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BIOBUTANOL ACCUMULATION USING ALTERNATIVE SUBSTRATES BY CULTIVATION OF *Clostridium acetobutylicum* STRAINS

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The aim of the research was to investigate the accumulation of biobutanol by strains *Clostridium acetobutylicum* using alternative substrates (Jerusalem artichoke juice, technical glycerol, crude glycerol, shredded biomass of soy, rape, wheat and switchgrass). In order to increase the accumulation of butanol in the process of cultivation, the cells of *C. acetobutylicum* were statically immobilized on carriers (belting strips, ferrite rings and Raschig rings) by the method of adsorption immobilization. The cells were precipitated by centrifugation, the supernatant was distilled and then fermentation products were determined. Gas chromatography was used to determine the presence of solvents in the culture fluid. The biggest accumulation of butanol (2 g/dm^3) was at concentration of crude glycerol 16 g/dm^3 in the medium, and complete inhibition of culture development — at glycerol concentration 25 g/dm^3. The accumulation of butanol by the strain *C. acetobutylicum* IMB B-7407 using fill and draw method depended on the amount of sequestered and infused medium. Immobilization of the culture using the Raschig rings allowed increasing the bioconversion to butanol twice. So it is shown the possibility to use non-traditional substrates for the production of biobutanol. The most accumulation of butanol was achieved using glycerol (11 g/dm^3) as water-soluble substrate, and using switchgrass as lignocelluloses substrate (2,6 g/dm^3). Immobilization of *C. acetobutylicum* culture cells on carriers increased the accumulation of butanol. The use of Raschig rings, as carriers for immobilization, allowed increasing the accumulation of butanol twice.

Key words: biobutanol, *Clostridium acetobutylicum* IMB B-7407, immobilization.

Immobilization of the producer cells is one of the ways to increase the accumulation of the target product in the process of cultivation [1]. Both organic and nonorganic carriers [2] are used to produce immobilized cells. The materials used as carriers should have the following properties: chemical and biological stability, mechanical integrity (primarily resistance to rubbing), ensure the interaction of enzymes of microorganisms with the substrate; significant specific surface, capacity and porosity; the possibility of obtaining technologically convenient forms (granules, membranes, pipes, sheets, etc.); easy transfer to the reaction form (activation); high hydrophilicity, which ensures the possibility of carrying out cytoadherence reactions with carrier in aqueous medium; low cost. The lack of carriers that meet all these requirements at once and the diversity of tasks stimulate the search of suitable materials for the immobilization of specific producer cells [2, 3].

The objective of this work was to investigate the accumulation of biobutanol by immobilized cells of strains *C. acetobutylicum* using alternative substrates.

Materials and Methods

For the research we used: butanol producer strains *Clostridium acetobutylicum* IMB B-7407 (IFBG C6H), *Clostridium* sp. IMB B-7570 (IFBG C6H 5M) from the "Collection of microorganisms' strains and plant lines for agricultural and industrial biotechnology", Government Entity "Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine"; Jerusalem artichoke juice *Helianthus tuberosus* (Florium,

Ukraine), technical glycerol and crude glycerol (Pharma, Belgium), shredded green biomass of soy *Glycine max*, rape *Brássica nápus*, wheat *Tríticum* sp. (all from National Research Center "Institute of Mechanization and Electrification of Agriculture" of the National Academy of Sciences of Ukraine) and switchgrass *Panicum virgatum* L. (Kyiv National Botanical Garden named after M.M. Hryshko). The following medium composition (g/dm^3) was used to determine the accumulation of butanol while using glycerol: crude glycerol (from 10.0 to 20.0), yeast extract — 1.0; $(NH_4)_2SO_4$ — 0.6; $(NH_4)_2HPO_4$ — 1.6; pH 6.5. The medium was sterilized for 30 min and at pressure of 1 atm. To determine the accumulation of butanol while Jerusalem artichoke juice (*Helianthus tuberosus*) the root tubers were cleaned, peeled, grinded and juiced. The juice was diluted to 19.6% of dry matter and added to (g/dm^3) $(NH_4)_2SO_4$ — 0.6; $(NH_4)_2HPO_4$ — 1.6; the pH was adjusted to 6.68.

Vynohradski medium and slices of chalk rubbed potatoes [4] were used as activation medium. The cultivation of *Clostridia* was carried out according to the procedure [4] in the anaerobic jar "AE 01" (RF) in nitrogen atmosphere. Anaerobic jar was placed in the thermostat at the temperature of 35 ± 1 °C.

Biomass of switchgrass, soy, rape, wheat was dried at the temperature of 30±1 °C during 48 h. The dried biomass (7% humidity) was shredded up to 200 mesh with the help of mill "Tsyklon MSH 1" (Ukraine). The moisture was determined by moisture analyzer RADWAG MA 50/C/1 (Poland). Lignin content was determined according to the procedure [4].

To immobilize cells the following carriers were used: belting strips (Promfiltr, Ukraine) with a surface area of 35.4 cm^2, ferrite rings (Epcos, FRG) — 2.1 cm^2, and Rushig rings (Antey, RF) — 0.5 cm^2 (Fig. 1).

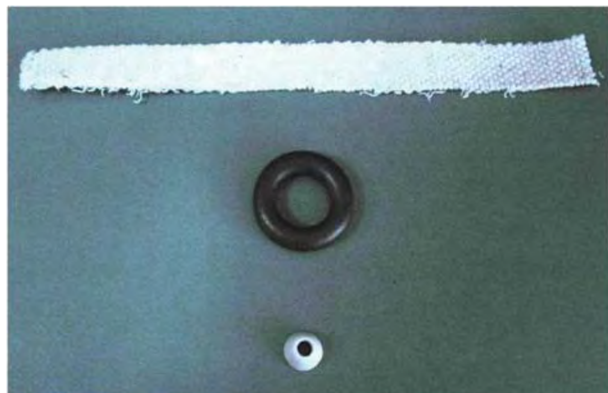

Fig. 1. **Carriers for immobilization (from up to down):**
belting strips, ferrite rings, Rushig rings

Immobilization of cells on carriers was carried out for 12 h by static method of adsorption immobilization. Cultivation of immobilized culture cells on carriers was carried out according to the procedure [4]. After 5 days cultivation was stopped, the cells were precipitated using an ultracentrifuge "Labofuge 400R" (Germany), the supernatant was distilled at 100 °C with a refrigerator until there is no output volume (100 cm^3) in the receiving flask and the fermentation products were determined.

Presence of ethanol, acetone and butanol in culture liquid was determined using gas chromatograph ("Kristall-5000 lux", RF) with flame-ionization detector and packed column 3 m in length, phase Carbowax 1500 on chromaton N-A-W-DMSC (0.20–0.25 mm). The column temperature was 60 ± 2 °C, the evaporator' temperature was 160 ± 5 °C, nitrogen-hydrogen-air ratio was 1:1:10 [5].

Statistical data analysis was performed using Microsoft Excel program. All experiments were done in three replicates. The difference between two averages was considered probable at $P < 0.05$ (these results are marked with *). In addition to Fig. 1–5, 7, the enzymatic medium without seed was as control (zero value), and in Fig. 6 for control was inimmobilized cells, so the control and rendered on the drawing.

Results and Discussions

In the process of biodiesel producing from renewable biomass, glycerol is produced in large quantities as a by-product of vegetable oils during esterification. Purification of glycerol is quite expensive energy-consuming process, although refined glycerol is widely used in pharmacology, cosmetology, food and other industries. To reduce the cost of microbial synthesis and increase the profitability of biobutanol technology, the crude glycerol was used as a substrate — an unpurified waste of biodiesel production. Investigation of butanol accumulation by the strain *C. acetobutylicum* IMB-7407 was carried out at different concentrations of crude glycerol (Fig. 2).

From the results shown in Fig. 2 it is evident, that bioconversion to butanol takes place when crude glycerol concentration is in range from 10 to 20 g/dm^3. The most accumulation of butanol (2 g/dm^3) took place when crude glycerol concentration in medium was equal to 16 g/dm^3, and complete inhibition of culture development at a concentration of 25 g/dm^3.

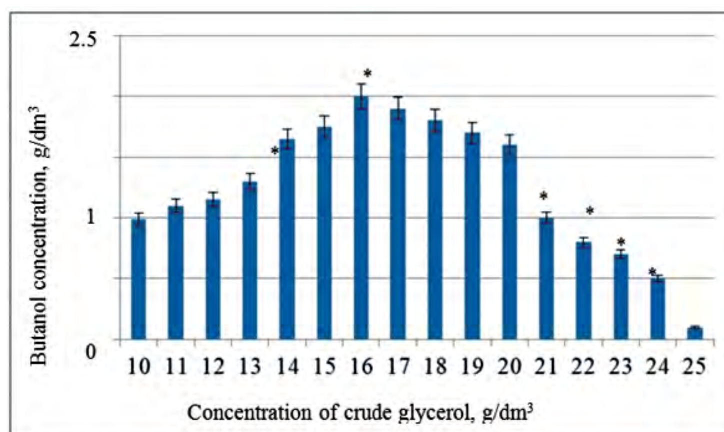

Fig. 2. **Butanol accumulation on different concentration of crude glycerol**

Note: in addition to Figure 6, the fermentation (enzymatic) medium without seed was as control, and in Figure No. 6 for control (zero value) was inimmobilized cells, so the control and rendered on the drawing.

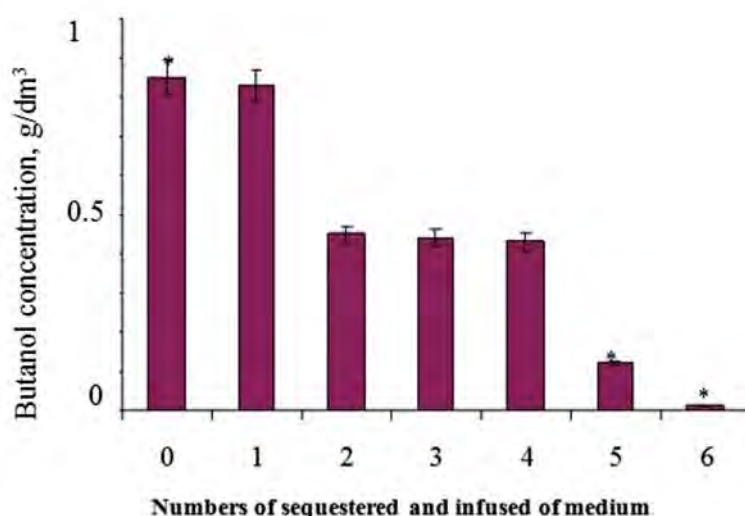

Fig. 3. **Accumulation of butanol in strain *C. acetobutylicum* IMB B-7407 using over-fill method**

In order to increase the accumulation of butanol at the minimum concentration of crude glycerol in the medium, we cultivated *C. acetobutylicum* IMB B-7407 by fill and draw method. Each period of sequestered and infused occurred after 96 h. The results are shown in (Fig. 3).

Accumulation of butanol in the culture liquid by strain *C. acetobutylicum* IMB B-7407 did not change in the process of cultivating during the first period of medium sequestering and infusing and from the second up to the fourth period the accumulation of butanol was reduced by half. The subsequent use of fill and draw method lead to decrease of butanol accumulation by 8 times and final stop in the sixth period [6–8].

To increase the accumulation of butanol by stain *C. acetobutylicum* IMB B-7407 using crude glycerol as a substrate, we carried out immobilization of cell cultures on different carriers, which had high adhesion activity relative to the culture. After immobilization, biomass of cells was attached to all carriers, however, according to cytological studies the morphology of the culture has changed (Fig. 4).

Figure 4 shows that cells of immobilized culture were attached to the carriers and formed long chains of about 20 cells. The initial non-immobilized culture did not form such chains [4]. To determine the number of cells on the carrier, we carried out washing of the carriers and drying the resulting microbial biomass to a completely dry weight (CDW). The obtained results are presented in Fig. 5.

Figure 5 shows that the largest number of cells was absorbed on the fibers of the belting strips. With the use of fabrics, the

CDW of strain *C. atsotobutylicum* IMB-7407 exceeded other carriers by almost five times. The previous studies [4] showed that technical glycerol was the best source of carbohydrate for the culture, therefore cultivation of immobilized cells was carried out using technical glycerol as a substrate and accumulation of butanol was determined. The results obtained are shown in Fig. 6.

Figure 6 shows that the accumulation of butanol has increased due to the use of immobilized cultures on Raschig rings and belting strips. Therefore, culture immobilized on ferrite rings lost its properties to the synthesis of solvents and accumulated only acids. According to the results of the study, we can conclude that the immobilization of stain *C. acetobutylicum* IMB B-7407 on the Raschig rings has allowed to double the accumulation of butanol.

Jerusalem artichoke juice [9] may be one of the promising substrates for obtaining biofuels. A study was conducted with the use of Jerusalem artichoke juice as a substrate for cultivating strains *C. acetobutylicum* IMB B-7407 and *Clostridium* sp. IMV B-7570. The results obtained are shown in Fig. 7.

Fig. 7 shows that the accumulation of butanol in the culture liquid of stain *C. acetobutylicum* IMB-7407 was 3.5 g/dm^3, ethanol was 0.2 g/dm^3, acetone was not produced. Alternatively, the strain *Clostridium* sp. IMB B-7570 almost did not convert the Jerusalem artichoke juice into alcohol.

We performed cultivation of strain *Clostridium* sp. IMB B-7570 using different lignocellulosic substrates — biomass of soy, rape, switchgrass and wheat, and determined accumulation of butanol. The results are presented in Fig. 8.

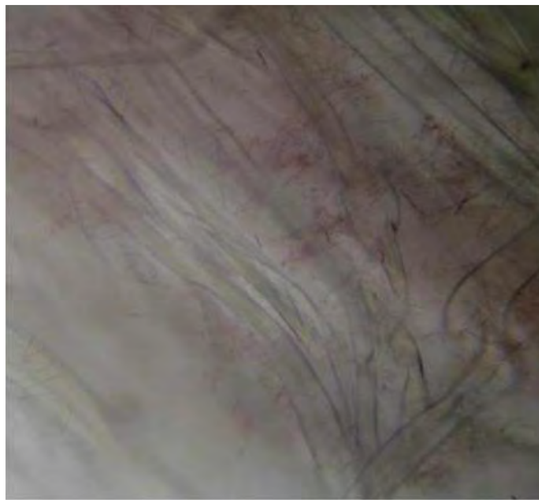

Fig. 4. **Microphoto (increase 900) of immobilized culture (colored brawn) on belting strips**

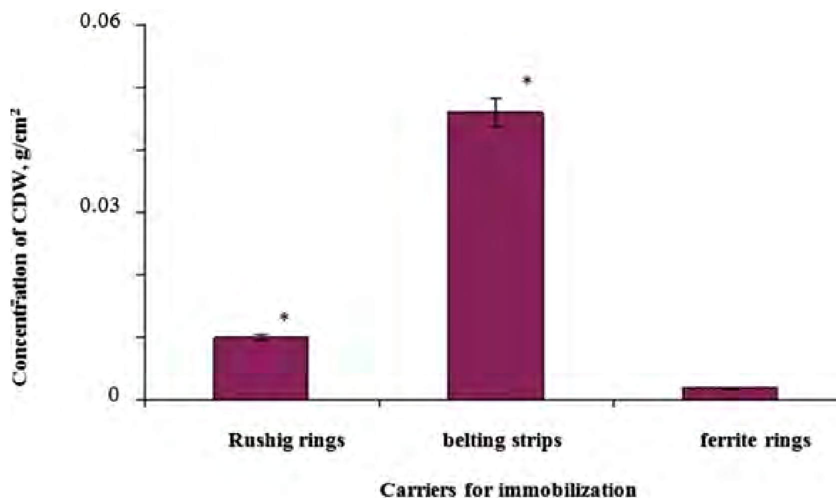

Fig. 5. **Concentration of biomass of *C. atsotobutylicum* IMB-7407 strain on different carriers**

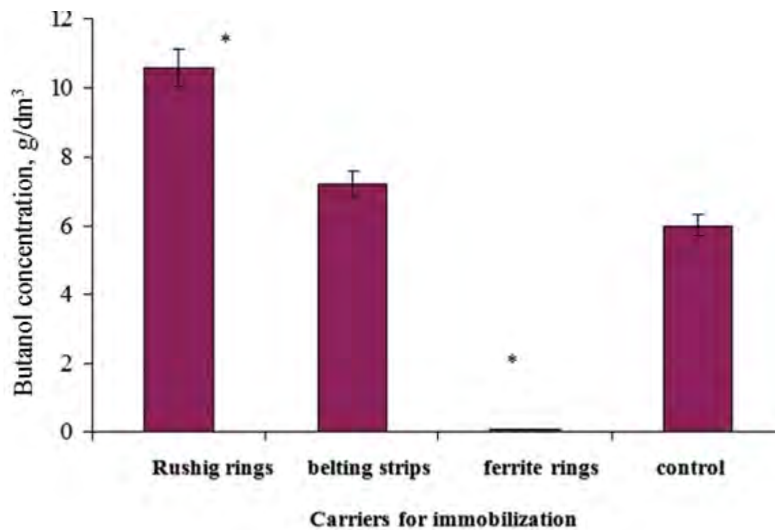

Fig. 6. **Accumulation of butanol by immobilized *C. acetobutylicum* IMB B-7407 stain on different carriers**

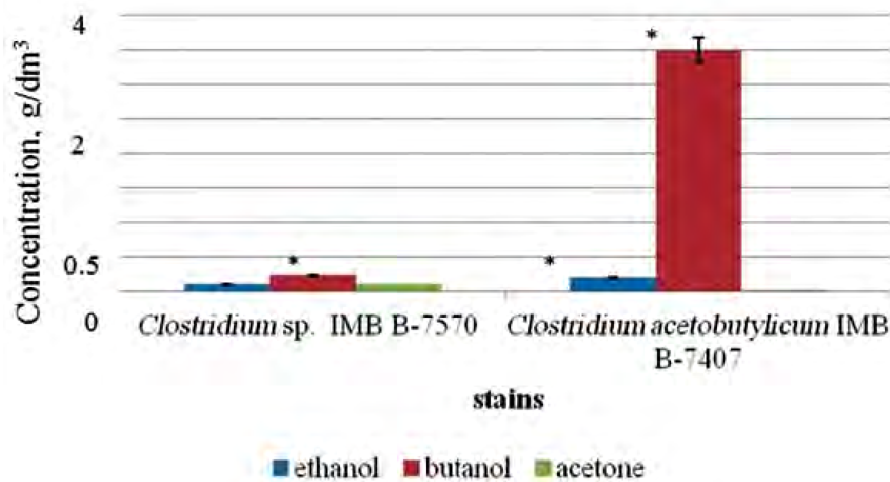

Fig. 7. **Accumulation of butanol using Jerusalem artichoke juice as substrate**

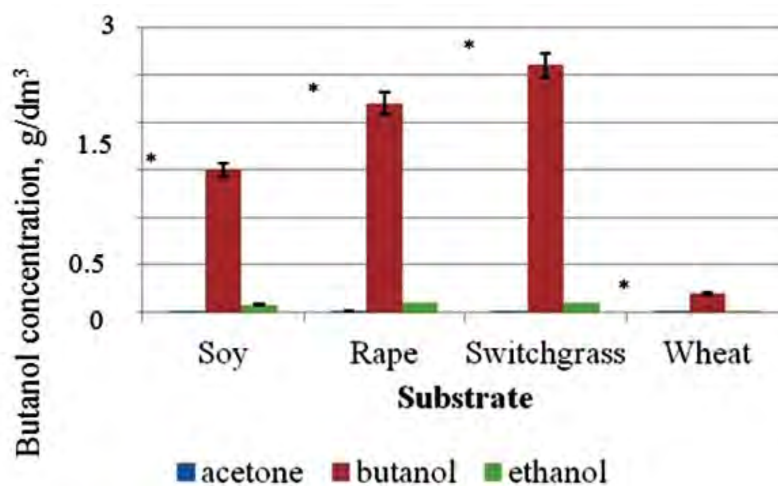

Fig. 8. **Accumulation of butanol on different substrates**

Fig. 8 shows that the biggest accumulation of butanol (2.6 g/dm^3) was when using switchgrass biomass as a substrate and the smallest when using wheat (0.2 g/dm^3).

Studies have shown that non-traditional substrates (shredded biomass of soy, rapeseed, switchgrass, wheat; Jerusalem artichoke juice, technical glycerol, crude glycerol) are converted into biobutanol. While using the fill and draw method the accumulation of butanol by the culture *C. acetobutylicum* IMB B-7407 on crude glycerol did not change during the first period of fill and draw of the medium, but during the second it decreased. Immobilization of cells *C. acetobutylicum* IMB B-7407 on Raschig rings, belting rings and ferrite rings as carriers showed that all carriers had high adhesion activity relative to culture. It was determined that accumulation of butanol doubled when using Raschig rings as a carrier for the immobilization of cells *C. acetobutylicum* of IMB B-7407.

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ВИКОРИСТАННЯ АЛЬТЕРНАТИВНИХ СУБСТРАТІВ ДЛЯ НАКОПИЧЕННЯ БІОБУТАНОЛУ ЗА КУЛЬТИВУВАННЯ ШТАМІВ *Clostridium acetobutylicum*

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Метою роботи було дослідити накопичення біобутанолу штамами *Clostridium acetobutylicum* з використанням альтернативних субстратів (соку топінамбуру, технічного гліцеролу, гліцеролу сирцю, подрібненої зеленої біомаси сої, ріпаку, дротоподібного проса, пшениці). Для підвищення накопичення бутанолу в процесі культивування проводили іммобілізацію клітин *C. acetobutylicum* на носіях (смужках бельтингу, феритових кільцях та кільцях Рашига) статичним способом — методом адсорбційної іммобілізації. Клітини осаджували за допомогою центрифугування, супернатант переганяли та визначали продукти бродіння. Наявність розчинників у культуральній рідині визначали за допомогою газової хроматографії. Максимальне накопичення бутанолу (2 г/дм^3) спостерігали за концентрації у середовищі гліцеролу-сирцю 16 г/дм^3, а повне інгібування розвитку культури — за 25 г/дм^3. Накопичення бутанолу штамом *C. acetobutylicum* ІМВ В-7407 за культивування від'ємно-доливним методом залежало від кількості вилучень та доливань середовища. Іммобілізація культури з використанням кілець Рашига дала змогу підвищити біоконверсію до бутанолу в 2 рази. Таким чином, було показано можливість використання нетрадиційних субстратів для отримання біобутанолу. Найбільшого накопичення було досягнено за використання гліцеролу (11 г/дм^3) як водорозчинного субстрату та лігноцелюлозного — дротоподібного проса(2,6 г/дм^3). Іммобілізація клітин культури *C. acetobutylicum* на носіях підвищувала накопичення бутанолу. Використання кілець Рашига як носіїв для іммобілізації дало змогу підвищити накопичення бутанолу в 2 рази.

Ключові слова: біобутанол, *Clostridium acetobutylicum* ІМВ В-7407, іммобілізація.

ИСПОЛЬЗОВАНИЕ АЛЬТЕРНАТИВНЫХ СУБСТРАТОВ ДЛЯ НАКОПЛЕНИЯ БИОБУТАНОЛА ПРИ КУЛЬТИВИРОВАНИИ ШТАММОВ *Clostridium acetobutylicum*

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Целью работы было исследовать накопление биобутанола штаммами *Clostridium acetobutylicum* с использованием альтернативных субстратов (сока топинамбура, технического глицерола, глицерола-сырца, измельченной зелёной биомасы сои, рапса, прутевидного проса, пшеницы). Для повышения накопления бутанола в процессе культивирования проводили иммобилизацию клеток *C. acetobutylicum* на носителях (полосках бельтинга, ферритовых кольцах, кольцах Рашига) статическим способом — методом адсорбционной иммобилизации. Клетки осаждали с помощью центрифугирования, супернатант перегоняли и определяли продукты брожения. Наличие растворителей в культуральной жидкости определяли с помощью газовой хроматографии. Максимальное накопление бутанола (2 г/дм^3) наблюдали при концентрации в среде глицерола-сырца 16 г/дм^3, а полное ингибирование развития культуры — при 25 г/дм^3. Накопление бутанола штаммом *C. acetobutylicum* ІМВ В-7407 при культивировании отъемно-доливным методом зависит от количества отъема и долива среды. Иммобилизация культуры с использованием колец Рашига дала возможность повысить биоконверсию до бутанола в 2 раза. Таким образом, для получения бутанола была показана возможность использования нетрадиционных субстратов. Наибольшее накопление бутанола было достигнуто при использовании глицерола (11 г/дм^3) как водорастворимого субстрата и как лигноцеллюлозного — прутевидного проса (2,6 г/дм^3). Иммобилизация клеток культуры *C. acetobutylicum* на носителях повышала накопление бутанола. Использование колец Рашига в качестве носителя для иммобилизации позволило повысить накопление бутанола в 2 раза.

Ключевые слова: биобутанол, *Clostridium acetobutylicum* ІМВ В-7407, иммобилизация.