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NEW STRAIN-PRODUCERS OF BIOBUTANOL. III. METHODS OF INCREASED BUTANOL ACCUMULATION FROM BIOMASS OF SWITCHGRASS *Panicum virgatum* L.

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The aim of this work was to enlarge accumulation of butanol from switchgrass *Panicum virgatum* L. biomass using strains-producers obtained from grounds and silts of Kyiv lakes. The objects of the study were strains of *C. acetobutylicum* IMB B-7407 (IFBG C6H), *Clostridium acetobutylicum* IFBG C6H 5M and *Clostridium tyrobutyricum* IFBG C4B from the "Collections of microbial strains and lines of plants for food and agricultural biotechnology" of the Public Institution "Institute of Food Biotechnology and Genomics" of the National Academy of Sciences of Ukraine. Gas chromatography was used to determine the alcohol concentration at the stage of solvent synthesis. To determine the effect of butanol precursors during cultivation, butyric, lactic and acetic acids were used. Optimization of processing parameters, which was based on the needs of cultures, allowed us to increase the yield by 20 and 50% for the initial and mutant strain respectively. Using synthetic precursors (such as lactic, butyric and acetic acid) during cultivation increased total concentration of butanol by 1.7 times. To optimize the process, a study was carried out using acetone- butyl grains. Using of acetone-butyl grains in concentrations up to 60% does not affect the synthesis of butanol by *C. acetobutylicum* IFBG C6H 5M. Increasing the concentration of grains led to decrease in accumulation of butanol. Almost double increase in accumulation of the target product (butanol) was achieved using two-stage fermentation and/or precursors of synthesis. It was shown the possibility of using acetone-butyl grains in fermentation. As a result the mass fraction of the waste was reduced.

Key words: butanol, switchgrass *Panicum virgatum* L., producer strains *Clostridium*.

Nowadays the world market of butanol exceeds 9.6 billion liters and is assessed at more than 6.0 billion US dollars [1]. There is a stable trend towards yearly increase (by 3.2%) of demand for butanol. Butanol is used as a solvent for industrial production, in production of melamine-formaldehyde resins and plasticizers, butyl acetate, butyl acrylate and in organic synthesis. Besides, it has begun to be actively used as fuel. Only 0.1 % of total butanol production goes to microbiological synthesis, and compared to other biofuel types its share is only 2%. Experts forecast that in the next 5–10 years this value will reach 30% [2]. Unfortunately, today the microbiological method of butanol production is not efficient due to formation of by-products such as ethanol and acetone during the cultivation, relatively high cost of feedstock, low strain productivity, and the problem of waste disposal — acetone-butyl spent grains. To optimize the process of metabolite production it is necessary to perform the following: carry out the primary selection of producer strains, make changes in genetic structure of strain-producer to increase the product accumulation, define the optimal technological parameters (pH, temperature, nutrient requirement) and conditions for nutrition and biomass accumulation and chose the way for cell-producer immobilization [3–10].

In case of using the reducing and considerably cheap plant biomass, it is necessary to create profitable technology of acetone-butyl fermentation and production of solventogenic strains.

The aim of this study was the increase of butanol accumulation from the biomass of switchgrass *Panicum virgatum* L. using purified producer strains.

Materials and Methods

We investigated strains of *C. acetobutylicum* IMB B-7407 (IFBG C6H), IFBG C4B and IFBG C6H 5M from the "Collections of microbial strains and lines of plants for food

and agricultural biotechnology" of the Public Institution "Institute of Food Biotechnology and Genomics" of the National Academy of Science of Ukraine; switchgrass *Panicum virgatum* L. biomass (it is the main model system of lignocellulose raw material; its genome is completely decoded) obtained from the Gryshko National Botanical Garden. Switchgrass is widely used in production of bioethanol. The microorganisms were cultivated and the solvents were extracted as it was shown before [5, 6]. To determine precursor effect on butanol formation process, butyric, lactic and acetic acids were used (all from "Setuza", China). As a source of lactic acid, lactic serum was added to the fermentation mixture ("Zarechye", Ukraine, TU46.39.11 — 93). The serum, butyric and acetic acids were added in relevant ratios (1:1:1, 1:2:1, 1:1:2, 1:3:1, 1:4:1, relatively) to the switchgrass mash, total acid concentration was 5 g/l. The plant mass was dried and ground into leaves of *Panicum virgatum* L. To determine the components of the of the wire-like millet i.e. lignin biomass we used The Ukrainian Standard ISO 13906:2013, cellulose — The Ukrainian Standard ISO 2470:2005, humidity — The Ukrainian Standard EN 13041:2005, protein — The Ukrainian Standard 4595:2006, hemicellulose — The Ukrainian Standard 3500-97.

The data were statistically processed using Microsoft Excel software. All the experiments were run in three repetitions. The difference between two average values was considered adequate at $P<0.05$ (the adequate results are marked with asterisk*).

Results and Discussion

When the plant raw material is planned to be chosen with its further use, the first step is the analysis of substrate content. It gives the possibility to measure and consider all the macro components with proper accuracy. It was done the chemical analysis of switchgrass *Panicum virgatum* L. to determine the component composition of the plant feedstock [11]. The results of the analysis are shown at Fig. 1.

The main components of the switchgrass biomass were: cellulose (35%), hemicellulose (29%), and lignin (26%). The study [6] showed how cellulose and hemicellulose sugars may be converted by *C. acetobutylicum* IFBG C6H strain. To compare productivity of *C. acetobutylicum* IFBG C6H (stock strain) and IFBG C6H 5M (mutant strain) fermentation was performed taking into consideration previous results of parameter optimization [12]. The obtained data are given in Fig. 2.

Optimization of the process parameters basing on the obtained data of the culture requirement, allowed us to increase the target product accumulation by 20% for the stock strain and by 50% for the mutant strain. Under these conditions, acetone concentration was insignificantly increased (less than1%), while that of ethanole was not changed. On the basis of the obtained data, the IFBG C6H 5M mutant strain was selected as the prospective one for investigations of butanol accumulation intensification. At the first stage of acetone-butanol-ethanol (ABE) fermentation, *C. acetobutylicum* bacteria produce butyric, propionic, acetic and lactic acids (the stage of acid formation). Then pH is decreased and the stage of solvent synthesis takes place such as butanol, acetone, ethanol and isopropanol. This stage is

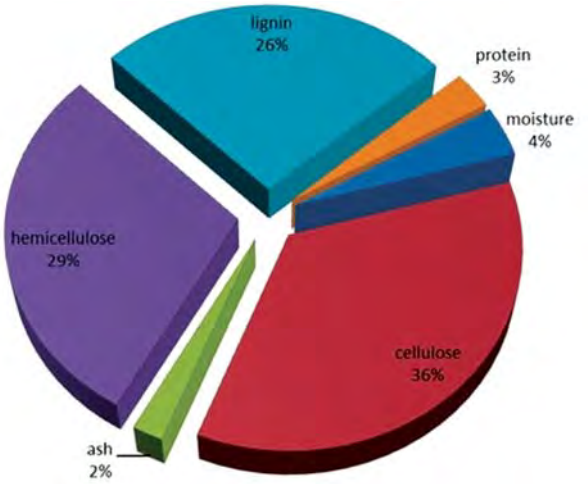

Fig. 1. **Component composition of the switchgrass *Panicum virgatum* L. biomass**

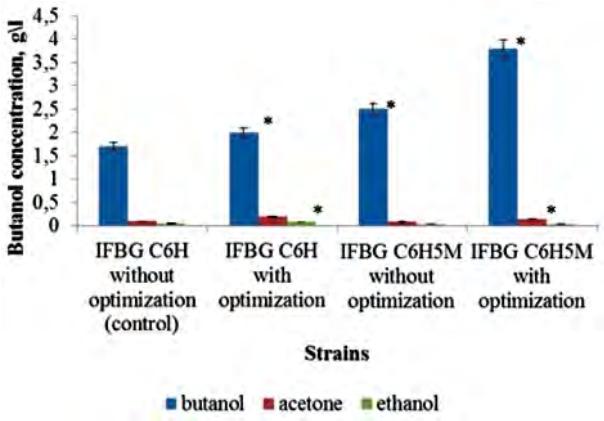

Fig. 2. **Solvent synthesis in the mash of the switchgrass biomass**

Note: here and after * — $P < 0.05$ as compared to control.

characterized with increased concentration of butyric acid and pH < 5. Generation of butanol is limited due to blocking of microorganism growth at 1 to 2% butanol concentration. Since the acetone-butyl fermentation is one of the types of butyric-acidic fermentation, to intensify the butanol accumulation the study was carried out using synthesis of precursors such as lactic, acetic and butyric acids. The correspondent acids were introduced into the enzymatic medium at the start of cultivation, first separately, then as the mixture. The fermentation was made for each case. The results of the investigation are presented in Fig. 3.

The obtained data proved that using the synthesis precursor such as lactic, butyric and acetic acid led to the increase of butanol accumulation. Insignificant butanol accumulation was observed when acetic acid (up to 3.7 g/l) and serum (up to 3.9 g/l) were introduced compared to the control value (3.5 g/l). Significant increase (in 1.7 times) of butanol concentration was noted in case of using all three precursors. The conversion of saccharides under these conditions was increased almost in two times, from 0.32 to 0.58 (Table). Addition of the precursors to the fermentation environment provided the growth of economic fermentation factors but had not influence on the biomass accumulation and specific growth rate of the culture. It should be noted that in case of precursor using, the ratio of butanol concentration to biomass grew from 1.2 to 2.0. The ratio of the biomass to the consumed substrate changed from 0.38 to 0.29 respectively. Such change of kinetic parameters shows the increase of butanol accumulation due to substrate conversion.

To determine the optimal concentration of precursors, fermentation was carried out with various ratios of precursor concentrations. The results are given in Fig 4.

The ratios of the butanol synthesis precursors, which were placed into the fermentation environment, raised the final concentration of the target product differently. The ratio 1:1:3 was optimal for serum, acetic and butyric acid respectively. Acid addition to the mash just in this ratio raised the concentration of the target product by 17%. Using engineering solutions, the authors [13] proposed methods of butanol obtaining with ABE process optimization. Double bioreactors with immobilized microorganisms were used in the process of fermentation, and the butanol synthesis process had two stages. At the first stage the butyric acid was synthesized, and then butanol. Such process led to faster butanol accumulation. Compared to the common ABE

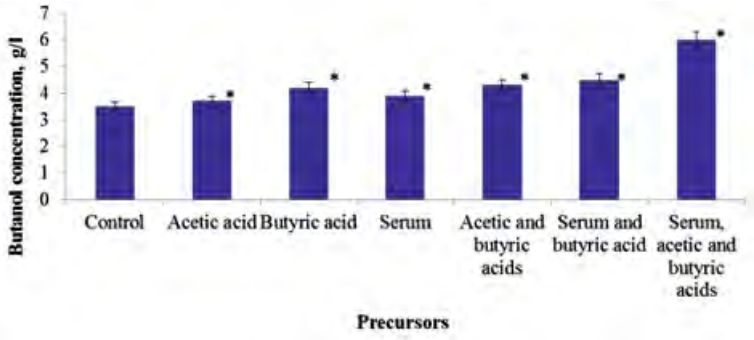

Fig. 3. **Synthesis of butanol with IFBG C6H 5M in the mash of the switchgrass using precursors**

Influence of precursors on the kinetic parameters of fermentation

Parameters	Reference	When precursors are used
Biomass, g/l	3.05±0.33	2.98 ±0.32
Y p/s*	0.32	0.58
Y p/x	1.2	2
Y x/s	0.38	0.29
μ, year^{-1}	0.13	0.12

* *Note.* Y — economic factors of cultivation; p — butanol concentration; x — biomass; s — spent substrate; μ — specific growth rate

process, such technology gave butyric acid, butanol, carbon dioxide, hydrogen, and excluded the formation of undesirable products (propionic acid, acetone, isopropanol and ethanol). Such process minimizes the alcohol inhibition of microorganisms, increases reactor production, and reduces energy expense for concentration and extraction of the target product. We used this two-stage scheme with the biomass of wire-like millet as the substrate (Fig. 5).

As it was shown in [6], cellulose or lignocellulose feedstock may be used for cultivation with the following production of acids and their subsequent using. When the acids are used with two bioreactors coupled in series (two-step fermentation, Fig. 5), unlike in the case of the classical fermentation, the strain productivity and butanol accumulation go up.

Following this scheme, the ground wire-like millet biomass enters as a substrate (lignocellulose raw material) to the first bioreacror. It is fermented by IFBG C4B strain, which is a butyric acid producer. The process runs under the following engineering parameters: inoculum — 20% , temperature — 35 °C, pH 6, duration — three days. Then, the fermentation mixture goes to the second bioreacror with non-hydrolyzed biomass of wire-like millet and it is fermented by IFBG C6H 5M strain with the production of butanol and other alcohols (engineering parameters: inoculum — 20% , temperature — 35 °C, pH 5, duration — from three to four days). Butanol was extracted from the cultural liquid by one of the methods described in [3]. This technology doubles the final butanol concentration.

An important problem of butanol production process is waste. Utilization (evaporation) of acetone butyl spent grains is rather expensive. To optimize the butanol production process it was suggested that the acetone butyl spent grains obtained in the course of fermentation should be returned to the fermentation process, it is the so-called recycled spent grains.

Using the recycled spent grains in concentration of 60% from the volume of the fermentation mixture did not affect significantly the butanol synthesis by *C. acetobutylicum* IFBG C6H 5M strain. With the increase of the spent grains concentration in the environment, it was observed the decrease of butanol accumulation (Fig. 6). It gives the possibility to return up to 60% of the spent grains to the enzymatic environment. The remaining part of the spent grains was divided into decantation liquid and slurry. The decantation liquid was used in the new fermentation instead of water ("recycled" water), and the slurry may be used for feeding farm animals or as a fertilizer enriched with riboflavin and B_{12} vitamin [14, 15].

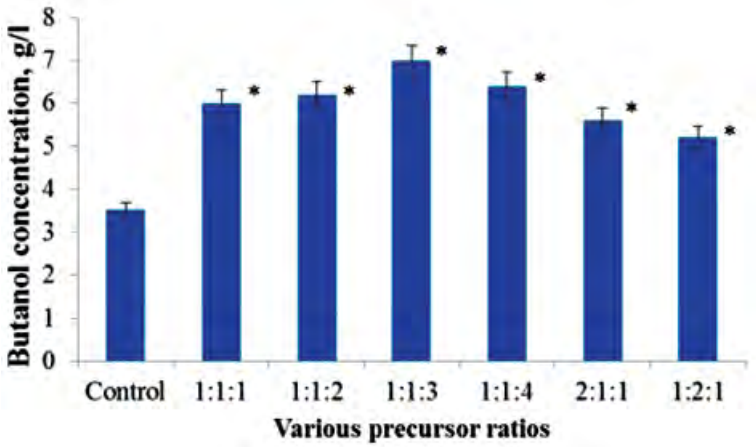

Fig. 4. **Butanol synthesis with IFBG C6H 5M strain in the mash of switchgrass using various precursor ratios**

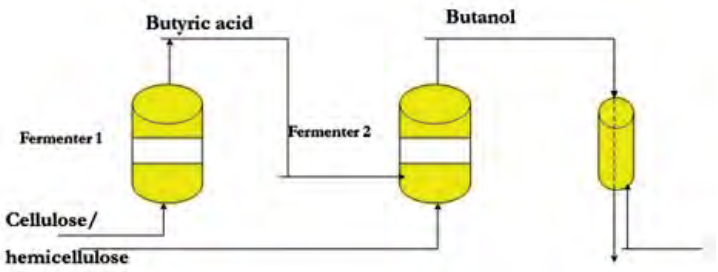

Fig. 5. **Schematic diagram of two-step butanol production using precursors**

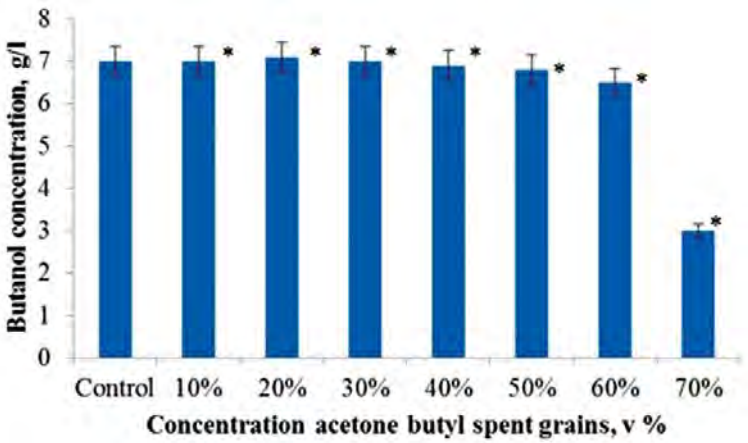

Fig. 6. **Butanol synthesis by IFBG C6H 5M strain in the mash of the switchgrass using acetone butyl spent grains**

It was demonstrated that the increase in accumulation of the target product (butanol) nearly by two times (from 3.8 to 7 g/l) was achieved by using two-step fermentation and/or synthesis precursors. Using acetone butyl (recycled) spent grains in the cultivation process resulted in the decrease of mass fraction of waste.

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НОВІ ШТАМИ-ПРОДУЦЕНТИ БІОБУТАНОЛУ. III. СПОСОБИ ПІДВИЩЕННЯ НАКОПИЧЕННЯ БУТАНОЛУ З БІОМАСИ ДРОТОПОДІБНОГО ПРОСА *Panicum virgatum* L.

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Метою роботи було збільшити накопичення бутанолу з біомаси дротоподібного проса *Panicum virgatum* L. з використанням штамів-продуцентів, виділених із ґрунтів і мулів озер Києва. Об'єктами дослідження слугували штами *Clostridium acetobutylicum* IMB B-7407 (IFBG C6H), *C. acetobutylicum* IFBG C6H 5M та *C. tyrobutyricum* IFBG C4B із «Колекції штамів мікроорганізмів та ліній рослин для харчової і сільськогосподарської біотехнології» ДУ «Інститут харчової біотехнології та геноміки» НАН України. Концентрацію спиртів на стадії синтезу розчинників визначали, застосовуючи газову хроматографію. Для визначення впливу попередників на накопичення бутанолу в процесі культивування використовували масляну, молочну та оцтову кислоти. Оптимізація технологічних параметрів з урахуванням потреб культур дала змогу підвищити вихід цільового продукту на 20 і 50% у вихідного та мутантного штамів, відповідно. Використання в процесі культивування попередників (зокрема, молочної, масляної та оцтової кислот) підвищувало кінцеву концентрацію бутанолу в 1,7 раза. З метою оптимізації процесу було проведено дослідження з використанням ацетоно-бутилової барди. Застосування ацетоно-бутилової барди в концентрації до 60% не впливало на синтез бутанолу штамом *C. acetobutylicum* IFBG C6H 5M. Зі зростанням концентрації барди накопичення бутанолу зменшувалось. Збільшення накопичення цільового продукту (бутанолу) майже вдвічі досягали, використовуючи двостадійну ферментацію та/або попередники синтезу. Показано можливість використання в процесі культивування ацетоно-бутилової барди. У результаті було досягнено зменшення масової частки відходів.

Ключові слова: бутанол, дротоподібне просо *Panicum virgatum* L., штами-продуценти *Clostridium*.

НОВЫЕ ШТАММЫ ПРОДУЦЕНТЫ БИОБУТАНОЛА. III. СПОСОБЫ ПОВЫШЕНИЯ НАКОПЛЕНИЯ БУТАНОЛА ИЗ БИОМАССЫ СТЕБЛЕВИДНОГО ПРОСА *Panicum virgatum* L.

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Целью работы было увеличить накопление бутанола из биомассы стеблевидного проса *Panicum virgatum* L. с использованием штаммов-продуцентов, выделенных из грунтов и илов озер Киева. Объектами исследования служили штаммы *Clostridium acetobutylicum* IMB B-7407 (IFBG C6H), *C. acetobutylicum* IFBG C6H 5M и *C. tyrobutylicum* IFBG C4B из «Коллекции штаммов микроорганизмов и линий растений для пищевой и сельскохозяйственной биотехнологии» ГУ «Институт пищевой биотехнологии и геномики» НАН Украины. Для определения концентрации спиртов на стадии синтеза растворителей использовали газовую хроматографию. Для определения влияния предшественников на накопление бутанола в процессе культивирования использовали масляную, молочную и уксусную кислоты. Оптимизация технологических параметров с учетом потребностей культур позволила повысить выход целевого продукта на 20 и 50% у исходного и мутантного штаммов, соответственно. Использование в процессе культивирования предшественников (в частности, молочной, масляной и уксусной кислот) повышало конечную концентрацию бутанола в 1,7 раза. С целью оптимизации процесса было проведено исследование с использованием ацетоно-бутиловой барды. Применение ацетоно-бутиловой барды в концентрации до 60% не влияло на синтез бутанола *C. acetobutylicum* IFBG C6H5M. С увеличением концентрации барды накопление бутанола уменьшалось. Увеличение накопления целевого продукта (бутанола) почти в два раза было достигнуто с использованием двухстадийной ферментации и/или предшественников синтеза. Показана возможность использования в процессе культивирования ацетоно-бутиловой барды. В результате было достигнуто уменьшение массовой доли отходов.

Ключевые слова: бутанол, стеблевидное просо *Panicum virgatum* L., штаммы-продуценты *Clostridium*.