Marine Biosurfactants, I. Screening for Biosurfactants among Crude Oil Degrading Marine Microorganisms from the North Sea

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Three bacterial strains of marine origin were isolated during a screening for biosurfactants among *n*-alkane degrading microorganisms. One strain – identified as *Alcaligenes* sp. MM 1 – produced a novel glucose lipid. In the case of *Arthrobacter* sp. EK 1 the well-known trehalose tetraester was found as major component. From another pure culture classified as *Arthrobacter* sp. SI 1, extracellular emulsifying agents with properties indicating high molecular weight substances were detected. Furthermore trehalose corynomycolates were found at up to 2 g/l. The isolated biosurfactants showed good interfacial and emulsifying properties.

Introduction

The oil pollution of the marine environment and the related shore has assumed alarming proportions. The ordinary self-cleaning of the sea involving evaporation or sedimentation of certain oil components, as well as biodegradation by marine microorganisms, is overburdened due to the permanent addition of hydrocarbons and especially large oil spills.

Therefore it is important to develop novel techniques to combat these.

In recent studies concerning the prevention of oil pollution in sensitive coastal areas surface and interfacial active agents of microbiological origin were taken into consideration for the limited local application. They seem to be well suited for the elimination of hydrocarbons and for a better distribution facilitating the usual but slow attack in the sea [1].

It is known that the application of some synthetic detergents has led to additional problems because of their toxicity and their persistence in nature [2–4]. Thus the use of a microbial surfactant could be helpful in removing an oil spill and would reduce long term pollution in the marine environment as well as the destruction of the habitats of aquatic animals and fish [5, 6].

Our intention has been to screen marine *n*-alkane utilizing microorganisms with the expectancy of finding novel biosurfactants of low toxicity, good biodegradability and a good efficiency in stimulating the local cleaning-up especially of sensitive coastal areas.

Materials and Methods

Microorganisms

Marine mixed cultures were obtained from the Biologische Anstalt Helgoland (F.R.G.) and were also taken from several polluted areas of the North Sea.

Cultivation conditions

The enrichment media for the marine microorganisms were as follows:

Sea-water medium: 0.5 g/l $Na_2HPO_4 \cdot 2H_2O$, 0.1 g/l K_2HPO_4, 1.5 g/l NH_4Cl or 2.4 g/l $NaNO_3$, 2 g/l yeast extract, 13 ml/l mihagol-S (*n*-alkanes: 89% C_{14}, 11% C_{15}), 100 ml/l $H_2O_{dest.}$, 900 ml/l sea-water, 0.5 ml/l solution of trace elements, pH 7.5.

Synthetic sea-water medium: 23 g/l NaCl, 6.16 g/l $MgSO_4 \cdot 7H_2O$, 5.08 g/l $MgCl_2 \cdot 6H_2O$, 0.75 g/l KCl, 1.47 g/l $CaCl_2 \cdot 2H_2O$, 0.89 g/l $Na_2HPO_4 \cdot 2H_2O$, 5 g/l $NaNO_3$, 13 ml/l mihagol-S, pH 7–7.5, supplemented with 30 mg/l $FeSO_4 \cdot 7H_2O$ or 2 g/l yeast extract and 0.5 ml/l of solution of trace elements.

Medium NA 93: 1 g/l yeast extract, 1.86 g/l citrate, 3.5 g/l $NaNO_3$, 0.75 g/l KH_2PO_4, 0.75 g/l

$Na_2HPO_4 \cdot 2H_2O$, 0.07 g/l $CaCl_2 \cdot 2H_2O$, 0.03 g/l $FeCl_3 \cdot 6H_2O$, 0.3 g/l $FeSO_4 \cdot 7H_2O$, 1.65 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l H_3PO_4, 13 ml/l mihagol-S, pH 6.5.

Solution of trace elements: 11 g/l $ZnSO_4 \cdot 7H_2O$, 0.04 g/l $CuSO_4 \cdot 5H_2O$, 0.01 g/l KJ, 6 g/l $MnSO_4 \cdot H_2O$, 1 g/l $FeSO_4 \cdot 7H_2O$, 0.3 g/l $CoSO_4 \cdot 7H_2O$, 0.06 g/l H_3BO_3, 5 g/l EDTA.

The cultures were incubated at 100 rpm on a rotary shaker at 18–27 °C.

Fermentation studies with *Arthrobacter* sp. SI 1 were carried out in a 50 l bioreactor (Braun, Melsungen, F.R.G.) equipped with an intensor system using a working volume of 30 l synthetic sea-water medium. Temperature was maintained at 20 °C and the pH value 7.2–7.5. Ethanol and $NaNO_3$ concentrations were measured enzymatically (UV Test, Boehringer, F.R.G.).

Isolation of n-alkane utilizing microorganisms

Marine isolates were obtained by direct plating of dilutions of enriched cultures on petri dishes containing sea-water medium supplemented with Bacto Agar at 18 g/l. The lid of the reversed petri dish held a sterile paper-filter soaked with mihagol-S. Subsequent incubation was carried out at 18–27 °C over a period of several days.

Screening for biosurfactants

The screening methods described were applied to the marine mixed or pure cultures.

Hemolysis of red blood cells [7]

Petri dishes with sheep blood agar (No. 13414, Merck, Darmstadt, F.R.G.) were inoculated with marine microorganisms and incubated at 27 °C. Hemolysis of red blood cells around the colonies indicated potential biosurfactant production.

Direct thin-layer chromatography [8]

1–10 mg of biomass were applied to silica gel plates (No. 5554, Merck, Darmstadt, F.R.G.) and at first developed for 10 min in the solvent system A (chloroform:methanol (2:1, v/v)) and after drying and careful removal of biomass the TLC was then developed in the solvent system

B: chloroform:methanol:water (65:15:2, v/v/v) or
C: chloroform:methanol:acetic acid (65:15:2, v/v/v).

Various spray reagents were used to determine the functional groups of the components after vertical development of the TLC. 4-Methoxybenzaldehyde or α-naphthol were used for sugar and 2,7-dichlorofluoresceine for lipid detection (No. 9677, Merck, Darmstadt, F.R.G.).

Biosurfactant recovery

Glycolipids

At the end of cultivation the whole culture broth was acidified with 10% H_2SO_4 to pH 2.5 and then extracted thrice with ethyl acetate. After rotary evaporation of the solvent the crude organic extract was used for glycolipid detection by TLC in the solvent system B and C and it was further purified by column and thick layer chromatography [9].

Emulsifier

After removal of the cells the supernatant was concentrated by ultrafiltration on an Amicon YM 100 filter which had a molecular weight exclusion limit of 100,000. The retentate was extensively dialyzed against deionized water and then lyophylized. Non-polar lipids were removed by extracting the dry material with methylene chloride.

Emulsion test

The detection of extracellular emulsifying agents was performed in 4.87 ml of the cell-free supernatant and 130 μl of mihagol-S. After vortexing at maximum speed in a test tube (16 mm × 160 mm) the turbidity of the solution was measured for up to 3 h at 623 nm using a Zeiss spectrometer PM 2 A.

Physicochemical properties

Surface and interfacial tension of purified glycolipids were measured by the ring method using a Lauda Autotensiomat (Lauda, Wobser KG, Königshofen, F.R.G.).

Enzyme digestion

1 mg/ml of the crude emulsifier was digested by chymotrypsin α (16 U/ml) for 5 h or by pronase (7 U/ml) for 24 h in 0.08 n phosphate buffer at pH 7.5 and 37 °C.

Digestion with α-Amylase (36 U/ml) was performed in 0.04 n phosphate buffer at pH 7.0 and 20 °C for 5 h. The remaining emulsifying activity was estimated with hexadecene.

Heat denaturation

1 mg/ml of crude emulsifier in 0.08 n phosphate buffer at pH 7.5 was incubated up to 5 h in a boiling water bath.

The protein content of the emulsifier was estimated by the method of Lowry *et al.* [10] using crystalline bovine albumin as the standard. Carbohydrates were determined by anthrone reagent [11].

Results and Discussion

Survey on marine bacteria and biosurfactants

During the screening for *n*-alkane utilizing and biosurfactant producing marine microorganisms three strains were isolated by special techniques mentioned previously. They were identified by the aid of Bergey's manual of determinative bacteriology [12].

Table I summarizes the isolated bacteria and the related biosurfactants produced after cultivation on *n*-alkanes and detection of glycolipids by direct TLC of colonies or after extraction of the whole culture broth with ethyl acetate. Estimation of emulsifying agents was carried out by vortexing the cell free supernatant for 1 min with mihagol-S.

In most cases the main product was the well-known trehalose tetraester (TL-4). This glycolipid was overproduced at up to 5 g/l by *Arthrobacter* sp. EK 1 in medium NA-93 under limiting conditions and was found to be cell-bound and contained in the culture supernatant. It consists of the disaccharide trehalose which is acylated by three fatty acids (C_8-C_{14}) and one succinoyl group [13]. In the case of *Arthrobacter* sp. SI 1 glycolipids as well as other components were detected. Using *n*-alkanes as carbon source extracellular emulsifying agents which appeared to be substances of high molecular weight were detected. When the carbon source was changed from mihagol-S to ethanol only the emulsifier was found as no TL-4 was produced due to the hydrophilic nature of ethanol. Instead of TL-4 trehalose dicorynomycolates (TL-2) were now produced at up to 2 g/l.

From another pure culture identified as *Alcaligenes* sp. MM 1 a novel glucose lipid (GL) was isolated. The production of this cellbound glycolipid is growth associated and the amount of glucose lipid in the culture broth reached 500 mg/l in synthetic sea water medium. The lipophilic component consisting of four β-hydroxydecanoic acids linked together by ester bonds is coupled glycosidically with C-1 of glucose [14]. The complete structure of the glycolipids described is shown in Fig. 1.

Properties of the glycolipids

The purified glycolipids were examined with regard to their surfactant properties by determining their surface and interfacial tension. The results are summarized in Table II. The minimum surface tension of TL-2 was 36 mN/m, while that of TL-4 reached 26 mN/m. The interfacial tension measured against *n*-hexadecane was 17 mN/m in the case of TL-2 and <5 mN/m using TL-4. The CMC values were 4 mg/l and 15 mg/l respectively. These results were in agreement reached with those achieved with the trehalose lipids from *Rhodococcus erythropolis* [16].

The novel glucose lipid shows very good surface and interfacial properties and is comparable to TL-4.

Table I. Isolated marine microorganisms and their biosurfactants.

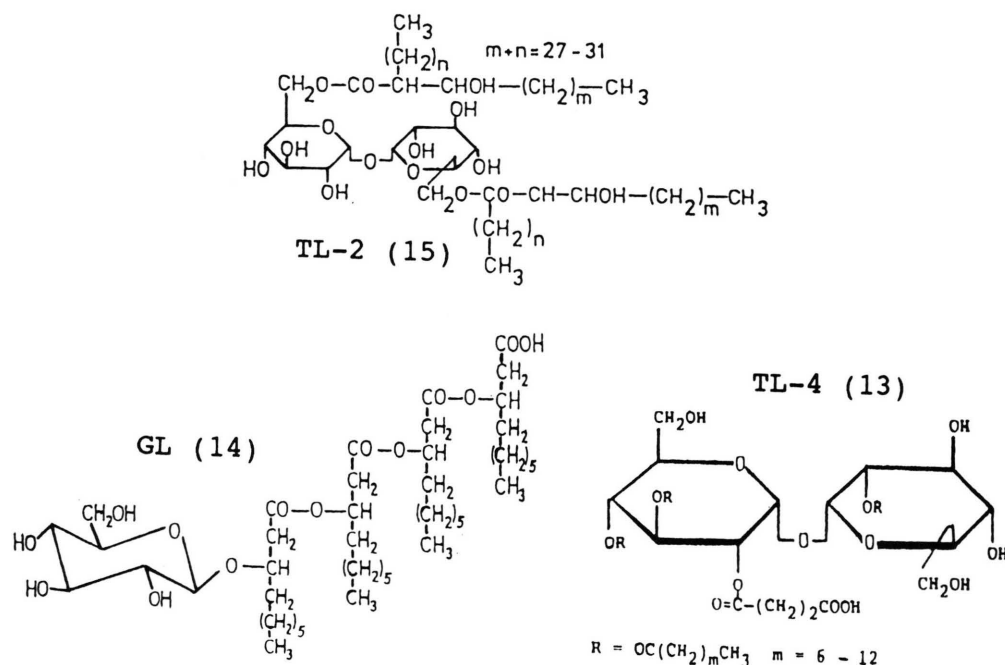| Carbon source	Microorganism	Biosurfactant
mihagol-S	*Arthrobacter* sp. EK 1	trehalose tetraester*
mihagol-S	*Alcaligenes* sp. MM 1	glucose lipid
mihagol-S	*Arthrobacter* sp. SI 1	trehalose tetraester* emulsifying agents
ethanol	*Arthrobacter* sp. SI 1	trehalose diester* emulsifying agents

* Main product.

Fig. 1. Molecular structures of the isolated glycolipids.

Table II. Surface and interfacial properties of the purified glycolipids from the marine isolates.

Biosurfactant	Minimum surface tension [mN/m]	Minimum interfacial tension* [mN/m]	CMC [mg/l]
TL-2	36	17	4
TL-4	26	<5	15
Glucose lipid	28	<5	25

* Against *n*-hexadecane.

Hemolysis of red blood cells

Another screening method for biosurfactants is the use of sheep blood agar and the possible hemolysis of red blood cells. Hemolysis depending on microbial cultures, biosurfactants and chemical surfactants is listed in Table III. It is surprising that not every surfactant/microorganism causes hemolysis. The biosurfactants of the marine isolates showed nearly no hemolytic activity, except for TL-4 which produces small colorless rings of damaged cells. Contrary to this the glycolipids of *Pseudomonas* sp. DSM 2874 − the rhamnolipids R 1 and R 3 − cause good hemolysis similar to the synthetic detergent Pril. Considering the toxicity of synthetic detergents, the non-hemolytic activity

Table III. Hemolytic activity of several surfactants/microorganisms after incubation for 120 h at 27 °C.

Microorganism/surfactant	Hemolysis (+/−)
Arthrobacter sp. EK 1	−
TL-4	+*
Alcaligenes sp. MM 1	−
Glucose lipid	−
Arthrobacter sp. SI 1	−
TL-2	−
Emulsifying agents of	−
Arthrobacter sp. SI 1	
Pseudomonas sp. DSM 2874	+
Rhamnolipids R 1 and R 3	+
Pril**	+

* Small colorless rings of hemolyzed red blood cells.
** Trade mark of Henkel (F.R.G.).

of biosurfactants might be an indication of their reduced toxicity.

Extracellular emulsifying agents

Extracellular emulsifying agents were detected after removal of the cells and concentration of the supernatant with an Amicon YM 100 filter. Only the retentate from the cultivation with *Arthrobacter* sp. SI 1 showed emulsifying activity. The cultivation conditions in shake flask experiments and the results of the production of these non-dialyzable substances are summarized in Table IV.

The best results were obtained by using *n*-alkanes or ethanol as carbon source supplemented with NaNO₃. In the case of mihagol-S the supernatant was often emulsified through coproduction of TL-4 which is an equally good emulsifier and can even affect the estimation of other extracellular emulsifying agents after ultrafiltration. After cultivation with ethanol no TL-4 was found. This is in agreement with other authors who postulated the necessity of hydrophobic substrates for the formation of biosurfactants [17, 18]. The remaining activity of the supernatant showed that ethanol is able to induce the production of the emulsifying factors and is therefore the best substrate. Instead of TL-4 another glycolipid was detected in large amounts. These trehalose dicorynomycolates are not able to stabilize emulsions with *n*-alkanes because of their low HLB-value. It was very surprising to find more than 1 g/l of this very insoluble substance in the supernatant. The emulsifier would probably facilitate the release of TL-2 from the cell wall.

Production in a 50 L bioreactor

Fig. 2 shows the time course of the formation of the emulsifying factors in a bioreactor cultivation with *Arthrobacter* sp. SI 1.

The exponential phase of growth occurred after a lag-phase of 24 h. The cell dry weight rose from 0.88 g/l to 11.85 g/l at the time of termination. The estimation of emulsifying activity runs parallel to growth and reached maximum values at termination. The formation of the emulsifier appeared to be partly growth associated. The yield of crude emulsifier was 1.5 g/l. As a polysaccharide affected the dispersion of *n*-alkanes, the emulsion test was performed with hexadecene which gave satisfactory results. In further studies it was possible to remove the polysaccharide, thereby improving activity of the remaining emulsifying substances. The formation of TL-2 increased to 700 mg/l. This was less than in experiments with shake flasks. The good aeration of the bioreactor might inhibit TL-2 formation. Such an influence on the formation of sugar corynomycolates by aeration has been observed [19].

Properties of the emulsifier

The ability of the crude emulsifier to stabilize oil in water emulsions was tested with several mixtures of hydrocarbons in natural sea water. In Fig. 3 the optical density of the emulsions are compared to those obtained with emulsan, a known lipopolysaccharide [20]. Mixtures containing aliphatics and aromatics such as mihagol-S/1-methylnaphthalene (1:1, v/v) or kerosene were efficiently emulsified. Less emulsification was ob-

Table IV. Formation of emulsifying agents by *Arthrobacter* sp. SI 1 in synthetic sea water medium under varying conditions. Estimation of the emulsifying activity after 1 h.

Carbon source	Nitrogen source	Cultivation time [d]	Emulsifying activity (OD_{623nm})
mihagol-S*	NaNO₃	7	1.25
mihagol-S	NH₄Cl	12	0.50
glycerine	NaNO₃	12	0.45
glucose	NaNO₃	10	0.13
n-tetradecane	NaNO₃	12	0.86
olive oil	NaNO₃	12	0.37
ethanol*	NaNO₃	10	1.18
ethanol	urea	9	0.54
ethanol	NH₄Cl	7	0.55

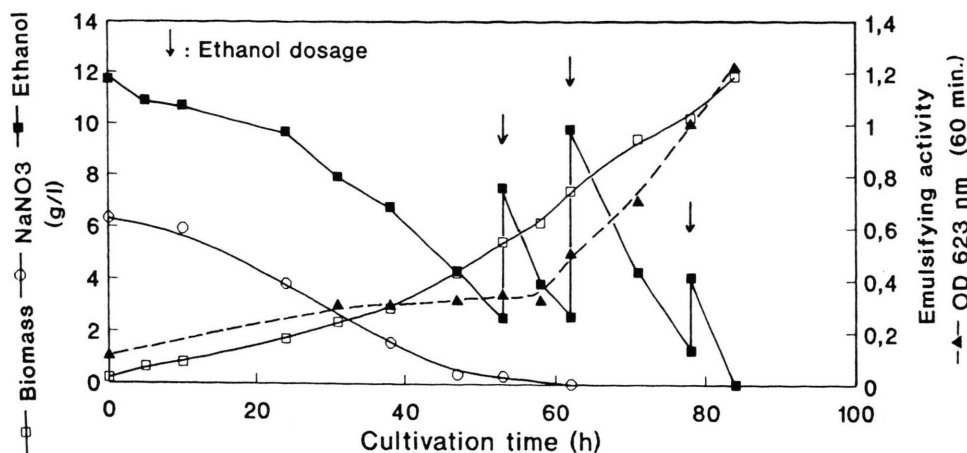* Average activity of different cultivations.

Fig. 2. Cultivation of *Arthrobacter* sp. SI 1 in a 50 l bioreactor with ethanol as sole carbon and energy source. Conditions: synthetic sea water with N/P/YE-supplementation, pH 7.2–7.5, 20 °C, intensor system, 800 rpm, aeration: 0.5 v/v/m.

tained in the case of *n*-alkanes. Furthermore it could be observed that divalent cations such as Mg^{2+} are necessary for emulsification. Exhaustive dialysis against distilled water brought about complete loss of emulsifying activity.

Heat stability

The emulsifier does not show heat stability. Their is a considerable loss of activity after boiling in a water bath for 5 h. The stability after boiling for 3.5 h is nevertheless remarkable (Fig. 4). This
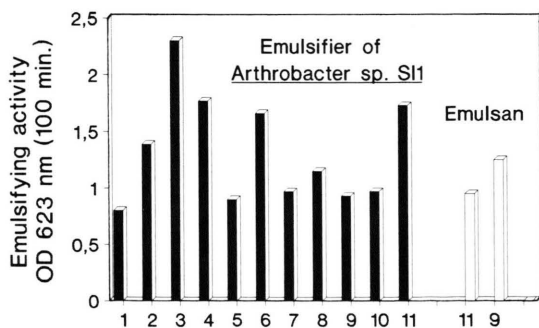

Fig. 4. Inactivation of the emulsifying agents from *Arthrobacter* sp. SI 1 by enzymatic and thermic treatments. Conditions: 1 mg emulsifier/ml 0.08 n phosphate buffer, pH 7.5, 2.5% hexadecene.

result is comparable to those obtained by Reddy *et al.* and Roy *et al.* [21, 22]. Their emulsifying factors described were also stable after heating for 90 min.

Enzyme digestion

The reduced emulsifying activity after digestion with proteases or amylase as shown in Fig. 4 might give a first indication of the nature of the emulsifier. Heat denaturation and digestion by proteases lead to the conclusion that the protein moiety of the crude isolate is very important for emulsification and that possibly lipoproteins were produced by *Arthrobacter* sp. SI 1. The protein content of the lyophylized crude emulsifier was 33% as deter-

Fig. 3. Influence of several hydrocarbons on the ability of the substances produced by *Arthrobacter* sp. SI 1 to stabilize emulsions compared to emulsan. Conditions: 0.5 mg emulsifier/ml sea water. 1. mihagol-S; 2. 1-methylnaphthalene; 3. mihagol-S/1-methylnaphthalene (1:1); 4. model oil; 5. cyclohexane; 6. 1,2,4-trimethylcyclohexane; 7. 1-phenyldecane; 8. hexadecene; 9. hexadecane; 10. decane; 11. kerosene.
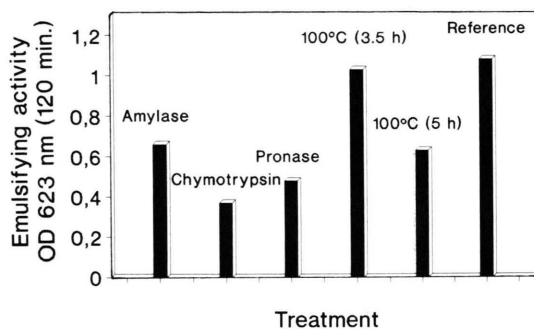
mined with the Lowry reagent. After continuous extraction of the lyophylized emulsifier with methylene chloride and removal of the polysaccharide by precipitation with isopropanol the protein content was 27.5% but only traces of compounds containing sugar of the order of 5% were found.

The exact nature and the structure of the emulsifier awaits further investigations.

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