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CHEMICAL MUTAGENESIS OF THE LYSINE-PRODUCING STRAIN *Brevibacterium* sp. IMV B-7447

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The aim of the work was to obtain a producer strain with increased lysine accumulation using the chemical mutagenesis method.

Methods. To achieve the goal, we used the method of treating the lysine-producing strain with the chemical mutagen N-methyl-N-nitro-N-nitrosoguanidine, cultivating the resulting clone and determining the accumulation of lysine in flasks and a bioreactor.

Results. The optimal concentrations and duration of mutagen action for the production of mutant microorganisms were found. Clones with the maximum lysine accumulation were selected. Mutagenesis was carried out consecutively three times. As a result, lysine-producing strain *Brevibacterium* sp. IMV B-7796 auxotrophic regarding leucine and threonine with maximum accumulation of the target amino acid was obtained.

Conclusions. The lysine producer *Brevibacterium* sp. IMV B-7796 was obtained, which produced 65.0 g/dm^3 of lysine in a bioreactor under conditions of periodic cultivation with feeding. The *Brevibacterium* sp. IMV B-7796 strain was proposed as a basis for the creation of a genetically modified strain with increased accumulation of lysine for further use in industrial lysine technology.

Key words: *Brevibacterium* sp., lysine, chemical mutagenesis, producer strain.

L-lysine is one of the essential amino acids, which is mainly used as feed additive. Traditional fodder crops such as corn, wheat or barley are low in lysine. The addition of 0.5% by weight of L-lysine increases the feed quality in the same way as the addition of 20% soybean meal [1]. The production of lysine globally was 2,200,000 tons per year as of 2014 [2]. As a feed additive, only L-form of lysine is effective, which is produced mainly by microbiological synthesis. In the microbiological production of α-amino acids and, in particular, lysine [3], the producer strains of *Brevibacterium* sp. (synonym of *C. glutamicum* [1]) are used.

Brevibacterium sp. are gram-positive, asporogenic, non-pathogenic bacteria, which by their nature are usually not capable of metabolites oversynthesis [2]. To further increase producer's productivity, traditional selection or genetic engineering are used. One of the ways to increase the strain's productivity is the selection of auxotrophic producers capable of oversynthesis of amino acids. Most of industrial lysine-producing strains are auxotrophic mutants [4, 5]. With the help of mutagenesis and selection, producers with auxotrophies for various amino acids, vitamins and resistance to antimetabolites were obtained. Producers with such mutations demonstrated a gradual increase in productivity [6, 7]. N-methyl-N′-nitro-N-nitrosoguanidine (NTG or MNNG) is one of the effective mutagens that leads to a change in the microorganism's phenotype, in particular, it induces auxotrophy and resistance to antibiotics [8–10].

Materials and Methods

The change in lysine accumulation by the producer strain *Brevibacterium* sp. IMV B-7447 [11] from the "Collection of microorganisms strains and plant lines for food and agricultural biotechnology of the State Enterprice "Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine" was studied for using a mutagen.

Cultivation conditions and mediums. The strain was grown in dipped meat-peptone agar enriched with glucose for 24 hours. Composition of enriched meat-peptone agar (MPAer.) (g/dm^3): nutrient broth — 23.0, glucose — 10.0, yeast extract — 5.0, agar — 30.0, distilled water, pH 7.0±0.1

For the selection of colonies and auxotrophic detection, a minimal medium (MM) with the following composition (g/dm^3) was used: glucose — 30.0, $(NH_4)_2SO_4$ — 10.0, KH_2PO_4 — 2.0, $MgSO_4$ — 0.4, agar — 30.0; with the antimetabolite S-(2-aminoethyl)-L-cysteine (AEC, thialisin) (Sigma, USA) or amino acids (leucine, isoleucine, threonine, homoserine, methionine).

As an inoculation medium, the following composition was used (g/dm^3): glucose — 70.0, corn extract (KE) — 30; KE hydrolysate — 40; chalk — 10. Subsequently, 10% (by volume) of the inoculum was added to the enzymatic medium. As an enzymatic medium, the following composition was used (g/dm^3): glucose — 120.0, KE — 35.0; salt hydrolysate KE — 45; $(NH_4)_2SO_4$ — 40; $MgSO_4$ — 0.5; KH_2PO_4 — 2.0 mg, biotin — 200 µg/ml, thiamine — 250 µg/ml, glucose, amino acids, and 2% dry sterile chalk were added after sterilization. Cultivation of bacteria was carried out for 72 h in a shaker-incubator "BIOSAN" ES-20 (Latvia) at a temperature of 30 °C and a speed of 240 rpm.

Cultivation of a lysine-producing strain in a bioreactor

Cultivation was carried out in a bioreactor "Sartorius Biostat B TWIN" (Germany) with a paddle stirrer and a flask volume of 10 dm^3. The inoculum (10% by volume) was introduced into the bioreactor to the enzymatic medium with the initial volume of the medium filling 30% of the bioreactor volume.

The inoculation medium composition (g/dm^3): molasses — 60, KE hydrolysate — 40, KE — 30, $CaCO_3$ — 10, tap water. A culture loop from slant agar grown for 24 h was added to the medium. One shoal was distributed into flasks (0.5 dm^3) containing 30 ml of inoculation medium. Cultivation was carried out in a shaker-incubator for 20–22 hours at a temperature of 30 °C and a speed of 250 rpm.

The enzymatic medium composition (g/dm^3): glucose — 120, KE hydrolysate — 45, KE — 35, $(NH_4)_2SO_4$ — 40; $MgSO_4$ — 0.5; KH_2PO_4 — 2.0, leucine — 0.7, biotin — 200 µg, thiamin — 250 µg, tap water. Glucose was added to the medium separately after sterilization. Biotin, thiamin and leucine were added to the sterile medium in the form of sterile solutions.

The carbohydrate nutrition composition: glucose, molasses, $MgSO_4$, $(NH_4)_2SO_4$, tap water. The growth nutrition composition: corn extract, KE hydrolysate, tap water, KH_2PO_4, $(NH_4)_2HPO_4$, solutions of biotin and thiamine. Ready feed in 2 dm^3 glass bottles (Simax, Czech Republic) was connected to the bioreactor. Ammonia water (25%) was used for maintain the pH, as a defoamer — "Propinol B400".

The inoculum was grown using orbital shaker-incubator for 24 hours, and 10% by volume was added to the bioreactor with the enzymatic medium (initial volume 3 dm^3). The optical density of the inoculum was 0.3 units, pH 5.6–5.8. Technological parameters of cultivation were as follows: stirrer revolutions — (700–800) rpm; temperature — 30 °C; pH 7.0; air supply — (1.0–1.2) vol/rpm.

Carbohydrate feeding was added depending on the drop in glucose level. Growth feeding was administered guided by cultivation parameters such as ECO_2 (concentration of carbon dioxide in the exhaust gas mixture) and pO_2 (oxygen concentration in the medium). pH was adjusted automatically with ammonia water. The defoamer was added when the foam amount in the bioreactor flask exceed the predetermined level.

Chemical mutagenesis. Bacterial suspension (cell titer 10^6–10^7) was prepared from daily shoal in sterile physiological solution. The suspension of the selected clones was kept in a shaker (220 min^{-1}) at a temperature of 30–32 °C for 5–30 min in a Tris-malate buffer (pH 6.0) containing 100, 200, 300, 400 and 500 µg/dm^3 NTG, respectively. Then the cells were washed in 0.1 M Tris-phosphate buffer with pH 7.2. The bacterial suspension obtained by the dilution method was sown in Petri dishes in the MC containing the lysine analog — S-(2-aminoethyl)-L-cysteine (AEC) at a concentration of 0.4 mg/dm^3. The number of surviving cells was determined by the number of brevibacteria strains colonies grown in MPAer.

Auxotrophy research was carried out according to the methodology [12], modified for the needs of bacterial producers and nutrient media. To determine the strains auxotrophy, a bacterial suspension was taken, which was prepared as follows: a two-day culture was selected from cultures of MPAer, and diluted in sterile physiological solution to a concentration of 1×10^5 colony-forming units (CFU)/dm^3, which corresponded to 0.5 units of optical density (OD). The obtained inoculum was transferred sterilely into: complete medium (MPAer), MM, MM with the investigated amino acid and MM with the investigated antimetabolite (MM+AEC), respectively. Auxotrophy was determined by the presence of bacterial growth on the selected medium.

The medium pH was determined using a pH-meter "pH-150" (RF). Determination of glucose concentration was carried out using Fehling's solutions according to the method [13]. The amount of ammonium nitrogen was determined according to the method [14]. OG was measured by a KFK-3 photoelectrocolorimeter (RF) in cuvettes with a size between the walls of d = 5 mm at a wavelength of 440 nm. The amount of amino acids was determined with the amino acid analyzer "AAA-400" (Ingos, Czech Republic). Cytological studies were performed using a microscope "Laboval4" (Carl Zeiss, Germany). Photographs were taken with a "Canon PowerShot A640" camera (Japan).

Statistical data processing was done using Microsoft Excel. All experiments were performed in three replicates. The difference between two mean values was considered significant at $P < 0.05$.

Results and Discussion

One of the ways to obtain high-performance lysine producers is to obtain regulatory mutants, which are selected by insensitivity of their homoserine dehydrogenase enzyme to threonine. S-(2-aminoethyl)-L-cysteine (thialysine) was used as a selective agent. The resulting mutants were resistant to AEC and had two regulatory mutations that disrupted the retroinhibition of both homoserine dehydrogenase and aspartate kinase. Mutation in the gene coding aspartate kinase synthesis resulted in the loss of sensitivity to the reciprocal inhibition by lysine and threonine. In this case, the maximum accumulation of threonine and lysine occurred [15]. The tendency to increase the rate of lysine accumulation in mutants was associated with inhibition of homoserine dehydrogenase and homoserine kinase activity, as well as insensitivity of aspartate kinase to retroinhibition [16].

Only those cells in which the mechanism of negative regulation of amino acid biosynthesis was disrupted, and which synthesized an excess of the target amino acid, survived and formed colonies on the minimal medium with the AEC antimetabolite; this served as a criterion for the selection of mutant clones.

The most efficient of chemical mutagens, NTG, creates alkylation of bases in the replication fork, and forms mutants with transitions, transversions, and deletions. Mutations are found in most lysine producers treated with NTG, that prevent inhibition of aspartate kinase activity through the feedback of the coordinated action of L-lysine and L-threonine (gene lysC) or reduce the activity of homoserine dehydrogenase and reduce the availability of L-threonine in cells and thus also reduce the activity of kinase (gene hom), which is important for the creation of genetically modified strains [9, 10].

Considering the above, we determined the effect of NTG on the cells bacterial suspension of the strain *Brevibacterium* sp. IMV B-7447 to increase the accumulation of lysine. The percentage of cells that survived was determined by the number of colonies that grew on MPAer with AEC. Cell survival under the influence of NTG varied depending on its concentration and duration of action. Within two minutes of NTG exposure at a concentration higher than 200 μg/dm^3, no living cells remained. The greatest mutagenic effect, in which cell survival ranged from 0.1 to 1%, was obtained using a concentration of NTG of 100 μg/dm^3 and an exposure time of 20 min (Fig. 1).

After cultivation of the obtained clones on MPAer medium, colonies of different colors (without pigments, yellow and pink) and sizes were obtained (Fig. 2), which were selected for further determination of lysine production.

After 72 hours cultivation on the enzymatic medium, the obtained clones were studied and analyzed. The lysine accumulation by the resulting colonies exceeded its accumulation by the original strain. Colonies with a pink color eventually returned (after 2–3 reseeding) to the original yellow color, which indicated the mutation instability. Further studies were carried out only for the colonies of yellow color according to the following scheme (Fig. 3).

NTG-1, NTG-2, NTG-3 clones were initially grown on the slant MPAer for 24 hours, then

a loop with the cells was transferred to the inoculation medium for cultivation. After 24 hours they were passed into the flasks with enzymatic medium to select the clone with the greatest lysine accumulation. Cultivation of clones in a glucose enzymatic medium was carried out under conditions of aeration and T = 31±1 °C for 72 h (Fig. 4).

All clones produced lysine, but NTG-3 had the maximum level of lysine accumulation (36.0 g/dm^3) after 72 h cultivation in flasks (medium with glucose).

Brevibacterium sp. NTG-3 clone obtained by chemical mutagenesis was an auxotroph for threonine and leucine, while the parent strain *Brevibacterium* sp. IMV B-7447 was an auxotroph for leucine and methionine.

The initial culture and the resulting clone were tested for sensitivity to antibiotics. Both strains have been shown to be susceptible to antibiotics such as azithromycin, ampicillin, ceftriaxone, benzylpenicillin, gentamicin, tetracycline, streptomycin, and kanamycin and resistant to chloramphenicol.

To establish the stability of the *Brevibacterium* sp. NTG-3 clone with a changed phenotype, the clone was passed (for two months with an interval of two weeks) on a solid and liquid medium, and the accumulation of lysine was determined. The production of lysine by the clone did not change during the passing and amounted to 36.0 — 36.2 g/dm^3. The *Brevibacterium* sp. NTG-3 culture was deposited in the Depository of the Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine as *Brevibacterium* sp. IMV B-7796.

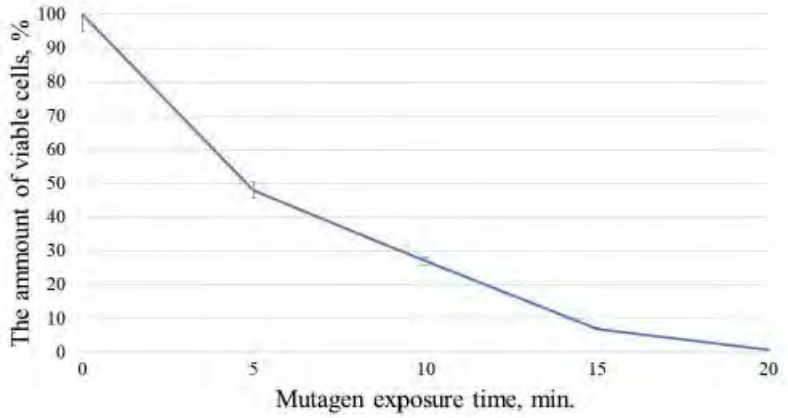

Fig. 1. **Cell survival of *Brevibacterium* sp. IMV B-7447 under the NTG influence at a concentration of 100 μg/dm^3**

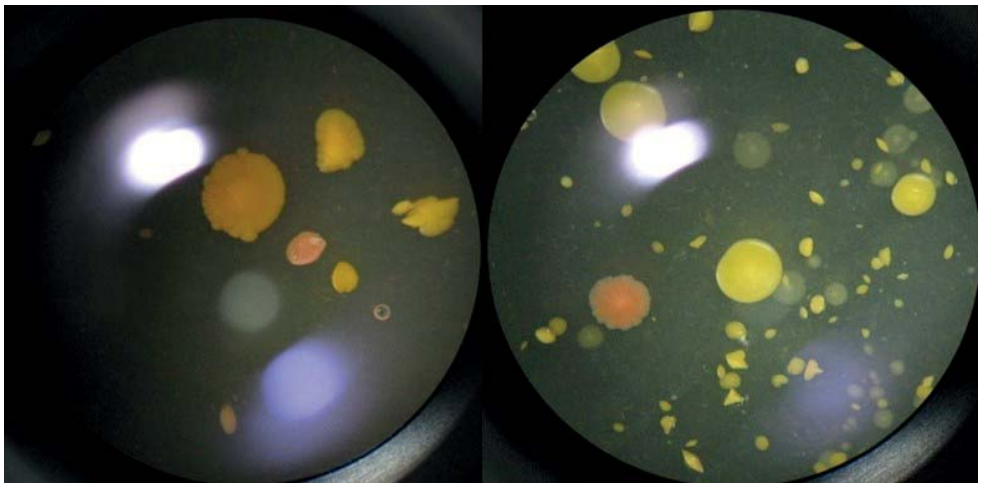

Fig. 2. **Colonies on MPAer medium after treatment with NTG mutant strain *Brevibacterium* sp. IMV B-7447**

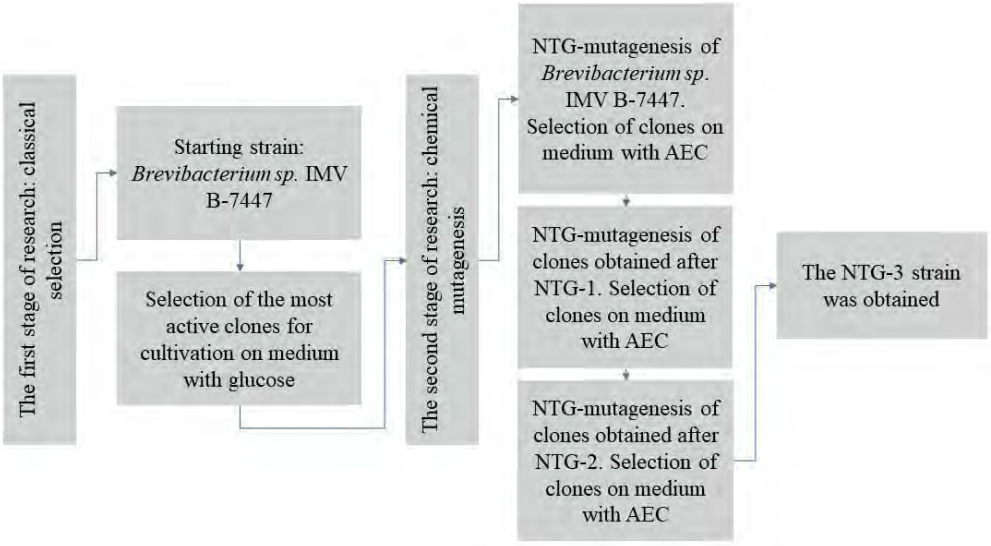

Fig. 3. **Scheme for the producing the strain with increased lysine accumulation**

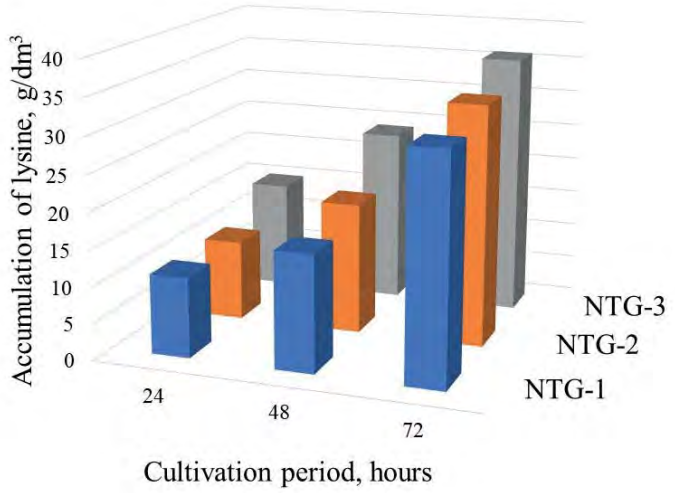

Fig. 4. **Dynamics of lysine accumulation by clones after chemical mutagenesis** ($P \leq 0{,}05$)

To develop the lysine technology and its further scaling, periodic cultivation with feeding was carried out in the Biostat B TWIN bioreactor.

During the cultivation of the strain, the accumulation of lysine in the culture medium was determined (Fig. 5).

On the first day of cultivation under periodic conditions, the producer strain used up to 25% of carbohydrates for biomass synthesis. Further, in spite of the background decrease in the growth rate, the cells actively synthesized lysine.

The rate of lysine accumulation by the producer strain *Brevibacterium* sp. IMV B-7796 was 0.96 g/dm^3/h. Within 69 hours, the strain accumulated 65±2 g/dm^3 lysine, and the bioconversion of carbohydrates was 48% . Further cultivation was not appropriate, the amount of lysine in the culture liquid decreased, which may be due to the use of lysine by the culture to accumulate biomass.

Conclusions

The effect of chemical mutagenesis (NTG) on cells of the *Brevibacterium* sp. IMV B-7797 strain was shown. Cell survival under the influence of NTG varied depending on its concentration and duration of action. Within two minutes of NTG exposure at a concentration higher than 200 μg/dm^3,

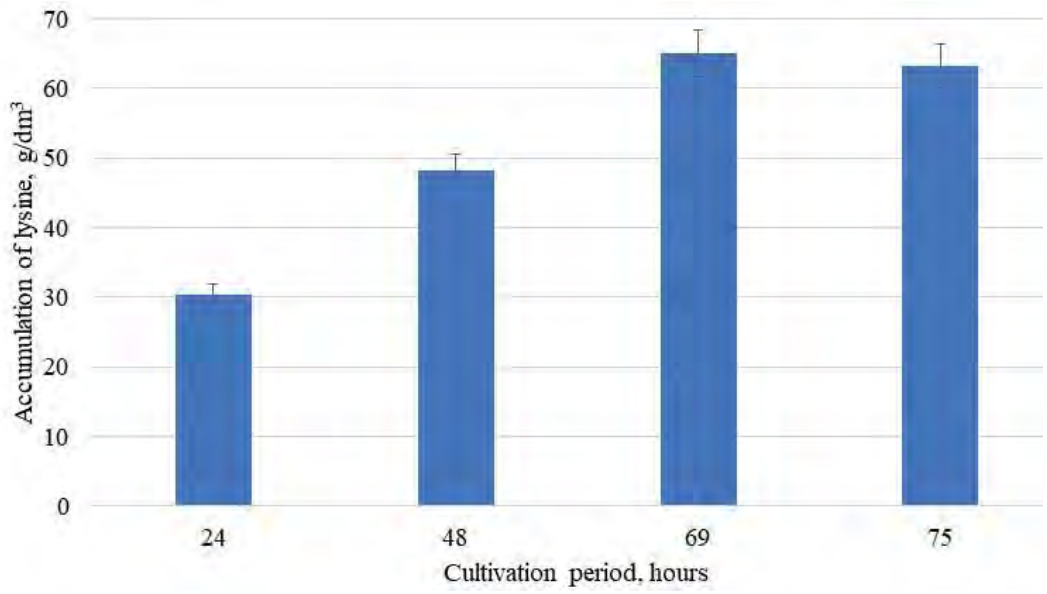

Fig. 5. **Accumulation of lysine by *Brevibacterium* sp. IMV B-7796 in a bioreactor**
Note: The initial concentration of lysine in the medium with the inoculum was 0.1 g/dm^3

no living cells remained. The greatest mutagenic effect was obtained using a NTG concentration of 100 μg/dm^3 and an exposure time of 20 minutes. In order to achieve the maximum accumulation of lysine, mutagenesis was carried out consecutively three times. A lysine-producing strain of *Brevibacterium* sp. IMV B-7796 with a lysine accumulation rate of 0.96 g/dm^3/h, which accumulated 65±2 g/dm^3 lysine within 69 hours, had a carbohydrate bioconversion of 48% under conditions of periodic cultivation with feeding. The strain *Brevibacterium* sp. IMV B-7796 is proposed to be used in the future to create a recombinant strain with increased accumulation of lysine, and to develop industrial lysine technology.

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This article contains no human or animal research conducted by any of the authors.

The authors declare no conflict of interest.

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ХІМІЧНИЙ МУТАГЕНЕЗ ШТАМУ-ПРОДУЦЕНТА ЛІЗИНУ *Brevibacterium sp.* ІМВ В-7447

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Мета роботи — отримати штам-продуцент із підвищеним накопиченням лізину за допомогою методу хімічного мутагенезу.

Методи. Для досягнення мети використовували метод оброблення хімічним мутагеном N-метил-N-нітро-N-нітрозогуанідином штаму-продуцента лізину з культивуванням отриманого клону та визначенням накопичення лізину в колбах і біореакторі.

Результати. Встановлено оптимальні концентрації та час дії мутагену для одержання мутантних мікроорганізмів і відібрано клони з максимальним накопиченням лізину. Мутагенез проводили послідовно тричі. Як результат, отримано ауксотрофний за лейцином та треоніном штам-продуцент лізину *Brevibacterium* sp. ІМВ В-7796 з максимальним накопиченням цільової амінокислоти.

Висновки. Отримано продуцент лізину *Brevibacterium* sp. ІМВ В-7796, що накопичував 65,0 г/дм^3 лізину в біореакторі за умов періодичного культивування з підживленням. Штам *Brevibacterium* sp. ІМВ В-7796 запропоновано для створення на його основі генетично-модифікованого штаму з підвищеним накопиченням лізину і подальшим використанням у промисловій технології лізину.

Ключові слова: *Brevibacterium* sp., лізин, хімічний мутагенез, штам-продуцент.