Production of Four Interfacial Active Rhamnolipids from *n*-Alkanes or Glycerol by Resting Cells of *Pseudomonas species* DSM 2874

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In a simple phosphate buffer or a sodium chloride solution resting cells of *Pseudomonas spec.* DSM 2874 produced up to 15 g/l of different rhamnolipids. The rhamnolipid composition of the organic crude extract depended on the temperature during the cultivation and on the C-source. The optimal sodium chloride concentration for rhamnolipid formation was about 100 mM/l and the optimal phosphate buffer concentration about 65 mM/l. The optimal pH-value for the production of rhamnolipids from *n*-alkanes or glycerol was in the range pH 6.0–7.2. While rhamnolipid formation with glycerol as the sole C-source showed a wide optimum ranging from 27° up to 37 °C, production of rhamnolipids from *n*-alkanes had a sharp optimum at 37 °C. The addition of multivalent cations, different N-sources and EDTA caused an inhibition of rhamnolipid formation, while the *n*-alkane concentration had no influence. Specific rhamnolipid formation decreased with increasing cell concentration. Various C-sources were suitable for the formation of rhamnolipids by resting cells of *Pseudomonas spec.* DSM 2874. Yields, which were comparable to those obtained on *n*-alkanes or glycerol, were found for stearic acid, fatty alcohols and vegetable oils.

A study of the time course of glycolipid production of resting cells was carried out in a 20 l-bioreactor with an intensor system and with *n*-tetradecane as the sole C-source.

Introduction

When grown on *n*-alkanes, glucose or glycerol, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* produce extracellular rhamnolipids [1–7]. These rhamnolipids show a good emulsifying power in comparison with chemical surfactants [3] and have significant surface and interfacial active properties [8, 9]. Although they are one of the important factors for the *n*-paraffin uptake of *Pseudomonas aeruginosa* [10], they are not produced in substantial amounts before the stationary phase of growth [11]. Evidently small amounts of rhamnolipids are sufficient to allow the growth of *Pseudomonas aeruginosa* on hydrocarbons because of the low critical micelle concentration values of these compounds [9].

Pseudomonas spec. DSM 2874 produced two rhamnolipids, R1 and R3, under nitrogen limitation [9, 12], which were identical with compounds described previously [1, 3, 4, 5, 7]. Experiments on medium optimization for the growth of *Pseudomonas spec.* DSM 2874 indicated that a limitation of multivalent cations also caused the formation of rhamnolipids. Thus the cell growth and production phases were separated in order to investigate the influence of different factors on the production of rhamnolipids by resting cells. During the growth phase two rhamnolipids, R1 and R3, were produced under growth limiting conditions, four rhamnolipids could be isolated from the culture broth of resting cells. Resting cells of *Pseudomonas spec.* DSM 2874 formed two new rhamnolipids, R2 and R4, which are more hydrophilic analogues of R1 and R3, under certain conditions. The structure of the four rhamnolipids is shown in Fig. 1. Extensive work has been carried out on the chemical and physical characterization of these four surface and interfacial active glycolipids and this will be discussed elsewhere [9].

This paper describes the formation of rhamnolipids by resting cells of *Pseudomonas spec.* DSM 2874 and factors influencing their production.

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Materials and Methods

Microorganism and growth conditions

Pseudomonas spec. DSM 2874 was isolated by enrichment culture techniques from a water sample. Morphological and physiological characteristics in-

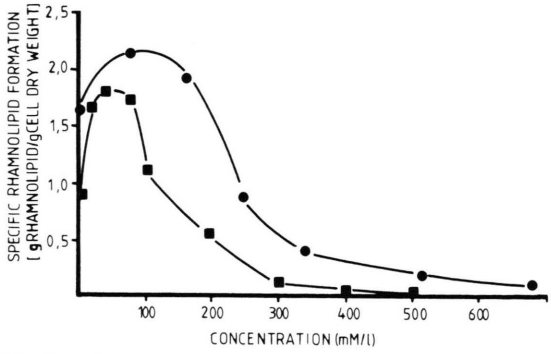

Fig. 2. Influence of the sodium chloride concentration (●) and the phosphate buffer concentration (■) on the rhamnolipid formation by resting cells of *Pseudomonas spec.* DSM 2874. Conditions: 4% *n*-alkanes as C-source, $T = 30\,°C$, pH adjusted to pH 7.0 every 24 h with 1 N NaOH (NaCl: 168 h, PO_4-buffer: 144 h).

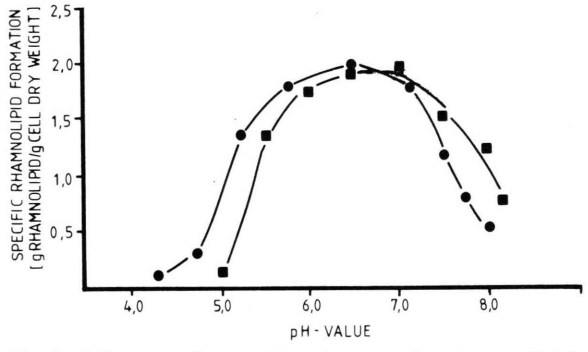

Fig. 3. Influence of the pH value on the rhamnolipid formation by resting cells of *Pseudomonas spec.* DSM 2874 after 144 h. Conditions: 4% *n*-alkanes (●) or 4% glycerol (■) as C-source, 100 mM PO_4-buffer, $T = 30\,°C$, pH adjusted with 1 N NaOH or 1 N H_3PO_4.

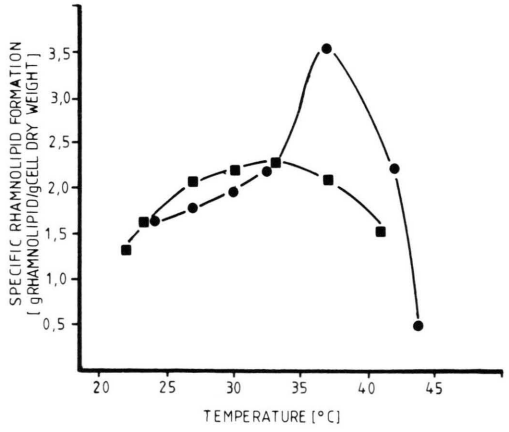

Fig. 4. Influence of the temperature on the rhamnolipid formation by resting cells of *Pseudomonas spec.* DSM 2874 after 168 h. Conditions: 6% *n*-alkanes (●) or 4% glycerol (■) as C-source, 65 mM PO_4-buffer, pH adjusted to pH 6.6 every 24 h with 1 N NaOH.

optimum at 37 °C. In this case a temperature shift from 30 °C to 37 °C also gives rise to a change of the product spectrum (Table I). The more hydrophilic rhamnolipids R2 and R4 are only formed at temperatures lower than 37 °C.

Ca^{2+}-, Mg^{2+}- and Fe^{2+}-ions are essential for the growth of *Pseudomonas spec.* DSM 2874. We tested the influence of these multivalent cations on the rhamnolipid formation by resting cells.

While Ca^{2+}-ions had no influence on the production of rhamnolipids up to 3 g/l $CaCl_2 \cdot 2\,H_2O$, Mg^{2+}-ions and Fe^{2+}-ions caused a slight inhibition of rhamnolipid formation even at small concentrations. Higher concentrations of the nutrients led to a distinct inhibition of the rhamnolipid production as with sodium-chloride and phosphate, probably through osmotic effects.

In case the of growing cells of *Pseudomonas spec.* DSM 2874 nitrogen-limitation causes an overproduction of rhamnolipids [9, 12]. Thus the influence of various N-sources on rhamnolipid formation was investigated. Table II summarizes the results of these experiments. The additions of 0.2% of different N-sources to the culture broth of *Pseudomonas spec.* DSM 2874 caused a distinct inhibition of rhamnolipid production, but led to no complete suppression.

Further the influence of the addition of EDTA (Titriplex III) was investigated. An EDTA addition had a positive effect on the production of anionic Trehalosetetraesters [18]. EDTA, which is a metal ion complexing agent [19], should be used to chelate metal ions which were probably introduced into the culture broth of resting cells from the cell wet mass after centrifugation. Increased EDTA-concentrations caused an intense inhibition of the rhamnolipid formation. Further the cell dry weight decreased with increasing EDTA-concentrations. Evidently EDTA led to damage of the cells. Although glycerol concentrations over 30 g/l led to an inhibition of rhamnolipid formation [20], alkane concentrations varying from 20 g/l – 120 g/l had no influence on the production of rhamnolipids by resting cells.

Table III sums up the results of experiments on the reincubation of resting cells. After the first incubation cells loose nearly 80% of their ability to form rhamnolipids. While in sodium chloride solution there is no visible rhamnolipid production after the third and fourth passage, resting cells show 13% of their initial activity in phosphate buffer after the

Table II. Influence of different N-sources on the rhamnolipid formation by resting cells of *Pseudomonas spec.* DSM 2874 after 168 h. Conditions: 8% *n*-alkanes as C-source, 65 mM PO_4-buffer, $T = 30\,°C$, pH adjusted every 24 h to pH 6.6 with 1 N NaOH or 1 N H_3PO_4.

N-source (0.2%)	Specific rhamnolipid formation [g rhamnolipid/g cell dry weight]
–	2.0
$(NH_4)_2SO_4$	1.1
$NaNO_3$	0.4
NH_4NO_3	0.2
Glutamate	1.5
Urea	1.5

Table III. Rhamnolipid formation by reincubated resting cells of *Pseudomonas spec.* DSM 2874. Conditions: 4% *n*-alkanes as C-source, $T = 30\,°C$, pH adjusted to pH 7.0 every 24 h with 1 N NaOH. Cells were separated after each run of 168 h by centrifugation.

Incubation runs	Rhamnolipid production (% initial production)	
	0.5% sodium chloride solution	65 mM PO_4-buffer
1	100%	100%
2	20%	23%
3	0.5%	13%
4	0.5%	25%

Table IV. Rhamnolipid formation by resting cells of *Pseudomonas spec.* DSM 2874 grown on *n*-alkanes or glucose with 4% of different C-sources after 168 h. Conditions: 100 mM PO_4-buffer, $T = 30\,°C$, pH adjusted every 24 h to pH 7.0 with 1 N NaOH or 1 N H_3PO_4.

C-source	Specific rhamnolipid formation [g rhamnolipid/g cell dry weight]	
	glucose-grown cells/ *n*-alkane-grown cells	
n-alkanes (89% C14, 9% C15)	1.2	2.0
n-octadecane	n.d.	1.0
n-tetradecane	n.d.	2.5
C14–C18-fatty alcohols	n.d.	1.3
stearic acid	n.d.	1.6
soj bean oil	1.5	1.4
olive oil	n.d.	1.0
glycerol	1.7	1.3
glucose	0.9	0.4
sucrose	0.2	0.1
citric acid	n.d.	0.3
glutamat	0.3	n.d.

(n.d. = not determined).

third and even 25% after the fourth passage. Evidently resting cells require phosphate for the production of rhamnolipids.

Several different C-sources were tested in shake flasks for the production of rhamnolipids. The results of this experiment are shown in Table IV. Various C-sources are suitable for the formation of rhamnolipids by resting cells. Yields, which were comparable to those obtained on *n*-alkanes or glycerol, were found for stearic acid, C14–C18-fatty alcohols and vegetable oils. The C-source used for growth of *Pseudomonas spec.* DSM 2874 had a distinct effect on the rhamnolipid content. Alkane grown cells had the highest affinity for *n*-alkanes as C-source for the formation of rhamnolipids, while glucose grown cells favoured glycerol as C-source.

In all experiments foam formation was observed when substantial amounts of rhamnolipids were produced.

Rhamnolipid production with resting cells in a bioreactor

After consideration of the shake flasks results, resting cells of *Pseudomonas spec.* DSM 2874 were incubated at 37 °C and pH 6.6 in a 20 l bioreactor in order to investigate the time course of rhamnolipid production. The medium contained 5 g/l sodium chloride and 100 g/l *n*-tetradecane as the sole C-source. The results of this experiment are shown in Fig. 5. To avoid excessive foam production, a

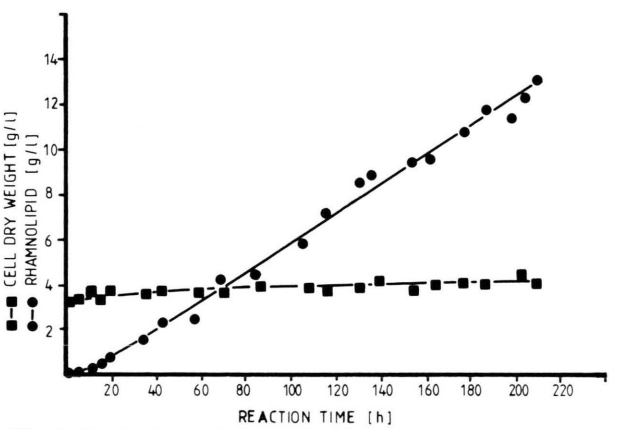

Fig. 5. Production of rhamnolipids with resting cells of *Pseudomonas spec.* DSM 2874 in a 20 l bioreactor equipped with an intensor system. Conditions: 10% *n*-tetradecane as C-source, 0.5% NaCl-solution, $T = 37\,°C$, pH automatically adjusted to pH 6.6 with 10% NaOH solution, aeration: 0.18 Nm^3/h with 30% O_2, rotation: 1000 rpm.

dicated that the organism belonged to the genus of *Pseudomonas*.

Pseudomonas spec. DSM 2874 was cultivated in a mineral salts medium, pH 6.8, containing (per liter) 4.42 g $Na_2HPO_4 \cdot 2H_2O$, 3.4 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.4 g/l $CaCl_2 \cdot 2H_2O$, 0.4 g citric acid $\cdot$ $1H_2O$, 0.04 g $FeSO_4 \cdot 7H_2O$, 0.005 g/l $MnSO_4 \cdot H_2O$, 0.0012 g NH_4-Heptamolybdate and 10 g of C14–C15 *n*-alkanes or 20 g glucose as the sole C-source at a temperature of 30 °C. Batch cultivations were carried out in a G20-Bioreactor (Fa. Braun-Melsungen AG, Melsungen, F.R.G.) equipped with flat blades. Physiological activity was monitored by the use of a pH-electrode, a pO_2-electrode and oxygen- and carbon-dioxide gas analyzers (Fa. Maihack AG, Hamburg, F.R.G.). pH was titrated to pH 6.8 by addition of 10% NH_4OH-solution. The time course of the cultivation was checked by measuring the dry mass-, alkane- or glucose-concentration and ammonium-ion-concentration. In the case of *n*-alkane as C-source, biomass concentration was determined by the method of Rapp *et al.* [13]. The ammonium-ion-concentration in the supernatant was determined by the method of Facwett *et al.* [14]. Alkane-concentration in the culture broth was monitored after extraction by gas chromatography. Glucose-concentration in the supernatant was determined by the dinitrosalicyl-acid-method [15]. Cells were harvested by centrifugation at 20000 rpm with a Padberg-centrifuge type 41 (Carl Padberg GmbH, F.R.G.) as soon as the C-source was consumed.

Conditions for resting cells

After harvesting the cells by centrifugation the wet biomass was used for various investigations in shake flasks or in a bioreactor.

Shake flask experiments were carried out in 500 ml shake flasks containing 100 ml of various aqueous solutions. The reaction mixtures usually contained around 5 g/l cell mass dry weight and were incubated at 100 rpm and 30 °C for 6–7 days.

For resting cell experiments in a b20 reactor (Giovanola Frères, Monthey, Switzerland) equipped with intensor system was used. Resting cells of *Pseudomonas spec.* DSM 2874 were incubated in a medium containing (per liter) only 5 g NaCl and 100 g *n*-tetradecane at 37 °C. The pH was titrated to pH 6.6 by addition of 10% NaOH solution. Physiological activity of the resting cells was monitored by the methods described under growth conditions.

Isolation of the rhamnolipids

After various incubation times 25 ml of the reaction suspensions were acidified with 10% H_2SO_4 to pH 2.0 and extracted twice with 100 ml ethyl acetate. After removing the solvent by rotary evaporation the residues were dissolved in 5 ml chloroform and used for the measurement of the whole rhamnolipid content by the anthrone method [16]. A detailed description of the separation of the rhamnolipids by column and thick layer chromatography and their characterization by physical and chemical methods is published elsewhere [9]. Analytical thin layer chromatography was carried out on Silica Gel plates (No. 5554, E. Merck AG, Darmstadt, F.R.G.) using the solvent system chloroform-methanol-acetic acid (65:15:2) (vol./vol./vol.). After horizontal development of the plates rhamnolipids were determined with 4-methoxy-benzaldehyde-spray reagent [17].

Results

After growth on 2% glucose ($Y_{x/s} = 0.45$, $\mu_{max} = 0.34$) or 1% *n*-alkanes ($Y_{x/s} = 0.84$, $\mu_{max} = 0.29$) cells of *Pseudomonas spec.* DSM 2874 were harvested by centrifugation at 20000 rpm and used for resting cell experiments.

Shake flasks experiments

Initial investigations showed that resting cells of *Pseudomonas spec.* DSM 2874 were able to form rhamnolipids in a Sörensen phosphate buffer or a

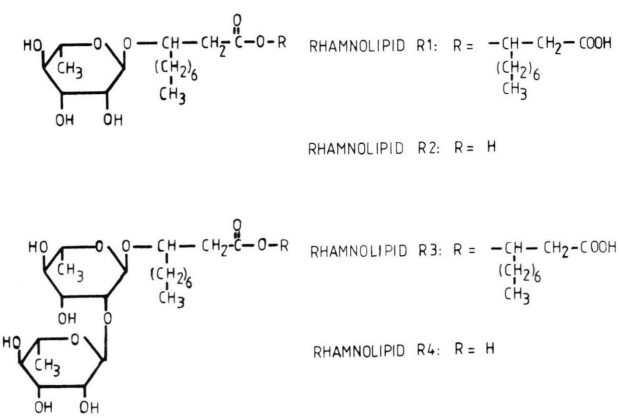

Fig. 1. Rhamnolipids produced by resting cells of *Pseudomonas spec.* DSM 2874.

Table I. Rhamnolipid formation by *Pseudomonas spec.* DSM 2874.

Cultivation conditions	Specific rhamnol. formation [g rhamnolipid/g cell dry weight]	Conversion rate [g rhamnolipid/g substrate]	Specific productivity [mg rhamnolipid/g cell dry weight × hour]	Yield [%] on			
				R1	R2	R3	R4
Growing cells C-source: glucose no limitation	0.08	0.04	9	50	–	50	–
Growing cells C-source: *n*-alkanes no limitation	0.12	0.11	10	50	–	50	–
Growing cells C-source: *n*-alkanes N-limitation (180 h)	0.6	0.18	4	65	–	35	–
Resting cells C-source: glycerol Temperature: 30 °C (168 h)	1.9	0.10 (0.09) [a]	12	22	15	62	1
Resting cells C-source: *n*-alkanes Temperature: 30 °C (168 h)	2.0	0.23 (0.21) [a]	13	42	15	41	2
Resting cells C-source: *n*-alkanes Temperature: 37 °C (168 h)	3.5	0.24 (0.21) [a]	22	57	–	43	–

[a] Conversion rate under consideration of the C-source used for the growth of resting cells of *Pseudomonas spec.* DSM 2874.

physiological sodium chloride solution at pH 7.0 from *n*-alkanes or glycerol as the sole C-source at 30 °C. While cells of *Pseudomonas spec.* DSM 2874 formed two rhamnolipids, R1 and R3, under growth limiting conditions [9, 12], two additional, more hydrophilic rhamnolipids, R2 and R4, could be isolated from the culture broth of resting cells [9]. Their structures are presented in Fig. 1. The separation of the crude products by column chromatography [9] indicated, that the rhamnolipid composition of the crude product depended on the C-source (Table I).

Different sodium-chloride- and phosphate-buffer-solutions were tested to find the most suitable system for the production of rhamnolipids with *n*-alkanes as the sole C-source. The results are shown in Fig. 2. The optimal concentration was about 100 mM in the case of sodium chloride and about 65 mM in the case of the Sörensen phosphate buffer.

To determine the optimal pH-value for the formation of rhamnolipids from *n*-alkanes and glycerol various Sörensen phosphate buffer solutions were tested ranging from pH 4.2 to pH 8.0. While pH-values of pH 5.5 to pH 8.2 allow optimal growth of *Pseudomonas spec.* DSM 2874 on *n*-alkanes, the rhamnolipid production is optimal in the pH range 6.0–7.2. As one can see in Fig. 3 there is hardly a difference between the formation of rhamnolipids from *n*-alkanes with alkane-grown cells and from glycerol with glucose-grown cells.

Fig. 4 shows the temperature optimum of rhamnolipid formation with *n*-alkanes or glycerol as the sole C-source. *Pseudomonas spec.* DSM 2874 is able to produce rhamnolipids at temperatures up to 43 °C. While the rhamnolipid-formation with glycerol as the sole C-source and glucose-grown cells shows a wide optimum reaching from 27 °C up to 37 °C, the production of rhamnolipids from *n*-alkanes with alkane-grown cells shows a sharp

reactor equipped with an intensor system was used, the aeration was only 0.15 VVm, but with 30% O_2 in the air. While biomass increased slightly from 3.6 to 4.1 g cell dry weight/l, rhamnolipid formation began after 8 h and continued linearily for 210 h, when 13.2 g rhamnolipid/l were formed from 56 g *n*-tetradecane. The specific formation amounted to 3.2 g rhamnolipid/g cell dry weight after 210 h and 0.24 g rhamnolipid were formed from 1 g *n*-tetradecane.

The composition of the crude product was the same during the whole cultivation time. Further experiments showed, that a degradation of rhamnolipids could be observed in the absence of a C-source. The degradation led to L-rhamnose, which could not be further consumed by *Pseudomonas spec.* DSM 2874, and to the free fatty acids, which were imediately used as C-source. In spite of the degradation of the rhamnolipid in absence of an energy-source there was no change in the composition of the crude product, so that one can say, that the more hydrophilic rhamnolipids R2 and R4 are no degradation products of the rhamnolipid R1 and R3.

Discussion

Resting cell experiments with *Pseudomonas spec.* DSM 2874 showed that the best production of rhamnolipid was observed in phosphate-buffer or a sodium-chloride solution without addition of any other nutrients. The addition of nutrients, which were essential for the growth of *Pseudomonas spec.* DSM 2874 in various concentrations to the culture broth of resting cells had no or even a negative effect on the production of rhamnolipids. Various N-sources caused a distinct inhibition of the rhamnolipid formation. Previous experiments with growing cells [9, 12] had already indicated, that nitrogen metabolism, which has a great influence on general metabolic pathways [21], appeared to be closely connected with the biosynthesis of the rhamnolipids.

Mineral elements can play an important role in the regulation of the production of microbial metabolites [22]. For example, pyvoderine synthesis in *Pseudomonas fluorescens* was found in highest yield at iron concentration that permitted only partial growth [22]. Addition of magnesium chloride and ferrous sulphate to the culture broth of resting cells of *Pseudomonas spec.* DSM 2874 caused inhibition of rhamnolipid formation even at low concentrations.

A phosphate-limitation can be an important factor for directing the metabolism of *Pseudomonas aeruginosa* towards production of pyocyanin [23]. Sodium chloride, potassium chloride and phosphate concentrations had a negative effect on the rhamnolipid formation only at higher concentrations, probably arising from osmotic effects, although phosphate was necessary for the repeated rhamnolipid production by resting cells of *Pseudomonas spec.* DSM 2874.

Temperature can be another important factor in the production of secondary metabolites [22]. In the case of *Pseudomonas spec.* DSM 2874 the production of rhamnolipids showed a sharp optimum at 37 °C with alkane-grown cells and *n*-alkanes as the sole C-source. The yield and composition of the crude product of rhamnolipids was temperature dependent, while the latter was also dependent on the C-source.

For *n*-alkane-grown cells the highest yields of rhamnolipids were attained with *n*-alkanes as C-source, while for glucose-grown cells the highest yields were attained with glycerol or vegetable oils as C-sources.

In future the biosurfactants will become an important factor in tertiary oil recovery [24]. Besides the chemical and physical properties of the surfactants, the production costs are decisive in the application of a surfactant [25]. So one precondition for a successful application of biosurfactants in tertiary oil recovery is the development of processes for their formation, which are competitive in costs to the production of chemically synthesized surfactants. The production of rhamnolipids with resting cells of *Pseudomonas spec.* DSM 2874 has various advantages. After separation of the cells by centrifugation the rhamnolipids are dissolved in the supernatant which contains only 5 g/l sodium chloride, while the purified rhamnolipids are only slightly soluble in aqueous solutions. There are few disturbing by products in this solution, thus it can be directly used for flooding experiments after dilution to the required concentration and salt addition to give the salt content of synthetic deposit water. The cells of *Pseudomonas spec.* DSM 2874 can be repeatedly used for the production of rhamnolipids.

The next step in our work will be the production of rhamnolipids with immobilized cells of *Pseudomonas spec.* DSM 2874 for which two problems have to be solved; namely the prevention of a possible end-product-inhibition and the prevention of foam production caused by the rhamnolipid formation. Initial experiments along those lines will be published elsewhere.

Acknowledgement

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