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RESEARCH ARTICLE

Solubilization of *n*-hexadecane by micellar solutions of trehalolipid—surfactants of biological origin

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Abstract

Objectives. To isolate biosurfactants of glycolipid nature produced by oil hydrocarbon degrading bacteria and to establish their ability to solubilize hydrophobic compounds in the case of *n*-hexadecane.

Methods. Trehalolipids were isolated from bacteria *Rhodococcus erythropolis* X5 (VKM Ac-2532 D) and *Rhodococcus erythropolis* S67 (VKM Ac-2533 D) included in the MikroBak biopreparation for the bioremediation of oil-contaminated territories. The genome of *R. erythropolis* X5 is deposited in the National Center for Biotechnology Information database under GenBank accession numbers CP044283 and CP044284, BioSample – SAMN12818508, BioProject – PRJNA573614, and SRA – PRJNA573614. The content of trehalolipid biosurfactants was estimated by the amount of trehalose in aqueous solutions of biosurfactants using the phenol-sulfur method. The surface tension of the obtained aqueous solutions of biosurfactants was determined by the du Noüy ring method using a Kruss K6 tensiometer (*Kruss*, Germany). The critical concentration of micelle formation was determined by the inflection point on the curves of surface tension dependence on the concentration of the biosurfactant solution. In order to establish the solubilizing ability of biosurfactants, the residual concentration of *n*-hexadecane in an aqueous sample of different concentrations was determined using a gas chromatographic method of analysis.

Results. At a constant surface tension of 24.2 mN/m and 25.0 mN/m for *R. erythropolis* X5 and *R. erythropolis* S67, respectively, the critical micelle concentration for both strains was 33 mg/L ($3.8 \cdot 10^{-5}$ mol/L). The solubilizing effect of *Rhodococcus* trehalolipid micellar solutions against hydrophobic *n*-hexadecane was demonstrated by gas chromatographic analysis. The solubilization process was characterized using molar solubilization capacity (S_m), molar solubilization ratio (MSR), micelle–water partition coefficient (K_m), and solubilization energy (ΔG_S^0). It was shown that the solubilization process of *n*-hexadecane proceeds spontaneously ($\Delta G_S^0 = -35.5$ kJ/mol) and more efficiently ($S_m = 4.3$ mol/mol, MSR = 4.7 mol/mol) than in comparison with other biosurfactants of glycolipid nature.

Conclusions. Based on the value of the molar solubilization coefficient, it can be concluded that trehalolipids of the *R. erythropolis* X5 strain solubilize *n*-hexadecane in aqueous solutions to a greater extent than compared to other biosurfactants of a glycolipid nature, but are inferior to synthetic surfactants.

Keywords

biosurfactants, solubilization, bacteria-destructors, surface tension, *Rhodococcus*, *n*-hexadecane, trehalolipids

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НАУЧНАЯ СТАТЬЯ

Солюбилизация *н*-гексадекана мицеллярными растворами трегалолипида — ПАВ биологического происхождения

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Аннотация

Цели. Выделить биосурфактанты гликолипидной природы, продуцируемые бактериями-деструкторами углеводородов нефти, и установить их способность к солюбилизации гидрофобных соединений на примере *н*-гексадекана.

Методы. Трегалолипиды выделяли из бактерий *Rhodococcus erythropolis* X5 (ВКМ Ac-2532 Д) и *Rhodococcus erythropolis* S67 (ВКМ Ac-2533 Д), входящих в биопрепарат «МикроБак» для биоремедиации нефтезагрязненных территорий. Геном *R. erythropolis* X5 депонирован в базе данных National Center for Biotechnology Information под номерами доступа GenBankCP044283 и CP044284, BioSample – SAMN12818508, BioProject – PRJNA573614 и SRA – PRJNA573614. Содержание трегалолипидных биосурфактантов оценивали по количеству трегалозы в водных растворах биосурфактантов с помощью фенольно-серного метода. Поверхностное натяжение полученных водных растворов биосурфактантов определяли методом отрыва кольца де Нуи с использованием тензиометра Kruss K6 (*Kruss*, Германия). Критическую концентрацию мицеллообразования определяли по точке перегиба на кривых зависимостях поверхностного натяжения от концентрации раствора биосурфактанта. Для установления солюбилизирующей способности биосурфактантов определяли остаточную концентрацию *н*-гексадекана в водной пробе различной концентрации с помощью газохроматографического метода анализа.

Результаты. При постоянном поверхностном натяжении 24.2 мН/м и 25.0 мН/м для *R. erythropolis* X5 и *R. erythropolis* S67 соответственно значение критической концентрации мицеллообразования для обоих штаммов составило 33 мг/л ($3.8 \cdot 10^{-5}$ моль/л). С помощью газохроматографического метода анализа показано солюбилизирующее действие мицеллярных растворов трегалолипидов родококков в отношении гидрофобного *н*-гексадекана. Процесс солюбилизации охарактеризовали с помощью молярной солюбилизирующей способности (molar solubilization capacity, S_m), молярного коэффициента солюбилизации (molar solubilization ratio, MSR), коэффициента распределения мицелла–вода (micelle–water partition coefficient, K_m) и энергии солюбилизации (ΔG_S^0). Показано, что процесс солюбилизации *н*-гексадекана протекает самопроизвольно (ΔG_S^0 = −35.5 кДж/моль) и более эффективно (S_m = 4.3 моль/моль, MSR = 4.7 моль/моль) по сравнению с другими биосурфактантами гликолипидной природы.

Выводы. На основании величины молярного коэффициента солюбилизации можно сделать вывод, что трегалолипиды штамма *R. erythropolis* X5 в большей степени солюбилизируют *н*-гексадекан в водных растворах по сравнению с другими биосурфактантами гликолипидной природы, однако уступают синтетическим поверхностно-активным соединениям.

Ключевые слова

биосурфактанты, солюбилизация, бактерии-деструкторы, поверхностное натяжение, *Rhodococcus*, *н*-гексадекан, трегалолипиды

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INTRODUCTION

Biosurfactants or surfactants of biological origin have significant advantages over synthetic surfactants due to their physicochemical and biological properties. They are rapidly occupying a significant niche in the production of so-called green products and displacing analogues of chemical origin from the market. Due to their structural diversity and properties, such as reduction of surface and interfacial tension, foaming, emulsification, wetting, stabilization of emulsions and solubilization of hydrophobic substances, biosurfactants can be used in the pharmaceutical and food industries [1], cosmetology [2], to increase oil recovery, in bioremediation of polluted areas [3], in bioelectrochemistry [4], in agriculture [5], and in wastewater treatment technologies. Biosurfactants are produced by microorganisms of various taxonomic groups, such as bacteria of the genera *Pseudomonas*, *Rhodococcus*, *Arthrobacter*, *Mycobacterium*, *Nocardia*, *Corynebacterium* and yeasts of the genera *Candida* and *Rhodotorula* [6].

Among bacteria capable of forming and releasing biosurfactants into the environment, special attention needs to be paid to the bacteria of the *Rhodococcus* genus. The metabolic activity of *Rhodococcus* is due to the presence of a large number of enzyme systems which enable them to degrade many natural and anthropogenic organic compounds, such as alkanes, cycloalkanes, aromatic compounds, phenols, polycyclic aromatic hydrocarbons, halogenated hydrocarbons, and polychlorinated compounds. The list of toxic environmental pollutant compounds which can be mineralized or transformed by *Rhodococci* is quite large. It also includes explosives, pharmaceuticals, plastics and difficult-to-degrade synthetic polymers [7].

Rhodococci are characterized by the formation of surfactants of a glycolipid nature in response to the presence of alkanes in the nutrient medium. Such substances are one or two nonreducing disaccharides of trehalose bound to mycolic acids: long-chain α-branched-α-hydroxylated fatty acids with different carbon chain lengths [8].

Due to their low critical micelle formation concentration (CMC), the ability to reduce surface and interfacial tension, high activity under extreme environmental conditions (temperature, pH), good emulsifying ability combined with high biodegradability, low toxicity and environmental safety, as well as the possibility of being obtained from renewable sources of raw materials [9], these biological surfactants are more promising for the development of environmentally friendly biotechnologies.

In connection with the above properties and the wide application of trehalolipids in a range of industrial fields, the study of the solubilizing ability of their micellar solutions is both topical and in demand.

The aim of the study was to isolate biosurfactants of a glycolipid nature produced by the bacteria-destructors of the *Rhodococcus* genus of petroleum hydrocarbons, and to evaluate their solubilizing ability with respect to hydrophobic compounds on the example of *n*-hexadecane.

MATERIALS AND METHODS

Rhodococcus erythropolis X5 (VKM Ac-2532 D) and *Rhodococcus erythropolis* S67 (VKM Ac-2533 D) strains were obtained from the collection of the Laboratory of Biology and Plasmids of the G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences. The MicroBak biopreparation contains these bacteria and is used for bioremediation of oil-contaminated territories [10]. The genome of *R. erythropolis* X5 is deposited in the NCBI database[1] under GenBank accession numbers CP044283 and CP044284, BioSample – SAMN12818508, BioProject – PRJNA573614 and SRA – PRJNA573614 [11].

Microorganisms were cultured in rocking flasks in 200 mL of liquid Evans mineral medium [12] with the addition of *n*-hexadecane (20 g/L) (*ECOS-1*, Russia) as the sole source of carbon and energy at 26°C and aeration at 180 rpm on an Excella 25 orbital rocker (*Eppendorf*, Germany) for 3 days.

Biosurfactants were isolated from culture fluid samples by means of liquid–liquid extraction [13]. Glycolipid components were separated by means of thin-layer chromatography on TLC plates Silica gel 60 F254 (*Merck*, Germany) [14].

The total biosurfactant content was determined by the concentration of carbohydrate in the sample spectrophotometrically using the phenol–sulfur method [15], after previously constructing a graduation relationship for the corresponding carbohydrate—trehalose. In order to calculate the concentration of trehalolipid, the obtained value of sugar concentration was multiplied by a coefficient equal to the ratio of the molecular weight of glycolipid (862) to the molecular weight of trehalose (342). This is respectively equal to 2.5.

[1] National Center for Biotechnology. Information https://www.ncbi.nlm.nih.gov. Accessed June 31, 2024.

The surface tension of the aqueous solutions of biosurfactants obtained was determined by means of the method [13] using a Kruss K6 tensiometer (*Kruss*, Germany). An initial aqueous solution with a concentration of 250 mg/L was prepared. A series of samples with biosurfactant concentrations in the range of 0–250 mg/L was obtained by dilution method, and the surface tension of each solution was measured. The CMC was determined by the inflection point on the curves of surface tension dependence on concentration.

In order to establish the solubilizing ability of biosurfactant, we determined the residual concentration of *n*-hexadecane in aqueous samples of biosurfactant of different concentrations according to the method [16] with our modifications. For this purpose, 300 μL of *n*-hexadecane and 10 mL of biosurfactant solution of the corresponding concentration were added to the tubes, which were corked and shaken vigorously for 1 min. Subsequently, the tubes were left at 28°C, and 180 rpm on an Excella 25 orbital rocker (*Eppendorf*, Germany) for 24 h. The contents of the tubes were poured into a separating funnel and left for 2 h for phase separation. Then the *n*-hexadecane layer was withdrawn into a separate tube. 10 mL of hexane was added to the tubes using a graduated pipette, corked and shaken vigorously for 1 min. The gas chromatographic determination of the residual concentration of *n*-hexadecane extracted with hexane was then carried out. The method used to determine oil product content in natural and waste waters was the gas chromatographic method[2] on a Chromatek Crystal 5000.2 gas chromatograph (*Chromatek*, Russia) with Varian Capillary Column CP-Sil 8 CB (50 m) and flame ionization detector. The concentration of hexadecane was calculated by means of the absolute graduation method.

Experiments were performed in triplicate, statistical processing was performed using Microsoft Office Excel 2010 and SigmaPlot® 2011 software. The average value ± confidence interval was calculated.

RESULTS AND DISCUSSION

Representatives of the *Rhodococcus* genus are efficient degraders of oil hydrocarbons. They easily adapt to extreme environmental conditions and are often included in the composition of biopreparations for cleaning from oil pollution[3]. They produce biosurfactants of glycolipid nature, namely trehalolipids. The hydrophobic nature of oil hydrocarbons is the reason for their low bioavailability in the process of biodegradation. However, the formation of micellar solutions of trehalolipids can contribute to its increase. In the present work, the solubilizing ability of *R. erythropolis* X5 and *R. erythropolis* S67 strains was evaluated with respect to a hydrophobic compound, using *n*-hexadecane as an example.

Biosurfactants of a glycolipid nature were isolated from the culture fluid of petroleum hydrocarbon-degrading bacteria *R. erythropolis* X5 and *R. erythropolis* S67 by means of extraction with a system of polar organic solvents. Previously, 2,3,4-succinyl-octanoyl-dodecanoyl-2'-decanoyl-trehalose and 2,3,4-succinyl-dioctanoyl-2'-decanoyl-trehalose had been found to be the main biosurfactants produced by these bacteria grown on *n*-hexadecane, both at 26°C and 10°C [13, 17].

In order to confirm the chemical structure of the isolated substances synthesized by the studied bacteria, the lipid extract obtained was analyzed by thin-layer chromatography. In order also to detect trehalose tetraethers, we used α-naphthol reagent which is a specific developer for sugars. This enabled us to identify glycolipids among other lipid components. When comparing the obtained data with date from the literature (Table 1), it can be noted that *Rhodococcus* biosurfactants appear on chromatograms with a different number of spots and retention values. However, the spot which appears to have the highest intensity with a retention factor of R_f 0.35–0.39 is present in all chromatograms, except for *R. erythropolis* A29-k1 strain and *R. ruber* IEGM 231 strain.

Table 1. Comparative characteristics of the R_f values of trehalolipids of various *Rhodococcus* strains

Microbial strain	R_{f1}	R_{f2}	R_{f3}
R. erythropolis X5	0.38	0.50	0.59
R. erythropolis S67	0.37	0.50	–
R. ruber IEGM 231 [18]	0.18	0.39	0.75
R. erythropolis A29-k1 [19]	0.46	0.54	–
R. sp. 3–2 [15]	0.35	0.53	0.56

[2] Methodology for measurement of oil product content in natural and waste waters by gas chromatographic method with flame ionization detector. MVI-05-94. M.: 1994.

[3] Neustroev M.M. *Ecological assessment of oil-contaminated permafrost soils and development of methods of their bioremediation*. Cand. Sci. Thesis. (Biol.). Yakutsk. 2016.

The trehalolipid components thus revealed may have structural differences, but they all contain carbohydrate residues. The results obtained are consistent with the results of earlier studies [20].

The amount of isolated trehalolipids of *R. erythropolis* X5 and *R. erythropolis* S67 bacteria after 3 days of cultivation was determined by means of measuring the trehalose content in the analyzed samples. Strain X5 produced more biosurfactants (0.31 g/L, $36 \cdot 10^{-5}$ mol/L) in contrast to strain S67 (0.25 g/L, $29 \cdot 10^{-5}$ mol/L). The results presented agree with the data of Luong [14], in which the content of trehalolipid biosurfactants was about 0.3 g/L. The same amount of trehalolipids was obtained by White [21] in the cell-free supernatant during cultivation of *Rhodococcus sp.* PLM026 on sunflower oil at 19°C.

One of the important colloidal-chemical properties of surfactants of both chemical and biological origin is the ability to reduce the surface tension (air–water). As a consequence, the effectiveness of biosurfactants can be evaluated by means of measuring the surface tension of their solutions and plotting the graphical dependence of surface tension on the biosurfactant content (Fig. 1). At low contents (up to 25 mg/L), a linear dependence can be observed. There is a sharp decrease in surface tension when the biosurfactant content is increased within the range of 30 to 35 g/L. With a further increase in the biosurfactant content, there is a slowing of the rate of surface tension. This is due to gradual saturation of the surface layer with biosurfactant molecules. When the CMC is reached, biosurfactants in aqueous solution begin to form micelles and the surface tension does not change further. The CMC of isolated trehalolipids of strains X5 and S67 was determined using the inflection point of the graphical dependence of surface tension on the biosurfactant concentration.

At a constant surface tension of 24.2 and 25 mN/m for *R. erythropolis* X5 and *R. erythropolis* S67, respectively, the CMC value for both strains was 33 mg/L ($3.8 \cdot 10^{-5}$ mol/L). Table 2 summarizes the surface tension and CMC values of biosurfactants isolated by other researchers.

The results obtained by us agree with the data of other authors (Table 2). The trehalolipids studied are not inferior to other types of glycolipids in terms of efficiency in reducing the surface tension of water. The advantage of the isolated trehalolipids of *R. erythropolis* X5 and *R. erythropolis* S67 over synthetic surfactants should be noted. For example, sodium dodecyl sulfate (CMC = 2.34 g/L) and Tween-80 (CMC = 0.016 g/L) reduce the surface tension of water from 72 to 37 and 34.8 mN/m, respectively [28], while natural surfactants reduce the surface tension of water to lower values.

An interesting objective was to evaluate the solubilizing ability of the trehalolipids to increase the bioavailability of hydrophobic pollutants to bacterial cells by increasing their hydrophilicity. The solubilization process can be described as colloidal dissolution of various substances in surfactant micelles, therefore, solutions with trehalolipid content multiple of CMC were used to determine the solubilizing ability of trehalolipids.

Solubilization can be quantitatively characterized by using the molar solubilizing capacity (S_m). This is the ratio of the number of moles of *n*-hexadecane to the number of moles of trehalolipid:

$$S_m = \frac{C_1}{C_2}, \quad (1)$$

wherein C_1 is the *n*-hexadecane concentration, mol/L, C_2 is the trehalolipid concentration, mol/L.

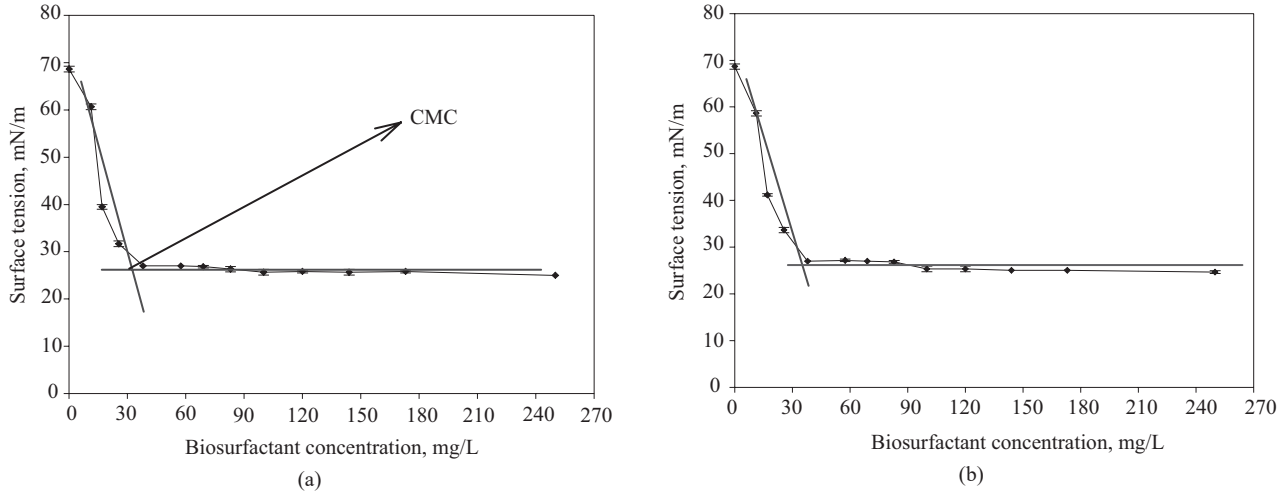

Fig. 1. Dependence of surface tension on biosurfactant content of *R. erythropolis* X5 (a) and *R. erythropolis* S67 strains (b)

Table 2. Characteristics of biosurfactants

Microorganism	Biosurfactant	Surface tension, mN/m	CMC, mg/L
Pseudomonas aeruginosa S6 [22]	Rhamnolipids	33.9	50.0
Staphulococcuss aprophyticus SBPS-15 [23]	Glycolipids (staphylosan)	30.9	24.0
Rhodococcus sp. HL-6 [24]	Glycolipids	30.7	40.0
Rhodococcus ruber IEGM 235 [25]		26.8	54.0
Rhodococcus erythropolis DSM 43215 [25]	Trehalolipids	26.0–32.0	4.0–15.0
Rhodococcus wratislaviensis BN38 [26]		24.4	5.0
Rhodococcus qingshengii FF [27]		–	85.0

With increased concentration of trehalolipid micellar solutions, the amount of solubilized *n*-hexadecane naturally increased in the range of trehalolipid contents above the CMC. This indicates solubilization of the hydrophobic compound in surfactant micelles. After 24 h, the average molar solubilization of *n*-hexadecane in aqueous solution of biosurfactant strain X5 was 4.3 mol of *n*-hexadecane per 1 mol of trehalolipid. The solubilization isotherm has a linear character at the concentration of trehalolipid above the CMC, indicating a constant micelle shape (Fig. 2).

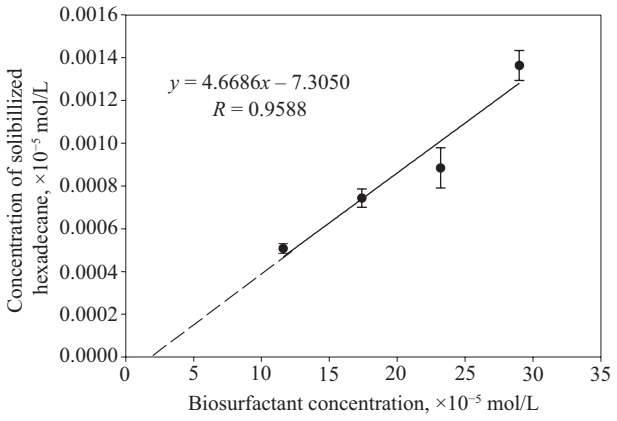

Fig. 2. Solubilization isotherm of *n*-hexadecane by trehalolipid micelles

Solubilization isotherms enable us to determine the values of CMC for which solubilization isotherms should be extrapolated to the concentration axis. Segments cut off on the concentration axis give the desired CMC values [28]. According to Fig. 2, the CMC value of trehalolipid biosurfactant produced by *R. erythropolis* X5 strain is $2.5 \cdot 10^{-5}$ mol/L. This value is slightly different from the value of CMC found from the surface tension isotherm ($3.8 \cdot 10^{-5}$ mol/L). This may be due to an error in the method of determining the solubilizing capacity of biosurfactants.

The solubilization isotherm presented also allows us to determine the molar solubilization ratio (MSR) as the tangent of the slope of this straight line (Fig. 2). MSR is the amount of *n*-hexadecane that can be solubilized by one mole of micellar solution of trehalolipid and characterizes in general the ability of surfactant to solubilize *n*-hexadecane.

We consider the binding constant or molar distribution coefficient as a parameter of interaction between *n*-hexadecane and trehalolipid. This is calculated according to the following formula:

$$K_m = \frac{\text{MSR}}{S_{CMC} V_w (1 + \text{MSR})}, \quad (2)$$

wherein MSR is the molar solubilization ratio, mol/mol; S_{CMC} is the solubility of *n*-hexadecane at CMC; V_w is the molar volume of water; V_w = 0.01805 dm^3/mol at 298 K.

A knowledge of thermodynamic parameters is necessary to achieve a better understanding of the solubilization mechanism. From the thermodynamic point of view, solubilization can be considered as the distribution of *n*-hexadecane between micellar and aqueous phases. The free energy of solubilization is calculated on the basis of the following equation:

$$\Delta G_S^0 = R \cdot T \cdot \left(\ln \frac{\text{CMC}}{mol_w} \right), \quad (3)$$

wherein ΔG_S^0 is solubilization free energy, kJ/mol; R is the gas constant, R = 8.31 J/mol·K; T is the temperature, K; mol_w is the solvent (water) molarity, mol_w = 55.5.

In order to describe the solubilizing ability of surfactants, MSR and CMC values are primarily established. Determination of the micelle–water distribution coefficient (K_m) and solubilization energy (ΔG_S^0) is less frequently used in research work. Among glycolipid biosurfactants (Table 3), isolated trehalolipids solubilize *n*-hexadecane in aqueous solutions more efficiently. However, they are inferior to synthetic surfactants, namely those surfactants of a nonionic nature (Tween-80 and Triton X-100). In [29–31], the difference in the solubilizing ability of synthetic surfactants of different chemical structure in relation to *n*-hexadecane and chlorine derivatives of hydrocarbons is shown. For nonionogenic surfactants, the solubilizing ability of *n*-hexadecane is higher than that of chlorine derivatives. For sodium dodecyl sulfate, an ionogenic surfactant, a lower solubilization capacity of *n*-hexadecane and chlorine derivatives can be observed. Thus, the value of molar solubilizing capacity may differ depending on the chemical structure of the surfactant itself and the solubilized substance. In [31] it is demonstrated that the solubility of naphthalene, phenanthrene and pyrene increased linearly with an increasing concentration of rhamnolipid biosurfactant of bacteria of the *Bacillus* genus. In addition, the values of molar solubilization coefficient decreased in the series: naphthalene > phenanthrene > pyrene.

With regards to the system which we studied, the free energy value has a negative value (ΔG_S^0 = −35.5 kJ/mol), indicating a spontaneous solubilization process. It was shown in [34] that the solubilization processes of nitrogen-containing heterocyclic substances (aniline, indole, quinolone), surfactants of chemical (sodium dodecyl sulfate (Tween-80, Span 20, and TX-100)) and biological (rhamnolipid) origin are spontaneous ($\Delta G_S^0 \leq 0$). The value of solubilization free energy for rhamnolipid is markedly lower than for synthetic surfactants. This proves the better solubilizing ability of rhamnolipid and is consistent with our data.

Table 3. Comparative table of data (obtained and from literature) of the physicochemical characteristics of solubilization of different substances by nature

Surfactant	Solubilizate	MSR, mol/mol	ΔG_S^0, kJ/mol	lgK_m	Reference
Trehalolipids	Hexadecane	4.7	−35.5	1.065	This work
Monorhamnolipids		0.89	–	8.25	[33]
Diramnolipids		3.8	–	–	
Sodiumdodecylsulfate	Hexadecane	0.018	−54.5	–	[29]
	Perchloroethylene	0.16	–	–	[30]
Twin-80	Hexadecane	15.1	−64.4	–	[29]
	Hexachloroethane	0.15	–	–	[31]
Triton X-100	Hexadecane	40	−64.6	–	[29]
	Hexachlorobutadiene	1.22	–	–	[31]

CONCLUSIONS

For *Rhodococcus* trehalolipids, their solubilizing effect on hydrophobic *n*-hexadecane was demonstrated using the following physicochemical values: molar solubilizing capacity; molar solubilization coefficient; micelle–water distribution coefficient; and solubilization energy. Based on the value of the molar solubilization coefficient, it can be concluded that trehalolipids of *R. erythropolis* X5 strain solubilize *n*-hexadecane in aqueous solutions to a greater extent than other biosurfactants of glycolipid nature, while they are inferior to synthetic surfactants.

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Authors' contributions

I.A. Nechaeva—formulating the research objectives and methodology, and writing the text of the article.

A.S. Parfenova—planning and conducting experiments, processing experimental data, and discussion of the results.

A.S. Filippova—conducting experiments, processing experimental data.

A.E. Filonov—scientific consulting, editing the article.

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