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Isolation and Characterization of Biosurfactant Producing Microorganisms from Soils

Izolacja i charakterystyka mikroorganizmów zdolnych do produkcji biosurfaktantów ze środowiska glebowego

Biosurfactant production is critical not only from the standpoint of obtaining innovative and relatively cheap surface-active agents but also due to impossibility or limited biodegradation of conventional surfactants obtained by means of chemical syntheses. The main goal of the project was to isolate and characterize (macroscopically and microscopically) microorganisms capable of biosurfactant production and to evaluate the potential of these strains for biosurfactant production from the soil environment contaminated with crude oil products using the methods of conventional microbiology. The focus of the study was on isolation of pure bacteria cultures from soil contaminated with crude oil products sampled from the areas of a petrol station, macroscopic and microscopic identification of the isolated strains and testing their capability of biosurfactant production. It was demonstrated that 2 of 21 strains isolated from soil which was most likely contaminated with crude oil products showed a potential for biosurfactant production. This potential was evaluated by means of the drop collapse, oil spreading and hemolytic assay tests. The study also discussed basic characteristics of biosurfactants and their application in various domains of science and industry.

Keywords: **biosurfactants, surface-active compounds of biological origin, microorganisms, drop collapse test, oil spreading test, strain hemolytic potential**

Introduction

Biosurfactants are defined as surface-active compounds produced by microorganisms or obtained using the enzymatic synthesis methods, capable of reducing surface tension and interfacial tension [1]. Surface-active compounds accumulate at interfaces between phases and, even for very low concentrations, modify surface properties of liquids they are dissolved in. Such substances are characterized by asymmetrical molecule structure, which usually contains nonpolar (hydrocarbons) and strongly polar part [2]. These compounds can be produced by such microorganisms as bacteria, fungi or moulds [3]. They represent substances which are secondary metabolites (intra- or extracellular) i.e. the compounds which are mostly

organic and not indispensable for microorganism life. However, they can perform an undefined role [4, 5].

Surfactants are chemical compounds with amphiphilic properties [6, 7]. They are capable of modification of other surface properties of liquids they are dissolved in [8]. Furthermore, they can reduce surface tension of the liquid, thereby allowing for wetting of solid surfaces by these liquids and mixing two liquids which naturally form two unmixable phases, such as water and oil [6].

Biosurfactants are produced as by-products of metabolisms, mainly of aerobic bacteria during their growth on water-soluble or hydrophobic substrates [9]. Studies have found [10, 11] that microorganisms capable of biosurfactant production are also capable of hemolysis. Due to industrially useful properties, *Pseudomonas* bacteria are popular producers of biosurfactants, mostly glycolipids. Furthermore, *Bacillus* genus bacteria, especially *Bacillus subtilis*, are described in the literature as efficient producers of biosurfactants. Among yeasts, the main species with such potential are *Candida bombicola* and *Candida lipolytica.* This group of microorganisms also includes such biosurfactant producers as *Sacharomyces cerevisiae* and *Kluyveromyces lactis* [12]. Microorganisms can produce both large and small molecular biosurfactants. Small molecular biosurfactants include glycolipids and lipopeptides, which are more effective in reducing surface and interfacial tension. Among large molecular biosurfactants are polysaccharides and lipopolysaccharides, which are effective in stabilization of emulsions such as oil with water. Molecular mass of biosurfactants ranges from 500 to 1500 Da, whereas their critical micelle concentration (CMC) ranges from 1 to 200 mg/l [13].

Biosurfactants show advantages over their synthetic equivalents since they are biodegradable and demonstrate stability at temperatures, pH values and salinity considered extreme for microorganisms. In addition to these properties, biosurfactants are characterized by low toxicity and a broad spectrum of action. With insignificant changes in the structure evoked by microorganism reaction to variable composition of the growth medium or chemical and enzymatic manipulations, functional characteristics of biosurfactants can be adjusted [3, 14]. Furthermore, biosurfactants do not accumulate in natural ecosystems and therefore are not hazardous to the environment [13]. Additionally, biosurfactants are characterized by capability of reducing surface and interfacial tensions, solubilization of hydrophobic compounds in water solutions, forming and destabilizing emulsions, forming foams and gels, extracting metals, wetting, cleaning, flocculation and inhibition or stimulation of microorganism growth [13]. This helps these compounds perform the roles of emulsifiers, demulsifiers, solvents, detergents, wetting agents or viscosity reducing agents.

Most researchers admit [1, 3, 8, 13, 15, 16] that the main function of biosurfactants is to allow microorganisms to grow on substrates which are immiscible in water, such as hydrocarbons and vegetable oils. Consequently, most microorganisms that produce surfactants are capable of decomposing hydrocarbons [17].

Surface-active agents of biological origin have been employed in various fields of science. They have been extensively used in medicine due to their unique antifungal, antibacterial, antiviral and antiadhesive effects. More and more studies

have pointed to biosurfactants as anticancer substances [1, 18]. Due to the capability of reducing surface tension on the boundaries of phases, biosurfactants can protect surfaces from adhesion of microbes [19]. They are also used in transport of drugs to the locations of their action as auxiliary emulsifying agents and as vaccine-enhancing agents [13].

Due to low toxicity and accelerated biodegradability, biosurfactants have been widely used in cosmetic industry. In the food sector, biosurfactants have been typically used as emulsifiers. Biosurfactants can be also applied in agriculture and many industrial sectors, including extraction and processing of petroleum and production of paints and varnishes. A substantial interest has been attracted to the use of these compounds in removal of heavy metals from soil or galvanic waste [11, 20].

Surfactants can be also used in environmental engineering and environmental protection for e.g. land bioremediation, with synthetic substances being more and more often replaced with their biological equivalents produced by certain bacteria or yeasts [21].

The aim of this study was to isolate and characterize (macroscopically and microscopically) microorganisms capable of biosurfactant production and to evaluate the potential of these strains for biosurfactant production from the soil environment contaminated with crude oil products using the methods of conventional microbiology.

1. Materials and methods

1.1. Taking samples for examinations and isolation of strains capable of biosurfactant production

The soil from the areas of the petrol station contaminated with crude oil products was used in the experiments. The samples were obtained in the spring season. Atmospheric conditions were 15°C on average during the warmest time of a day and no precipitation over the several (more than ten) days before sampling.

Several decimal dilutions of the soil sample were performed in sterile saline solutions in order to isolate bacteria which are potentially capable of biosurfactant production. Another stage of the isolation procedure was based on spread plate technique using Petri dishes with solid nutrient broth medium with glucose (BTL, Łódź). The dishes were incubated at temperature of 20 ±2°C for 96 hours. In order to obtain bacteria capable of sporulation, the test tubes with bacterial suspension were pasteurized in the dilution series before culture by placing the tubes in a water bath at temperature of 80°C for 20 minutes. After pasteurization, spread plate technique was used on the dishes with solid medium and incubation was conducted analogically as in the previous case. After completion of incubation time, the grown bacterial colonies were counted for each dish and characterized macroscopically: bacterial colonies with different morphologies were streaked on the broth medium with glucose and incubated for 72 hours at temperature 20 ±2°C. The strains that had grown were Gram-stained in order to determine their association with individual groups of Gram-positive and Gram-negative bacteria. Furthermore, macroscopic

morphology of colonies of the examined strains was also characterized. After incubation, all strains were stored for further examinations by freezing at temperature of –20°C in glycerol in the ratio 1:1.

All isolated strains were tested for capability of biosurfactant production. Strains were streaked on solid nutrient broth with glucose (BTL, Łódź). The dishes were incubated for 24 hours at room temperature. Next, in sterile conditions, biological material was transferred using the inoculation loop to Erlenmeyer flasks, where 50 ml of liquid growth medium was previously prepared (enriched broth, BTL, Łódź). The flasks were plugged with a cotton plug and covered with aluminium foil. The content was incubated in the laboratory shaker at room temperature and 150 rpm for 7 days. After the incubation period, growth medium with microorganisms was filtered through cellulose qualitative filter paper with medium filtering rate (BN-67/7327-04, POCh Gliwice).

1.2. Testing of isolated strains in terms of capability of biosurfactant production

1.2.1. Drop collapse method

After a week of incubation, the isolated strains grown in the liquid medium were tested for potential biosurfactant production using the drop collapse test [10, 11, 13, 22-25]. The method consists in observation of the behaviour of the drop shape of a liquid after growth compared to the oil medium, provided in this case by Diesel oil manufactured by Miles. The positive control was 10% solution of SDS (sodium dodecyl sulfate) (Sigma), whereas negative control was water. For 24 hours before performing the experiment, the Eppendorf flasks were filled to perform tests with Diesel oil of 40 µl and left at room temperature until completion of the test in order to stabilize the oil level. Performing the test consisted in applying drops of the liquid filtrate after growth with volume of 5 µl using the automatic pipette to ca. 0.5 cm over the oil surface (40 µl) in the 250 µl Eppendorf test tube and observation of the drop. If the drop fell and created an apparent emulsion on the bottom similar to SDS, the strain was considered potentially producing surface-active compounds. On the other hand, if the drop resembled a water drop i.e. it was floating on the surface of oil for the first 4 seconds and had a shape similar to a sphere and, after falling down, did not form an apparent emulsion on the bottom, these strains were considered as not producing biosurfactants. Observation of the shape and behaviour of the drop was performed for 1 minute. For each isolated strain, the above test was conducted with ten repetitions, with negative and positive controls.

1.2.2. Modified oil spreading method

A modified oil spreading test was performed to confirm the results obtained from the drop collapse test [11, 13, 22, 23, 26]. The experiment consisted in observation of the behaviour of a drop of the liquid after growth on a flat surface of the medium, which was provided by Diesel oil (Miles). A drop without capability of reducing surface tension remains convex, has a spherical shape and does not

increase its diameter following the application of the drop. A drop with capability of reducing surface tension becomes flat after application, with frayed shape and increased diameter. This allows for identification of the strains that would potentially produce biosurfactants. The experiment was performed on Petri dishes with 5 ml of Diesel oil. Next, 40 µl of filtrate after growth was applied from the height of 0.5 cm at the angle of 45° using the automatic pipette and its behaviour on the medium surface was observed. The positive control was 10% solution of SDS (Sigma), whereas the negative control was distilled water. For each isolated strain, the modified test was conducted with ten repetitions, with negative and positive controls.

1.2.3. Assessment of strain hemolytic potential

Assessment of strain hemolytic potential was made using the dish method on the Columbia solid growth medium with 5% sheep blood (BTL, Łódź). Strains were streaked on the growth medium, with dishes incubated for 24 hours at room temperature. Noticeably brighter zones around the grown bacterial colonies confirmed hemolytic potential of the strain [10, 13, 22].

2. Results

2.1. Evaluation of bacterial count in the soil sample

Large number of microorganisms in soil suggests dynamic soil processes, including removal of the hardly biodegradable compounds, to which bacteria are capable of adapting through modification of the enzymatic apparatus. This modification can lead to production of biosurfactants, which facilitate intake and metabolism of hydrocarbons in the cell. The study evaluated total bacterial count and spore-forming bacteria count (Table 1).

Table 1. **Mean count of microorganisms in 1 g of humid soil and dry soil for general number of bacteria and mean number of spore-forming microorganisms in 1 g of humid soil and dry soil**

Dilution	Mean count of microorganisms in 1 g of humid soil	Mean count of microorganisms in 1 g of dry soil (assuming sample humidity at the level of 30%)	Mean count of spore-forming microorganisms in 1 g of humid soil	Mean count of spore-forming microorganisms in 1 g of dry soil
10^{-2}	$1.3 \cdot 10^{5}$	$9.1 \cdot 10^{5}$	0.0	0.0
10^{-3}	$5.9 \cdot 10^{5}$	$5.6 \cdot 10^{5}$	0.0	0.0
10^{-4}	$1.2 \cdot 10^{6}$	$1.7 \cdot 10^{6}$	$2.0 \cdot 10^{5}$	$2.9 \cdot 10^{5}$
10^{-5}	$2.0 \cdot 10^{6}$	$2.8 \cdot 10^{6}$	$1.3 \cdot 10^{6}$	$1.9 \cdot 10^{6}$
10^{-6}	$1.3 \cdot 10^{6}$	$1.9 \cdot 10^{7}$	$1.3 \cdot 10^{7}$	$1.9 \cdot 10^{7}$
10^{-7}	$6.6 \cdot 10^{6}$	0.0	$2.3 \cdot 10^{8}$	$3.2 \cdot 10^{8}$
10^{-8}	$1.3 \cdot 10^{8}$	$1.9 \cdot 10^{9}$	0.0	0.0
Mean	$2.0 \cdot 10^{7}$	$2.7 \cdot 10^{5}$	$3.5 \cdot 10^{7}$	$4.9 \cdot 10^{7}$

Mean total bacterial count in 1g of humid soil was $2.7 \cdot 10^7$, whereas in the case of spore-forming bacteria, this count was $4.9 \cdot 10^7$.

2.2. Microscopic and macroscopic characterization of isolated strains

After 24 h of incubation on the solid growth medium (solid broth) growth of microorganism colonies was insufficient. Therefore, incubation was extended to 96 h. Each colony distinguished in the macroscopic assessment was streaked on the solid broth medium and the strains were stored in agar slants. Furthermore, the colonies of microorganisms were subjected to macroscopic assessment in terms of the colony shape and characterization of its edge, surface, colour and size. Growth in agar slants was also characterized after 96 h of incubation, whereas growth in the liquid culture was examined after 168 h. The isolated strains were evaluated microscopically and Gram stained. All the isolated strains belong to the group of Gram-positive bacteria. Bacilli shapes and spherical forms were found in the isolated bacteria. Results are presented in Table 2.

Table 2. **Characteristics of liquid culture of isolated strains (+/++ amount of the bacterial flocs on the bottom); results of Gram staining: G+ means Gram-positive bacteria, G - means Gram-negative bacteria)**

No.	Strain	Microscopic characterization			Macroscopic characterization				
		Gram staining	Bacteria shape	Notes	On the solid medium following 96 h of incubation	On the agar slant following 96 h of incubation	in the liquid culture following 168 h		
							Medium turbidity	Medium colour	Presence of flocs on the bottom
1.	AP_1	G+	Bacilli	Numerous, with noticeable endospores located centrally			YES	Yellow-orange	–
2.	AP_2	G+	Spherical shape	Cocci	Yellow, round, convex and glossy colony	Yellow colonies located along the streak	YES	Yellow-orange	+
3.	AP_3	G+	Spherical shape	–	Yellow, round, convex and opaque colony	Yellow colonies located along the streak	YES	Brown	+
4.	AP_4	G+	Spherical shape	–	White, milky, convex, round, glossy colony	White colonies growing in clusters	YES	Yellow-orange	+
5.	AP_5	G+	Spherical shape	Cocci	White, milky, round, large compared to others, with smooth border	Milky colonies growing in large clusters, very diffuse, glossy	YES	Yellow-orange	–
6.	AP_6	G+	Spherical shape	–	White, milky, glossy, flat, irregular colony with smooth border, sticky, humid	Milky colonies growing along the streak in clusters	YES	Brown	–
7.	AP_7	G+	Spherical shape	Large cluster	Orange, round, glossy colony, small compared to others	Glossy yellow colonies located along the streak	YES	Yellow-orange	+

8.	AP_8	G+	Spherical shape	Large cluster	White, round, sticky, large compared to others	White colonies growing in clusters	YES	Brown	–
9.	AP_9	G+	Spherical shape	Cocci	Yellow-purple, irregular, glossy, convex, wrinkled and small compared to others	Glossy yellow colonies growing in clusters	YES	Yellow-orange	–
10.	AP_10	G+	Spherical shape	Cocci	White, round, glossy, convex, with smooth edge, mean size compared to others	Substantially grown slant	NO	yellow, clear	–
11.	AP_11	G+	Bacilli	Numerous, with noticeable endospores located centrally	Intensive growth in the form of white strands on the entire plate surface	Growth on the entire slant surface in the form of white dry flocs	YES	Yellow-orange	++
12.	AP_12	G+	Spherical shape	Growth in the chain form	White, wrinkled, flat with smooth edge	Growth in the cluster on the bottom of the slant	YES	Yellow	–
13.	AP_13	G+	Bacilli	Growth in the chain form	White, round, with smooth edge, large compared to others, humid, sticky	Intensive growth on the entire surface of the slant	NO	Yellow, clear	–
14.	AP_14	G+	Bacilli	Growth in the chain form	Pink, round, flat with smooth edge, semitransparent border, small compared to others	Pinkish colonies, growing outside the streak in small clusters	YES	Yellow-orange	–
15.	AP_15	G+	Bacilli	Growth in the chain form	Pink, round, flat with smooth edge, small compared to others	Pink colonies growing along the streak in clusters	YES	Orange	–
16.	AP_16	G+	Bacilli	Numerous, with noticeable endospores	White, round, large compared to others, with smooth edge, relatively convex	Intensive growth of white colonies on the entire surface of the slant	YES	Orange	–
17.	AP_17	G+	Bacilli	Growth in the chain form	White, round, with smooth border, convex	Intensive growth on the entire surface of the slant	YES	Yellow-orange	–
18.	AP_18	G+	Spherical shape	–	Orange-yellow, semitransparent border, very glossy	Yellow colonies growing along the streak	YES	Yellow-orange	–
19.	AP_19	G+	Spherical shape	–	White, glossy, flat, with smooth edge, humid, with dark point in the middle of the colony	White, glossy colonies growing over the entire surface of the slant, very intensive growth	YES	Yellow-orange	–
20.	AP_20	G+	Spherical shape	–	White, round, with smooth edge, flat, humid, producing yellow stain	White colonies growing over the entire surface of the slant	YES	Yellow-orange	–
21.	AP_21	G+	Spherical shape	–	White, round, with frayed edge, flat	Intensive growth on the entire surface of the slant	YES	Yellow-orange	–

2.3. Biosurfactant production tests: drop collapse test, modified oil spreading test and hemolytic assay

The potential of the isolated strains for biosurfactant production were examined using three tests: drop collapse test, modified oil spreading test and hemolytic assay on the Columbia medium with 5% sheep blood. The results obtained were used to assess whether a strain is potentially capable of biosurfactant production. The positive control in oil spreading and drop collapse tests was 10% solution of SDS (Sigma), whereas the negative control was distilled water. The results obtained in the study are presented in Table 3.

Table 3. **Results of hemolytic assay, drop collapse and oil spreading tests; * [%] means a percentage of repetitions with positive results; 1-10 repetitions; strains capable of biosurfactant production confirmed in all three tests are marked in grey**

No.	Strain	Hemo-lytic assay	Drop collapse test											Oil spreading test									
			1	2	3	4	5	6	7	8	9	10	* [%]	1	2	3	4	5	6	7	8	9	10	* [%]
1.	AP_1	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
2.	AP_2	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
3.	AP_3	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
4.	AP_4	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
5.	AP_5	+	+	+	+	+	+	+	+	+	+	+	100	+	+	+	+	+	+	+	+	+	+	100
6.	AP_6	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
7.	AP_7	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
8.	AP_8	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
9.	AP_9	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
10.	AP_10	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
11.	AP_11	+	–	–	+	–	+	–	–	+	–	–	30	–	–	–	–	–	–	–	–	–	–	0
12.	AP_12	+	+	+	+	+	+	+	+	+	+	+	100	+	+	+	+	+	+	+	+	+	+	100
13.	AP_13	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
14.	AP_14	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
15.	AP_15	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
16.	AP_16	–	–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0
17.	AP_17	–	+	+	+	+	+	+	+	+	+	+	100	+	+	+	+	+	+	+	+	+	+	100
18.	AP_18	–	+	+	+	+	+	+	+	+	+	+	100	+	+	+	+	+	+	+	+	+	+	100
19.	AP_19	–	+	–	+	+	+	+	+	+	+	+	90	+	+	+	+	+	+	+	+	+	+	100
20.	AP_20	–	+	+	–	+	+	+	+	–	+	+	80	+	+	+	+	+	+	+	+	+	+	100
21.	AP_21	–	+	–	+	–	–	–	–	–	–	+	30	–	–	–	–	–	–	–	–	–	–	0
K+	SDS		+	+	+	+	+	+	+	+	+	+	100	+	+	+	+	+	+	+	+	+	+	100
K–	distilled water		–	–	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	–	–	0

3. Discussion

The experiment consisted in isolation and initial characterization of strains with potential for biosurfactant production. Screening of bacteria capable of biosurfactant

production was started from the choice of the environment where the soil was likely to be contaminated with crude oil products. In this sample, the likelihood of the presence of microorganisms capable of decomposition of hydrocarbons used as carbon sources is very high, resulting in the need for biosurfactant production that facilitates the use of these substrates in metabolism.

Mean total bacterial count in 1 g of humid soil was $2.7 \cdot 10^7$, whereas in the case of spore-forming bacteria, this count was $4.9 \cdot 10^7$ (Table 1). Compared to the literature data, with the bacteria count in the relatively fertile soils documented as ranging from $1 \cdot 10^9$ do $5 \cdot 10^9$ [27], this value is twice lower. Microorganism count in the soil sample may vary depending on many factors, including the season of the year, climate and amount of pollutants [28]. Microorganism count in the soil may reflect its condition and capability of self-purification as a result of activity of microorganisms such as bacteria or fungi. It was demonstrated in the literature [29] that diesel oil does not lead to substantial modifications in soil microorganism count. However, it has a significant effect on biological balance. The bacteria count in the non-pasteurized and pasteurized samples should be emphasized. Pasteurization was performed in order to verify whether spore-forming bacteria were present in the soil sample, especially aerobic Bacillus genus bacteria, which have been widely used in biotechnological production, including formation of secondary metabolites as substances demanded in the market e.g. enzymes. They are also abundant in nature, with broad ranges of pH and temperature and high growth rate [30]. The experiment demonstrated presence of bacteria capable of endospore formation: bacterial colonies grew on agar plates whereas in one sample that was pasteurized, endospores (strain AP_1) were observable in the microscopic examinations. Aerobic *Bacillus* genus bacteria are very interesting microorganisms due to their extensive use in enzyme syntheses, production of antibiotics and insecticides [5]. All the spore-forming bacteria are of cylindrical shape and are Gram-positive which was confirmed by the experiment: strains AP_1, AP_12 and AP_17 were stained in purple during Gram-staining, being the spore-forming strains, had an extended shape and sporulated forms noticeable in microscopic observation, which refracted light due to large amounts of condensed protein materials [31].

The isolated strains were tested for hemolytic potential on Columbia blood agar with 5% content of sheep blood. The test results are presented in Table 3. According to the literature [10, 11, 13], strains that produce biosurfactants also have hemolytic potential. The findings of the study are fully consistent with the literature data because part of strains tested in the experiment that yielded positive results in drop collapse and oil spreading tests did not show hemolytic potential. The results of the test that examined hemolytic potential was performed after 48 hours from application. This may mean that isolated strains are characterized by extended growth time since they needed slightly more time for growth both on agar plates with glucose and on the medium with sheep blood compared to manufacturer recommendations - in the case of broth medium this was 96 hours compared to expected time of 24÷48 h, whereas for the Columbia agar, this was 48 h with incubation time of 24 h. A growth and brighter zones around the grown colonies were observed on

the medium with sheep blood. This was used to identify the hemolysis type. Of 21 isolated strains, only 3 had hemolytic potential. In the case of hemolytic potential, noticeable light yellow and transparent zones were observed around the colonies due to complete lysis of blood cells. Furthermore, hemolytic potential does not occur when bacterial colonies are incapable of inducing hemolytic processes. This can be observed on the dishes as the lack of changes in the growth medium [10]. The test revealed that three strains had hemolytic potential: AP_5, AP_11 and AP_12 (see Table 3), which suggests that these strains are capable of biosurfactant production. Other strains, without hemolytic potential, were not identified as the strains capable of biosurfactant production. It should be emphasized that excessively long storage in laboratory settings may lead to losing primary properties, including hemolytic potential. The experiment yielded strains with positive results in drop collapse and oil spreading tests. However, they did not show hemolytic potential (AP_17, AP_18_, AP_19 and AP_20). These strains were not qualified as strains that produce biosurfactants.

A liquid culture on the enriched broth medium was grown in the experiment using previously isolated strains frozen in glycerol in order to obtain suspension after culture, with its behaviour observed during drop collapse and modified oil spreading tests. These methods were chosen as tests for biosurfactant production due to their broad description in the literature concerning the problems of microbiological production of biosurfactants [13, 24-26, 32]. Furthermore, the appearance of the liquid culture, which can be an important element in organism classification, was monitored after 7 days from starting the culture [33]. Growth on the liquid medium may suggest preferences concerning access to oxygen. In this case, the increase in the form of the flocs on the bottom may also show that certain microorganisms need anaerobic environment for growth. The study did not find either presence of the floating skin at the liquid level, which may point to the presence of microorganisms that need a high concentration of oxygen to grow (such a growth can be observed in Bacillus genus bacteria [33]) or diffusion growth, characteristic of anaerobic bacteria, manifested by growth media turbidity [34]. Observation of the liquid culture found that strains AP_2, AP_3, AP_4 and AP_7 and AP_9 can be categorized as relatively anaerobic microorganisms due to the increase in the form of flocs accumulated on the bottom. Other strains did not reveal a characteristic flocs or skin. However, it should be mentioned that reduction in the surface tension in the culture caused by presence of surfactants can lead to failure to form the skin and cause a change in the type of growth into the diffusion growth [33]. Therefore, such changes are possible in the liquid medium in the case of strains that produce biosurfactants.

It was found that strains which grew in the liquid culture without forming the flocs but showing the diffusion growth during the assessment by means of drop collapse test, oil spreading tests and hemolytic assay were classified as strains potentially producing biosurfactants (AP_5 and AP_12). Duration of the incubation of the liquid culture was 7 days since, according to the literature [4], biosurfactants as secondary metabolites are produced in the stationary growth phase. The results

of biosurfactant production tests are not fully consistent. The drop collapse test is very sensitive to the method it is performed, especially to applying a small volume of suspension after culture on the oil surface. Both positive and negative controls were falling down the medium. The only differences were the shape of the drop and duration of falling of individual volumes applied [10]. Therefore, it is hard to state whether the drop can be considered as potentially containing surface-active compounds, leading to the discrepancies in the case of several strains: part of repetitions pointed to the production of surface-active compounds, whereas other repetitions did not confirm this phenomenon. Such a situation was observed for the strains AP_11, AP_19, AP_20 and AP_21. Performing the test was also difficult due to the consistency of the suspension after culture. Many of the isolated strains were characterized by very sticky and thick suspensions after culture which, due to small volumes of application, represented a substantial difficulty: the suspension stuck to the disposable tip of the automatic pipette instead of being dropped to the oil. Therefore, performing the test required substantial precision, attention and competencies to evaluate the obtained results. The modified oil spreading method turned out to be helpful: it was easier to perform the test and analyse the obtained results, which were unequivocal. Part of results from the drop collapse test was confirmed (AP_5, AP_12, AP_17, AP_18, AP_19 and AP_20) and therefore it can be considered that these strains are actually capable of biosurfactant production. However, hemolytic assay confirmed only some of the results obtained: strain AP_5 and AP_12. This led to the conclusion that only these two of 21 isolated strains are capable of production of surface-active compounds.

With regards to the strains capable of biosurfactant production, it is possible to obtain such strains from the contaminated soil. This points to the opportunities for biosurfactant production by soil bacteria. This potential may result from the deficiency of carbon sources other than hydrophobic, which leads to the necessity of adaptation of bacteria to conditions in the area of soil environment, polluted with crude oil products. It was found after completion of the experiment that the modified oil spreading test is the fastest and relatively simple to evaluate ability of biosurfactant production since it is characterized by the easiness of elimination of procedural mistakes, whereas the results obtained using this method provide the answer to the question whether a strain is capable of biosurfactant production. The experiment can be continued by taking into consideration the methods of surfactant isolation such as membrane filtration or precipitation with solvents in order achieve more accurate results of pure products and determine their type and amount, using for example spectroscopic or chromatographic methods. The results of this project should be treated as initial analyses.

Conclusions

The following conclusions were drawn based on the experiment:

- The microscopic observation of the strains isolated from soil environment from pasteurized and non-pasteurized samples allowed for numbering them among

Gram-positive bacteria. This suggests that this structure of the cell wall is demanded under conditions of growth in soil.

- Performing the hemolytic assay revealed presence of potentially pathogenic bacteria with hemolysin enzyme. Furthermore, the test confirmed the ability of biosurfactant production only for two strains (AP_5, AP_12), which yielded positive results, also during the drop collapse and modified oil spreading tests. However, hemolytic potential is not synonymous with capability of biosurfactant production.
- The tests for biosurfactant production demonstrated that 2 of 21 isolated strains were potentially capable of biosurfactant production. The isolated strains are likely to have a potential of their use in production and in petroleum industries and during bioremediation of soils and agriculture. However, further research is needed to confirm such opportunities.

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Streszczenie

Produkcja biosurfaktantów jest istotna z punktu widzenia nie tylko wytwarzania nowatorskich i względnie tanich środków powierzchniowo czynnych, ale także ze względu na brak możliwości lub bardzo ograniczoną biodegradację tradycyjnych surfaktantów wytwarzanych metodami syntez chemicznych. Głównym celem projektu było wyizolowanie oraz charakterystyka mikro- i makroskopowa mikroorganizmów zdolnych do produkcji biosurfaktantów wraz z oceną zdolności tych szczepów do produkcji biosurfaktantów ze środowiska glebowego zanieczyszczonego substancjami ropopochodnymi z użyciem metod klasycznej mikrobiologii. Zakres badań obejmował izolację czystych kultur bakteryjnych z gleby, zanieczyszczonej substancjami ropopochodnymi, pochodzącej z okolic stacji benzynowej, makro- i mikroskopową identyfikację wyizolowanych szczepów oraz testowanie ich zdolności do produkcji biosurfaktantów. Wykazano, że dwa spośród 21 wyizolowanych szczepów z gleby prawdopodobnie zanieczyszczonej związkami ropopochodnymi ma potencjał w produkcji biosurfaktantów. Zdolność tę oceniano z zastosowaniem testów „drop-collapse", „oil-spreading" oraz zdolności szczepu do hemolizy. W pracy opisano również podstawowe cechy biosurfaktantów oraz ich zastosowanie w różnych dziedzinach nauki i przemysłu.

Słowa kluczowe: **biosurfaktanty, związki powierzchniowo czynne pochodzenia biologicznego, mikroorganizmy, drop-collapse, oil-spreading, zdolność szczepu do hemolizy**