Rhamnolipid Biosurfactants Produced by *Renibacterium salmoninarum 27BN* During Growth on *n*-Hexadecane

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A new strain *Renibacterium salmoninarum 27BN* was isolated for its capacity to utilize *n*-hexadecane as sole substrate. Growth on *n*-hexadecane was accompanied with the production of glycolipid surface active substances detected by surface pressure lowering and emulsifying activity. Glycolipid detection by thin layer chromatography and infrared spectra analyses showed for the first time that *Renibacterium salmoninarum 27BN* secretes the two rhamnolipids RLL and RRLL typical for *Pseudomonas aeruginosa.* Growth of *Renibacterium salmoninarum 27BN* on *n*-hexadecane depended on the bioavailability of the substrate and the secreted rhamnolipids appeared to be efficient in increasing hexadecane availability for the cells.

Key words: Renibacterium salmoninarum, Biosurfactants, Rhamnolipids

Introduction

Many prokaryotic and eukaryotic microorganisms can grow on compounds that are poorly soluble in aqueous media. The growth on such substrates, like hydrocarbons, is often associated with the production of surface-active compounds. Surface-active molecules contain hydrophilic and hydrophobic moieties which enable them to concentrate at interfaces and to reduce the surface tensions of aqueous media. The structures, properties and production of biosurfactants have been reviewed extensively (Desai and Banat, 1997; Fiechter, 1992). Probably the best characterized of these are rhamnolipids, which belong to the glycolipid class of biosurfacatants. Rhamnolipids have been identified predominantly from *Pseudomonas* (Burger *et al.*, 1963; Zhang and Miller, 1995; Beal and Betts, 2000; Tuleva *et al.*, 2001).

In the present study we show the ability of the newly isolated *Renibacterium salmoninarum 27BN* strain to produce rhamnolipids when grown on *n*-hexadecane.

Material and Methods

Microorganism, identification and maintenance

The strain used in this study was isolated from hydrocarbon contaminated industrial waste water samples. The isolate was plated on mineral salt agar containing 2% hexadecane as the sole carbon source. Single colonies obtained were transfered into 100 ml Erlenmeyer flasks containing 10 ml liquid mineral salt medium supplemented with 2% *n*-hexadecane or *n*-paraffins and cultivated at 28 °C and 130 rpm. The selected strain was characterized by using the Vitek system (bioMerieux, Montalieu-Vercieu, France) and several physiological and biochemical tests following directions of the latest edition of Bergey's Manual (Holt *et al.*, 1994). The strain was maintained on Nutrient Broth agar (Merck) slants at 4 °C and subcultures were made every 2 weeks. Inocula were prepared by growing cells at 28 °C for 18 h in Nutrient Broth in an orbital incubator at 130 rpm.

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Growth conditions

The composition of the mineral salt medium used in this study was the following (g l^{-1}): $K_2HPO_4 \cdot 3H_2O$ (4.8); KH_2PO_4 (1.5); $(NH_4)_2SO_4$ (1.0); $Na_3(C_6H_5O_7) \cdot 2H_2O$ (0.5); $MgSO_4 \cdot 7H_2O$ (0.2); yeast extract (0.1); supplemented with trace element solution with the following composition (mg l^{-1}): $CaCl_2 \cdot 2H_2O$ (2.0); $MnCl_2 \cdot 4H_2O$ (0.4); $NiCl_2 \cdot 6H_2O$ (0.4); $ZnSO_4 \cdot 7H_2O$ (0.4); $FeCl_3 \cdot 6H_2O$ (0.2); $Na_2MoO_4 \cdot 2H_2O$ (0.2); and 2% hexadecane or 2% mannitol as sole carbon source, pH 7.2. Hexadecane was sterilized through 0.2 μm membrane filters (Milipore Corp., Bedford, MA). Growth was monitored by measuring the $A_{600.}$ When *n*-hexadecane was used as the carbon source 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.

Detection of biosurfactant activity

Samples of the culture media of the selected strain were centrifuged at 8000 × *g* for 20 min. Surface tension 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 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.

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 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 from the supernatant fluid (5 ml) with previous acidification with HCl to pH 2. Supernatant fluid was shaken twice for 5 min with 15 ml of diethyl ether. The organic extracts were concentrated on a rotary evaporator and analyzed by thin layer chromatography (TLC) on silica gel 60 plates (5553, Merck). Chromatograms were developed with chloroform/methanol/acetic acid (15:5:1) 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, USA).

The orcinol assay (Chandrasekaran and Bemiller, 1980) was used for direct assess of the amount of glycolipids in the sample. Extracellular glycolipids concentration was evaluated in triplicate by measuring the concentration of rhamnose: 333 μl of the culture supernatant was extracted twice with 1 ml diethyl ether. The ether fractions were evaporated to dryness and 0.5 ml of H_2O was added. To 100 μl of each sample 900 μl of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80 °C the samples were cooled to room temperature and the OD_{421} was measured. The rhamnolipid concentrations were calculated from a 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 4000–400 cm^{-1} spectral region at a resolution of 2 cm^{-1} and 50 scans.

Determination of residual hexadecane

Residual *n*-hexadecane was extracted from whole cultures with equal volume of *n*-hexane and analyzed with a gas chromatograph (Hewlett-Packard model 5859) equipped with 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 mineral salt medium with 2% *n*-hexadecane (Rosenberg *et al.*, 1980).

Bacteria were harvested from growth cultures by centrifugation at 8000 × *g* for 10 min at 4 °C, washed twice, and suspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 4H_2O$; 7.26 g KH_2PO_4; 1.8 g urea

and 0.2 g $MgSO_4 \cdot 7H_2O$ in 1 l distilled water, pH 7.2) to an initial absorbance at 400 nm to 1.0. Hexadecane (0.5 ml) and cell suspensions (2.0 ml) were vortexed in a test tube for 2 min and equilibrated for 15 min. The bottom aqueous phase was carefully removed with a Pasteur pipette and the A_{400} was measured. The adherence was expressed as the percentage decrease in optical absorbance of the lower aqueous phase following the mixing procedure, compared with that of the cell suspension before mixing.

Results and Discussion

Microbial isolation and identification

The Vitek automated system was used for isolate identification and the strain was identified as *Renibacterium salmoninarum 27BN*. The strain showed the ability to use carbon sources as glucose, glycerol and mannitol, which are known as good substrates for glycolipid production (Robert *et al.*, 1989).

Detection and quantification of the surface active compounds

The newly isolated strain formed halos on blue agar plates which detected the production of extracellular glycolipids (Siegmund and Wagner, 1991). Thin-layer chromatography (Itoch *et al.*, 1971) and infrared spectral analyses were used for direct detection and quantification of the glycolipids.

In the thin-layer chromatography two typical glycolipid spots were revealed after the orcinol-sulfuric staining at R_f 0.83 and 0.42 corresponding to the reference rhamnolipids RLL and RRLL from *Pseudomonas aeruginosa* (data not shown). Further identification of the sugar moiety after acidic hydrolysis confirmed it as rhamnose. This result suggests that the newly isolated strain *Renibacterium salmoninarum 27BN* produces both types of rhamnolipids that are usually secreted by *Pseudomonas* spp.

Consistent with this finding is the infrared spectra (IR) analysis of the organic extract of *Renibacterium salmoninarum 27BN* surface active compounds and of the referent rhamnolipids. As seen from Fig. 1. bands characteristic of rhamnolipids (appearance of carbonyl absorption arising from ester and carboxylic groups) were observed at 1739 and 1718 cm^{-1}. The absorption bands of higher frequencies (1739 cm^{-1}) are assigned to the ester groups while those at about 1718 cm^{-1} originate from carboxylic groups. In the region 3000–2700 cm^{-1} several C-H stretching bands of CH_2 and CH_3 groups were also observed.

Biosurfactant production

Maximal rhamnolipid production on mannitol occurred in the stationary growth, indicating its characteristics as a secondary metabolite (Fig. 2). This was expected, since growth limitating conditions are required for rhamnolipid production (Venkata Ramana and Karanth,1989).

In the study of biosurfactant production on *n*-hexadecane we started the cultures with two different inocula-washed cells and whole culture broth. This affected growth kinetics of the cultures and the pattern of biosurfactant production

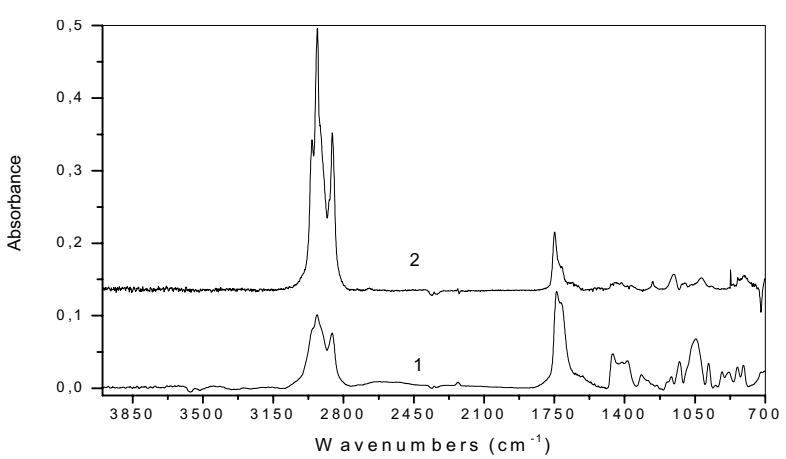

Fig. 1. Infrared spectra of the reference rhamnolipids from *Ps. aeruginosa* (1) and *R. salmoninarum 27BN* rhamnolipids (2).

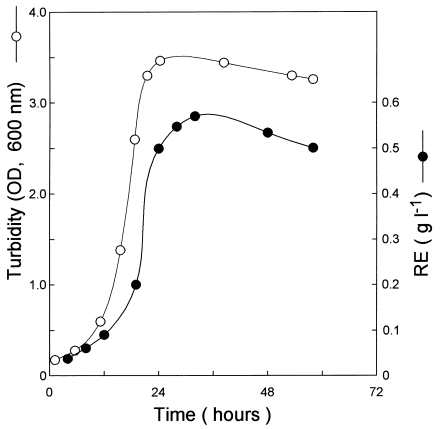

Fig. 2. Production of biosurfactants (glycolipids) by *R. salmoninarum 27BN* in mineral salt medium with 2% mannitol. Incubation was done at 28 °C with shaking at 130 rpm. OD, optical density. Biosurfactant levels are expressed as rhamnose equivalents (RE). Values are averages from three cultures.

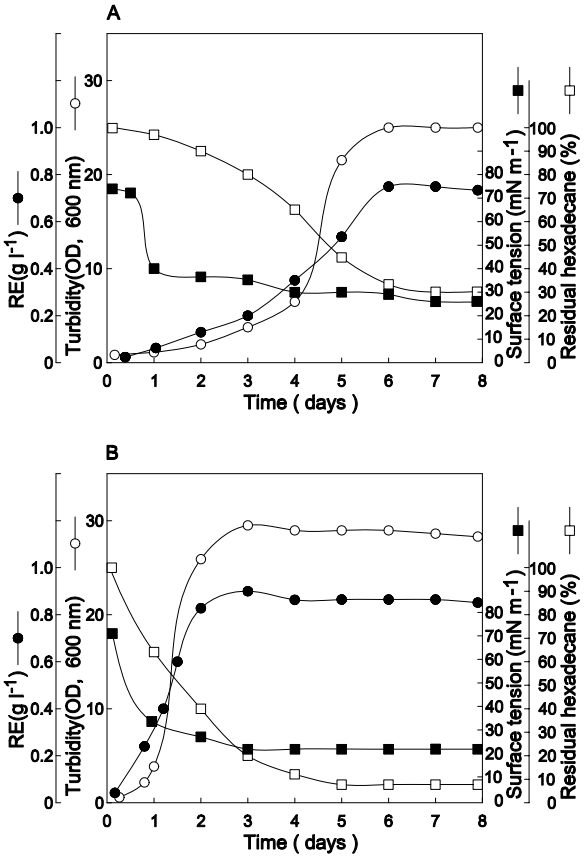

Fig. 3. Production of biosurfactants by *R. salmoninarum 27BN* grown on mineral salt medium with 2% hexadecane as substrate. (A) Inoculation with 5 ml washed cells from an overnight culture on nutrient broth. (B) Inoculation with 5 ml from an overnight culture on nutrient broth. Incubation was done at 28 °C with shaking at 130 rpm. Biosurfactant levels are expressed as rhamnose equivalents (RE). Values are averages from three cultures.

were different. Cultures started with washed cells (Fig. 3A) compared with the whole broth (Fig. 3B), required longer adaptation expressed as a delayed lag phase. Since biosurfactant production, like cell growth, depends on the availability of the substrate, rhamnolipid accumulation (expressed as rhamnose equivalents) was also delayed in the case when washed cells were used as inoculum. The lowered surface tension (Fig. 3A and B) and stabilized kerosene-water emulsions indicated the presence of surface active compounds even after 24 h of cultivation. In both cases maximal surfactant concentration was reached in the stationary growth with values of 0.78 and 0.92 g l^{-1} for cultures inoculated with washed cells and with the whole broth, respectively. At the end of cultivation (192 h), only 9.3 ± 2.1% residual hexadecane was registered in the cultures inoculated with the whole broth while it was higher (29.7 ± 3%) for the washed cells. These results confirm that the activity of the strain depends on the bioavailability of the hydrophobic substrate in the aqueous phase and the secreted rhamnolipids appear to be efficient in increasing hexadecane availability for the cells.

Increasing in cell hydrophobicity from 37 ± 3.8% in the logarithmic growth to 69.4 ± 2.7% was observed in the beginning of the stationary phase in the case when washed cells were used as inoculum. This may facilitate cell adhesion and access to the substrate, as suggested by the subsequent maximal surfactant secretion. In the case when the whole broth was used as inoculum cell hydrophobicity did not change significantly during growth (from 58 ± 1.7% to 67.3 ± 2.4%, respectively).

In summary, we have isolated a new strain with the capacity to grow on and degrade *n*-hexadecane and at the same time to produce surface-active compounds. To our knowledge, this is the first report of *Renibacterium salmoninarum* strain that produces the two typical for *Pseudomonas aeruginosa* rhamnolipids. There are several reports of pathogenic bacteria that were found to produce biosurfactants (Burd and Ward, 1996; Iglewski,

1989). Their exact role is not clear – may be they assist the colonization of host tissues or participate in increasing the bioavailability and degradation of hydrophobic organic contaminants by the host bacteria (Finnerty, 1994; Rosenberg, 1986; Rouse *et al.*, 1994). Hence, such organisms may play an important role in the natural degradation of hydrocarbon contaminants in the environment and have potential use in accelerated bioremediation processes.

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