III. MICROORGANISMS AND ENVIRONMENT

INFLUENCE OF FOOD INDUSTRY WASTES AS SUBSTRATES ON THE YIELD OF BIOSURFACTANTS OF THE STRAIN *PSEUDOMONAS SP.* PS-17

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Abstract.The effectiveness of the application of economically-sound substrates (glycerol, used frying oil and phosphatide concentrate) for the synthesis of rhamnolipid surfactants by the strain *Pseudomonas* sp. PS-17 was established. It was also shown that the use of combined (mixed) substrates contributed to the increased concentrations of rhamnolipids if compared to the media with monosubstrates. When using a mixture of glycerol and used frying oil or phosphatide concentrate as carbon sources the concentration of rhamnolipids can be increased to 16 g/l. The possibility of application of the obtained biosurfactants in agriculture as plant growth regulators was established. The developed approaches to the synthesis of microbial surfactants will help to balance the overall cost of biosurfactant production.

Keywords: rhamnolipid biosurfactants, *Pseudomonas*, phosphatide concentrate, food industry waste, mixed substrates.

INTRODUCTION

The study of the synthesis of biosurfactants becomes increasingly important. Compared with chemical surfactants, these compounds have a number of advantages, such as low toxicity, biodegradability and effectiveness at the extreme values of temperature and pH. The unique properties of biosurfactants (emulsification of fats and carbohydrates, reduction of the surface tension of solutions, regulation of wetting, effect on capillary processes, increase in the permeability of cell membranes) [1,2] determine the prospects for their use in agriculture, medicine, environmental protection, enhanced oil recovery etc. Biosurfactants can be used as stand-alone products, as well as for the creation of complex agents.

Despite the potential benefits and applicability of biogenic surfactants, they are still not widely available due to a number of technological and economic factors, including self-cost. The topical task in this direction may be the search for cheap raw materials, since it is known that the cost of raw material constitute 10-30 % of cost of a finished product [3]. Thus, the use of cost-effective substrates can represent effective strategy to reduce the cost of the final product.

The source of carbon is an important factor in the production of biosurfactants and has a great impact on their cost. That is why much attention is paid to the use of renewable resources, including by-products of agriculture, food industry and biodiesel production as carbon sources in the process of biosurfactant production [4]

The utilization of the plant oil wastes – phosphatide concentrates is a considerable problem taking into account the scope of the industry. An important problem of the food industry and public food service is the utilization of used frying oil, which is accumulated at production facilities, food service facilities etc. The increase in capacity of world production of biodiesel generates great amounts of glycerol as by-product of this process. Use of waste substrates in the production processes is likely to increase its influence on the field of microorganism-based production, since they are usually cheaper, maximize the utilization efficiency regarding the overall production process and makes the process more environmentally friendly. Therefore, these substrates (sunflower waste, fried oil and glycerol) were selected for the present study.

Earlier it was shown that the strain *Pseudomonas* sp. PS-17 is active and technologically promising producer of surfactants (rhamnolipids, rhamnolipid biocomplex) [5], the possible application areas of the obtained compounds were established [6-8]. In our view, the target products obtained by culturing the producer on various substrates and grown under different conditions, may have specific properties and, consequently, the appropriate application [9, 10].

In this work the optimization of the biosurfactant production process by the strain *Pseudomonas* sp. PS-17 on the inexpensive substrates based on low-cost raw materials was studied.

The aim of the present study is the selection of cost-effective substrates for biosurfactant synthesis by the strain *Pseudomonas* sp. PS-17, as well as optimization of the production process using the fractional substrate addition and production of various forms of target products for purposeful applications in various fields.

MATERIALS AND METHODS

Bacterial strain and the method of its cultivation. The object of the research was the producer of surfactants - strain *Pseudomonas* sp. PS-17 from the collection of microorganisms of the Department of Physical Chemistry of Fossil Fuels, L.M.Lytvynenko Institute of Physical-Organic and Coal Chemistry NAS of Ukraine, the registration number in the Depository of Zabolotny Institute of Microbiology and Virology *Pseudomonas* sp. IMB B-7434). The cultivation of microorganisms was performed in Erlenmeyer flasks (750 ml) with a working volume 150 ml on a rotary shaker (220 rpm) at 30 °C in liquid nutrient medium with the following composition (g/l): $NaNO_3$ - 4,0; $K_2HPO_4 \times 3H_2O$ - 2,0; KH_2PO_4 - 1,2; $MgSO_4 \times 7H_2O$ - 0,5; sodium citrate - 5,0. Waste products of agricultural industry, namely glycerol, sunflower waste, fried oil in concentration 30-60 g/l were used as carbon sources. Cultivation time – 5 days.

Isolation and purification of products. Surface-active rhamnolipid biocomplex (RBC) was isolated from the culture liquid supernatant (CLS) via precipitation with 10% HCl solution at pH 3 and heating to 85 °C. The precipitate was kept at 4 °C overnight, separated by centrifugation (8000 rpm, 20 min) and dried under vacuum to constant weight.

Rhamnolipids were isolated by extraction from the CLS with Folch mixture (chloroform/methanol 2:1), followed by the evaporation of the extract under vacuum. The concentration of rhamnolipids in the culture liquid was determined by orcinol method using spectrophotometer Shimadzu UVmini-1240 (Shimadzu Corp., Japan) [11].

Qualitative analysis of lipids was performed by thin layer chromatography (TLC) using plates DC-Alufolien Kieselgel 60 (Merck, Germany), mobile phase chloroform-methanol-water 65:25:4. Visualization of the chromatograms was performed with 5% alcoholic solution of phosphorus-molybdic acid (total lipids) and orcinol reagent (rhamnolipids).

Isolation of lipids from the rhamnolipid biocomplex. The isolation of lipids from biocomplex was performed via double extraction of 1 g of dry complex with the mixture chloroform-methanol in different ratios in the amount of 20 cm^3 with the consequent extract evaporation under vacuum.

Determination of surface activity. Surface tension and critical micellar dilution (CMD) of culture liquid was determined by Du Noüy method with a platinum ring on Krüss K6 tensiometer [12] with a Kruss K6 digital tensiometer (“Kruss” Gmbh, Germany) by the ring method.

Determination of the influence of biosurfactants on the germination of sunflower seeds. Pre-sowing treatment of seeds was carried using generally accepted method [13]: calibrated seeds were soaked for 1 h in CLS solutions with dilution 1:200. Germination and seed germ inability were determined according to Ukrainian State Standard DSTU 4138-2002.

RESULTS

From previous studies [14] it is known that the strain *Pseudomonas* sp. PS-17 synthesizes extracellular surfactants, namely homologous rhamnolipids and polysaccharides that form rhamnolipid biocomplex with surface-active and emulsifying properties. The ability of the strain to synthesize surfactants on nutrient media with cost-effective substrates for reduction of the cost of end products was investigated; the cultivation conditions for higher yield of surfactants were chosen.

The first stage included the determination of the influence of natural carbon sources – glycerol, used frying oil and phosphatide concentrate – on the growth of *Pseudomonas* sp. PS-17, and the biomass accumulation. The results are shown in Fig. 1.

As it seen from the above data, the optimal concentration of glycerol for biomass accumulation was 50 g/l, but for higher substrate concentrations (60 g/l) the biomass accumulation values were similar to the options using glycerol 30-40 g/l. Similar regularities were observed with use of frying oil as a substrate - 50 g/l was optimal concentration. In options using phosphatide concentrate the amount of formed biomass was much higher than in the options with other substrates. In our opinion, this is due to incomplete assimilation of sunflower oil waste. Dosed addition of all substrates didn’t affect the biomass accumulation compared to options with a single addition.

The influence of natural substrates on the synthesis of surfactant products by the strain *Pseudomonas* sp. PS-17 was also studied (Fig. 2-3)

It was shown that the highest yield of rhamnolipid biosurfactants was observed when using glycerol in concentration 50 g/l. Higher concentration apparently caused inhibition of bacterial growth, resulting in significantly lower concentrations of biosurfactants. These results correlate with the data on biomass accumulation. The concentration of surfactants in options using phosphatide concentrate appeared to be inflated due to the presence of undigested substrate residues in the end product. When fried sunflower oil was used the final concentration of surfactants was on average 40% lower than with glycerol. It is known that composition of biosurfactants can be adjusted for specific applications by changing the substrate [15].

The research of production stages towards increasing the product yield, reducing harmful effects on the environment and efficient use of agricultural wastes adequately reflects modern problems of industry, including biotechnology. Therefore, the next stage consisted in the investigation the influence of dosed addition of substrates. Since secondary metabolites are mainly synthesized in the stationary phase, a carbon source was added on the 72 hour of the process. This enabled the increase in the amount of synthesized biosurfactants – when using fried oil it was higher on 18-22%. Dosed addition of glycerol did not contribute to increasing the concentration of surfactant products.

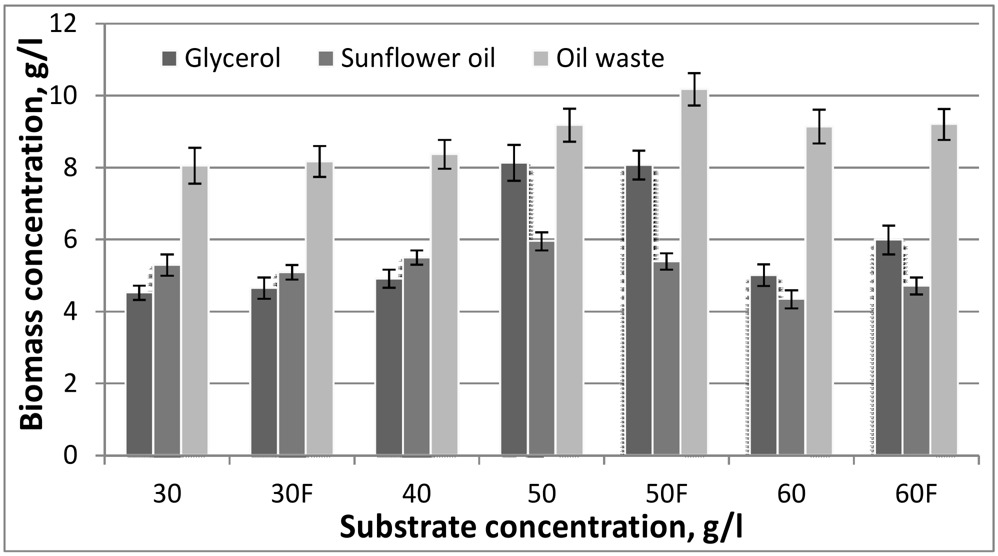

Fig. 1. Biomass accumulation by the strain *Pseudomonas* sp. PS-17 when cultivated on glycerol, sunflower oil and sunflower oil waste

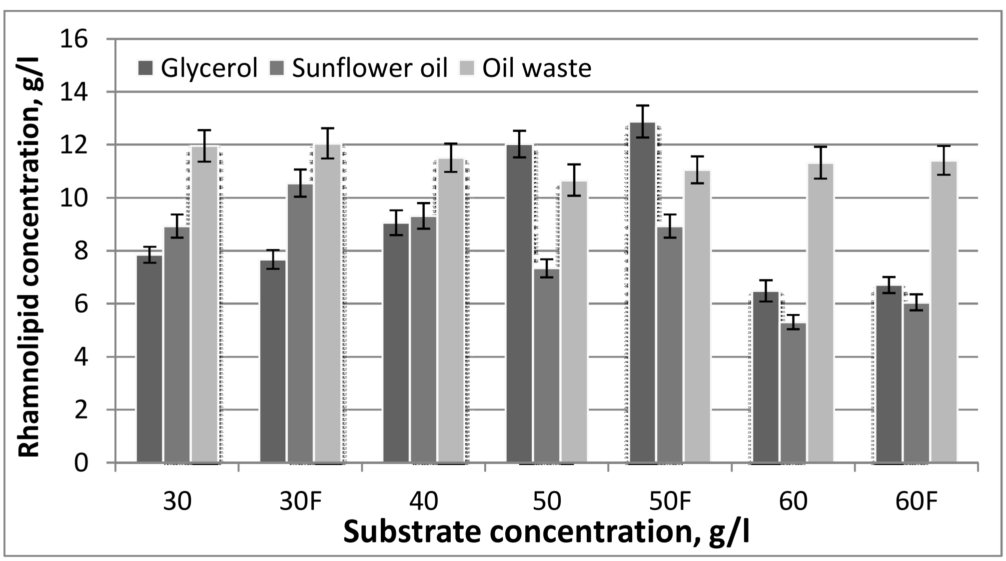

Fig. 2. Synthesis of rhamnolipids by the strain *Pseudomonas* sp. PS-17 when cultivated on glycerol, sunflower oil and sunflower oil waste

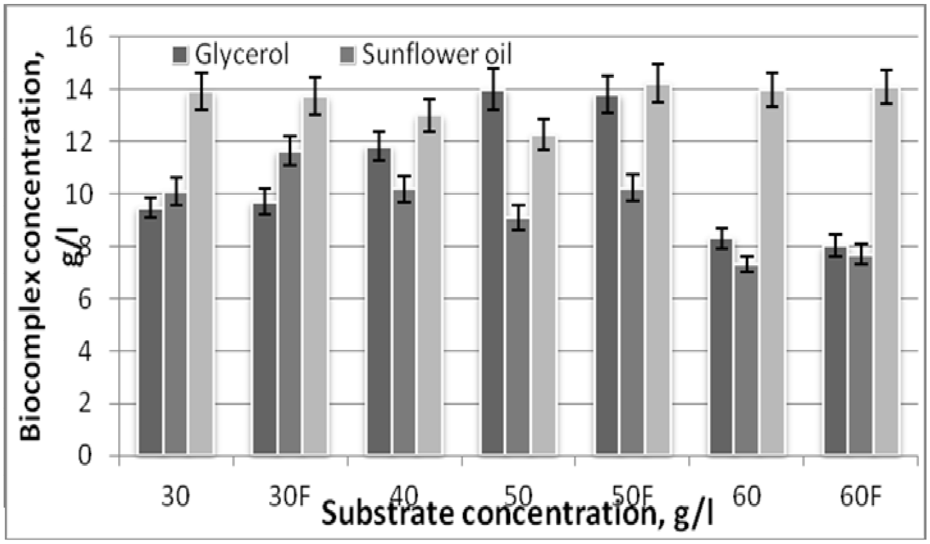

Fig. 3. Synthesis of rhamnolipid biocomplex by the strain *Pseudomonas* sp. PS-17 when cultivated on glycerol, sunflower oil and sunflower oil waste

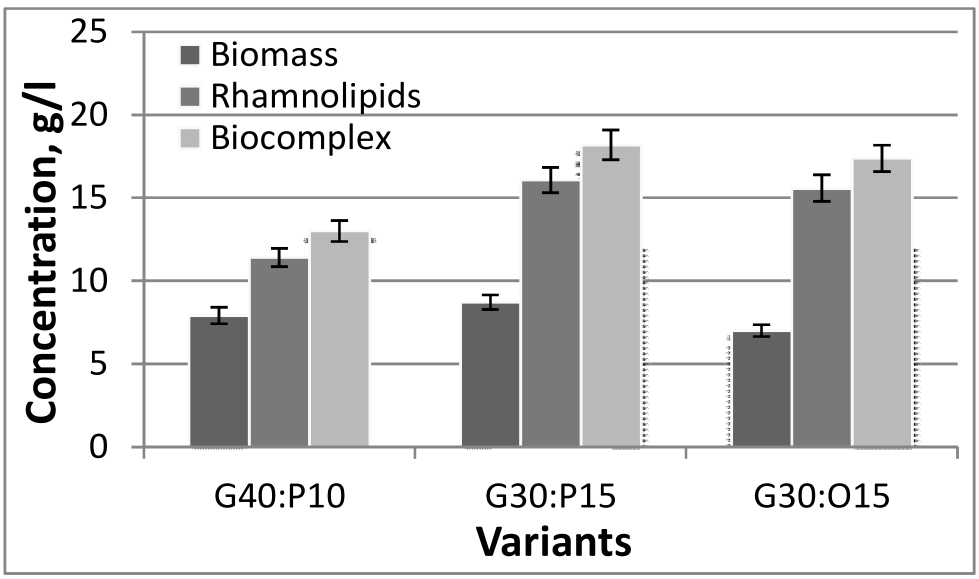

Fig. 4. Characteristics of the culture liquid supernatant of *Pseudomonas* sp. PS-17 cultivated on medium with combined substrates

With the aim of further increase of the yield of the final product the feasibility of the addition of hydrophobic carbon source in the form of composite substrates was investigated. The optimal concentrations of substrates that have the most significant effect on the synthesis of biogenic surfactants are shown on Fig. 4.

It was established that the cultivation of the strain-producer on the medium with combined substrates contributed to improvement of the yield of the products. The optimum substrate concentration was 30 g/l of glycerol at the beginning of cultivation with the following addition of sunflower oil waste in concentration 15 g/L after 72 hours of cultivation. Reduction or increase in the concentration of carbon substrates resulted in the decreased synthesis of end products, as well as incomplete assimilation of water-insoluble substrates.

The qualitative analysis of the surfactants was conducted. Thin layer chromatograms of the lipid extract obtained from CLS is shown in Fig.5.

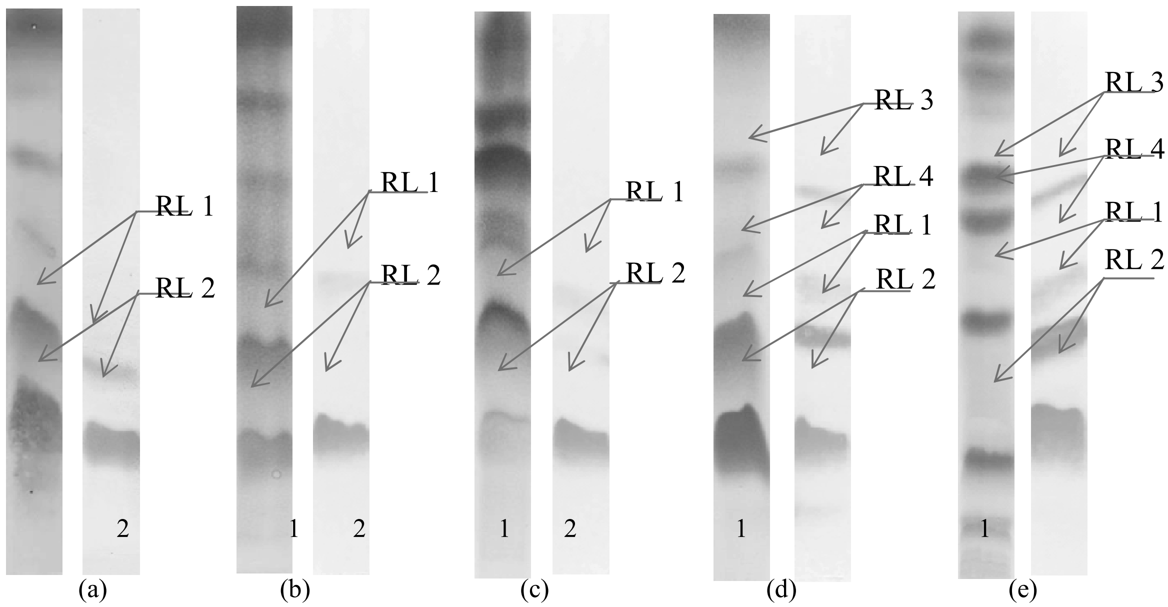

Fig. 5. TLC chromatograms of lipid extracts from CLS of the strain Pseudomonas sp. PS-17 cultivated on different carbon sources. Options: a) glycerol, b) oil, c) sunflower oil waste d) glycerol / oil, e) glycerol / sunflower oil waste; visualization with 5% alcoholic solution of phosphorus-molybdic acid (1) and orcinol reagent (2).

As it is seen from the chromatogram, the greatest content of lipid fractions (RL1, RL2) was obtained when the strain was cultured on the media with glycerol and mixed substrates glycerol/sunflower oil waste and glycerol/oil (2:1). When using mixed substrates and their dosed addition a higher yield of RL1 and RL2 was obtained, if compared with mono-substrates.

It was also determined that when culturing the strain *Pseudomonas* sp. PS-17 on media with a mixture of glycerol with phosphatide concentrate or with fried oil four homologous rhamnolipids were obtained as opposed to the cultivation on the medium with glycerol (Fig. 5) Such influence of fatty acids in substrates on the distribution of rhamnolipid homologues was confirmed by the literature data [16]. In the article [17] it was shown that the use of fatty acids as cosubstrates influenced the ratio between the rhamnolipid homologues.

Since the most technologically promising biosynthesis product of the strain *Pseudomonas* sp. PS-17 is rhamnolipid biocomplex, it is important to investigate the effect of substrates on its composition. It was determined that high concentrations of fried sunflower oil and phosphatide concentrate resulted in higher total content of the product due to the remains of undigested substrate in the extract. In options with the combined carbon sources with dosed addition, the amount of biocomplex was lower, but its composition corresponded to the RBC composition after the cultivation on glycerol.

Table 3. Characteristics of rhamnolipid biocomplex of *Pseudomonas* sp. PS-17 when cultivating on combined substrates

Variants	Biocomplex concentration in CLS, g/l	Content of lipids in the complex, %
Glycerol 50 g/l	10,01	81,02
Glycerol 30 g/l + Sunflower oil waste 15 g/l	13,19	79,45
Glycerol 30 g/l + Fried oil 15 g/l	12,37	78,66

It is shown that the use of mixed substrates can provide the necessary lipid composition of rhamnolipid biocomplex. Thus, the results showed the possibility of the application and prospects of various cheap substrates for the production of biosurfactants.

In recent years biosurfactants attracted attention as a potential alternative to synthetic surfactants, including the application in the agricultural sector. In particular, in crop production biosurfactants can play an important role in increasing the permeability of cell membranes of plants thus improving the bioavailability of nutrients and water [18].

Considering the topicality of the development of effective environmentally friendly substances for plants, the influence of culture liquid supernatants of *Pseudomonas* sp. PS-17 (CLS), produced on the media with different substrates, on the growth rates of sunflower was studied. Previously it has been shown that CLS of *Pseudomonas* sp. PS-17, obtained via cultivation with glycerol stimulates the development of seedlings and increases the germination energy [19]. This is caused by the ability of biosurfactants to change the properties of the root surface and contribute to their colonization by bacteria [20]. It is also known that biosurfactants better soil wettability and maintain the desired distribution of nutrients in it thus helping the improvement of the plant growth [21] (Table 4).

Table 4. Morphometric indices of sunflower after the pre-sowing seed treatment with culture liquid supernatants, produced on different substrates

Options	Shoot		Root		Germinability, %
	Length, cm	Mass, g	Length, cm	Mass, g	
Control	3,67	0,23	7,23	0,52	89
CLS	5,73	0,34	10,86	0,84	95
CLS sunflower waste + glycerol	5,90	0,38	9,78	0,83	96
CLS sunflower oil + glycerol	6,00	0,38	9,48	0,77	95

It was found that pre-sowing treatment of sunflower seeds with CLS, obtained from the cultivation in medium with glycerol, contributed to the increase of shoot and root growth from 47-56% and 50-61% respectively if compared with the control. A similar effect was observed when using mixed substrates, such as CLS (sunflower waste / glycerol): root and shoot growth increased on 60-65% and 35-59% respectively, and CLS (sunflower oil / glycerol) – on 63-65% and 31-48% respectively. Thus, the use of CLS obtained via cultivation on mixed substrates has shown similar results on the germ inability with results for CLS produced with glycerol.

CONCLUSION

Cost-effective substrates were selected, namely rapeseed oil waste, fried oil and glycerol, and their efficiency for the biosynthesis of rhamnolipid biosurfactants by the strain *Pseudomonas* sp. PS-17 was shown.

The feasibility of the application of the combined carbon sources for the production of rhamnolipid surfactants was determined. To enhance the final product yield the optimal conditions were selected: addition of oil waste or fried oil in concentration 15 g/l on 72 h of cultivation of the strain on glycerol as the main carbon source (30 g/l).

The conducted studies have shown the prospects of the use of waste from food and biodiesel as feedstock for the commercial production of biogenic surfactants and thus reduce production costs. The application of the produced biogenic surfactants in agriculture as an alternative environmentally friendly plant growth regulators was also established.

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ВЛИЯНИЕ НА ОТПАДЪЦИ ОТ ХРАНИТЕЛНАТА ПРОМИШЛЕНОСТ ВЪРХУ ДОБИВА НА БИОСЪРФАКТАНТИ ОТ ЩАМ *PSEUDOMONAS SP.* PS -17

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Резюме: Изследвана е ефективността от приложението на икономически изгодни субстрати – глицерол, използвано за пържене олио, фосфатиден концентрат, за синтез на рамнолипидни сърфактанти от щам *Pseudomonas sp*. PS -17. Установено е, че използването на среда със смесени субстрати допринася за повишаването на количеството на рамнолипиди в сравнение със среда с моносубстрати. При използване на смес от глицерол и олио, употребявано за пържене, или фосфатиден концентрат като въглеродни източници, концентрацията на рамнолипидите може да нарасне до 16 г/л.Изследвана беше и възможността от приложението на получените биосърфактанти в земеделието като регулатори на растежа.

Разработените подходи за синтез на микробни сърфактанти ще допринесат за балансиране на общите разходи за продукция на биосърфактанти.

Ключови думи: рамнолипид биосърфактант, *Pseudomonas*, фосфатиден концентрат, отпадъци от хранителната индустрия, смесени субстрати.

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