UDK 582.282.23:[604.2:547.458 *https://doi.org/10.31861/biosystems2023.01.013*

ACCUMULATION OF EXOPOLYSACCHARIDES BY YEASTS OF *RHODOTORULA* SP.

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The work is devoted to the search and assessment of the possibility of using alternative carbon sources for the production of exopolysaccharides by yeasts of the genus Rhodotorula sp. Exopolysaccharides (EPS) are high-molecular polymer metabolites of microorganisms produced on the outside of cells. They have a high ability to gel, emulsify, and suspend. The ability to synthesize EPS has been found in many microorganisms, but their level varies widely both for different EPS producers and for one producer under different cultivation conditions. Therefore, the search for active producer strains, alternative nutrient media, and the development of effective microbial exopolysaccharide technologies is an urgent task of biotechnology.

The capabilities of three species of the genus Rhodotorula were evaluated: R.rubra, R.minuta, R.glutinis to secrete and accumulate exopolysaccharides (EPS) on classical Sabouraud's medium with glucose was evaluated. The maximum amount of EPS in the culture fluid of R. minuta was determined.

Differential diagnostic Hiss media with maltose, lactose and mannitol were used to determine the use of different carbon-containing substrates by yeast cultures. The ability of all three studied yeast species to use maltose and mannitol as a carbon source was established. Accordingly, these substrates were added to the Sabouraud medium in the amount of 20 g/l, 40 g/l or 60 g/l. It was noted that the maximum amount of EPS in the culture liquid of R. rubra and R. minuta accumulates under the conditions of using 60 g/l of mannitol as a carbon source. For R.glutinis, the highest EPS indicator was established on a medium with 60 g/l of glucose.

Keywords: Rhodotorula, exopolysaccharides (EPS), Sabouraud medium, Hiss medium with maltose, lactose and mannitol.

Introduction. In recent decades, biogenic exopolysaccharides (EPS) of microbial origin have been intensively studied as potential agents in food technology and pharmaceutical development. Although high cost currently limits the production of EPS, their properties, in particular, low toxicity, biodegradability, environmental safety, and the possibility of synthesis from cheap substrates create the prospect of their use in various industries and agriculture (Hamidi et al., 2020; Donot et al., 2012). Changes in the rheological characteristics of water systems have led to the use of these biopolymers in the oil and mining, textile, food, pharmaceutical, chemical, agricultural, and medical industries (Hamidi et al., 2019). Microbial EPS have a number of advantages over plant-based polysaccharides. They can be produced in the required volumes regardless of the season and climatic conditions. The economic feasibility of using microbial EPS is due to their extracellular nature and high synthesis productivity on cheap substrates. Unlike chemical polymers (polyacrylamide), microbial EPS are resistant to temperature, oxidative, and mechanical degradation, but are subject to biological degradation and are non-toxic, which makes their use environmentally safe (Mahapatra and Banerjee, 2013).

In Ukraine, the industrial production of EPS is not sufficiently developed and cannot meet the needs of target consumers. Today, the principle of "reuse" - the repeated use of resources for the purpose of greening production - is attracting the attention of society. This concept can also be applied to biotechnological industrial production, when various wastes and by-products are reused as a nutrient medium to obtain valuable metabolites.

The ability to synthesize EPS has been found in many microorganisms, but the level of synthesis of these polymers varies widely both for different EPS producers and for one producer under different cultivation conditions (Okoro et al., 2021; Osemwegie et al., 2020; Leroy and Vuyst, 2016). The development of highly efficient technologies for the production of metabolites important for practical use is based on the targeted regulation of the biosynthesis process.

The production of microbial EPS, as well as most secondary metabolites, is determined by the composition of the culture medium (the nature of the carbon and nitrogen source, their concentration and ratio) (Schmid et al., 2016; Pavlova et al., 2004). The method of substrate supply and the physicochemical conditions of cultivation, such as temperature, pH, and aeration level, are important. The duration of the

cultivation process, its frequency, and the density of the medium also have a direct impact on the final result of cultivation. Changing the conditions of cultivation of the product leads to changes in the chemical composition of the metabolite spectrum. In the case of exopolysaccharides, this is manifested in changes in the molecular weight and ratio of several polysaccharides, which necessarily affects the rheological properties of the resulting EPS (Silambarasan et al., 2019; Sugumaran et al., 2017; Torres et al., 2012).

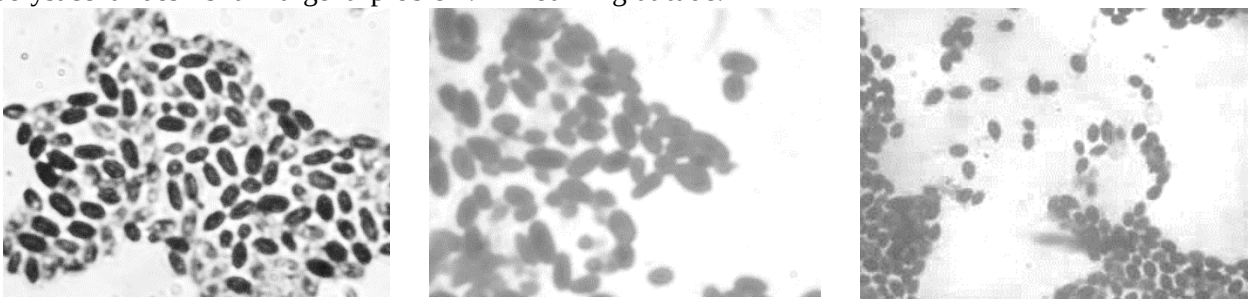
The search for the most productive EPS biosynthetics is carried out among microorganisms with low growth efficiency, and optimization of the technology for the production of microbial EPS should be associated with the correct choice of substrate (or mixture of substrates) and cultivation conditions. Most microbial EPS synthetics use carbohydrates as a source of carbon and energy. In the industrial production of EPS, products derived from sugar beet: molasses, sugar syrup, sucrose, or from corn: starch, hydrolyzed starch, glucose syrup, glucose are commonly used as substrates (Wang et al., 2021; Smelcerovic et al., 2008).

In this regard, the search for active producer strains, alternative culture media, and the development of effective technologies for microbial exopolysaccharides is an urgent problem. In our opinion, the yeast of the genus *Rhodotorula* deserves attention, as it synthesizes polysaccharides on typical substrates and quickly adapts to new cultivation conditions.

Rhodotorula species are widespread saprophytic yeasts that can be isolated from many environmental sources. *Rhodotorula* belongs to the genus of imperfect yeasts in the Sporidiobolaceae family, which are anamorphic yeasts. The vegetative form of the organisms usually has a single spheroidal, oval or elongated cell in malt extracts. Colonies are often reddish, orange, or yellow as a result of carotenoid synthesis. The genus *Rhodotorula* includes eight species, of which *R. rubra, R. glutinis* and *R. minuta* are the most common (Silambarasan et al., 2019; Bhama et al., 2014; Thakur et al., 2007).

The aim of the work was to evaluate the peculiarities of EPS secretion and accumulation by *Rhodotorula* yeast cultures under the conditions of using different carbon-containing substrates.

Materials and methods. Cultures of three species of the Rhodotorula genus served as research material: *R. rubra, R. minuta, R. glutinis* (Fig. 1.). The museum cultures of microorganisms were kindly provided by the staff of the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, for which we express our gratitude.



R. rubra ***R. minuta*** ***R. glutinis***

Fig. 1. Photomicrographs of Rhodotorula species (Bhama et al., 2014; Kot A. et al., 2016; Thakur et al., 2007)

The growth of the yeast inoculum lasted for 48 h at a temperature of 28 °C and intense stirring at 160 rpm on Sabouraud medium containing: 40 g/l of glucose and 10 g/l of peptone. The main fermentation was carried out under similar conditions for 120 hours on Sabouraud medium.

Upon completion of the cultivation, the amount of exopolysaccharides (EPS) in the culture liquid was determined. To do this, the yeast biomass was separated from the culture liquid by centrifugation for 20 min at 3000 rpm on a CM-3M centrifuge. The amount of exopolysaccharides was determined in the fugate by ethanol precipitation. For this purpose, 96% ethanol (in a ratio of 1:2) was added to the fugate. The resulting precipitate was then washed with ethanol after centrifugation (10 min at 6000 rpm) and centrifuged again and dried until dry weight was reached, and the value was recorded gravimetrically (Ziadi et al., 2018).

To determine the ability of yeast to metabolize different carbon sources, Hiss medium with maltose, lactose and mannitol was used. For this aim, variants of the Hiss medium were prepared according to the prescription. Sterile medium was poured into sterile test tubes, the column of liquid was 6-7 cm, (10 ml of medium). In the thickness of the medium, culture was sown by injection. The color change of the medium was observed after 3 days of cultivation. The appearance of a pink-purple color indicates the use of this carbohydrate as a carbon source by the yeast culture. Thus, the optimal carbon source for the studied yeast species was determined.

After determining the carbon source, Sabouraud's medium was prepared, where glucose was replaced by either maltose or mannitol. The amount of carbon-containing substrate was 20 g/l, 40 g/l and 60 g/l.

Yeast cultivation was carried out in 250 ml conical flasks. The yeast culture was introduced in the amount of 2.5 ml, having previously standardized it according to McFarland to 0.5. In each variant of the study, the EPS content was determined as described above.

Statistical processing of the results was carried out using Microsoft Excel software. Differences in the results discussed in the paper are significant at the level of reliability $p \leq 0.05$ according to the Student's test.

Results and discussion. We carried out measures to determine the peculiarities of secretion and accumulation of EPS by cultures of yeasts of the genus *Rhodotorula*.

The basis for choosing this yeast is a number of characteristic features inherent in these microorganisms: high growth rate, ability to absorb various carbon substrates, undemanding to the mineral composition of the nutrient medium, synthesis of a wide range of BAR, relatively easy process of extracting useful metabolites.

We have previously determined the amount of exopolycharides produced by this yeast under normal conditions on Sabouraud medium (Fig. 2).

It was noted that among the three studied yeast species, *R. minuta* produces the maximum amount of EPS - 10.35 mg/ml of culture liquid. The minimum value recorded for *R. glutinis* is 8.56 mg/ml.

The ability to produce EPS is a typical property of representatives of various classes of fungi, including yeast (Osemwegie et al., 2020). And the main differences in the number under normal cultivation conditions or under natural growing conditions are related to the peculiarities of the biology of a particular species or even a strain of a microorganism.

It is known that the carbon source will have a direct effect on both the accumulation of yeast biomass and the production of major metabolites (Wang et al., 2021; Sugumaran et al., 2017; Torres et al., 2012). Therefore, our next step was to identify an alternative carbon source. For this purpose, we used the Hiss differential diagnostic test with various carbon-containing substrates: maltose, lactose, and mannitol (Table 1).

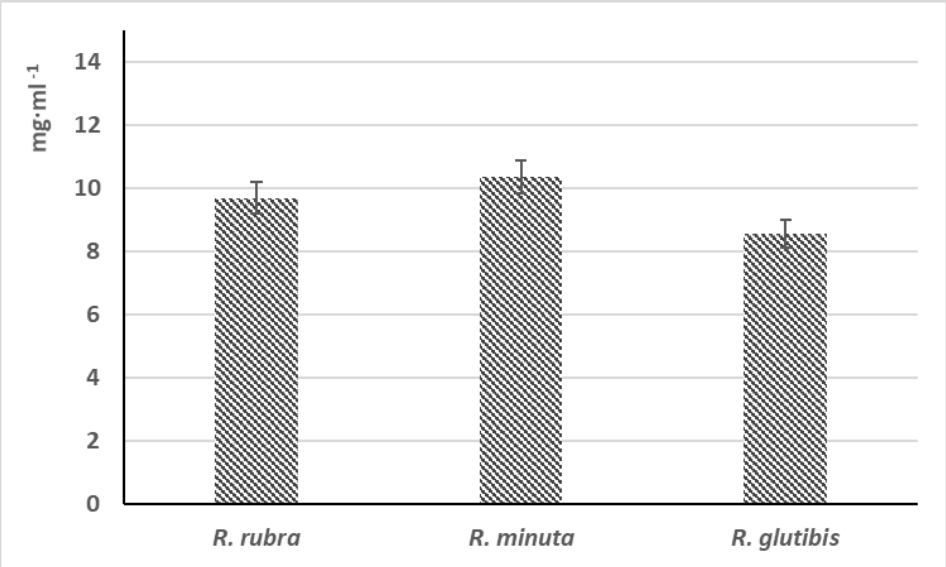


Fig. 2. Content of exopolysaccharides in the culture liquid of Rhodotorula yeast

Table 1.

The results of cultivation of representatives of the genus Rhodotorula on Hiss differential diagnostic medium with different carbohydrates

(- missing color, + present color of different intensity)

Variants of the Hiss medium	Representatives of the genus Rhodotorula		
	R. rubra	***R. minuta***	***R. glutinis***
with lactose	-	-	-
with maltose	++	+	++
with mannitol	++	+++	+++

These components of the Hiss medium are not too expensive and therefore will not significantly affect the final cost of the finished product. Also, they can become a good alternative for obtaining EPS and replacing glucose in the standard Sabouraud medium.

All three studied yeast species do not use lactose as a carbon source, the medium did not change color and remained yellow. The same variants of Hiss medium, containing maltose and mannitol, changed the color to a greater or lesser extent to pink-purple, indicating the use of these carbon sources by all three species of *Rhodotorula* yeast.

It is known that the ability to synthesize EPS varies widely both for different EPS producers and for one producer under different conditions of its cultivation (Okoro et al., 2021; Sugumaran et al., 2017). Therefore, for each yeast species, we evaluated the ability to secrete and accumulate EPS in the culture liquid on media with the classical carbon source - glucose and with alternative sources - maltose and mannitol. The classical Sabouraud medium contains 40 g/L of glucose, so we chose this amount of carbon-containing substrates and also varied their amount to 20 and 60 g/L.

We obtained the following results. Thus, for *R. rubra*, it was noted that the minimum amount of EPS accumulates in the culture liquid on medium with maltose (Fig. 3 A).

Regardless of the amount of maltose in the nutrient medium, the amount of EPS did not exceed 9 mg/ml of the culture liquid. Maltose, also known as "malt sugar", is a natural disaccharide consisting of two glucose residues. It is found in large quantities in sprouted grains of barley, rye and other grain crops. It is also found in tomatoes, in the pollen and nectar of a number of plants. Especially a lot of maltose is contained in malt and malt extracts. Thus, the production wastes of different types of beer can be considered as a promising alternative medium for the production of EPS.

On the classical Sabouraud medium with glucose, a clear dependence between the amount of EPS and the amount of substrate added was observed.

Changing the carbon source from glucose to mannitol led to an increase in EPS accumulation in the R. rubra culture fluid: 8.69 mg/l at 20 g/l mannitol and up to 12.67 mg/ml at 60 g/l mannitol.

A similar dependence was found for *R. minuta* (Fig. 3B). Under the conditions of using both glucose and maltose, an almost identical level of EPS accumulation in the culture liquid was observed. Under both conditions, there is a clear dependence between the concentration of the substrate in the culture medium and the amount of EPS. An increase in the substrate concentration does not lead to a significant increase in EPS. Under the action of both substrates, the maximum amount of EPS was recorded at 11 mg/ml of culture fluid.

The addition of mannitol allows to increase this indicator to a maximum of 14.7 mg/ml, provided that 60 g/l of mannitol is used in the culture medium. This is significantly higher than the results obtained with other substrates, as well as with lower concentrations of mannitol in the medium.

Mannitol is a hexahydric alcohol related to mannose, an isomer of sorbitol. It is naturally found in small amounts in almost all vegetables. Thus, plant meals and sludges after fruit and vegetable juice extraction can be considered as an alternative source of carbon for the production of targeted biotechnological products.

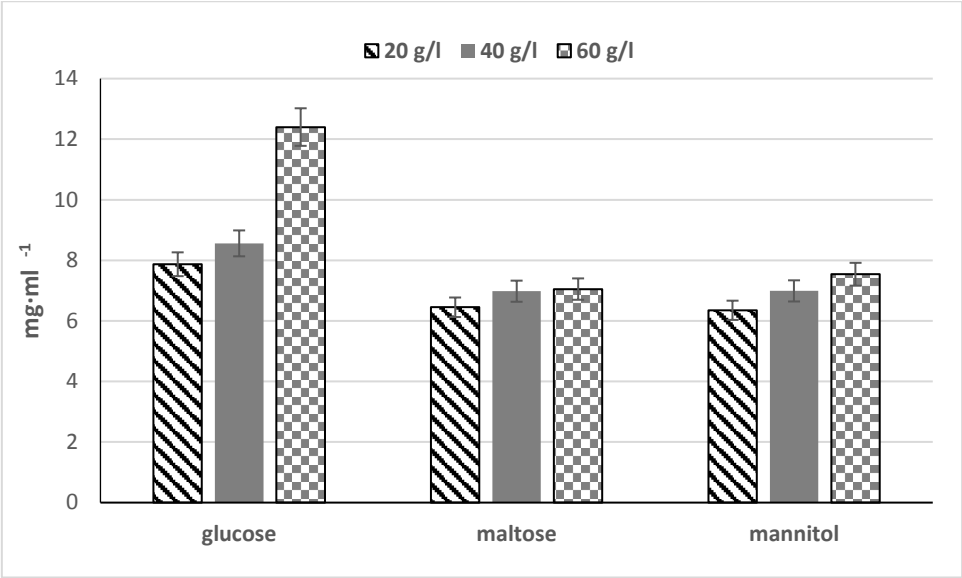
As for *R. glutinis*, no positive effect of mannitol was observed for this yeast species (Fig. 3C).

When both mannitol and maltose were used, the level of EPS was recorded to be the same, ranging from 6.5 to 7.5 mg/ml of culture fluid. However, the use of classical Sabouraud medium compositions with glucose resulted in the maximum amount of EPS of *R. glutinis*. Moreover, the introduction of glucose in the amount of 20 g/l or 40 g/l did not lead to reliable changes in the amount of EPS, but increasing glucose to 60 g/l of the medium made it possible to obtain 1.6 times more of the target metabolite.

Therefore, the secretion and accumulation of EPS in the culture liquid of the yeast genus *Rhodotorula* directly depends on both the biology of the species and the nature of the carbon-containing substrate and its amount in the nutrient medium.

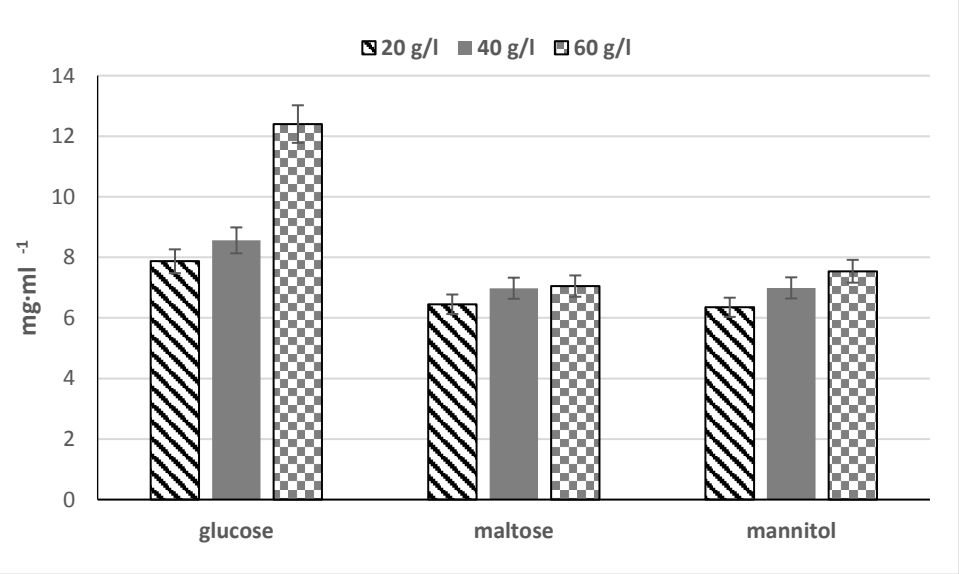
Our research on the example of yeasts of the genus *Rhodotorula*: *R.rubra, R.minuta, R.glutinis* made it possible to evaluate the ability of these three species to secrete and accumulate exopolysaccharides (EPS) both on the classical Sabouraud medium with glucose and on mediums containing maltose and mannitol

We are convinced that representatives of the yeast genus *Rhodotorula* can become promising producers of exopolysaccharides. And the use of various alternative carbon-containing substrates will allow the development of an effective and economically viable technology for obtaining EPS both on a laboratory and industrial scale.



A

B



C

Fig. 3. Accumulation of EPS in the culture fluid of R. rubra (A), R. minuta (B) and R. glutinis (C) under the conditions of using different carbon-containing substrates

Conclusions

1. The ability of three species of the genus *Rhodotorula* (*R.rubra, R.minuta, R.glutinis*) to secrete and accumulate exopolysaccharides (EPS) on the classical Sabouraud nutrient medium with glucose was evaluated. The maximum amount of EPS in the culture fluid of *R. minuta* was determined

2. The ability of all three researched yeast species to use maltose and mannitol as a carbon source was established using Hiss differential diagnostic media with maltose, lactose and mannitol.

3. The dependence between the amount of EPS accumulated in the yeast culture liquid and the nature and amount of the carbon source was established. It was noted that the maximum amount of EPS in the culture liquid of *R. rubra* and *R. minuta* accumulates under the conditions of using 60 g/l of mannitol as a carbon source. For *R.glutinis*, the highest EPS indicator was established on a medium with 60 g/l of glucose.

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НАКОПИЧЕННЯ ЕКЗОПОЛІСАХАРИДІВ ДРІЖДЖАМИ *RHODOTORULA* SP.

Л. М. Чебан, Л. М. Васіна

Робота присвячена оцінці можливості використання альтернативних джерел карбону для продукції екзополісахаридів дріжджами роду Rhodotorula sp. Екзополісахариди (ЕПС) – високомолекулярні полімерні метаболіти мікроорганізмів, що продукуються назовні клітини. Вони володіють високою здатністю до гелеутворення, емульгування, суспендування. Здатність до синтезу ЕПС виявлено у багатьох мікроорганізмів, проте їх рівень коливається в широких межах як для різних продуцентів ЕПС, так і для одного продуцента за різних умов культивування. Тому пошук активних штамів-продуцентів, альтернативних живильних середовищ та розробка ефективних технологій отримання мікробних екзополісахаридів є актуальним завданням біотехнологій.

Оцінено здатність трьох видів роду Rhodotorula: R.rubra, R.minuta, R.glutinis секретувати та накопичувати екзополісахариди (ЕПС) на класичному середовищі Сабуро з глюкозою. Встановлено максимальну кількість ЕПС у культуральній рідині R. minuta.

В роботі використано диференційно-діагностичні середовищ Гісса з мальтозою, лактозою та манітом задля встановлення можливості використання культурами дріжджів різних карбонвмісних субстратів. Встановлена здатність всіх трьох досліджуваних видів дріжджів використовувати як джерело карбону мальтозу та маніт. Відповідно ці субстрати було внесено до складу середовища Сабуро у кількості 20 г/л, 40 г/л чи 60 г/л. Відмічено, що максимальна кількість ЕПС у культуральній рідині R.rubra та R.minuta накопичується за умов використання 60 г/л маніту як джерела карбону. Для R.glutinis найвищий показник ЕПС встановлений на середовищі з 60 г/л глюкози.

Ключові слова: Rhodotorula, екзополісахариди (ЕПС), середовище Сабуро, середовища Гісса з мальтозою, лактозою та манітом.

Отримано редколегією 12.02.2023 р.