

LYOPHILIZATION EFFECT ON PRODUCTIVITY OF BUTANOL-PRODUCING STRAINS

O. O. Tiginova
N. E. Beiko
A. S. Andriiash
S. G. Priymov
S. M. Shulga

SO “Institute of Food Biotechnology and Genomics”
of the National Academy of Sciences of Ukraine, Kyiv

E-mail: Shulga5@i.ua

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Investigation of lyophilization effect on the productivity of butanol-producing strains was the aim of our research. For this purpose we used butanol-producing strains; technical glycerol; biomass of switchgrass *Panicum virgatum* L. Lyophilization was performed using a lyophilization-drying. The effect of the protective medium on residual moisture of freeze-drying cultures suspensions depending on the concentration of glucose and sucrose was studied. It was shown that the lowest residual moisture was attained by using glucose and sucrose in amount of 10% and if the samples of freeze-drying bacteria had been saved for one month at 4 °C the productivity did not decrease. As temperature preservation was increased the productivity of the cultures was gradually decreased and it was greatly reduced at 30 °C. So the protective medium composition was optimized for lyophilization of butanol-producing strains as follows: sucrose 10.0%; gelatin 10.0%; agar 0.02%. It was shown that the preservation of samples of freeze-drying bacteria for six months at a temperature of 4 °C did not affect the productivity of strains. It was found that cultures could use glycerol as a carbon source for butanol accumulation before lyophilization.

Key words: butanol, producing bacteria, lyophilization.

The main problem of preservation of microorganisms in collections (museums) is the variability of biological material. During storage microorganisms on artificial media, there is a danger of a significant reduction or even loss of their physiological and enzymatic properties. For the “conservation” materials of biological origin the method of lyophilization has received prevalence. In the process of lyophilization of biological materials free water contained therein is removed by sublimation — evaporation directly from the solid state (ice). The temperature of the material that undergoes dehydration during the whole period of free water removal remains below freezing point resulting proteins do not denature at elevated concentrations of electrolytes [1–3].

The structure of the material after lyophilization drying is equally important. It should be dry, porous, without free water mass that almost kept the volume and structure of the original substance. Freezing followed by

drying does not guarantee the full preservation of all properties of biological material but this method significantly increases time of storage of cultures of microorganisms, live vaccine, serum and plasma [4–6].

When freezing any material that contains water and dissolved salts, there is an eutectic separation of solution and elevated concentrations of salt which can cause denaturation of proteins or cell destruction. The degree of damage depends primarily on the nature of dissolved salts, cooling rate and initial concentration of the suspension material. If the cooling is quicker the effect of concentrated salt solutions is lower. Through the selection and use of so-called “protective” medium of different composition during freezing and lyophilization drying it can prevent negative effects of eutectic concentration on microorganisms [7–11].

The aim of this work was to study the effect of lyophilization drying on productivity of butanol-producing microorganisms' strains.

Materials and Methods

For the study they were used butanol-producing strains *Clostridium acetobutylicum* IMB B-7407 (IFBG C6H) and its mutant *Clostridium* sp. IMB B-7570 (IFBG C6H 5M) from "Collection microorganism's stains and plants line for food and agriculture biotechnology" of the Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine; technical glycerol; biomass of switchgrass *Panicum virgatum* L. (Gryshko National Botanic Garden).

To prepare mashes of switchgrass, samples of 50.0 g of the substance were taken per 1 l of water, and sterilized for 2 h at a pressure 0.2 MPa. Biomass of switchgrass was dried at 30 ± 1 °C during 168 h. The dried biomass was ground using mill Cyclone MSh 1 (Ukraine) to particles sized 200 mesh. The moisture was determined by moisture analyzer RADWAG MA 50/C/1 (Poland).

To determine the optimal concentration of glycerol medium of the following composition we used (g/l): glycerol (from 30.0 to 180.0), yeast extract — 1.0; $(\text{NH}_4)_2\text{SO}_4$ — 0.6; $(\text{NH}_4)_2\text{HPO}_4$ — 1.6; pH 6.5. It was sterilized during 30 min at 0.1 MPa.

Culture of microorganisms at solid medium was performed following [12] in anaerobic culture apparatus AE 01 (RF) under a nitrogen atmosphere. The apparatus was kept in thermostat at 35 ± 1 °C. In five days, the fermentation was stopped and the cells were precipitated using ultracentrifuge Labofuge 400R (Germany), then the supernatant was distilled and fermentation products defined.

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 NAW-DMSC (0.20–0.25 mm). The column temperature was 60 ± 2 °C, the evaporator's 160 ± 5 °C, nitrogen: hydrogen: air ratio was 1:1:10.

To confirm the effect of protection medium on cell viability after lyophilization drying, medium of the following composition was used (%): glucose or sucrose at appropriate concentrations — 1.0; 10.0; 30.0; gelatin — 10.0; agar — 0.2.

Winogradsky medium at first was used for the accumulation of cultures of butanol producing stains [12]. Bacteria carried in a protective medium at the rate of cell concentration $4 \cdot 10^9$ cell/ml, then in the amount of 5 ml contributed carry in to penicillin bottles.

Samples were frozen in low-temperature refrigerator LAB 11/EL19LT (Elcold, Denmark) at -80 °C. Frozen samples were transferred into special cassettes in the pre-cooled camera (temperature condenser -50 °C) freeze drying CRUODOS-50 (TELSTAR, Spain). Lyophilization was performed at constant temperature -50 °C (drying temperature is not regulated) to stable residual pressure in the chamber 0.0018 Pa. Drying time was 72 h. The residual moisture was determined by moisture analyzer.

Preparing freeze-drying material for the study of productivity was necessary to fulfill two conditions: as accurately as possible restore first (to dry) volume of material and to derive living cells from a state of anabiosis. To fulfill these conditions freeze-drying material was adjusted with distilled water to a volume of 5 ml and kept at room temperature for 30 min.

Statistical data analysis was performed using Microsoft Excel program. All experiments were done in triplicate. The difference between two averages was considered α_2 probable at $P < 0.05$ (significant results marked with *) [13]. Aseptic environment was taken as control.

Results and Discussion

For the preparation of *Clostridium acetobutylicum* IMV B-7407 (IFBG C6H) and *Clostridium* sp. IMB B-7570 (IFBG C6H 5M) before lyophilization the dependence on residual moisture of freeze-drying cultures was first investigated, after lyophilization, on the concentration of glucose or sucrose in a protective medium (Table 1).

As follows from the data, under the same conditions of lyophilization the residual moisture of freeze-drying cultures after lyophilization depends on the type and concentration of carbohydrates. Lowest rates of residual moisture were obtained using glucose or sucrose at a concentration of 10%. The protective medium according to the research composition was optimized for lyophilization butanol-producing strains (10.0% sucrose; 10.0% gelatin; 0.02% agar).

Temperature conditions were particular important in the process of storage microorganisms after lyophilization drying. With increasing of the temperature of storage the number of viable cells of microorganisms and their productivity were decreased. Strains were cultivated on mashes of switchgrass to determine their productivity. The productivity (accumulation butanol) of freeze-

Table 1. Residual moisture of freeze-drying cultures dependence on the concentration of glucose or sucrose

| Carbohydrate | Concentration, % | Residual moisture of suspension, % | |
|--------------|------------------|------------------------------------|--------------|
| | | IFBG C6H | IFBG C6H 5M |
| Glucose | 1 | 18.60 ± 0.01 | 18.00 ± 0.01 |
| | 10 | 3.00 ± 0.01 | 2.63 ± 0.01 |
| | 30 | 45.40 ± 0.01 | 43.22 ± 0.01 |
| Sucrose | 1 | 18.11 ± 0.01 | 17.99 ± 0.01 |
| | 10 | 2.00 ± 0.01 | 1.68 ± 0.01 |
| | 30 | 48.33 ± 0.01 | 50.44 ± 0.01 |

drying microorganisms dependence by storage temperature after one month are shown in Fig. 1.

Productivity of the cultures did not decreased if the samples of freeze-drying bacteria preserve for one month at 4 °C. With increasing temperature of preservation the productivity of bacteria was gradually decreased, and at a temperature of 30 °C it was greatly reduced.

Further storage freeze-drying cultures was carried out at a temperature of 4 °C for 6 months, exploring their productivity through every month on mashes of switchgrass (Table 2).

It is seen from the table 2 that even after storage at a temperature of 4 °C for 6 months, the renewed freeze-drying culture (strain IFBG C6H 5M) was viable and the accumulation of butanol in cultural liquid after cultivation was hardly changed compared to accumulation before lyophilization 2.7 g/l [14]. Similar results were obtained for strain IFBG C6H.

One of the main factors that effect on lyophilization was a stage of culture development. As a source of energy the different carbohydrates and carbohydrate containing compounds for the development of microorganisms were used. Technical glycerol could be one of these sources of energy. To optimize the nutrient medium before lyophilization we researched alcohols accumulation by strain IFBG C6N using technical glycerol as a carbon source (Fig. 2).

The study results show that the greatest accumulation of butanol (10.0 g/l) in cultural liquid was at the glycerol concentration 100.0 g/l. Most bioconversion of substrate was observed at concentrations of glycerol 50.0 g/l with the accumulation of butanol 8.0 g/l.

Fig. 3 shows alcohols accumulation (productivity) strain IFBG C6H 5M at different concentrations of glycerol.

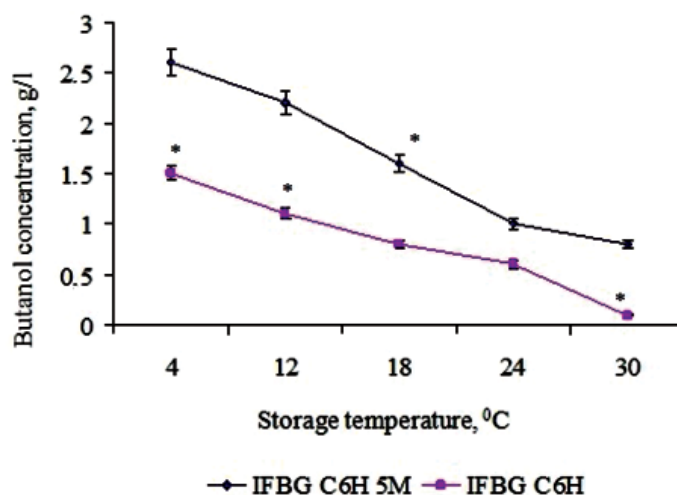


Fig. 1. Effect of storage temperature on the accumulation of butanol

Table 2. Butanol production by strain IFBG C6H 5M during storage

| Months of storage | Technological parameters | | |
|-------------------|--------------------------|---------------|--------------|
| | pH | Dry matter, % | Butanol, g/l |
| 0 | 6.65 ± 0.05 | 11.0 ± 0.1 | 2.60 ± 0.01 |
| 1 | 6.68 ± 0.05 | 11.1 ± 0.1 | 2.56 ± 0.01 |
| 2 | 6.63 ± 0.05 | 11.2 ± 0.1 | 2.54 ± 0.03 |
| 3 | 6.65 ± 0.05 | 11.3 ± 0.1 | 2.50 ± 0.01 |
| 4 | 6.68 ± 0.05 | 11.4 ± 0.1 | 2.49 ± 0.02 |
| 5 | 6.64 ± 0.05 | 11.3 ± 0.1 | 2.46 ± 0.01 |
| 6 | 6.63 ± 0.05 | 11.5 ± 0.1 | 2.40 ± 0.01 |

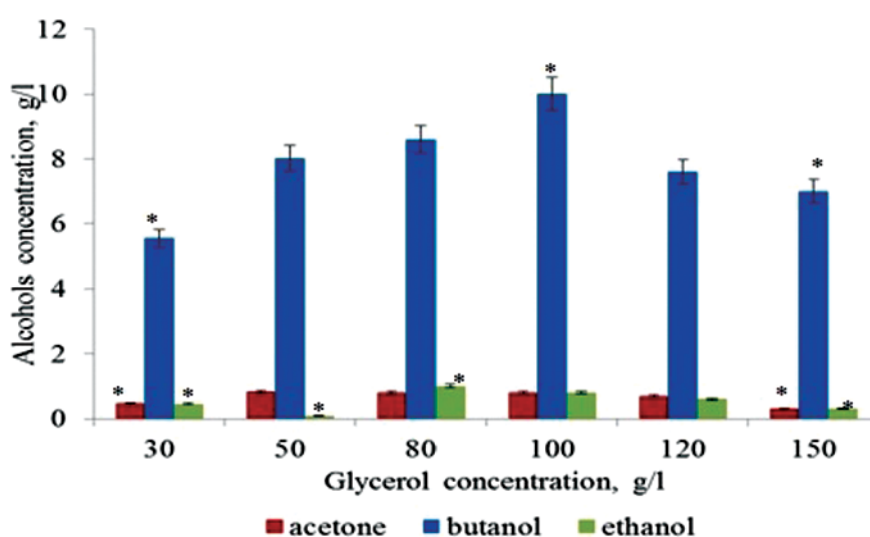


Fig. 2. Effect of glycerol concentration on the accumulation of alcohols by strain IFBG C6H

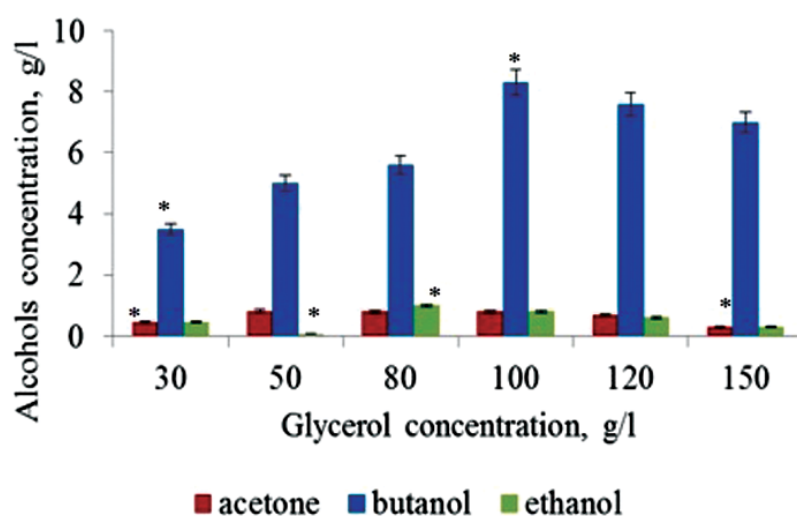


Fig. 3. Effect of glycerol concentration on the accumulation of alcohols by strain IFBG C6H 5M

Mutant strain IFBG C6H 5M had the changed properties concerning glycerol fermentation, compared with the original strain. Like with the original strain the most accumulation of butanol by strain IFBG C6H 5M was observed at concentrations of glycerol 100 g/l and exactly such concentration of glycerol was optimal for the development of culture before lyophilization. However, the accumulation of butanol by mutant strain IFBG C6H 5M was decreased to 8 g/l in comparison with the original strain IFBG C6H 10 g/l. From the analysis of the data and the results of [14], we can conclude that the strain

IFBG C6H 5M was characterized by higher cellulose activity and reduced bioconversion of glycerol.

So, composition of the protective medium (sucrose 10.0%; gelatin 10.0%; agar 0.02%) for lyophilization butanol-producing strains was optimized. The preservation of samples of freeze-drying cultures for 6 months at 4 °C does not effect on the productivity of strains. It is found that glycerol can be used as a carbon source for accumulation of butanol by cultures before lyophilization.

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ЕФЕКТ ЛІОФІЛІЗАЦІЇ НА ПРОДУКТИВНІСТЬ ШТАМІВ-ПРОДУЦЕНТІВ БУТАНОЛУ

О. О. Тігунова
Н. Є. Бейко
Г. С. Андріяш
С. Г. Прийомов
С. М. Шульга

ДУ «Інститут харчової біотехнології
та геноміки НАН України», Київ

E-mail: Shulga5@i.ua

Метою роботи було дослідити вплив ліофілізації на продуктивність штамів-продуцентів бутанолу. Для досліджень використовували штами-продуценти бутанолу; технічний гліцерол; біомасу дрогоподібного проса *Panicum virgatum* L. Ліофілізацію проводили за допомогою ліофільної сушарки. Досліджено вплив захисного середовища на залишкову вологість ліофілізованих культур залежно від концентрації глюкози і сахарози. Найнижчі показники залишкової вологості спостерігали у разі використання 10% -х глюкози та цукрози. Під час зберігання зразків ліофілізованих бактерій протягом 1 міс за 4 °С їхня продуктивність не зменшувалась. Із підвищенням температури зберігання продуктивність культур поступово знижувалась і за 30 °С значно зменшувалась. Таким чином, оптимізовано склад захисного середовища: сахароза — 10,0%; желатин — 10,0%; агар — 0,02% для ліофілізації штамів-продуцентів бутанолу. Показано, що зберігання зразків ліофілізованих бактерій упродовж 6 міс за 4 °С не впливало на продуктивність штамів. Встановлено, що для накопичення бутанолу культури перед ліофілізацією як джерело вуглецю можна використовувати гліцерол.

Ключові слова: бутанол, бактерії-продуценти, ліофілізація.

ЭФФЕКТ ЛИОФИЛИЗАЦИИ НА ПРОДУКТИВНОСТЬ ШТАММОВ-ПРОДУЦЕНТОВ БУТАНОЛА

Е. А. Тигунова
Н. Е. Бейко
А. С. Андряш
С. И. Приемов
С. М. Шульга

ГУ «Институт пищевой биотехнологии
и геномики НАН Украины», Киев

E-mail: Shulga5@i.ua

Целью работы было исследование влияния лиофилизации на продуктивность штаммов-продуцентов бутанолу. Для исследования использовали штаммы-продуценты бутанолу; технический глицерол; биомассу стеблевидного проса *Panicum virgatum* L. Лиофилизацию проводили с помощью лиофильной сушки. Изучено влияние защитной среды на остаточную влажность лиофилизированных культур в зависимости от концентрации в них глюкозы и сахарозы. Самый низкий показатель остаточной влажности наблюдали при использовании 10% -х глюкозы и сахарозы. При сохранении образцов лиофилизированных бактерий в течение 1 мес при 4 °С продуктивность не уменьшалась. С повышением температуры сохранения продуктивность культур постепенно понижалась и при 30 °С значительно уменьшалась. Таким образом, был оптимизирован состав защитной среды: сахароза — 10,0%; желатин — 10,0%; агар — 0,02% для лиофилизации штаммов-продуцентов бутанолу. Показано, что сохранение образцов лиофилизированных бактерий на протяжении 6 мес при 4 °С не влияет на продуктивность штаммов. Установлено, что для накопления бутанолу культуры перед лиофилизацией в качестве источника углерода можно использовать глицерол.

Ключевые слова: бутанол, бактерии-продуценты, лиофилизация.