UDC: 579.66:663.16:577.164.12

PROSPECTS OF USING GLUCOSE-FRUCTOSE SYRUP IN THE RIBOFLAVIN BIOTECHNOLOGY

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DOI: https://doi.org/10.15673/fst.v14i2.1512

Article history

Received 26.11.2019
Reviewed 21.12.2019
Revised 11.03.2020
Approved 02.06.2020

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Cite as Vancuver style citation

Polishchuk V, Dugan O. Prospects of using glucose-fructose syrup in the riboflavin biotechnology. Food science and technology. 2020;14(2):25-32. DOI: https://doi.org/10.15673/fst.v14i2.1512

Цитування згідно ДСТУ 8302:2015

Polishchuk V., Dugan O. Prospects of using glucose-fructose syrup in the riboflavin biotechnology // Food science and technology. 2020. Vol. 14, Issue 2. P. 25-32 DOI: https://doi.org/10.15673/fst.v14i1.1512

Abstract. Riboflavin is an important vitamin widely used in the food industry to enrich food and as a colourant. An important problem in the implementation of the riboflavin biotechnology is selecting rational, i. e. low-cost and technologically simple sources of carbon and nitrogen. It can significantly increase the efficiency of this technology. Fungi of the genus *Eremothecium* are known to be capable of synthesising aromatic substances. Studying the level of essential oil accumulation in the carbon and nitrogen sources suggested will allow creating technologies for the simultaneous production of riboflavin and essential oil. The object of study was the ascomycete *Eremothecium ashbyi* Guillierm. F-340. The biosynthetic activity of the selected producer strain of riboflavin on media with different carbon and nitrogen sources has been studied, as well as the composition of a rational growth medium to achieve the maximum riboflavin accumulation when culturing the strain, and the producer's ability to synthesise aromatic compounds on this medium. It has been shown how various carbon and nitrogen sources influence biomass accumulation and riboflavin synthesis by the strain *E. ashbyi* F-340. Monosaccharides (fructose, galactose) and hexatomic alcohol sorbitol have been found to be best suited for the maximum riboflavin accumulation. The best source of nitrogen was yeast extract. The data obtained prove the effectiveness of glucose-fructose syrup with the fructose content 8–12% on a dry basis (GFS-10). It has been shown that the largest vitamin amount (140 mg/dm^3) is synthesised when using GFS-10. Wide-ranging variation in the content of the synthesised essential oil has been revealed, the most of it being observed in the medium containing GFS-10 (273–453 mg/dm^3) as a carbon source. The use of GFS-10 has made it possible to increase the riboflavin yield by 6.7 times as compared to the medium with glucose, and by 3.7 times as compared to the medium with fructose. The yield of essential oil has increased by a factor of 5. The data obtained can be considered a prerequisite of optimising the nutrient medium.

Keywords: riboflavin, *Eremothecium ashbyi*, carbon and nitrogen sources, glucose-fructose syrup, essential oil.

Introduction. Formulation of the problem

Vitamin production in Ukraine is a branch which is not paid attention it deserves. Vitamin B_2 enters the human body with food and performs extremely important functions participating in redox reactions. However, only some foods are rich sources of riboflavin [1]. Obtaining riboflavin biotechnologically is attractive due to its simplicity: it is a one-stage process, cultivation is performed on simple growth media and under normal conditions, and there are no harmful impurities and atmospheric emissions [2]. The biosynthetic capacity of a riboflavin producer can be increased by selecting effective and low-cost components of a nutrient medium [3,4]. It is economically practical to introduce a production technology which will allow obtaining a wide range of target products (riboflavin for the food and agricultural industries, essential oil for the food and perfume and cosmetic industries).

Analysis of recent research and publications

Riboflavin is one of the most important and widespread vitamins. Vitamin B_2 is thermostable, but due to the high sensitivity of riboflavin to light, it can be rapidly destroyed. The standard intake of vitamin B_2 recommended for different groups of people is 1.5–2.0 mg a day [5,6]. Usually, food products contain but small amounts of riboflavin, so even with a varied diet and unrefined foods, it is difficult to get the recommended daily amount of riboflavin. Riboflavin is widely used to enrich food: to vitaminise milk, cereals, dietetic products, baby food. In the food industry, riboflavin is also used as a colourant [7]. Its advantages over many artificial colourants are safety and organicity for humans. So far, riboflavin has not been

identified as toxic. Any excess of vitamin B_2 is excreted in the urine, which can give it a bright yellow colour.

Riboflavin is industrially produced in three ways: chemical, microbiological, and mixed. The latter includes microbial synthesis of ribose followed by its chemical modification into riboflavin. Obtaining riboflavin by microbiological synthesis using micromycetes of the genus *Eremothecium* can simultaneously produce several high-value end products. Firstly, it is an important vitamin that is not enough in the human diet. Riboflavin of the quality that allows using it in the food industry can be obtained by certain refining methods [3,4]. Secondly, there is technical riboflavin that can be produced to be used in premixes to enrich the feed of farm animals. Crude vitamin, along with biomass, can be used as a feed supplement, which will contain protein as well. Thirdly, since fungi of the genus *Eremothecium* can synthesise essential oil with a rose aroma, development of co-production technology will make it possible to obtain high value products for the food and perfume and cosmetics industries [8,9].

Traditionally, a rose oil, which is one of the most expensive essential oils, is obtained from essential-oil-bearing rose species [10,11]. However, this method has a number of major deficiencies. The only known biotechnological method of producing essential oil with a rose aroma is the one using fungi of the genus *Eremothecium*. The composition of this essential oil is: β-phenylethanol (20–57%), geraniol (31–81%), citronellol (2.5–11%), nerol (1.1–6.8%) [12-18]. The biosynthesis of riboflavin and that of monoterpene alcohols were shown as conjugated processes. Accumulation of vitamin B_2 and aroma-forming compounds is accompanied by filling the fungus vacuoles with lipophilic compounds [14]. Oil obtained from fungi of the genus *Eremothecium* has antimicrobial and toxic effects on microorganisms of various taxonomic groups, depending on the content of aromatic compounds and their combination. These properties are characteristic of essential oil produced from rose petals [9].

When developing any technology, primary importance is attached to creating a growth medium that should be economically feasible and ensure the maximum accumulation of target products. Mono- and disaccharides (glucose, sucrose, maltose) are used as the sources of carbon and energy in the cultivation media of fungi *Eremothecium* [4]. Media that include molasses, a by-product of sugar production, which is often used in the microbiological industry, have the industrial value [2]. Peptone, yeast extract or autolysate, maize extract, soya flour are used as nitrogen sources [2-4].

In today's Ukrainian food industry, sugar-containing starch products (molasses, glucose and glucose-fructose syrups) are manufactured in high volumes. Glucose-fructose syrups (GFS), by their sensory, physicochemical, and technological parameters, are competitive with cane and beet sugar, and are considered the most promising sugar replacement products [19].

In Ukraine, the main starch-containing raw material is maize and maize-derived products. Glucose-fructose syrup is produced from maize starch by subjecting it to enzymatic hydrolysis, thus converting it into high glucose syrup. Then, some glucose is isomerised to fructose, purified with activated carbon and ion-exchange resins, disinfected with bactericidal filters (the pore size 0.45 μm), and concentrated [20].

Glucose-fructose syrups are produced at the PJSC "Interkorn Corn Processing Industry" (Dnipro) launched in 2005. To manufacture syrups, not acid, but enzyme preparations are used as starch liquefiers, which makes it possible to obtain a stable product with the required carbohydrate composition. Syrups are produced according to Specifications (Ukraine) 15.6-32616426-009:2005 "Glucose-fructose syrup."

Glucose-fructose syrup is a viscous odourless sweet liquid, with no foreign tastes. Syrups differ in their fructose content, as evidenced by the digital index in their names: GFS-10, GFS-42 (Table 1).

Table 1 – Quality indicator of glucose-fructose syrups

Physicochemical properties	**GFS-10**	**GFS-42**
Dextrose equivalent	65	97
Dry matter	75.0–77.0	70.5–71.5
pH	4.0–6.0	4.0–6.0
Specific carbohydrate composition (% of dry matter)		
Fructose	8–12	40–44
Glucose	22–26	50–54
Maltose	37–45	2–3
Maltotriose	6–10	2
Other sugars	10–15	1

Now, there are great opportunities of using GFS in biotechnology, but it has only been reported about using it to produce ethyl alcohol [21].

The purpose of the work was to establish whether glucose-fructose syrups could be used in industrial production of riboflavin and essential oil. To achieve this purpose, the following objectives were set**:**

1. To analyse the level of riboflavin accumulation during cultivation of the producer strain on media with different sources of carbon and nitrogen.

2. To study the effect of glucose-fructose syrups with different fructose concentrations on the accumulation level of the target product.

3. To examine the ability of *Eremothecium ashbyi* F-340 to synthesise aroma-forming components on media of different composition.

Research materials and methods

The object under study was the producer of riboflavin *Eremothecium ashbyi* Guillierm. 1935 RNCIM F-340. Submerged cultivation was carried out on orbital shakers LAB-PU-01 (LOIP LS-110) in

conical flasks with 50 ml of the culture medium under the following conditions: stirring 180 rpm, temperature 28°C, cultivation time 6 days.

To determine the most favourable carbon sources for biomass and riboflavin accumulation, we used a medium that contained 0.5% of yeast extract and 0.3% of peptone as the "background." No other carbon sources (equivalent to 10 g/dm^3 of glucose) were added to this medium, but fructose, galactose, xylose, maltose, lactose, sucrose, sorbitol, inositol, mannitol, dulcin, glycerine, potato starch, as well as glucose syrup and the glucose-fructose syrups GFS-10 and GFS-42.

The favourable nitrogen sources were determined on a medium of the following composition: glucose – 10 g/dm^3, K_2HPO_4 – 1 g/dm^3, KH_2PO_4 – 1 g/dm^3, $MgSO_4 \cdot 7H_2O$ – 0.5 g/dm^3, KCl – 0.5 g/dm^3. The inorganic compounds added to the medium as nitrogen sources were ammonium chloride, sodium nitrate and sodium nitrite, ammonium nitrate, urea, and some natural medium components that, too, are a nitrogen source: peptone, yeast extract, soya flour (in the equivalent of 3 g/dm^3 of NH_4Cl).

The biomass was quantified by weighing it after it was separated from the culture fluid by filtration and dried in the oven to a constant weight at 105°C [22].

The riboflavin accumulation level was studied by the spectrophotometric method at 450 nm preceded by hydrolysis of FAD to FMN for 12 hours in 10% TCA [23,24].

Aroma-forming compounds were isolated from the culture fluid by triple extraction with the organic solvent hexane (in the ratio 3**:**1), followed by removing it with a rotary evaporator under vacuum. The aroma-forming compounds were quantified by weighing the residue on an analytical balance [12,14,16,17].

Results of the research and their discussion

Mono- and disaccharides as well as carbohydrate-containing waste from various industries are traditionally used in biotechnology of vitamins as a source of carbon and energy in nutrient media [25,26]. However, the former are quite expensive to use, and the latter does not always satisfy all needs of a producer strain and does not lead to accumulation of a target product in large quantities.

It has been established that the object under study, the producer strain of riboflavin *Eremothecium ashbyi* Guillierm. F-340, as a carbon source, can assimilate carbon compounds of various nature (Table 2). However, it accumulates riboflavin best when there is hexabasic sorbitol, fructose, and galactose in the medium; the medium level of riboflavin is observed when glucose and maltose are used. The basal medium (indicated as "no carbon source" in Table 2) contained yeast extract and peptone as nitrogen sources.

On studying how the riboflavin producer grows on media with different carbon sources, it has been found advisable to use sorbitol, fructose, galactose, glucose, and maltose to develop the culture media composition in future.

Table 3 shows how the culture *Eremothecium ashbyi* grows on media containing inorganic compounds and organic substances as nitrogen sources. As a carbon source, 10 g/dm^3 of glucose was added to the media. No culture growth was observed with sodium nitrate and sodium nitrite.

The best source of nitrogen for *E. ashbyi* F-340 was yeast extract. There was by 2.7 times more riboflavin synthesised on the medium with yeast extract than on the medium with peptone. The positive effect on the growth of the producer is due to the complex composition of yeast extract. It is rich in vitamins, especially those of group B, in protein breakdown products (peptides and amino acids), and in growth factors, which makes it of great interest as a source of nitrogenous nutrition. Other authors, too, confirm a significant positive effect of yeast extract on riboflavin biosynthesis [4].

So far, no cost-effective medium containing these components and having appropriate technological characteristics has been suggested for cultivating *Eremothecium ashbyi*.

Table 2 – Level of riboflavin biosynthesis (strain *E. ashbyi* F-340) on media with different carbon sources

Carbon sources	Riboflavin concentration, mg/dm^3	Carbon sources	Riboflavin concentration, mg/dm^3
no carbon source	5.19±0.87	glucose	20.51±1.78
inositol	25.74±2.43	glucose syrup	18.78±0.92
sorbitol	52.0±1.56	lactose	9.13±1.71
mannitol	28.62±2.6	dulcite	9.77±1.04
fructose	37.69±2.27	glycerol	8.23±0.95
galactose	37.79±2.32	potato starch	6.0±0.21
maltose	18.67±0.72	ethanol	8.27±0.45
sucrose	10.85±1.88	citrate	8.43±0.52
xylose	13.77±1.6	carboxymethyl cellulose	1.93±0.27

Table 3 – Level of riboflavin biosynthesis (strain *E. ashbyi* F-340) on media with different nitrogen sources

Nitrogen source	Riboflavin concentration, mg/dm^3
Ammonium chloride	3.51±0.28
Ammonium nitrate	5.67±0.46
Urea	5.9±0.39
Peptone	7.92±0.8
Yeast extract	21.0±0.64

To solve this problem, it has been studied how glucose-fructose syrups with different fructose concentrations can be used in the industrial production of riboflavin. The tasks were to establish the level of riboflavin accumulation on growth media with different glucose-fructose syrups (GFS-10 and GFS-42) as the carbon source, and to find a nitrogen source most favourable for vitamin B_2 synthesis.

While studying how the strain *E. ashbyi* F-340 accumulated riboflavin and biomass when cultivated on GFS media, similar growth media with glucose as the carbon source were used for comparison (Fig. 1). It has been found that the greatest amount of the vitamin is synthesised using GFS-10 (140 mg/dm^3). This is 6.7 times as much as it is on the medium with glucose, and 3.7 times as much as on the medium with fructose (see Table 2). The glucose-fructose syrup GFS-10 contains the least amount of fructose, but significant amounts of maltose and oligosaccharides (Table 1). It can be assumed that oligosaccharides contribute to such a significant increase in the yield of riboflavin. With GFS-42, which contains a small amount of oligosaccharides, the synthesis of the target product has shown no significant difference from that in the reference media.

Studying the well-known nitrogen sources used as growth medium components (peptone, yeast extract, soya flour, and yeast extract+peptone) has shown that using the combination yeast extract+peptone with GFS results in the highest yield of riboflavin. This may be due to the fact that these components of the medium contain simultaneously large amounts of vitamins and growth factors necessary for the producer strain to accumulate the maximum of the target product.

In the previous studies, GFS was added to the medium in an amount equivalent to 10 g/dm^3 of glucose. On studying the effect of using GFS-10 in concentrations ranging 10 to 50 g/dm^3 in terms of glucose, it has been found that with higher concentrations of GFS-10, the concentration of riboflavin synthesised by the producer decreases significantly, which can be explained by catabolite repression, and there is a considerable increase in biomass (Fig. 2). A decrease in riboflavin accompanied by an increase in biomass may be due to the close relation between flavin biosynthesis and purine metabolism [27].

Another valuable target product synthesised by the producer strain is essential oil. Other authors have shown that on various media. *E. ashbyi* can synthesise 120 to 370 mg/dm^3 of essential oil [13,14,16]. Table 4 presents a wide range of variations in the essential oil amount depending on the medium used for cultivation.

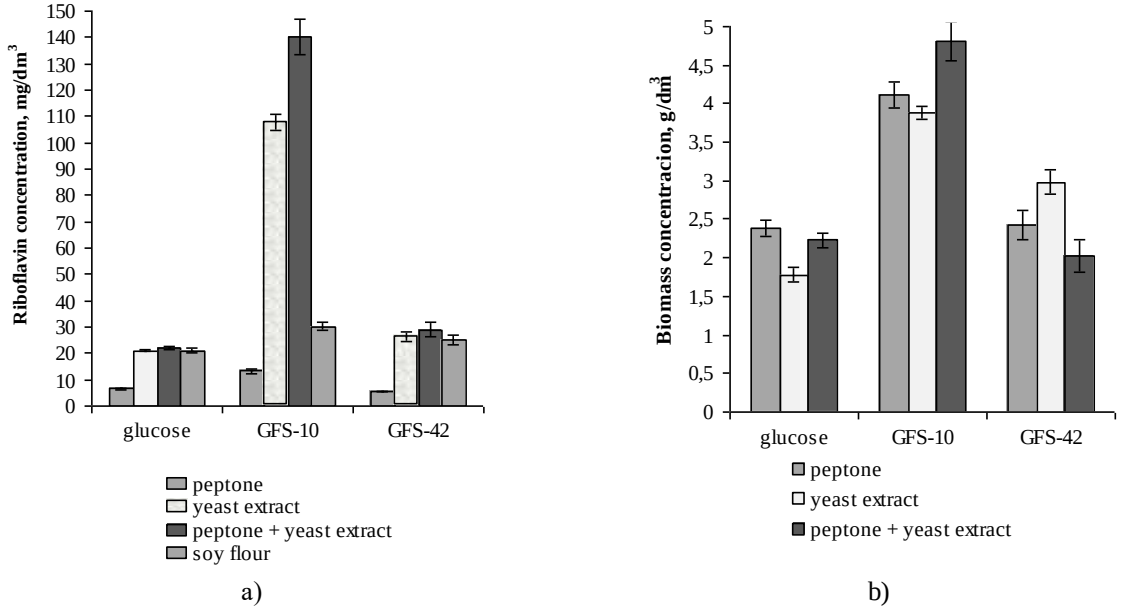
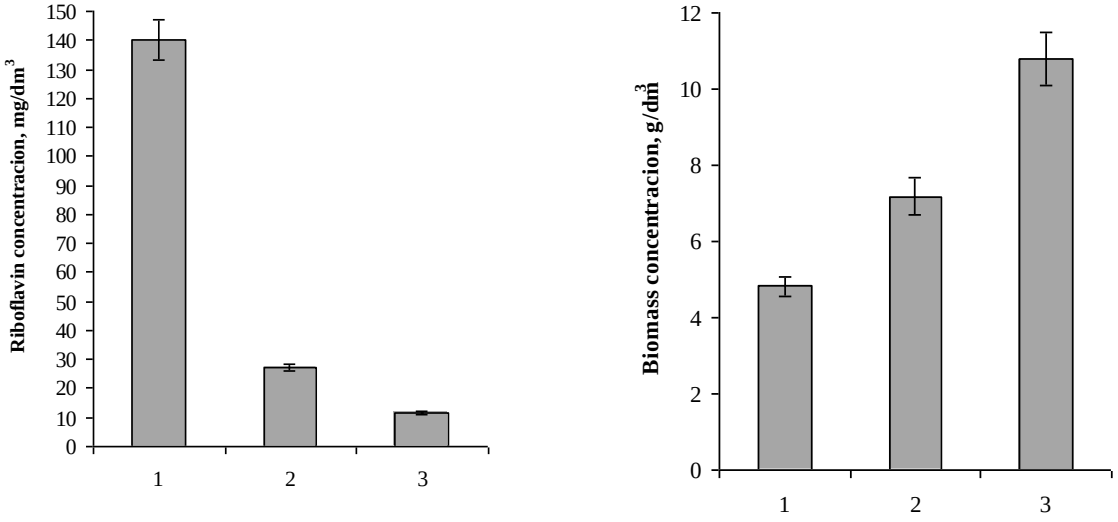


Fig. 1. Accumulation of riboflavin (a) and biomass (b) by the strain *E. ashbyi* F-340 in the course of culturing on the medium with glucose and the GFS medium ($p < 0.05$)



a) b)

Fig. 2. Accumulation of riboflavin (a) and biomass (b) by the strain *E. ashbyi* F-340 in the course of culturing on the medium with different concentrations of GFS-10 (in terms of glucose): 1 – 10 mg/cm^3, 2 – 30 mg/cm^3, 3 – 50 mg/cm^3 (p<0.05)

Table 4 – Level of essential oil accumulation by *E. ashbyi* depending on the culture medium

Media	Essential oil conbtent, mg/dm^3
Glucose+peptone+yeast extract	31±2
GFS-10+ peptone+yeast extract	160±11
GFS-42+ peptone+yeast extract	80±6
Glucose+yeast extract	73±5
GFS-10+ yeast extract	252±12
Glucose+peptone	60±2
GFS-10+peptone	140±7
GFS-10 (10 g/dm^3 in terms of glucose)	160±11
GFS-10 (30 g/dm^3 in terms of glucose)	273±13
GFS-10 (50 g/dm^3 in terms of glucose)	420±18

The largest amount of aroma-forming substances is synthesised on the medium containing GFS-10 (160–420 mg/dm^3) as the carbon source. It has been found that yeast extract has a positive effect on essential oil synthesis. The amount of essential oil increases with higher concentrations of GFS-10 in the medium.

Conclusion

It has been proved that the level of riboflavin accumulation by the culture *E. ashbyi* depends on the carbon sources in the cultivation medium. Monosaccharides (fructose, galactose) and hexabasic sorbitol are the best components for riboflavin biosynthesis. The best nitrogen source for *E. ashbyi* F-340 is yeast extract: the amount of riboflavin synthesised on the medium with yeast extract is 2.7 times as high as it is on the medium with peptone.

For the first time, it has been shown that the producer can be cultivated on the medium with glucose-fructose syrup (GFS-10) as the carbon source. The best results have been obtained with the combination yeast extract+peptone used as the nitrogen source. The use of GFS-10 increased the yield of riboflavin by 6.7 times and that of essential oil by 5 times, as compared to the glucose-peptone-yeast medium.

It has been proved that *Eremothecium ashbyi* can synthesise essential oil with the rose aroma in a wide variation range. The largest amount of aromatic substances is synthesised on the medium with GFS-10 (273–420 mg/dm^3).

The results of this work can be based on to optimise the growth medium composition by the three components: GFS, yeast extract, and peptone in order to maximise riboflavin accumulation by the producer strain *Eremothecium ashbyi*.

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ПЕРСПЕКТИВИ ВИКОРИСТАННЯ ГЛЮКОЗО-ФРУКТОЗНОГО СИРОПУ В БІОТЕХНОЛОГІЇ РИБОФЛАВІНУ

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Анотація. Рибофлавін є важливим вітаміном, що широко застосовується у харчовій промисловості для збагачення харчових продуктів та в якості барвника. Важливою проблемою реалізації біотехнології рибофлавіну є підбір раціональних джерел карбону та нітрогену, що є дешевими та технологічними. Це дасть можливість значно підвищити ефективність даної технології. Відомо, що гриби роду *Eremothecium* здатні до синтезу ароматичних

речовин, дослідження рівня накопичення ефірної олії на запропонованих джерелах карбону та нітрогену дасть можливість створення технології одночасного виробництва рибофлавіну та ефірної олії. Об'єктом дослідження був аскоміцет *Eremothecium ashbyi Guillierm*. F-340. Досліджували біосинтетичну активність обраного штаму-продуценту рибофлавіну на середовищах з різними джерелами карбону та нітрогену, компонентний склад раціонального поживного середовища для культивування продуценту з метою максимального накопичення рибофлавіну, здатність продуценту до синтезу ароматичних сполук на запропонованому середовищі. Встановлено вплив різних джерел карбону та нітрогену на накопичення біомаси та синтез рибофлавіну штамом *E. ashbyi* F-340, для максимального накопичення рибофлавіну краще підходять моносахариди (фруктоза, галактоза) та шестиатомний спирт сорбіт. Кращим джерелом нітрогену виявився дріжджовий екстракт. Отримані експериментальні дані свідчать про ефективність застосування глюкозо-фруктозного сиропу з вмістом фруктози від 8 до 12% на суху речовину (ГФС-10). Показано, що саме при використанні ГФС-10 синтезується найбільша кількість вітаміну (140 мг/дм3). Показаний широкий діапазон варіювання кількості синтезованої ефірної олії. Найбільша кількість спостерігається на середовищі, що містить в якості джерела карбону ГФС-10 (273–453 мг/дм^3). Використання ГФС-10 дозволило збільшити вихід рибофлавіну у 6.7 рази, порівняно з середовищем з глюкозою, та у 3.7 рази, порівняно з середовищем з фруктозою. Вихід ефірної олії збільшився у 5 раз. Отримані данні є передумовою для оптимізації поживного середовища.

Ключові слова: рибофлавін, *Eremothecium ashbyi*, джерела карбону та нітрогену, глюкозо-фруктозний сироп, ефірна олія.

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