poor availability of hydrophobic pollutants. Hydrocarbon-degrading microorganisms, mostly bacteria, can produce biosurfactants, amphiphilic molecules with effective surface and biological properties (Desai and Banat, 1997; Rosenberg and Ron, 1999). They can reduce the surface tension and enhance emulsification of hydrophobic

substrates thus increasing their availability for microbial degradation (Ron and Rosenberg, 2002). Because of their biodegradability, ecological safety, production on renewable resources and functionality under extreme conditions, biosurfactants are very attractive alternatives to the chemical surfactants (Lang and Wullbtandt, 1999; Banat *et al*., 2000; Rahman *et al*., 2002).

Biosurfactants are structurally diverse group of surface-active molecules and could have more or less specific role in different ecological niches (Ron and Rosenberg, 1999). It is therefore promising to isolate indigenous microorganisms from contaminated sites able to grow on different hydrocarbons and produce surfactants. Co-treatment with adapted isolates and surface active complexes of their own specific ecological niches or other related environments would be more effective for bioremediation.

In a previous work, 15 bacterial strains were isolated from lubricating oil contaminated wastewater, and a model consortium was proposed for bioaugmented clean-up of wastewater (Vasileva-Tonkova and Galabova, 2003). Several isolates grew well on hexadecane as a sole carbon source producing glycolipids. Among them, isolate marked as HW-6 showed the best growth and glycolipid secretion and was selected for further experiments. However, no investigations on its surface activity were made.

The aim of this work was to characterize the surface active compounds and its…. CMC value produced by the selected strain during growth on hexadecane as a model hydrocarbon used as a sole source of carbon and energy. An assessment was made also for possible application of secreted biosurfactants for waste treatment of hydrocarbon polluted environments.

MATERIAL AND METHODS

Microorganism and growth conditions

Bacterial strain HW-6 was isolated in this laboratory from lubricating oil polluted water (Vasileva-Tonkova and Galabova, 2003). It was identified as *Pseudomonas fluorescens* by routine morphological, microbiological and biochemical methods according to the latest edition of Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Isolate was maintained by monthly transfers onto slants containing meat-peptone broth (MPB), and after growth, stored at 4 ^{o}C. Stock culture was also prepared as cells suspended in a cryoprotective agent (20% sterile glycerol, final concentration, 10% v/v) and stored at -18 ^{o}C.

P. fluorescens HW-6 was cultivated in 500-ml Erlenmeyer flasks containing 50 ml mineral salt (MS) medium with composition as described earlier (Vasileva-Tonkova and Galabova, 2003). The medium was supplemented with 1.5% hexadecane or glucose as a sole carbon source. Hexadecane was sterilized through 0.2-µm Millipore membrane filters. Starter cultures were grown for 20 h at 28 ^{o}C in MPB. Experimental cultures were inoculated (1% v/v of the starter culture) and incubated in the dark at 28 ^{o}C in a rotary shaker at 130 rpm. Growth was monitored by optical density at 570 nm (OD_{570}).

Detection and isolation of surface-active compounds

The following tests were used for detection of surface-active compounds: hemolysis of erythrocytes (Johnson and Boese-Marazzo, 1980), growth inhibition of *Bacillus subtilis* (Itoch *et al*., 1971) and thin-layer chromatography (TLC) (Kates, 1972). For hemolysis of erythrocytes and growth inhibition of *B. subtilis,* culture supernatants were concentrated as described by Koch *et al*. (1991). The blood agar plates were incubated at 28 ^{o}C for 2 days, and *B. subtilis* plates at 28 ^{o}C overnight.

Surface activity assay

The surface tension of biosurfactant solutions was determined with an automatic tensiometer (Austria) at room temperature by the ring method. The organic extract was dried and redissolved in 0.14 M NaCl. Appropriate dilutions were prepared with 0.14 M NaCl. The critical micelle concentration (CMC) value is defined as that point at which biosurfactant no longer aggregated to form micelles. Values reached after continuous measurement were plotted against biosurfactant concentration and the CMC was determined graphically.

RESULTS AND DISCUSSION

Biosurfactant production

P. fluorescens HW-6 produced surface active compounds during growth on MS medium with 1.5% hexadecane as a sole source of carbon and energy. The secretion of biosurfactants was estimated using both an increase in emulsifying activity and in terms of rhamnose concentration. As can be seen in Fig.1, biosurfactant secretion started in the late-exponential growth phase and reached glycolipid production of 1.4 ± 0.22 g l^{-1} in the stationary growth phase. Simultaneously an increase in emulsifying activity was observed. Disappearance of hexadecane droplets was observed and biosurfactant production was accompanied by appearance of white turbidity of the culture medium at the third day of growth.

As the biosurfactants are secondary metabolites, maximal production occurs in the stationary growth phase (Bodour and Maier, 2002). Limiting addition of inorganic nutrients, including phosphate, nitrogen, iron, and carbon excess, has been reported to increase biosynthesis of lipids, polysaccharides or secondary metabolites (Boulton and Ratledge, 1987; Bodour and Maier, 2002; Ron and Rosenberg, 2002).

Detection of surface active compounds

The orcinol assay was used for quantification of glycolipids in organic extracts (Koch *et al.*, 1991). Rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose.

The supernatant fluid of *P. fluorescens* HW-6 after 10 days of growth on hexadecane lowered the surface tension of the medium below 35 mN m^{-1}. It was measured 34-mm in diameter zone of hemolysis on blood agar of 100-fold concentrated supernatant fluid after 2 days of incubation (Fig. 2A). After overnight incubation, 18-mm in diameter zone of inhibition of the growth of *B. subtilis* by10-fold concentrated supernatant was measured (Fig. 2B).

Surface tension and the CMC

For practical purposes, there is difference between an efficient surfactant and an effective surfactant. Efficiency is measured by the surfactant concentration required to produce a significant reduction in the surface tension of water, whereas effectiveness is measured by the minimum value, to which the surface tension can be reduced (Kim *et al.*, 2000). Therefore important properties of surfactants are their abilities to lower the surface tension in aqueous solutions, and to possess a low CMC (Sheppard and Mulligan, 1987).

It is known that the individual rhamnolipids are able to lower the surface tension of water from 72 mN m^{-1} to 25-30 mN m^{-1}, at concentrations of 10-200 mg l^{-1} (Lang and Wullbtandt, 1999; Bodour and Maier, 2002). Rhamnolipids secreted by *P. fluorescens* HW-6 reduced the surface tension of the water from 72 mN m^{-1} to nearly 32 mN m^{-1}. As shown in Fig. 4, the CMC was found to be approximately 20 mg l^{-1}, and the surface tension at this point was 37 mN m^{-1}. These results indicated that rhamnolipids from *P. fluorescens* are both efficient and effective surfactants.

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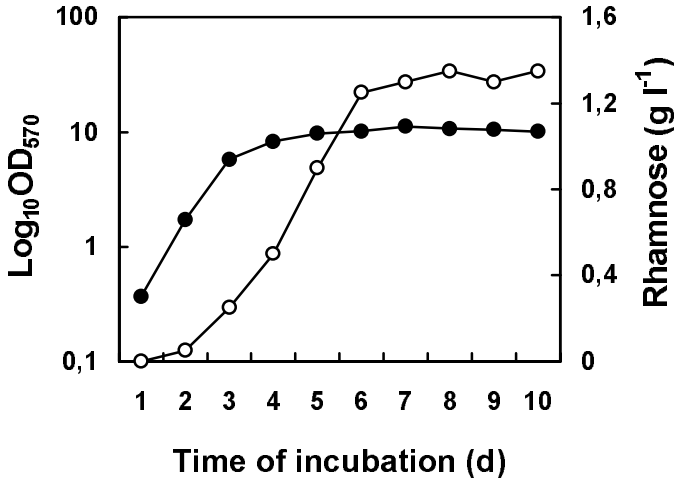

Fig. 1. *Time-course of glycolipid production during growth (measured as the optical density at 570 nm) of Pseudomonas fluorescens HW-6 cells in a batch culture with 1.5% hexadecane as a sole carbon source. Glycolipid levels are expressed as rhamnose equivalents. Mean values from three separate experiments are given.*
(o) rhamnose concentration; (●) OD_{570}

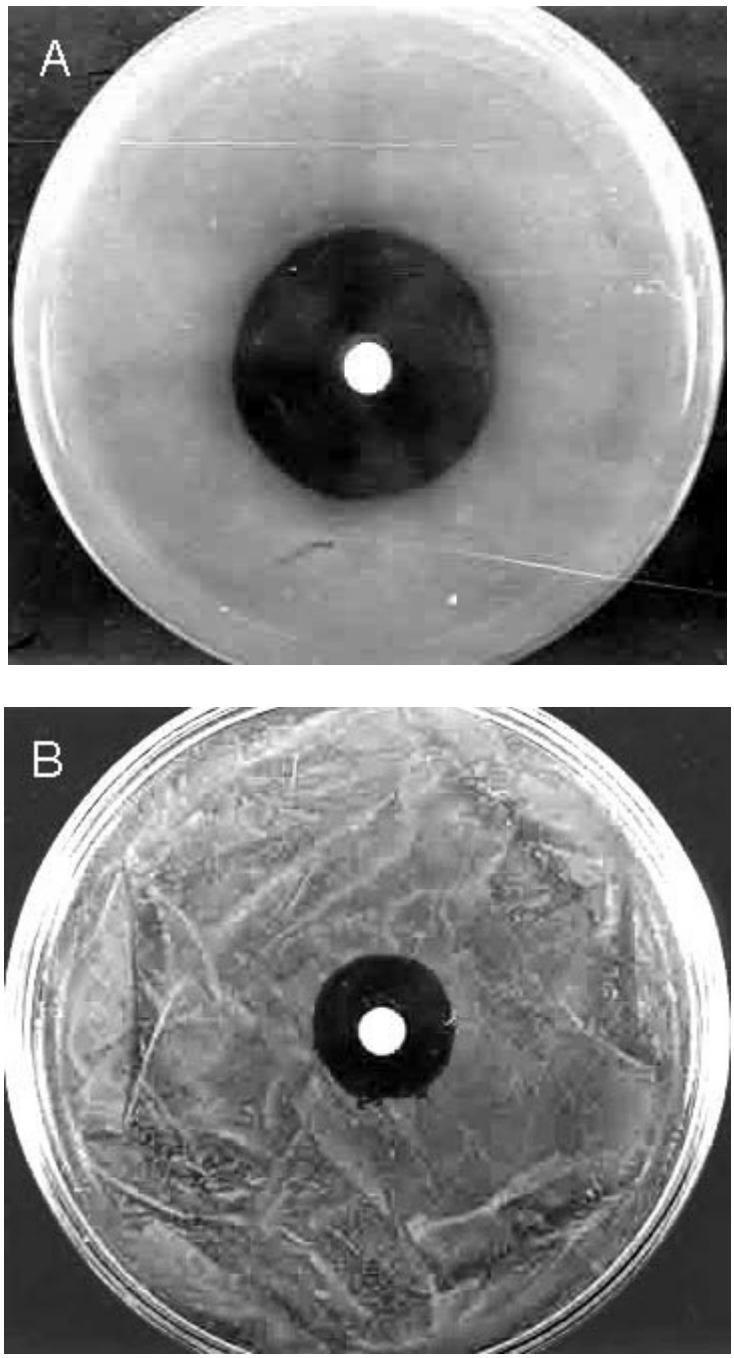

Fig. 2. *Detection of glycolipids produced by hexadecane-grown Pseudomonas fluorescens HW-6: hemolytic activity of a 100-fold concentrated supernatant fluid (plate A) and growth inhibition of Bacillus subtilis by 10-fold concentrated supernatant fluid (plate B).*

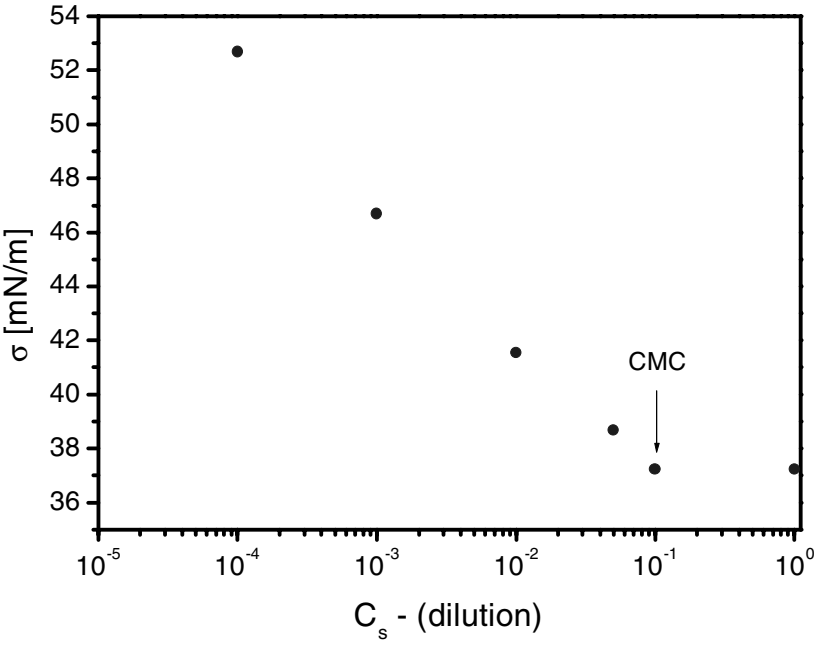

Fig. 3. *Critical micelle concentration (CMC) of P. fluorescens HW-6 rhamnolipids. Surface tension of the organic extract was measured after dilutions with 0.14 M NaCl, and the CMC was determined graphically.*