Enhanced Hydrocarbon Biodegradation by a Newly Isolated *Bacillus subtilis* Strain

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The relation between hydrocarbon degradation and biosurfactant (rhamnolipid) production by a new *Bacillus subtilis 22BN* strain was investigated. The strain was isolated for its capacity to utilize *n*-hexadecane and naphthalene and at the same time to produce surface-active compound at high concentrations (1.5 – 2.0 g l^{-1}). Biosurfactant production was detected by surface tension lowering and emulsifying activity. The strain is a good degrader of both hydrocarbons used with degradability of 98.3 ± 1% and 75 ± 2% for *n*-hexadecane and naphthalene, respectively. Measurement of cell hydrophobicity showed that the combination of slightly soluble substrate and rhamnolipid developed higher hydrophobicity correlated with increased utilization of both hydrocarbon substrates. To our knowledge, this is the first report of *Bacillus subtilis* strain that degrades hydrophobic compounds and at the same time produces rhamnolipid biosurfactant.

Key words: Hydrocarbon Degradation, Biosurfactants, *Bacillus subtilis*

Introduction

Organic compounds with limited water solubility are biodegraded very slowly because of their low availability to microbial cells. The availability of slightly soluble organic compounds can be enhanced by microbially produced surfactants which increase aqueous dispersion by many orders of magnitude (Zhang and Miller, 1992). In many cases, biosurfactants also stimulate the biodegradation of organic compounds. For example, alkane degradation is stimulated by rhamnolipids (Zhang and Miller, 1992), sophorose lipids (Oberbremer and Muller-Hurtig, 1990) and phospholipids (Kappeli and Finnerty, 1979).

The objective of this research was to investigate whether the enhanced degradation of hydrocarbons by the newly isolated strain *Bacillus subtilis 22BN* could be related to the registered during growth on *n*-hexadecane and naphthalene secretion of rhamnolipid surfactant.

Material and Methods

Microorganism and medium

The strain *Bacillus subtilis 22BN* used in this study was isolated from hydrocarbon contaminated industrial waste water samples. The organism was selected by means of enrichment culture techniques for its ability to grow on *n*-hexadecane and/or on naphthalene as single sources 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, *n*-hexadecane and crystalline naphthalene (Aldrich Chemical Co., Steinheim, Germany), was added at the concentration of 20 g l^{-1}. The experiments were started by inoculation with 5% log phase culture pregrown on meat peptone broth. All cultures were performed in the dark at 28 ± 1 °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

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are entirely due to biodegradation. Growth was monitored by measuring the A_{610}.

Detection of biosurfactant activity

Samples of the culture media of the selected strain were centrifuged at $8000 \times g$ for 20 min. Surface tension (ST) of the supernatant fluid of the culture was measured by the ring method using automatic Wilhelmy tensiometer (Biegler Electronic, Mauerbach, Austria). The emulsifying activity of the supernatant culture 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.

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

Detection and quantification of rhamnolipids

The surface active compound was extracted by liquid-liquid extraction with 3 volumes diethyl ether from the supernatant fluid which was previous acidified 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 ($C_{26}H_{48}O_9$) and RRLL ($C_{32}H_{58}O_{13}$) from *Pseudomonas aeruginosa* as reference substances (Jeneil Biosurfactant Company, Saukville, USA).

Further identification of the sugar moiety after acidic hydrolysis confirmed it as rhamnose.

The orcinol assay (Chandrasekaran and Bemiller, 1980) was used for direct assessment of 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}).

Determination of residual n-hexadecane and naphthalene concentrations

Biodegradation was measured as substrate disappearance. Residual *n*-hexadecane and naphthalene were extracted from whole cultures with two volumes of *n*-hexane and analyzed by gas chromatography using Hewlett-Packard gas chromatograph model 5890 equipped with a 30 m HP-5 capillary column and a flame ionization detector.

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% *n*-hexadecane or 2% naphthalene (Rosenberg *et al*., 1980).

Analysis of naphthalene intermediates

The concentration of hydroxylated aromatic metabolites from naphthalene degradation was determined by 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 l^{-1}.

Results and Discussion

Detection of the surface active compound

The newly isolated strain *B. subtilis 22BN* formed halos on blue agar plates which detect the production of extracellular anionic glycolipids (Siegmund and Wagner, 1991). In the thin-layer chromatogram one glycolipid spot was revealed after the orcinol/sulfuric acid staining at R_f 0.86 corresponding to the reference monorhamnolipid RLL from *Pseudomonas aeruginosa*. Further identification of the sugar moiety after acidic hydrolysis confirmed it as rhamnose. It is interesting to point out that the rhamnolipid is produced during growth on hydrophobic carbon sources (hexadecane and naphthalene), while synthesis of the well characterized lipopeptide biosurfactant surfactin from *B. subtilis* or *B. licheniformis* is inhibited by hydrophobic substrates (Cooper *et al*, 1981).

Surfactant production

B. subtilis 22BN produced rhamnolipid biosurfactant on both hydrocarbons we used (*n*-hexadecane and naphthalene). In both cases, the surface tension (ST) of the medium decreased at the beginning of exponential growth. During growth on *n*-hexadecane (Fig. 1), there was a drop from 71 to

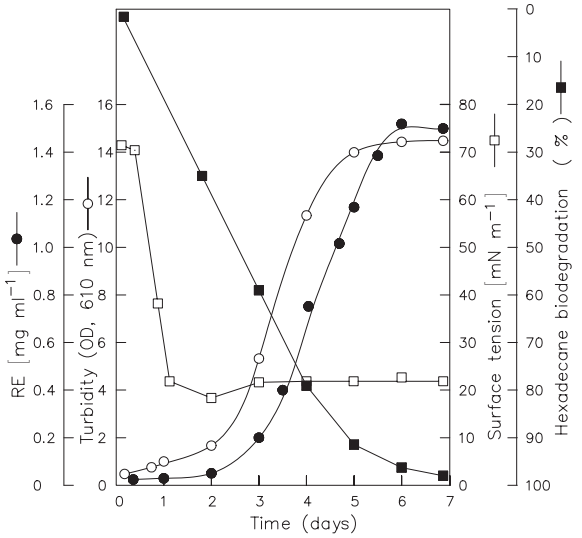

Fig. 1. Biosurfactant production and *n*-hexadecane degradation by *Bacillus subtilis 22BN* grown on mineral salt medium with 2% *n*-hexadecane as substrate. Incubation was done at 28 °C with shaking at 130 rpm. OD, optical density. Biosurfactant levels are expressed as rhamnose equivalents (RE). Biodegradation is expressed as % residual *n*-hexadecane. Values are averages from triplicate flasks.

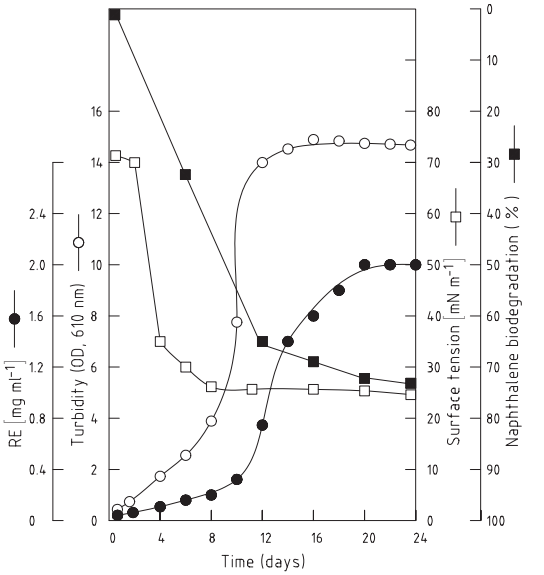

Fig. 2. Biosurfactant production and naphthalene degradation by *Bacillus subtilis 22BN* grown on mineral salt medium with 2% naphthalene as substrate. Incubation was done at 28 °C with shaking at 130 rpm. OD, optical density. Biosurfactant levels are expressed as rhamnose equivalents (RE). Biodegradation is expressed as % residual naphthalene. Values are averages from triplicate flasks.

38.3 mN m^{-1} within 24 h of cultivation, then for only 4 h ST declined to 21.1 mN m^{-1} finally reaching a minimum of 19 mN m^{-1} within 48 h of incubation. This finding was consistent with the results obtained by Zhang and Miller (1992). They indicated that low rhamnolipid concentrations cause sharp lowering in the surface tension and may be this is the dominant mechanism to enhance octadecane dispersion. Growth on naphthalene was not accompanied by such dramatic changes in ST (Fig. 2). It decreased to 35.5 mN m^{-1} within 4 d of cultivation reaching a minimum of 26.8 mN m^{-1} and did not decline further on. In addition, the drop in the surface tension in both cases 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. High levels of rhamnolipid at a concentration of 1.5 and 2.0 g l^{-1} were estimated in the stationary phase during growth on *n*-hexadecane and naphthalene, respectively.

Hydrocarbon degradation

The impact of biosurfactant production was assessed when the kinetics of degradation of the two hydrocarbon substrates were analyzed. As seen in Fig. 1, the biodegradation of *n*-hexadecane rapidly increased after 3 d of cultivation and the biodegradation percentage values (mean ± SD%, $n = 3$) after 4 d of cultivation were already 79 ± 2% and at the end of the incubation (7 d) only 1.7 ± 1% of the initial *n*-hexadecane was present.

Polycyclic aromatic hydrocarbons are utilized only in the dissolved state. So, naphthalene degradation by *B. subtilis 22BN* (Fig. 2) was slower compared to that of *n*-hexadecane and accumulation of naphthalene degradation metabolites was observed when the concentration of rhamnolipids in the medium increased sharply. The major metabolite, salicylic acid, reached maximal value of 450 mg l^{-1} in the beginning of stationary growth and did not change till the end of incubation. The biodegradation percentage values (mean ± SD%, $n = 3$) after 6 d of cultivation were 32 ± 4% and at the end of the incubation (24 d) 75 ± 2% of the initial naphthalene was biodegraded.

These results suggest that in both cases hydrocarbon degradation was related with the accumulation of the rhamnolipid biosurfactant in the medium. By increasing the solubility of the hydrophobic substrates it facilitated their transport to the microbial cells and enhanced their metabolism. When *n*-hexadecane was used as the substrate, concentration of the rhamnolipid of 0.4 g l^{-1} was enough to allow access of the cells to the substrate and to achieve 80% biodegradation within only 4 d. If the strain utilized naphthalene higher concentration of the biosurfactant (1.4 g l^{-1}) was necessary to achieve 65% biodegradation for a longer period (12 d) of cultivation.

Cell hydrophobicity during growth on hydrocarbons

It has been suggested previously that cell surface hydrophobicity is an important factor in predicting adhesion to surfaces (van Loosdrecht *et al.,* 1987). Thus, cell hydrophobicity was used as a measure of potential cell affinity for hydrophobic substrates and was determined by bacterial adherence to hydrocarbon (BATH) assay.

During growth on *n*-hexadecane, the transition from exponential to stationary growth was accompanied by an important increase in cell surface hydrophobicity (from 41 ± 3% to 75 ± 1%, respectively). When grown on naphthalene, the hydrophobicity of *B. subtilis 22BN* cells changed from 27 ± 5% in the early exponential phase to 66 ± 3% in the late exponential phase and then to 79 ± 2% in the beginning of stationary growth. These results are consistent with those of Zhang and Miller (1994) who described that cell hydrophobicity can be induced to change in the presence of a combination of both rhamnolipid and a slightly soluble substrate. The importance of cell surface properties for the biodegradation of hydrophobic organic substrates was indicated previously by Rosenberg and Rosenberg (1981). They showed that the rate of hydrocarbon degradation by the bacterial cells was dependent on cell affinity. Cells with high affinity for hydrocarbons utilized hexadecane more effectively than those with low affinity. Similarly in this study, development of higher hydrophobicity correlated with increased utilization of both hydrocarbon substrates.

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