

Naphthalene Degradation and Biosurfactant Activity by *Bacillus cereus* 28BN

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Z. Naturforsch. **60c**, 577–582 (2005); received Dezember 8, 2004/February 3, 2005

Biosurfactant activity and naphthalene degradation by a new strain identified as *Bacillus cereus* 28BN were studied. The strain grew well and produced effective biosurfactants in the presence of *n*-alkanes, naphthalene, crude oil and vegetable oils. The biosurfactants were detected by the surface tension lowering of the medium, thin layer chromatography and infrared spectra analysis. With (2%) naphthalene as the sole carbon source, high levels of rhamnolipids at a concentration of 2.3 g l^{-1} were determined in the stationary growth. After 20 d of incubation $72 \pm 4\%$ of the initial naphthalene was degraded. This is the first report for a *Bacillus cereus* rhamnolipid producing strain that utilized naphthalene under aerobic conditions. The strain looks promising for application in environmental technologies.

Key words: Naphthalene Degradation, Rhamnolipids, *Bacillus cereus*

Introduction

Organic hydrophobic pollutants include polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons, polychlorinated biphenyls and a range of biocides. PAHs are common environmental contaminants due to industries involved in coal and crude oil processing. They include low molecular weight substances (naphthalene) and high molecular weight components (pyrene, fluorene). Since many of them are known to be carcinogens or mutagens, PAHs represent a significant risk for human health (White, 1986).

Natural biological degradation processes of PAHs in contaminated environments are limited by the poor availability of these hydrophobic substrates to microorganisms. Surfactants can help, by solubilization or emulsification, to release hydrocarbons sorbed to organic matter and to increase their aqueous concentration. Several researchers have investigated the addition of biosurfactants to enhance hydrocarbon biodegradation (Oberbremer and Muller-Hurtig, 1990; Zhang and Miller, 1992; Hunt *et al.*, 1994).

On the other hand, more knowledge about the specific mechanisms used by microorganisms to get access to PAHs will be useful to improve PAH biodegradation applications. The objective of this study was to investigate the capacity of the PAH-

utilizing strain *Bacillus cereus* 28BN to produce biosurfactants and to determine if the production of surfactants is a part of its strategy to grow on poorly available substrates.

Materials and Methods

Microorganism

The strain *Bacillus cereus* 28BN used in this study was isolated from hydrocarbon contaminated industrial wastes. The organism was selected by means of enrichment culture techniques for its ability to grow on naphthalene as the single source of carbon and energy. The selected strain was identified on the basis of Gram reaction, cell morphology and several physiological and biochemical tests following directions of the latest edition of Bergey's Manual (Holt *et al.*, 1994). The composition of the mineral salt medium used in this study was described by Tuleva *et al.* (2002).

Growth conditions

Batch growth experiments were performed in 500 ml Erlenmeyer flasks containing 100 ml mineral salt media, pH 7.2. The carbon source crystalline naphthalene (Aldrich Chemical Co., Steinheim, Germany) and other carbon sources were added at the concentration of 20 g l^{-1} . The experi-

ments were started by inoculation with 5% log phase culture pregrown on meat peptone broth. All cultures were performed in the dark at $28 \pm 1^\circ\text{C}$ in an orbital incubator at 130 rpm. Control flasks without bacteria were incubated in the same conditions to quantify losses due to abiotic processes. As no significant losses (less than 1%) were found in the abiotic flasks, it is therefore assumed that losses are entirely due to biodegradation. The growth was monitored by measuring the absorbance at 610 nm (A_{610}) and in terms of whole cell protein, for which cells were harvested by centrifugation ($5,000 \times g$, 10 min), digested in 1 N NaOH (boiling waterbath, 10 min) and protein was estimated by the method of Lowry *et al.* (1951).

Detection of biosurfactant activity

Blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg ml^{-1} ; Sigma Chemical Co., Poole, UK) and methylene blue ($5 \mu\text{g ml}^{-1}$) were used to detect extracellular glycolipid production (Siegmond and Wagner, 1991). Biosurfactants were observed by the formation of dark blue halos around the colonies.

Samples of the culture media of the selected strain were centrifuged at $8,000 \times g$ for 20 min. The surface tension of the supernatant fluid of the culture was measured by the ring method using an automatic Wilhelmy tensiometer (Biegler Electronic, Mauerbach, Austria). The emulsifying activity of the culture supernatant was estimated by adding 0.5 ml of sample fluid and 0.5 ml of kerosene to 4.0 ml of distilled water. The tube was vortexed for 10 s, held stationary for 1 min, and then visually examined for turbidity of a stable emulsion.

The surface active compounds were extracted by liquid-liquid extraction with 3 volumes of diethyl ether from the supernatant fluid with previous acidification with HCl to pH 2. The organic extracts were analyzed by thin layer chromatography (TLC) on silica gel 60 plates (5553, Merck). Chromatograms were developed with chloroform/methanol/acetic acid (15:5:1 v/v/v) and visualized by orcinol/sulfuric acid staining as described by Itoch *et al.* (1971) using rhamnolipids RLL ($\text{C}_{26}\text{H}_{48}\text{O}_9$) and RLL ($\text{C}_{32}\text{H}_{58}\text{O}_{13}$) from *Pseudomonas aeruginosa* as the reference substances (Jeneil Biosurfactant Company, USA). Further identification of the sugar moiety after acidic hydrolysis confirmed it as rhamnose.

The orcinol assay (Chandrasekaran and BeMiller, 1980) was used to directly assess the amount of glycolipids in the sample. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg ml^{-1}).

Infrared spectra (IR)

The biosurfactants were extracted from the supernatant fluid (2 ml) with chloroform (2 ml), dried with Na_2SO_4 and evaporated on a rotary evaporator. The isolated substance was again dissolved in CHCl_3 and casted on a KBr window. The IR spectra were recorded on a Bruker IFS113v FTIR spectrometer, in the $4,000\text{--}400 \text{ cm}^{-1}$ spectral region at a resolution of 2 cm^{-1} and 50 scans.

Determination of residual naphthalene concentration

Residual naphthalene was extracted from whole cultures with two volumes of *n*-hexane and analyzed with a gas chromatograph (Hewlett-Packard model 5859) equipped with a flame ionization detector.

Determination of the critical micelle concentration (CMC)

The determination of CMC was performed by several dilutions of the supernatant fluid containing rhamnolipids after 20 d of cultivation. The surface tension of the supernatant fluid of the culture was measured by the ring method using an automatic Wilhelmy tensiometer (Biegler Electronic) at room temperature.

Cell surface hydrophobicity test

The bacterial adhesion to hydrocarbons (BATH) assay was used to determine changes in cell surface hydrophobicity during growth on minimal salt medium with 2% naphthalene (Rosenberg *et al.*, 1980).

Analysis of naphthalene intermediates

The concentration of hydroxylated aromatic metabolites from naphthalene degradation was determined by an adaptation of the method of Box (1983) which uses the Folin-Ciocalteu reagent. As it was assumed that the major metabolite is salicylic acid, a standard curve was prepared with sodium salicylate and the concentration of hydroxylated metabolic intermediates was estimated as salicylate equivalents in mg ml^{-1} .

Results and Discussion

Detection of the surface active compounds

The assay for the detection of extracellular rhamnolipids (Siegmund and Wagner, 1991) is based on the property that the concentration of anionic surfactants in aqueous solutions can be determined by the formation of insoluble ion pairs with various cationic substrates. The formation of this ion pair precipitate in the agar plate containing methylene blue is visualized by a dark blue region against the light blue background. The diameter of the dark blue region has been previously shown to be semiquantitatively proportional to the concentration of the anionic surfactant. Accordingly, the newly isolated strain *B. cereus* 28BN formed dark blue halos on the agar plates which are typical for the production of extracellular glycolipids (Siegmund and Wagner, 1991).

The thin-layer chromatography analysis showed that *B. cereus* 28BN biosurfactants contained one major component with the retardation factor (R_f) of 0.82. Further identification of the sugar moiety after acidic hydrolysis confirmed it as rhamnose.

Consistent with this finding is the infrared spectra analysis of the organic extract of *B. cereus* 28BN surface active compounds. As seen from Fig. 1, the carbonyl stretching band was observed at $1,745\text{ cm}^{-1}$, confirming the presence of an ester compound. In the region $3,000\text{--}2,700\text{ cm}^{-1}$ were also observed several C-H stretching bands of CH_2 and CH_3 groups.

Table I. Growth and surfactant production by *B. cereus* 28BN on water immiscible substrates.

Carbon source 2%	Whole cell protein [mg ml ⁻¹]	Surface tension [mN m ⁻¹]	Rhamnose [mg ml ⁻¹]
<i>n</i> -Hexadecane	0.92	23.5	1.72*
<i>n</i> -Paraffin	0.87	24.7	1.46*
Naphthalene	0.74	25.0	2.30**
Crude oil	0.62	31.0	0.84**
Olive oil	0.37	29.8	0.68*
Sunflower oil	0.32	25.5	0.57*

* Estimated after 7 d of incubation
** Estimated after 20 d of incubation.

Growth and surfactant activity on water immiscible substrates

Bacillus cereus 28BN showed the ability to produce effective biosurfactants during growth on water insoluble substrates (Table I). Water immiscible substrates like *n*-alkanes, naphthalene, hydrocarbon mixture (crude oil) and vegetable oils supported the surfactant production. Utilization of *n*-hexadecane, *n*-paraffin and naphthalene resulted in high production of rhamnolipids and significant reduction of surface tension. When vegetable oils were used as the sole carbon source, the surfactant concentration was lower but the growth was accompanied by the formation of stable oil in water emulsions.

As seen from Table I, the organism grew equally well on aliphatic and aromatic hydrocarbons.

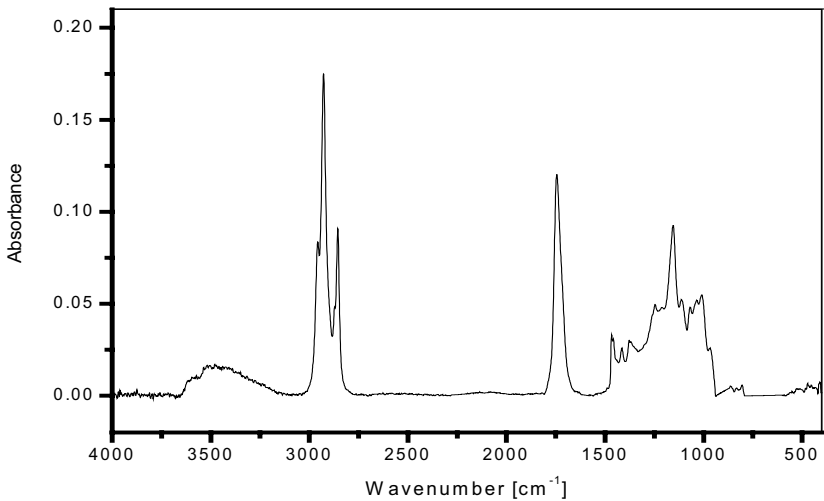


Fig. 1. Infrared spectrum of *Bacillus cereus* 28BN rhamnolipid surfactant.

However, the strain showed a great capacity for rhamnolipid production associated with good surface active characteristics on 2% naphthalene, so further investigations were conducted using this substrate.

*Biosurfactant production during growth of *B. cereus* 28BN on naphthalene*

When *B. cereus* 28BN was cultured on 2% naphthalene as the sole carbon source the surface tension decreased to 25 mN m^{-1} during the exponential growth phase (Fig. 2). This was accompanied by the formation of stable emulsions of the cell-free culture broth with kerosene, which indirectly implies the production of a biosurfactant or a mixture of biosurfactants. As expected, since rhamnolipids are secondary metabolites, the surfactant concentration started to increase more rapidly near the end of the exponential growth and high levels of rhamnolipids (2.3 g l^{-1}) were estimated in the late stationary phase.

Determination of the critical micelle concentration

One of the main characteristics of surfactants is their tendency to absorb at interfaces in an oriented fashion as a consequence of their amphipathic structure. As the surfactant concentration increases, the surface tension of the solution initially decreases and then becomes almost constant due to the interface saturation with the surfactant. The concentration at which this phenomenon occurs is known as the critical micelle concentration (CMC) and is determined from the break point of the surface tension *versus* concentration curve (Rosen, 1989). The measurement of surface tension as a function of rhamnolipid concentration (Fig. 3) showed that the tension decreased gradually until a minimum constant value of 25 mN m^{-1} was reached. The break point of the experimental curve yielded a CMC of 100 mg l^{-1} and is consistent with those reported in the literature for some rhamnolipid surfactants from *Pseudomonas aeruginosa* (Abalos and Pinazo, 2001; Haba *et al.*, 2003).

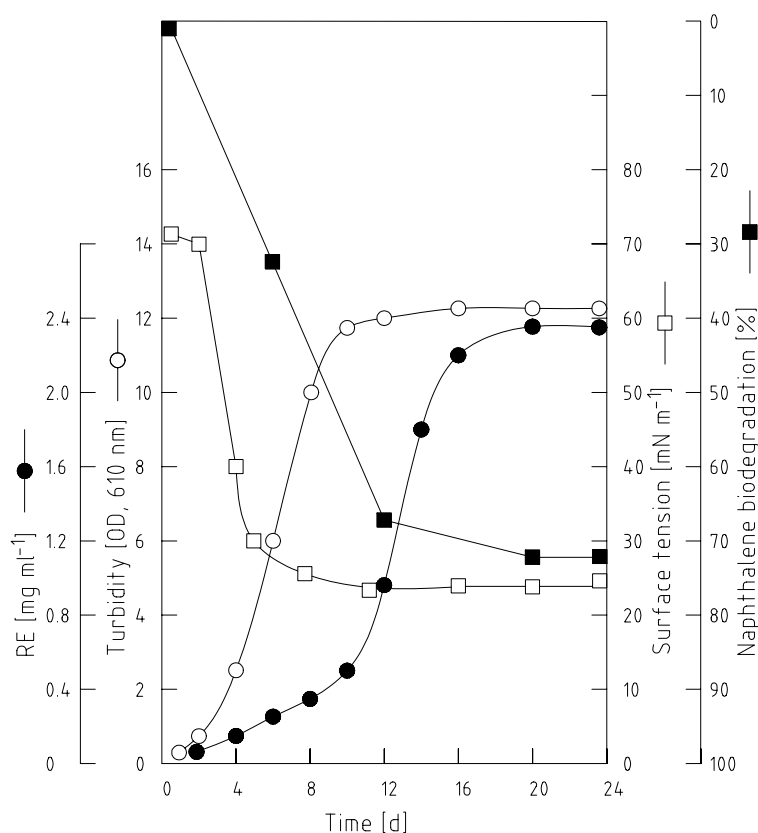


Fig. 2. Biosurfactant production and naphthalene degradation by *Bacillus cereus* 28BN grown on mineral salt medium with 2% naphthalene as the substrate. Incubation was done at 28 °C with shaking at 130 rpm. The biosurfactant levels are expressed as rhamnose equivalents (RE). OD, optical density. Biodegradation is expressed as % residual naphthalene. The values are averages from three cultures.

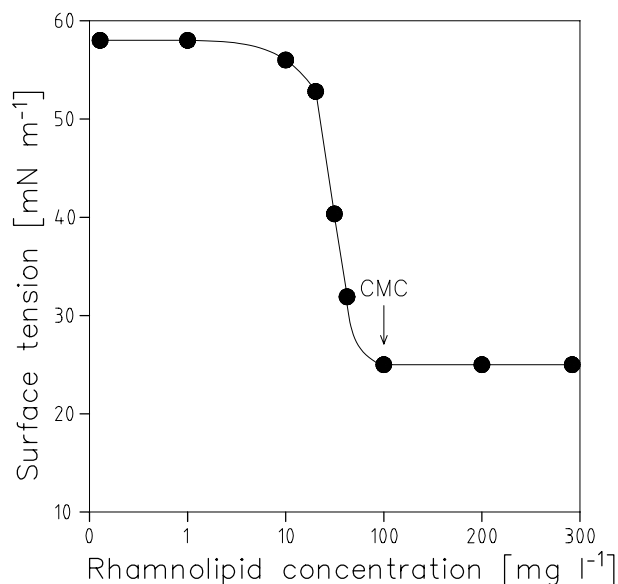


Fig. 3. Effect of increasing rhamnolipid concentrations on surface tension. The rhamnolipid solutions were prepared by several dilutions of the supernatant fluid after 20 d of cultivation on 2% naphthalene. The surface tension was measured at room temperature.

Degradation of naphthalene

Bacterial degradation of naphthalene (Fig. 2.) was quantified by two ways – indirectly, by the determination of naphthalene polar intermediates and directly, by measuring the concentration of residual naphthalene. Accumulation of the major metabolite, salicylic acid, started in the middle of the exponential growth and reached a maximal value of 760 mg l⁻¹ in the beginning of the stationary phase. The biodegradation percentage values expressed as residual naphthalene (mean ± SD%, $n = 3$) show that at the end of incubation $72 \pm 4\%$ of the initial naphthalene was degraded (Fig. 2).

The effective production of biosurfactants is usually viewed as the obvious criterion for the existence of biosurfactant-mediated hydrocarbon transfer. In fact, the question of the mode of action of biosurfactants in promoting emulsification (Rosenberg and Rosenberg, 1981; Neu and Poralla, 1990) or solubilization (Rosenberg and Rosenberg, 1981; Zhang and Miller, 1994) of the substrate, or in modifying cell hydrophobicity (Zhang and Miller, 1994) is long standing. According to Bouchez-Naitali *et al.* (1999) in biosurfactant-mediated transfer, cell contact takes place with hydrocarbons emulsified or solubilized by biosurfactants. They have observed for a large group of biosurfactant producing bacteria with high or medium hydrophobicity, that the hydrocarbon transfer mechanism involved both emulsification and solu-

bilization of the substrate but one or the other usually being privileged, depending on the strain concerned. However, the data on bacterial cell hydrophobicity suggested that for hydrophobic biosurfactant-producing bacteria, the hydrocarbon transfer mechanism involved primarily substrate emulsification thus providing emulsified hydrocarbon droplets containing hydrophobic regions on their surface. When grown on naphthalene as the substrate, *B. cereus* 28BN acted in a similar way. The strain exhibited medium cell surface hydrophobicity in the exponential growth which changed to high in the stationary phase (from $53 \pm 2\%$ to $90 \pm 1\%$, respectively). Most probably, in the case of *B. cereus* 28BN biosurfactant-enhanced naphthalene uptake involved primarily emulsification rather than solubilization. This is in line with the observation that a stable emulsion in the culture medium was formed at surfactant concentrations above the CMC and after that the percentage values of residual naphthalene started to decrease rapidly.

Bacillus is one of the common hydrocarbon-degrading microorganisms found in many different environments. Thus, Shimura *et al.* (1999), Zhuang *et al.* (2002), and Calvo *et al.* (2004) have reported the isolation of novel naphthalene-degrading *Bacillus* strains. To our knowledge this is the first report of rhamnolipid production by a *Bacillus cereus* strain resulting from naphthalene me-

tabolism. The surfactant activity showed by this microorganism facilitates the substrate uptake and consequently its degradation. This positive effect of biosurfactants on the biodegradation rate represents their general advantage over the chemical surfactants, since many of them may inhibit biodegradation (Laha and Luthy, 1992; Thiem, 1994; Stelmack *et al.*, 1999). So, it can be concluded that strains like *B. cereus* 28BN with the capacity to degrade naphthalene and at the same time to pro-

duce surface active compounds, can find application in bioremediation.

Acknowledgements

The first two authors contributed equally to this study. This work was supported by a Grant X-1103 from the National Fund for Scientific Investigations, Bulgarian Ministry of Education and Sciences.

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