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Physicochemical Properties of Biosurfactants Produced by Oil Destructor Microorganisms

L. A. BELOVEZHETS[1], M. S. TRETYAKOVA[2], YU. A. MARKOVA[2], L. P. OZNOBIKHINA[1]

[1] *A. E. Favorsky Irkutsk Institute of Chemistry, Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russia*

[2] *Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russia*

E-mail: lyu-sya@yandex.ru

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Abstract

The physicochemical properties and structure of biosurfactants produced by oil destructor microorganisms represented by three strains – *Rhodococcus* 108, *Acinetobacter* 112, *Acinetobacter* 114 – are investigated. It is shown that all these microorganisms are capable of synthesizing both cell-bound and extracellular biosurfactants. The amount of free biosurfactants produced by *Rhodococcus* 108 strain is higher than the amount produced by the bacteria belonging to *Acinetobacter* genus. All the studied compounds contain carbohydrate and lipid components. According to the data of IR spectroscopy, the biosurfactant isolated from the *Rhodococcus* 108 strain is a compound bearing long-chain aliphatic hydrocarbons, ester bonds, carbonyl and OH groups, while lipopolysaccharide isolated from *Acinetobacter* 114 strain consists of a trisaccharide skeleton, to which the residues of fatty acids are added (C_{10}–C_{22}) via ester and amide bonds.

Keywords: Oil destructor microorganisms, biosurfactants, emulsifying properties

INTRODUCTION

Biosurfactants represent a structurally diverse group of surface-active substances (SAS) synthesized by various microorganisms. These substances are not worse in their ability to form emulsions than synthetic SAS but they possess a number of advantages: the ability to get rapidly decomposed in the environment, the absence of toxicity, ecological safety, stable activity under extreme conditions [1, 2], efficiency in low concentrations [3].

Biogenic SAS are widely used in bioremediation of the environment from xenobiotics and to enhance oil recovery from soil strata. It was demonstrated that oil recovery from underground sandstone increases by up to 30 % with the use of trehalolipids from *Nocardia rhodochrous* [4]. During the experimental-industrial testing of this method at one of the sites of the Bondyuzhskoe oil field (PC Tatneft), an additional amount of 47 thousand tons of oil was obtained during 5 years since the start of testing, which accounted for about 30 % of the total amount of oil produced at this site within the indicated time interval [5]. Analysis of the efficiency of methods that were used to enhance oil recovery from strata at the Mancharovo deposit revealed that the most efficient methods were those based on microbiological action. During the years 1990–1998, the additional amount of oil produced due to the introduction of dry sludge was 43 515 t. The average amount of oil produced per one treatment over

the LC NGDU Chekmagushneft was 640 t. The specific increment was 296 t per 1 t of the reagent [6]. An essential direction of the use of microorganisms forming surfactants is the technology of bioremediation of soil contaminated with oil components [7, 8]. For instance, rhamnolipid produced by *Pseudomonas aeruginosa* is able to remove up to 25–70 and 40–80 % of hydrocarbons from contaminated sandy clay and clayey soil, respectively [9]. In addition, biosurfactants exhibit high efficiency also in soil remediation from heavy metals [10, 11], in particular uranium, cadmium, nickel [12] and lead [13].

All the above-listed advantages make biosurfactants promising objects to study and elaborate new ecologically friendly technologies related to oil production or elimination of the consequences of oil and oil product spills [14–16].

The goal of the work was to study the composition and properties of biosurfactants produced by oil-destroying microorganisms, for the evaluation of the possibility to use them in industry.

EXPERIMENTAL

Microorganisms were isolated from the rhizosphere of *Elytrigia repens* growing at the oil-polluted territory of the Irkutsk Region. To accumulate biosurfactants, microorganisms were cultivated in a shaker in the dark at 22 °C and pH 7 on the liquid culture medium 8E with the composition, g/L: $NaNO_3$ – 3.0, $K_2HPO_4 \cdot 3H_2O$ – 1.0, $FeSO_4 \cdot 7H_2O$ – 0.01, $MgSO_4 \cdot 7H_2O$ – 0.5, KCI – 0.5, using 2 % *n*-hexadecane as the only source of carbon and energy. The bacterial suspension was separated into cell biomass and supernatant by centrifuging at 6000 *g* with the help of a CM-6M centrifuge (ELMY, Latvia). Biosurfactants were extracted after cultivation for 6 days from the supernatant using a mixture of chloroform and methanol at the volume ratio of 3 : 1, after preliminary acidification of each sample with hydrochloric acid to achieve pH 2.

The emulsifying activity of biosurfactants was determined using Cooper's method [17]. A mixture of 4 mL of bacterial suspension and 3 mL of diesel fuel was stirred in an orbital stirrer at 200 r.p.m. and then settled in the vertical position for 1 h to achieve the separation of the aqueous phase from the hydrocarbon phase. The presence or absence of emulsion at the interface was determined visually. If the emulsion was present after settling for 24 h, emulsification index (E_{24}, %) was calculated according to the equation:

$$E_{24} = (V_e/V)\ 100$$

where V is the total volume of the mixture (7 mL); V_e is the volume of dense emulsion formed during mixing the bacterial suspension and diesel fuel, mL.

The ability of bacteria to cause a decrease in the surface tension of oil was determined in a Petri dish 15 cm in diameter by placing 20 mL of distilled water in the dish, and then adding 1 μL of crude oil. Then 100 μL of the supernatant of the culture of microorganisms was added into the centre of the oil surface, and the diameter of the formed clean zone was measured after 30 s [18].

The hydrophobicity parameter of bacterial cells was determined using the method described in [19].

To determine cell-bound surfactants, the cell biomass was suspended in phosphate buffer (0.1 M, pH 6.75). Then 0.5 mL of *n*-hexadecane was added to 3 ml of the suspension, the mixture was stirred for 3 min and left for 10 min at a temperature of 37 °C. Then the lower aqueous layer was sampled, and its optical density was measured with the help of a P-5400UF spectrophotometer (LC EKROSKHIM, Russia) at the wavelength of 585 nm in a cell 1 cm long. The content of cell-bound surfactants was determined using the equation:

$$H = (1 - (D_1/D_0))\ 100$$

where H is the amount of cell-bound surfactants, %; D_0 is the initial optical density of the bacterial suspension; D_1 is the optical density of bacterial suspension after the sorption of the cells on *n*-hexadecane.

To determine the component composition of biosurfactants, we carried out a number of qualitative reactions according to [20]. The presence of protein was detected using the reaction with ninhydrin, the presence of carbohydrates was determined by means of the Trommer test, lipids – by Goldman's reaction, and starch – by its reaction with iodine. The presence of peptide bonds was revealed with the help of the biuret test. The elemental composition of biosurfactants is, mass %: C – 70.72, N – traces, H – 12.81, ash – 5.4 (*Rhodococcus* 108); C – 76.31, N – 0.94, H – 14.2 (*Acinetobacter* 114).

Examination of biosurfactants by means of thin layer chromatography (TLC) was carried out with Silufol plates in the system chloroform/methanol/water (65 : 15 : 2). The developing agent was a naphthol reagent (α-naphthol 0.5 g in 100 mL of methanol/water mixture (1 : 1 by vol-

ume), with subsequent treatment with 10 % sulphuric acid under heating till the maximum colouring appeared.

The IR spectra of biosurfactants in thin film were recorded with a Varian 3100 IR Fourier spectrometer (Varian Inc., USA).

RESULTS AND DISCUSSION

For the efficient application of biogenic SAS, it is necessary to study their structure, physicochemical and physiological properties. We demonstrated previously that *Rhodococcus* 108 emulsifies oil film from the roots of the plant, which is due to the presence of biosurfactants in the cultural liquid [21]. In the present work, we studied three strains of microorganisms – *Rhodococcus* 108, *Acinetobacter* 112, *Acinetobacter* 114. The primary evaluation of their ability to form emulsion was based on the value of the emulsification index. It was determined that all these strains form stable emulsions but the efficiencies differed. The emulsification index varied from 13 to 73 %. A clean zone was formed after the addition of supernatant on the surface of the oil, and the diameter of this clean zone depended on the strain of bacteria (Table 1). To increase the amount of synthesized biosurfactants, all microorganisms were grown on the nutritional medium with *n*-hexadecane as the only source of carbon. The amount of cell-bound biosurfactants was approximately the same for all strains, while *Rhodococcus* 108 formed a substantially larger amount of extracellular forms (the content of free biosurfactants per 1 L of the nutritional medium was 1.523 g), which corresponds to the literature data [1, 22].

Qualitative reactions helped us to reveal the presence of carbohydrate and lipid components in the biosurfactants; the absence of peptides and high-molecular carbohydrates was determined (Table 2).

One compact spot (mobility R_f = 0.78) is detected on the thin layer chromatogram of the surfactants of *Rhodococcus* 108 extracted from the supernatant. This is the evidence of the presence of only one biosurfactant or several compounds that are very close in structure to each other, which does not allow their separation by means of TLC. Spots obtained for *Acinetobacter* 112 and *Acinetobacter* 114 merged into one weakly coloured band, which did not allow us to isolate individual compounds in the extract.

For biosurfactants produced by *Rhodococcus* 108 and *Acinetobacter* 114, thin-film IR spectra were recorded (Fig. 1, 2). The most intense absorption bands (a. b.) in the spectra of both compounds are those of the stretching vibrations of CH_2 groups observed within the region 2950–2855 cm^{-1}, and bending vibrations of these groups at 1460 and 722 cm^{-1}. In the IR spectrum of biosurfactant produced by the *Rhodococcus* 108 strain (see Fig. 1), a broad a. b. within 3400–2600 cm^{-1} characterizes the vibrations of the associated hydroxyl groups – ν(OH). The absorption band of associated carbonyl group ν(C=O) is ob-

TABLE 1

Parameters of the capacity of oil destructor bacteria to produce surface-active substances

Strain	Diameter of the formed clean zones, cm	Emulsifying activity of liquid cultures	Emulsification index, %	Content of biosurfactants	
				cell-bound, %	extracellular, g/L
Rhodococcus 108	4.0±0.2	++	73	37.0	1.523
Acinetobacter 112	3.2±0.4	+	57	31.5	0.177
Acinetobacter 114	1.5±0.2	+	13	31.2	0.166

TABLE 2

Component composition of biosurfactants (qualitative reactions)

Qualitative reaction	Strain of microorganism		
	Rhodococcus 108	*Acinetobacter* 112	*Acinetobacter* 114
Ninhydrin	–	–	–
Biuret	–	–	–
Iodine	–	–	–
Trommer reaction	+	+	+
Goldman reaction	+	+	+

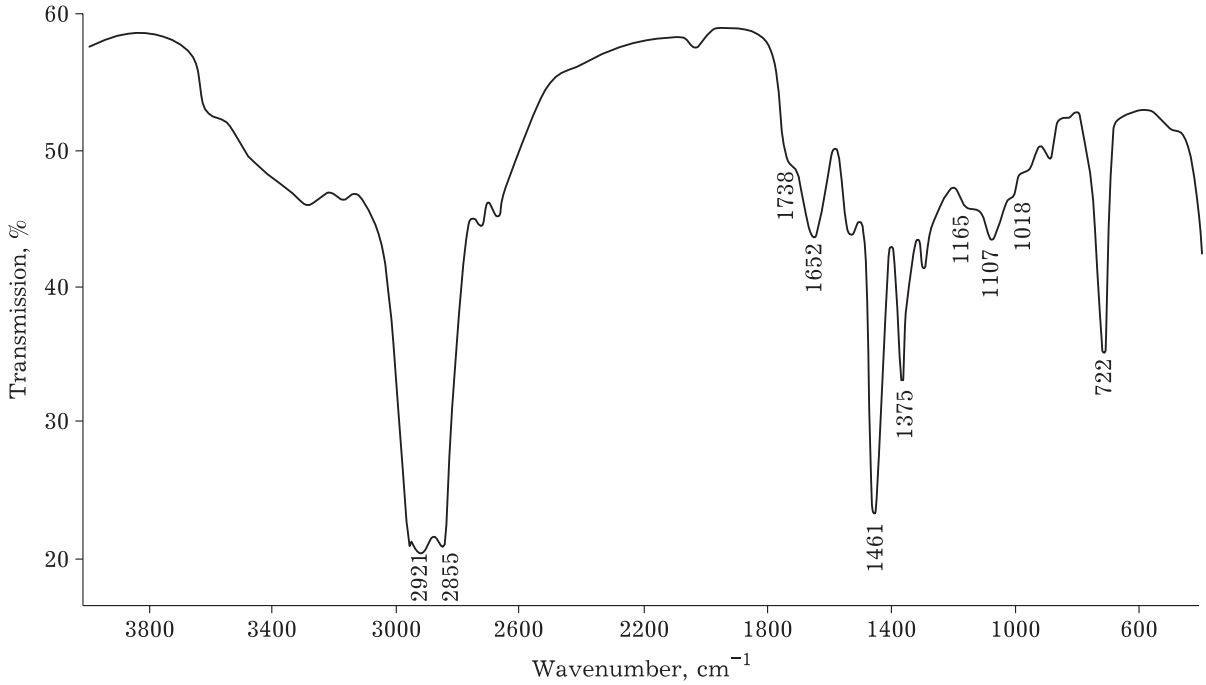

Fig. 1. IR spectrum of biosurfactant produced by *Rhodococcus* 108.

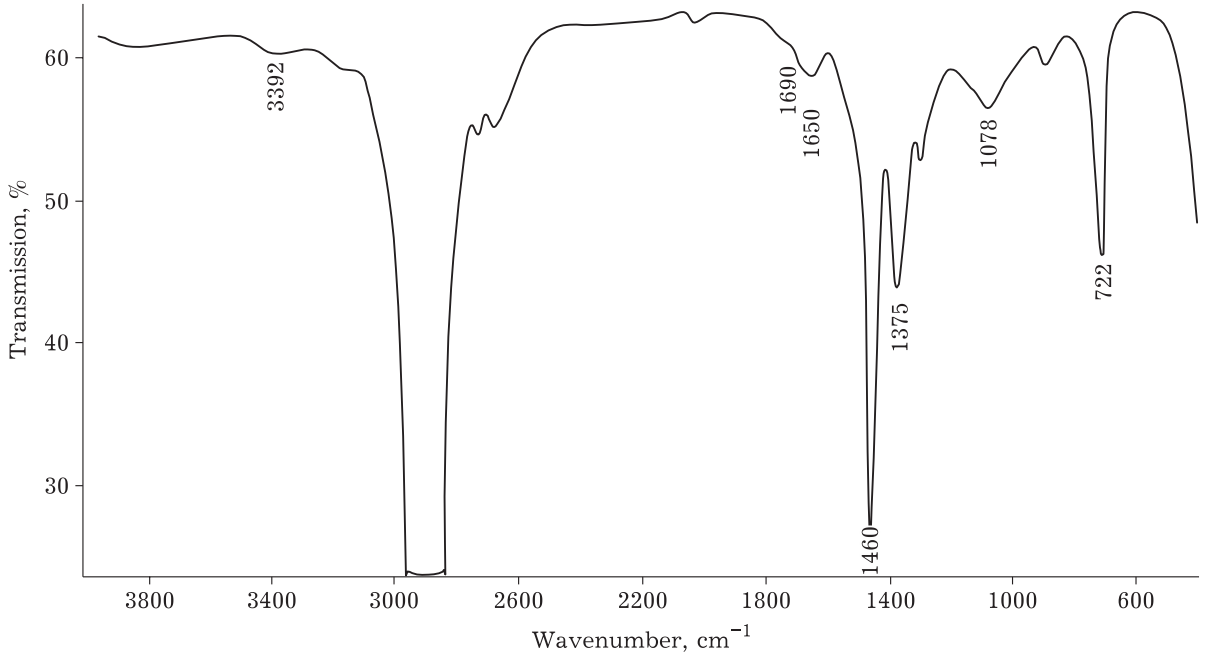

Fig. 2. IR spectrum of biosurfactant produced by *Acinetobacter* 114.

served at 1652 cm^{-1}. Ether and ester groups are characterized by a. b. with maxima within the range 1165–1018 cm^{-1}, which may be due to the presence of CH–O–CH and CH_2–O–CH_2 fragments. A weak shoulder at 1738 cm^{-1} on the high-frequency wing of the a. b. at 1652 cm^{-1} corresponds to the vibration of the free carbonyl group ν(C=O). A doublet vibration band δ(CH_3) at 1375 cm^{-1} is the evidence of the possible presence of branched C(CH_3)$_2$ groups. Therefore, the biosurfactant produced by *Rhodococcus* 108 possesses a complicated structure which involves long-chain aliphatic hydrocarbons, ether, carbonyl and hydroxyl groups. The obtained data allow us to assume that this is an ester of trehalose and mycolic acids.

The IR spectrum of biosurfactant produced by *Acinetobacter* 114 strain (see Fig. 2) contains, along with intense a. b. $\nu(CH_2)$, $\delta(CH_2)$ and $\delta(CH_3)$, also characteristic bands similar to those observed in the spectrum of biosurfactant produced by *Rhodococcus* 108, but less intense. Thus, a weak broad a. b. of associated hydroxyl groups ν(OH) is within the region 3500–3200 cm^{-1} and has a weakly pronounced maximum at 3390 cm^{-1}. The vibrations of associated and free carbonyl groups ν(C=O) are characterized by a weak a. b. at 1650 cm^{-1} and a shoulder on its wing at 1690 cm^{-1}, respectively. The band with the maximum at 1078 cm^{-1} is due to the vibrations of the ether fragment.

The presence of nitrogen (0.94 %) in the elemental composition allows us to assume that the biosurfactant is lipopolysaccharide composed of a trisaccharide basis (*D*-galactozamine + *D*-galactozaminuric acid + dioxyaminohexose) to which the residues of fatty acids (C_{10}–C_{22}) are connected through the ester and amide bonds.

CONCLUSION

The presence of both extracellular and cell-bound biosurfactants was revealed in all the studied strains of microorganisms: *Rhodococcus* 108, *Acinetobacter* 112, *Acinetobacter* 114. It was established that biosurfactants contain carbohydrate and lipid components. Groups corresponding to long-chain aliphatic hydrocarbons, ether, carbonyl and hydroxyl groups were revealed by means of TLC and IR spectroscopy. It is concluded on this basis that the possible structure of the extracted substances is trehalolipid for *Rhodococcus* 108, lipopolysaccharide for *Acinetobacter* 114. The obtained data allow us to consider *Rhodococcus* 108 as the producers of biosurfactants that are promising for further use in the industrial mining of highly viscous oil.

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