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Biodegradation of polychlorinated biphenyls (PCBs) in the presence of a bioemulsifier produced on sunflower oil

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Abstract

A bioemulsifier excreting bacterium of the species *Peudomonas cepacia* was isolated after a screening procedure using *n*-dodecane as carbon source. The partly purified bioemulsifier was preliminarily identified as a mixture of glycolipids. A decrease of the surface tension to 37 mN/m and a CMC of 5 mg/l could be measured with the bioemulsifier GL-K12. Using sunflower oil as main carbon source, up to 7.1 g/l bioemulsifier could be produced in oxygen and nitrogen limited fermentations on a scale of 300 l. The biodegradation of Aroclor 1242 in liquid cultures by a bacterial mixed population was enhanced by GL-K12 when added at biosurfactant concentrations of 0.2 g/l or more. The most positive effect was noted in the degradation of PCB congeners with 3 Cl atoms with an increase of up to 100%.

Introduction

Bioremediation of contaminated soils is limited by recalcitrance and a lack of availability of soil-sorbed hydrocarbons. In *ex-situ* soil washing procedures synthetic surfactants are often used to improve the separation of contaminants from soil particles. For in-site applications the use of most of such surfactants is problematic because of their toxicity and lack of biodegradability (Fiechter 1992; Georgiou et al. 1992). Biosurfactants primarily produced and excreted by microorganisms growing on hydrocarbons are found to be environmentally and biologically more compatible (Van Bernem 1984). To get an acceptable biosurfactants-enhanced bioremediation process some prerequisites have to be fullfilled. The first of those is the selection of biosurfactants able to enhance the bioavailability of a broad spectrum of contaminants often found in soils or of special species of contaminants hardly to attack like polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs). Second, the use of biosurfactants for bioremediation has to be economically acceptable. That means biosurfactants have to be supplied cheaply in high quantities and they must be efficient at low concentrations. The use of crude plant oil as a cheap carbon source for biosurfactant production has been demonstrated previously (Robert et al. 1989; Shabtai & Wang 1990; Davila et al. 1992). Significant enhancement of biosurfactant production is a well known phenomenon for different strains of microorganisms when essential medium components for growth are limiting. Medium composition has to be optimized for every culture (Syldatk & Wagner 1987). Repeated biosurfactant production by resting cells limited by nitrogen, oxygen or essential trace elements can lead to maximization of biosurfactant productivity as shown with rhamnolipid production by *Pseudomonas* sp. (Wagner 1987). Last but not least, the environmental harmlessness of selected biosurfactants at concentrations needed for practical application has to be proven.

The intention of this work was the selection and economical production of an environmental compatible bioemulsifier able to increase the bioavailability of PCBs. Therefore several bacterial strains should be isolated from a contaminated industrial site and screened for production of an emulsifier with a good emulsifying capacity and enhancing PCBs degradation. After the optimization of fermentation conditions the biosurfactant should be produced for practical application on a technical scale.

This project is part of a programme dealing with the feasibility of degradation of PCBs in contaminated soils. Preceding activities centred on the isolation of PCBs degrading microorganisms and the optimization of processes for bioremediation of soils contaminated with PCBs. Next, shall be confirmed that the bioemulsifier obtained will improve bioremediation of PCBs contaminated soils using *ex-situ* soil washing procedures and in-site applications. Cooperation with German and Korean companies active in this field is planned.

Materials and methods

Basal medium

The composition of the basal medium adjusted to pH 7.2 was according to Fiebig et al. (1993).

Microorganisms and culture condition

The soil used in the experiment was derived from an industrial site in Berlin (FRG). The soil was of sandy structure without humus fraction (humic material), contaminated with mineral oil containing polyaromatic hydrocarbons (PAHs) in addition to PCBs. 10 g soil were suspended in 100 ml of 0.9% (w/v) saline solution and agitated thoroughly for 30 min on a laboratory shaker. After sedimentation of solid materials each of 1 ml supernatant was transferred to a 500 ml Erlenmeyer flask and filled up with 100 ml basal medium. This preparation was used for the isolation of PCBs degrading and biosurfactant producing microorganisms and for testing biosurfactant degradation.

Isolation of a PCBs degrading mixed population. For the isolation of a PCBs degrading mixed population 1000 mg/l biphenyl was added as carbon and energy source and supplemented with 10 mg/l Aroclor 1242 (Promochem, Wesel, FRG) as an inductor for specific enzymes. After 12 weeks of incubation at ambient temperature on a laboratory shaker at 100 rpm 1 ml of suspended cells was transferred into fresh growth medium every 2 weeks. The enriched mixed culture was used for PCBs degradation experiments.

Screening for biosurfactants producing microorganisms. For the enrichment of biosurfactant producing microorganisms the basal medium was supplemented with 3 g/l *n*-dodecane as carbon and energy source. Cultivation occurred at room temperature on a laboratory shaker at 100 rpm. Every 4 to 5 days 1 ml of suspended cells was transferred into fresh growth medium. For the isolation of pure bacterial strains 0.1 ml of the enriched mixed culture was streaked on basal medium agar. The agar medium was prepared with 3 g/l *n*-dodecane and solidified with 0.8% (w/v) agar (Serva, Heidelberg, FRG). After incubation at 30 °C single colonies were picked up and subcultured several times to yield pure cultures.

The isolated pure cultures were screened for their ability to produce and excrete biosurfactants by (1) measuring the emulsifying capacity of the supernatant using the emulsifying test described below; (2) testing the hemolysis of red blood cells by the method of Schulz et al. (1991); and (3) using CTAB (cetyltrimethylammonium bromide) and methylene blue in a semiquantitative agar plate test according to Siegmund & Wagner (1991) which should be specific for anionic biosurfactants.

Identification of microorganisms

Single colonies were cultivated twice on plate count agar (Difco, Detroit, USA). Identification of bacteria was based on a commercially available diagnostic kit (Api system; Bio Merieux, Ghent, Belgium). The results were obtained by computer surveys available from this supplier.

Biosurfactant production

For biosurfactant production reactors of different scale were used, equipped with turbine stirrer and pH- and oxygen-measuring and control devices: 1 l reactor (L.H. Engineering, Stoke Poges, UK), 10 l reactor (Braun, Melsungen, FRG), 300 l reactor (Bioengineering, Wald, Switzerland).

Biosurfactant production was optimized in the 1 l reactor by variation of pH, temperature and oxygen saturation. Optimized fermentation conditions for bio-

surfactant production were as follows: pH 7.0, 30 °C, stirring 100 rpm, oxygen saturation 20%. According to fed batch mode each fermenter was prefilled by 95% of final volume with basic mineral medium supplemented with 3 g/l sodium acetate. For further cell proliferation, the remaining medium supplemented with 100 g/l acetic acid as carbon source was fed pH auxostatically (medium was added automatically when pH exceeded the preset value following acetic acid consumption). After depletion of acetic acid and ammonium 10 g/l sunflower oil was added as carbon source for biosurfactant production.

Biosurfactant isolation

For biosurfactant isolation the culture broth was centrifuged for 30 min at 12.000 g to separate cells. The supernatant was evaporated to dryness at 40 °C. The residue was resuspended in distilled H_2O (2% of the original volume) and extracted three times by sonification with an equal volume of ethylacetate. After drying by gassing with air the extract was stored at −18 °C until use.

Biosurfactant degradation test

Biosurfactant degradation was tested by measuring the reduction of total carbohydrate concentration and chemical oxygen demand (COD) in the cellfree culture liquid. Therefore 1000 ml Erlenmeyer flasks in duplicate were filled with 200 ml basal medium and 40 mg isolated biosurfactant GL-K12. After inoculation with 2 ml suspended cells from basic preparation, cultivation occurred at ambient temperature on a laboratory shaker at 100 rpm for 5 days.

Emulsifying test

The procedure was according to the method of Neu & Poralla (1990) and modified as follows: 3 ml of cellfree culture broth was extracted three times with an equal volume of ethylacetate by sonification. After evaporation of the organic phase the residue was resolved in 3 ml Tris-Mg-buffer in glass tubes (10 mm diameter with screw caps). Tris-Mg-buffer without extract was used as blank. After addition of 0.4 ml *n*-dodecane the tubes were vortexed horizontally at 2000 rpm on a IKA Vibrax (Janke & Kunkel, Staufen, FRG) for 5 min and equilibrated at room temperature for 10 min. The emulsifying capacity (EC_{620}) is given as the difference of the optical density (O.D.) at 620 nm measured in the tubes before and after agitation.

PCBs degradation experiments

All experiments were carried out in 500 ml Erlenmeyer flasks filled up with 100 ml basal medium and agitated at 100 rpm at ambient temperature. The medium was completed with 10 mg/l Aroclor 1242 and 1000 mg/l biphenyl. The inoculum was 1% (v/v).

PCBs mobilization experiments

1 mg Aroclor 1242 dissolved in 10 ml of *n*-hexane was sorbed to the bottom of 500 ml Erlenmeyer flasks. After the evaporation of *n*-hexane the flasks were exposed to 50 ml distilled H_2O with and without 20 mg/l biosurfactant and agitated at 50 rpm for 24 h at room temperature. The flasks were depleted and dried at 40 °C. The remaining PCBs were removed with 5 ml *n*-hexane and analysed gaschromatographically.

PCBs extraction and analysis

The extraction and analysis of PCBs was performed as described previously (Fiebig et al. 1993). The identification of single PCBs was ensured by comparison with chromatograms of single congeners and published chromatographic data (Ballschmiter et al. 1987; Krupc'ik et al. 1992).

Surface activity assay

Surface tension and interfacial tension (against *n*-hexadecane) were determined with a ring tensiometer (Krüss, Hamburg, FRG) using distilled water at 20 °C. The critical micel concentration (CMC) was estimated according to Cooper et al. (1979).

TLC analysis of the bioemulsifier

5 μl extract (see biosurfactant isolation) were applied to silica gel plates (silica gel 60; Merck, Darmstadt, FRG) and developed in the solvent system chloroform-methanol/water (65 : 25 : 4, v/v/v/). After air drying a mixture of ethanol/anisaldehyde/sulfuric acid/acetic acid (9 : 0.5 : 0.5 : 0.2, v/v/v/v) and 0.05% ethanolic rhodamin B solution were used as spray reagents for sugar and lipid detection respectively. After heating at 100 °C for 5–10 min the different spots of sugar and lipid moieties became registrable at visible light and

Table 1. Prescreening of isolated bacteria for emulsification potential

	Strain																
	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	K13	K14	K15	K16	K17
CTAB-agar																	
growth	+	+	+	+	++	+	+	+	+	+	+	++	+	+	+	++	+
blue halo	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hemolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EC_{620}*	0.07	0.03	0.06	0.02	0.08	0.05	0.02	0.05	0.15	0.04	0.03	0.14	0.03	0.07	0.07	0.09	0.01

* Emulsifying capacity of the supernatant after 5 days.

by UV radiation (254 nm) respectively. Rhamnose and lecithine were used as standards for sugar and lipid detection.

Acetic acid determination

Acetic acid was determined gaschromatographically as described previously (Fiebig & Dellweg 1985).

Ammonium analysis

Ammonium was analysed with a test kit using Neßler reagent (LCW 003; Dr. Lange, Düsseldorf, FRG).

Carbohydrate analysis

Total carbohydrate concentration in the culture supernatant was determined by the phenolsulfuric acid method of Dubois et al. (1956). Standard: glucose.

Chemical oxygen demand (COD) analysis

COD was measured by the dichromate method (test kit LCK 314; Dr. Lange, Düsseldorf, FRG).

Optical density measurement (OD)

Optical density (absorbance) was measured at 620 nm. During bioemulsifier production on sunflower oil OD of the homogeneous culture broth was measured after sampling and dilution using cell-free medium as blank. Decrease of OD was a measure of oil depletion. In the PCB degradation experiments biomass growth was monitored by OD measurement. To prevent PCB contamination Erlenmeyer flasks equipped with a side tube allowing direct photometrical measurement of the culture broth without opening and sample preparation were used. Before measurement solid biphenyl was allowed to settle in the side tube for a few seconds.

Results

Seventeen isolated bacteria were screened for their ability to produce biosurfactants. The results are shown in Table 1. All bacteria grew well on CTAB agar but did not show a dark blue halo around the colonies. Hemolysis of red blood cells was also not evident by any colony. The emulsifying capacity in the supernatant of the culture broth (EC_{620}) of the different isolates varied and was at maximum in the cultures K9 and K12 after 5 days of cultivation.

Production of the emulsifiers GL-K9 and GL-K12 was optimized in the 1 l fermenter varying pH, temperature and oxygen concentration. Testing partly purified emulsifiers GL-K9 and GL-K12 for fuel oil emulsification, the EC_{620} was 0.12 and 0.38 respectively. This was the reason why GL-K12 was chosen for further investigations.

The course of fermentation of biosurfactant GL-K12 in the 10 l reactor is presented in Figure 1. Acetic acid was used as carbon source, ammonium as nitrogen source for accelerated biomass growth during the start up of the process.

After 50 h ammonium and acetic acid were exhausted and sunflower oil was added. The fermentation continued under nitrogen and oxygen limitation. At that time the optical density of the culture broth reached about 2 and climbed up to 5.6 during the following 20 h caused by enhanced oil emulsification.

Dodecane emulsification and carbohydrate concentration in the cell-free culture liquid continuously increased until hour 170. In the meantime, the optical density of culture broth decreased to 4 accompanied by oil droplets becoming smaller and smaller, as observed

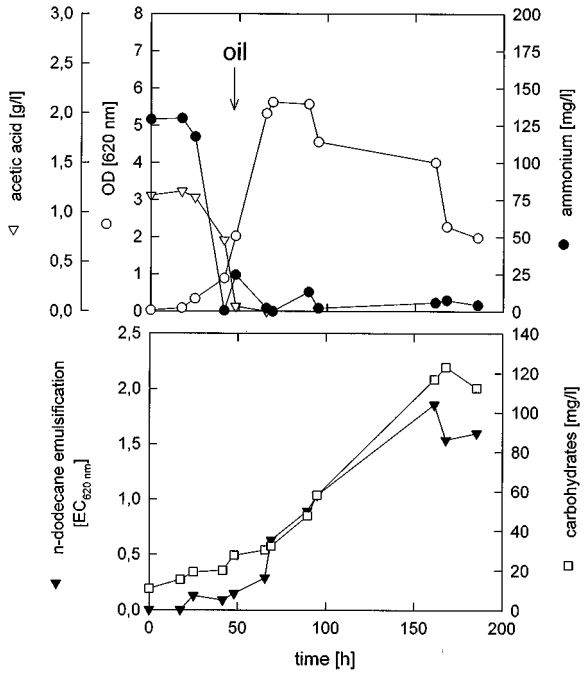

Figure 1. Batch fermentation of *Pseudomonas cepacia* K12 for biosurfactant production using sunflower oil.

Table 2. Biodegradation of bioemulsifier GL-K12 by a bacterial mixed population from soil

Time [day]	0	1	2	3	4	5
COD [mg/l]	148	116	36	20	< 15	< 15
Carbohydrates [mg/l]	3.5	2.0	0.6	0.1	0.1	< 0.1

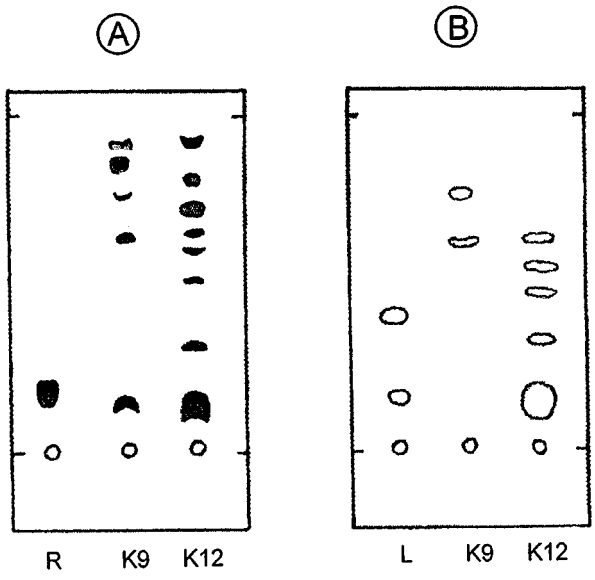

Figure 2. TLC analysis of bioemulsifier GL-K9 and GL-K12. (A) sugar detection, (B) lipid detection; R: rhamnose, L: lecithine.

Table 3. Effect of bioemulsifier GL-K12 on microbial degradation of different congeners of Aroclor 1242 by a mixed culture in mineral medium

Congeners of Aroclor 1242		Average values of degradation [%]	
Number of Cl atoms	Percentage [%]	No emulsifier	GL-K12, 200 mg/l
2	16	100	100
3	50	43	78
4	25	11	12
5	8	0	0
6	1	0	0
7	< 0.1	0	0

microscopically. The following sharp decrease of optical density is attributed to complete depletion of oil. At the same time, emulsifying capacity and carbohydrate concentration in the culture liquid also declined, presumably according to biosurfactant degradation after substrate depletion. During lab scale fermentation the final concentration of bioemulsifier GL-K12 was in the range of 8–13 g/l. In the 300 l fermenter a final yield of 7.1 g/l GL-K12 could be obtained.

The partly purified bioemulsifiers were preliminarily identified by TLC as a mixture of glycolipids using rhamnose and lecithine as standards for the detection of sugar and lipid moieties (Figure 2). For bioemulsifier GL-K12 R_f-values of 0.14, 0.33, 0.47, 0.54, 0.63 can be obtained from TLC.

GL-K12 decreased the surface tension of water from 72.6–37 mN/m. CMC and minimum interfacial tension of GL-K12 were 5 mg/l and 5 mN/m respectively.

Biodegradability of biosurfactant was investigated using the bacterial mixed population original enriched from soil and GL-K12 as the only carbon source. Based on reduction of COD and carbohydrate concentration, the bioemulsifier is completely degraded after 4 days of cultivation as shown in Table 2.

In a further experiment GL-K12 was tested for its ability to mobilize PCBs from surfaces. After shaking for 24 h at room temperature all congeners of Aroclor 1242 were nearly completely removed from the glass surface in the presence of GL-K12 (Figure 3).

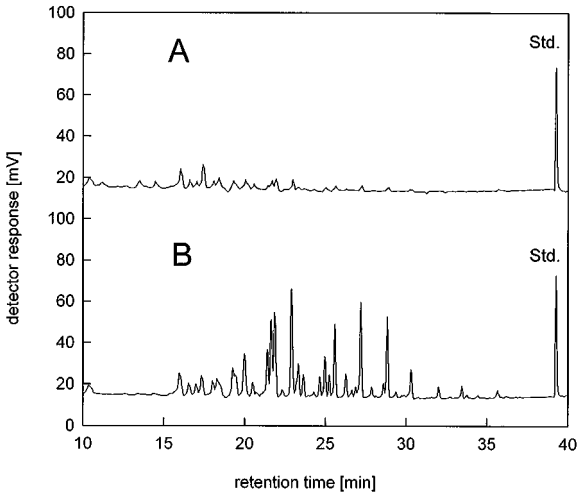

Figure 3. Gaschromatographic analysis of Aroclor 1242 removed from glass surface after treatment with and without bioemulsifier. (A) 20 mg/l GL–K12, (B) distilled H_2O; Std: standard.

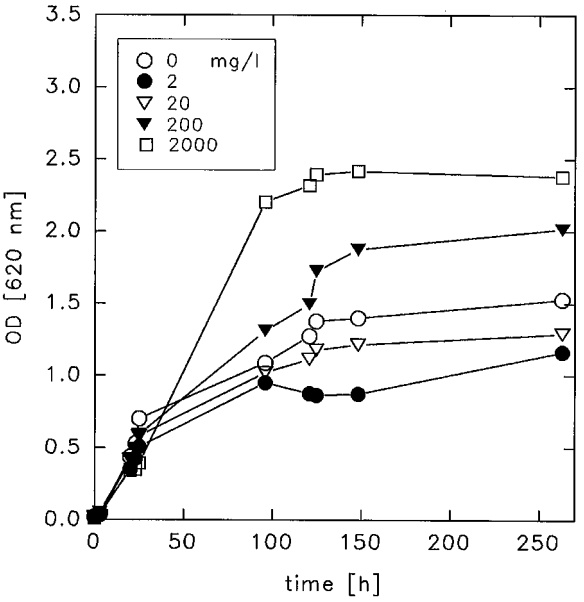

Figure 4. Bacterial growth of a mixed population on biphenyl supplemented with Aroclor 1242 in the presence of different concentrations of bioemulsifier GL-K12.

The influence of GL-K12 addition on PCBs degradation by microorganisms was tested primarily in liquid cultures. The growth of a PCBs degrading bacterial mixed population on biphenyl supplemented with Aroclor 1242 was slightly inhibited by addition of 2 and 20 mg/l glycolipid GL-K12 but significantly enhanced

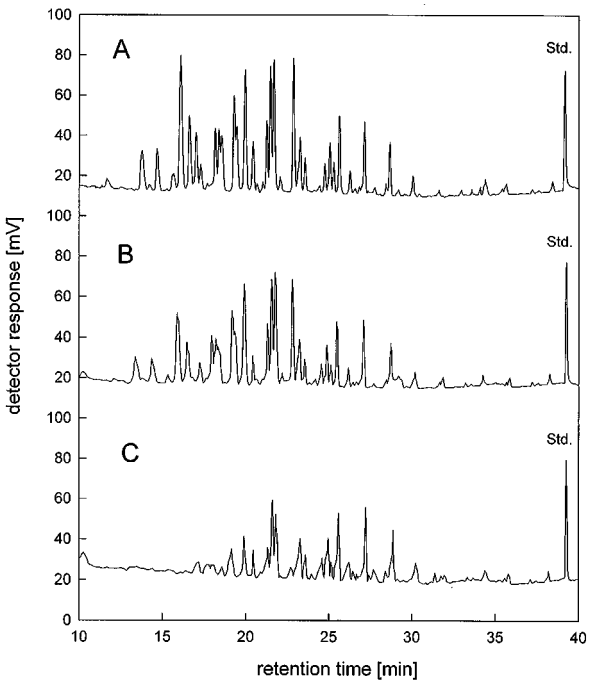

Figure 5. Degradation of Aroclor 1242 by a bacterial mixed culture in mineral medium. (A) Aroclor 1242, (B) without surfactant, (C) with 200 mg/l GL-K12; Std.: standard.

by the addition of 200 and 2000 mg/l biosurfactant respectively. This effect was registrable, exceeding a cultivation time of 100 h (Figure 4). According to the gaschromatographic analysis (Figure 5), an average degree of degradation of PCB congeners with the identical number of Cl substitution was calculated. In conclusion, congeners with 2 Cl atoms were completely degraded independent of surfactant addition. By the addition of GL-K12 the degradation of PCB congeners with 3 Cl atoms was nearly enhanced by 100% compared to the culture without biosurfactant supplementation. Higher chlorinated PCB congeners were either degraded at the same degree or not attacked at all (Table 3).

Discussion

When grown on *n*-alkanes different strains of bacteria produce extracellular biosurfactants with significant surface and interfacial active properties (Zajic et al. 1977; Syldatk et al. 1985b). This and simple analytical determination by GC was the reason why *n*-dodecane was used for screening microorganisms pro-

ducing biosurfactants. In the agar plate tests isolates showed neither hemolysis of red blood cells nor a dark blue halo around the colonies, although emulsifying capacity in the supernatant of some liquid cultures was significant. According to Siegmund & Wagner (1991), a dark blue halo around colonies should be attributed to the reaction of the cationic agent CTAB with anionic surfactants. Mulligan et al. (1984) stated that hemolytic zones around colonies on blood agar plates could be related to the ability of the microbes to produce surfactants. Schulz et al. (1991), however, demonstrated that not every surfactant-producing microorganism causes hemolysis. While rhamnolipids R1 and R2 of *Pseudomonas* sp. DSM 2874 cause good hemolysis several marine isolates showed nearly no hemolytic activity. Considering the toxicity of synthetic detergents showing significant hemolysis the non-hemolytic activity of biosurfactants was interpreted as an indication of their reduced toxicity.

The extracted bioemulsifiers GL-K9 and GL-K12 were tentatively identified by TLC as a mixture of glycolipids indicated by sugar and lipid moieties of the same spots. Among others, Syldatk et al. (1985b) found that the biosurfactants produced by pseudomonades in higher quantities are primarily rhamnolipids. Decreasing the surface tension of water from 72.6–37 mN/m GL-K12 is not quite as effective as rhamnolipids R1, R3 with a value of 31 described previously (Syldatk et al. 1985a). On the other hand the CMC of GL-K12 is 5 mg/l compared to 20 mg/l of the rhamnolipids R1 and R3. The minimum interfacial tension of GL-K12 (5 mN/m), a measure of the hydrocarbon/water emulsification potential, is comparable to that obtained with rhamnolipids R1 (8 mN/m) and R3 (3 mN/m).

The final yield of 7.1 g/l GL-K12 on a 300 l fermentation scale is in the same order reported with rhamnolipids produced on hexadecane or pentadecane. A yield of 320 g/l sophorose lipid reached with *Candida bombicola* using plant oil and glucose for biosurfactant production is, up to now, unparalleled (Davila et al. 1992).

The apparent decrease of surfactant activity after substrate depletion was already an indicator of their good biodegradability as could be confirmed in a separate experiment. This has also been observed with different biosurfactants. On average they were found more degradable than most synthetic surfactants available at the time (Poremba et al. 1991). Oberbremer et al. (1990) could also demonstrate immediate degradation of different biosurfactants after consumption of the carbon source treating a mixture of hydrocarbons in the presence of suspended soil in a stirred reactor. Degradability of biosurfactants is an important criterion allowing application for in-site bioremediation.

Aroclor 1242 has been nearly quantitatively removed from a glass surface in the presence of GL-K12, though it is taken into account that only mobilization of separate phase pollutants, not desorption from the surface, may have been the primary effect in the experiment performed. Effective solubilization of Aroclor 1260 has been reported in the presence of different surfactants. In repeated batch extraction processes the synthetic surfactants SDS (Clarke et al. 1991) or sodium dodecyl benzene sulfonate (SDBS) (McDermott et al. 1989) were used. Applying a solution of 10 g/l SDS, the batch washing experiment with soil containing about 1000 mg/kg PCBs yielded a removal exceeding 99% in a 2-week period (Clarke et al. 1991). Van Dyke et al. (1993a) compared the efficiency of different biosurfactants for removing hexachlorobiphenyl from soil slurries, gaining best results with surfactants produced by *Pseudomonas aeruginosa* UG2 and *Acinetobacter calcoaceticus* RAG-1. Increased recovery of hydrocarbons with UG2 rhamnolipids occurred only at surfactant concentrations greater than 0.5 g/l (Van Dyke et al. 1993b). Because of the adsorption of surfactants to soil particles the concentration for effective use normally exceeded the CMC. Further experiments have to be carried out to demonstrate whether GL-K12 is as effective in PCBs mobilization from actual site soil.

It is well known that PCB congeners containing more than 5 Cl atoms in the molecule are aerobically hardly to attack (Abramowicz 1990). As indicated in our experiment, it is not likely that the degradation of such molecules can be significantly improved even in the presence of biosurfactants enhancing their solubilization. Increased solubilization of crude oil in liquid medium by Emulsan even led to inhibition of biodegradation of the different hydrocarbons (Foght et al. 1989). In this respect the reason for the inhibition of PCBs degradation at low concentrations of GL-K12 is not known. The addition of different glycolipids like nonionic sophorose and trehalose lipids improved the availability of PAHs like naphthalene, phenanthrene and anthracene without inhibition of biodegradation by *Pseudomonas aeruginosa* (Brandes 1988). Enhanced bioavailability of octadecane by a factor of four could be obtained by Zhang & Miller (1992) after adding a rhamnolipid biosurfactant up to 300 mg/l.

In further experiments the degradation of soil sorbed PCBs have to be tested in the presence of added (bio)-surfactants. An investigation of fuel oil degradation in soil in the presence of a biosurfactant or a special mixture of synthetic surfactants gave the best results in the second case (Häusler et al. 1992). Further research is needed to get clear answers about the potential benefits of biosurfactants or synthetic surfactants either single or in optimal mixtures for their application in bioremediation processes.

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