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DEVELOPMENT OF CULTIVATION TECHNOLOGY FOR THE *ESCHERICHIA COLI* RECOMBINANT STRAIN PRODUCING ARGININE DEIMINASE OF *MYCOPLASMA HOMINIS*

The Escherichia coli recombinant strain producing arginine deiminase of Mycoplasma hominis has been designed. Storage conditions that provide stabilization of most productive clones of the producer were found. Terms for cultivation of the arginine deiminase producer using bioreactors of different volume were optimized. Highly purified samples of arginine deiminase were obtained and their long-term storage conditions were selected.

Key words: arginine deiminase, producer, bacteria Escherichia coli, Mycoplasma hominis.

The normal mammalian cells can synthesize L-arginine from citrulline in reactions catalyzed by arginine succinate synthase and arginine succinate lyase. However, in some types of tumor cells of humans and animals, these enzymes are not expressed [1, 2], so the presence of L-arginine in the blood is a necessary condition for their growth. It is known that for tumor cells of melanoma, hepatocarcinoma, certain types of sarcoma and others the arginine deficit induces their death as a result of apoptosis [1, 3, and 4]. The enzymes driving the arginine degradation may cause a significant decrease in its level in the bloodstream and inhibit the growth of human cancer cells [4]. One of these enzymes is arginine deiminase (ADI) (EC3.5.3.6) which catalyzes the reaction of L-arginine hydrolysis into L-citrulline and ammonia. The ADI antitumor effectiveness has been proved *in vitro* and *in vivo* [5]. Also, the ADI has been established to be a suppressant of human immunodeficiency virus (HIV) replication [6]. The authors of [7] reported that ADI is about 100 times more effective as inhibitor of lymphatic leukemia cell proliferation in comparison with L-asparaginase used in clinical oncology.

For a long time, ADI high immunogenicity was an obstacle for its application. However, the enzyme covalent modification with the help of activated polyethylene glycol allowed the researchers to overcome this obstacle [8]. An ADI dosage of 20—640 a.u./m^2 has been established not to cause serious side effects and not to be toxic to the human body, in general. The leading cancer hospitals around the world have been conducting clinical trials of cancer enzyme therapy using ADI and got a significant therapeutic effect [9, 10]. However, very high price of enzyme drugs prevents wide use of ADI in oncological practice.

The methods for obtaining recombinant ADI from E. coli bacteria were described [11] and patented [12]. However, these methods provide for culturing the bacteria using rich LB medium, with enzyme expression being induced using a relatively expensive reagent, the isopropyl-β-thiogalactoside (IPTG). Therefore, an objective was to develop methods for culturing producers and inducing ADI synthesis, which would make the enzyme production cheaper.

MATERIALS AND METHODS

For obtaining a recombinant producer strain a pre-designed plasmid (derivative of pET32 vector which contains ADI optimized gene *M. hominis*) was injected into the cells of *Escherichia coli* BL-21 strain [11, 13]. Transformants were selected in LB rich medium (1.5 % bacterial peptone, 0.5% yeast extract, 1% NaCl; *pH* = 7) containing ampicillin (100 μg/ml) and agar (2 %). The recombinant bacteria were grown at a temperature of 28—37 °C in LB rich medium of the following mineral composition (g/l): Na_2HPO_4 — 7.1; KH_2PO_4 — 6.8; NH_4Cl — 2.7; Na_2SO_4 — 0.71; $MgSO_4x7H_2O$ — 0.45; glycerol — 12.5; glucose — 0.5; lactose (expression inducer) — 0.1; pantothenic acid — 0.002; thiamine — 0.002; pyridoxine — 0.002; and biotin — 0.002 [14].

The microelements were added in the following amount (g/l): $FeCl_3x6H_2O$ — 12.6; $CaCl_2$ — 2.2; $MnCl_2$ — 2.0; $ZnSO_4xH_2O$ — 2.9; $CoCl_2$ — 0.5; $CuCl_2x2H_2O$ — 0.5; $NiCl_2$ — 0.26; Na_2MoO_4 — 2.3; H_3BO_3 — 0.12; and Na_2SO_3 — 0.34 [15]; *pH* of the medium was controlled and additionally titrated up to 7.2.

When growing the producer strain clones in the LB rich medium the ADI gene expression was induced using a generally accepted method (with the help of IPTG); in the mineral medium the cells were induced by lactose [14, 15].

The cell biomass was measured by turbidity method at a wavelength of 600 nm. The cells were pelleted at 6000 *g* during 20 minutes; the cell paste was stored at a temperature of –20 °C.

The ADI activity was determined by the amount of citrulline formed during the reaction as described previously [11, 16]. The unit of activity is the amount of enzyme that makes 1 μmol of L-citrulline of 1 μmol of L-arginine for 1 minute. To estimate the ADI content the cells with 0.1—1 ml of culture were pelleted for 5 minutes at 7000 *g* in 1.5 ml polypropylene tubes. The cells were washed with twice distilled water, pelleted again and resuspended in 0.4 ml of 12.5% trichloroacetic acid. The samples were incubated at T= –20 °C for 0.5—2 hours and centrifuged at 12 000 *g* for 5—7 minutes at T = 4 °C. The precipitate was washed twice with 0.5 ml of cold 80% acetone. The supernatant was poured off; the precipitate was dried and added with 10 ml of water and in 10—15 minutes with 5 ml of 0.1% NaOH. The mixture was kept for 10—15 minutes more, periodically shaken and added with 15 μml of 2-fold buffer for application (120 mmol Tris-HCl, *pH* = 6.8; 4% SDS, 20% glycerol, 0.02% bromphenol blue, 2.5% β-mercaptoethanol). The samples were boiled in water bath for 8—10 minutes, cooled to room temperature, pelleted (at 12 000 *g* for 1 minute) and used for electrophoresis or stored at T = –70 °C.

The proteins were separated by electrophoresis in 10 % polyacrylamide gels by conventional method. Having been treated with dye (Coomassie Brilliant Blue R250) the gels were scanned and measured with the use of density meter. The ADI content was calculated on the basis of data obtained.

The enzyme was extracted as described earlier [16]. The protein concentration was determined by Lowry technique. The statistical analysis of results was performed using standard methods. All experiments were repeated three times with three parallel values of reproducibility in each version.

RESULTS AND DISCUSSION

Getting a Producer Strain

The ADI encoding gene, *M. hominis*, was amplified, modified, and cloned as described in [16]. The designed plasmid pET3d-ADI containing *M. hominis* ADI optimized gene controlled by T7 promoter was introduced into *E. coli* BL-21 cells using electroporation. The transformants were selected on a medium with ampicillin. Thus, ADI

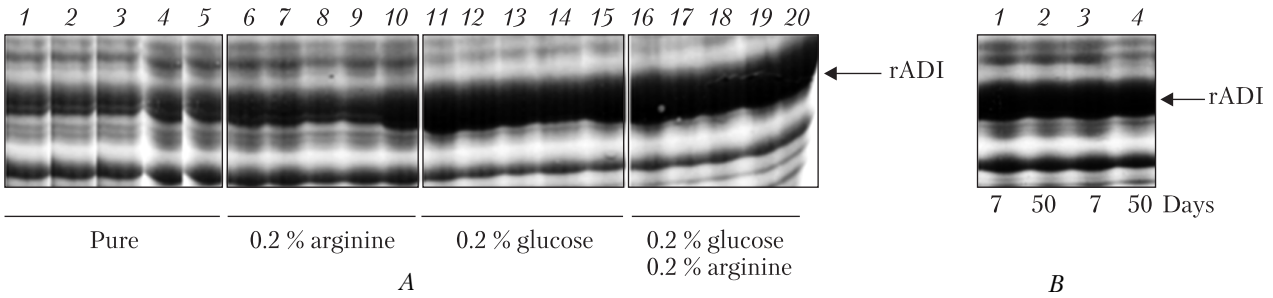

Fig. 1. Effect of arginine and glucose in the medium for selection and storage of producer clones on further ADI production in the mineral medium: A) analysis of production by ADI clones obtained in media with different admixtures (samples *1—5*: independent clones (transformants) obtained and stored on standard LB medium with ampicillin; samples *6—10*: independent clones (transformants) obtained on standard LB medium with ampicillin containing 0.2% arginine; samples *11—15*: independent clones (transformants) obtained on standard LB medium with ampicillin containing 0.2% glucose; samples *16—20*: independent clones (transformants) obtained on standard LB medium with ampicillin containing 0.2% arginine and 0.2% glucose); B) Analysis of ADI production by clones obtained in standard LB medium with ampicillin containing 0.2% arginine and 0.5% glucose (samples 1 and 3: independent clones (transformants) that were stored for 7 days at T = 5 °C; samples 2 and 4: the same independent clones (transformants) that were kept for 50 days at T = 5 °C)

producer colonies were obtained. Under conditions of IPTG induction they could accumulate enzyme in insoluble form as microbodies.

It should be noted that IPTG is quite expensive (10 g costs about 90 euros; induction of 1 liter of culture costs about 1 euro) which significantly increases the cost of ADI production. Since the cost of rich medium used in this case is also quite high, it has been decided to study possibilities of using a cheaper mineral medium in which natural sugar, lactose, can be a cheap inductor [14, 15].

At the initial stage, the fermentation was carried out in flasks. A suspension of cells grown in LB rich medium with ampicillin up to A_{600} = 1.0–2.5 was inoculated to mineral medium containing 0.4% solution of glycerol up to an optical density of A_{600} = = 0.3; 50 ml culture was incubated in 250 ml flasks on a shaker (200 rev./min at T = 30 °C) during 36—40 hours at T = 30 °C. Lactose was added up to concentration of 0.05; 0.1 and 0.5 g/l. These experiments showed that given the cost of reagents, lactose concentration of 0.1 g/l is optimal for auto-inductive ADI production (the data are not presented). The level of induction of ADI synthesis in the case of mineral medium with lactose (auto-induction) was slightly lower (by 10—20%) as compared with 20—25% of the total protein in the case of IPTG induction. However, the number of cells from the mineral medium was significantly greater than that of from the LB rich medium. It is obvious that the protein expression auto-induction method proposed earlier can be used as a basis, but the maximum and stable ADI production can be achieved only after the selection of optimal expression conditions.

To achieve a higher output of targeted heterologous protein it is necessary to stabilize highly effective producer strain clones and to study the effect of glycerol concentration in the medium, as well as the effect of aeration, time, and temperature of cultivation.

Stabilization of Producer Strain Based on ADI Induced Synthesis

When storing producer strain in LB agar medium during seven days at T = 4 °C the majority of clones induced by lactose showed a significant decline in ADI production (Fig. 1, A). In cell-free producer extracts stored under these conditions there was reported the ADI activity that could be caused by basal gene expression. As a result, this could lead to a decrease in the concentration of available arginine and to decreased viability of highly clones.

It was assumed that the addition of arginine or glucose which reduces the basal level of cloned

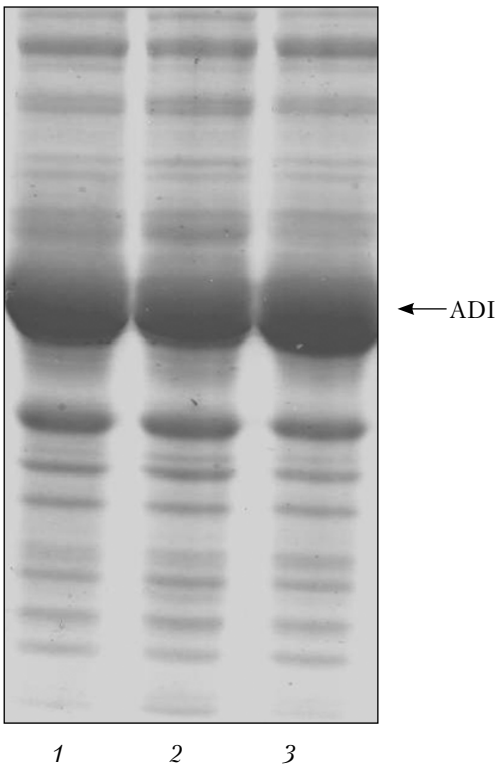

Fig. 2. Analysis of ADI production for various density of inoculation of producer strain cells in mineral medium: *1*) A_{600} = = 0.01; *2*) A_{600} = 0.1; *3*) A_{600} = 0.3

gene expression to the clone cultivation and storage medium would make it possible to raise the stability of highly productive clones and to maintain high effectiveness of ADI synthesis auto-induction. It was found that the addition of arginine to the medium provided better storage of highly productive clones, but only some clones had a high level of stabilization. In most cases (70—80% of clones tested) this effect was not pronounced enough (Fig. 1, A), while the addition of glucose led to the fact that the vast majority (80—90% of the tested clones) retained the ability to induce ADI synthesis during seven days of storage at these conditions (Fig. 1, A). However, during the further storage of clones within 2—3 weeks the level of ADI synthesis induction decreased (data are not showed). When storing highly productive clones in a medium with 0.2% arginine and 0.2% glucose the level of ADI synthesis was established to be a slightly higher as compared with the clones kept only with glucose (Fig. 1, A).

However, during the further storage, as in the previous cases, the level of ADI synthesis induction decreased (data are not showed). This problem was solved by increasing the concentration of glucose in

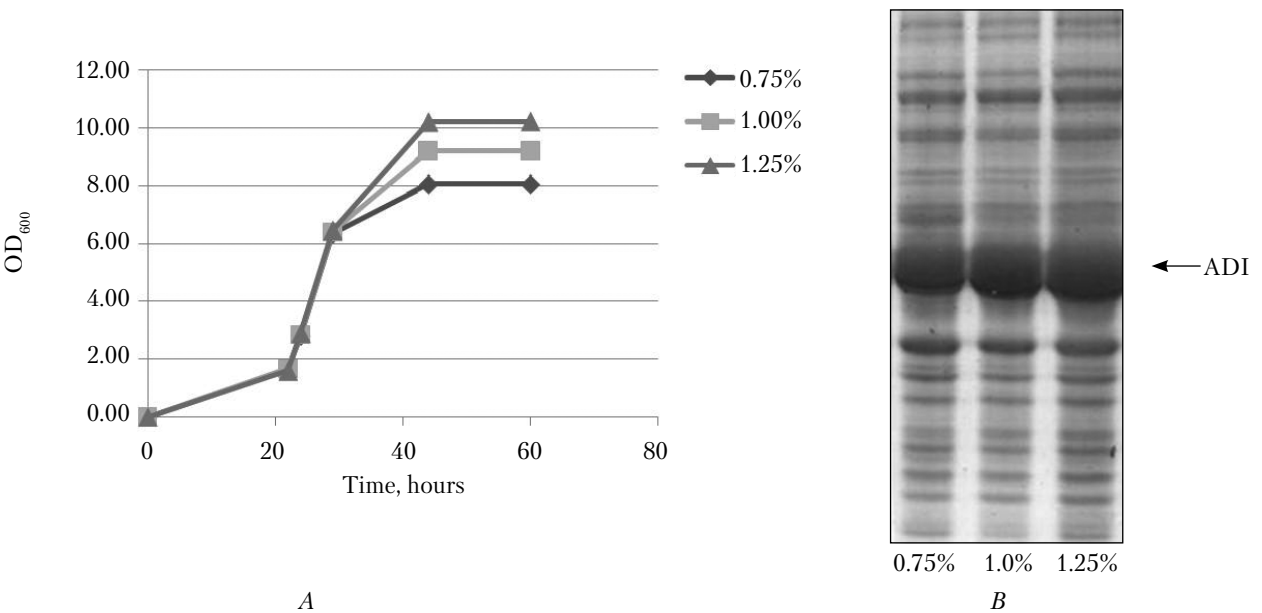

Fig. 3. Effect of glycerol concentration on producer biomass yield (*A*) and on induction of ADI synthesis (*B*)

the medium for up to 0.5%. In LB rich medium with ampicillin, 0.2% arginine, and 0.5% glucose, the clones stored at T = 4 °C during 50 days have been established to be capable of doing effective auto-induction of ADI synthesis (Fig. 1, B).

Optimization of Periodic Cultivation and Induction of ADI Synthesis

Under the above conditions of culturing the producer, the effect of temperature on the level of targeted protein expression has been studied as well.

The deviation from the optimal temperature (32 °C) by 4—5 °C has been found to lead to a decrease in production of targeted protein by 20-25% (data are not presented). It has been established that as concentration of main carbon source (glycerol) increases the producer grows better. The experiments showed that concentration of glycerol increased from 0.75 to 1.25% it was possible to get 1.2—1.3 times more cells without reducing ADI production (Fig. 2). The further increase in concentration of glycerol did not yield positive results.

It should be noted that in these experiments the maximum ADI output was observed in 30—35 hours of cultivation, whereas that of biomass was reported in 38—46 hours after the start.

When culturing the highly productive clones the pH level was controlled. As pH downed below 6.0 a significant decline in ADI production was reported. It should be noted also that increase in aeration by escalating intensity of mixing (250 rev./min) led to an increase in the output of targeted protein, with subsequent growth in frequency of mixing having no effect on the ADI output (data are not showed). For the purpose of scaling and simplification of technology for getting ADI we have studied the enzyme production using the cells together with medium (without precipitation) and different densities of inoculation in mineral medium (A_{600} = 0.01—0.3) (see Fig. 3).

The results showed that the volume of inoculum can be reduced down to 0.3—0.5% of the volume of fermentation without a decrease in ADI output, with the cells not having to be separated

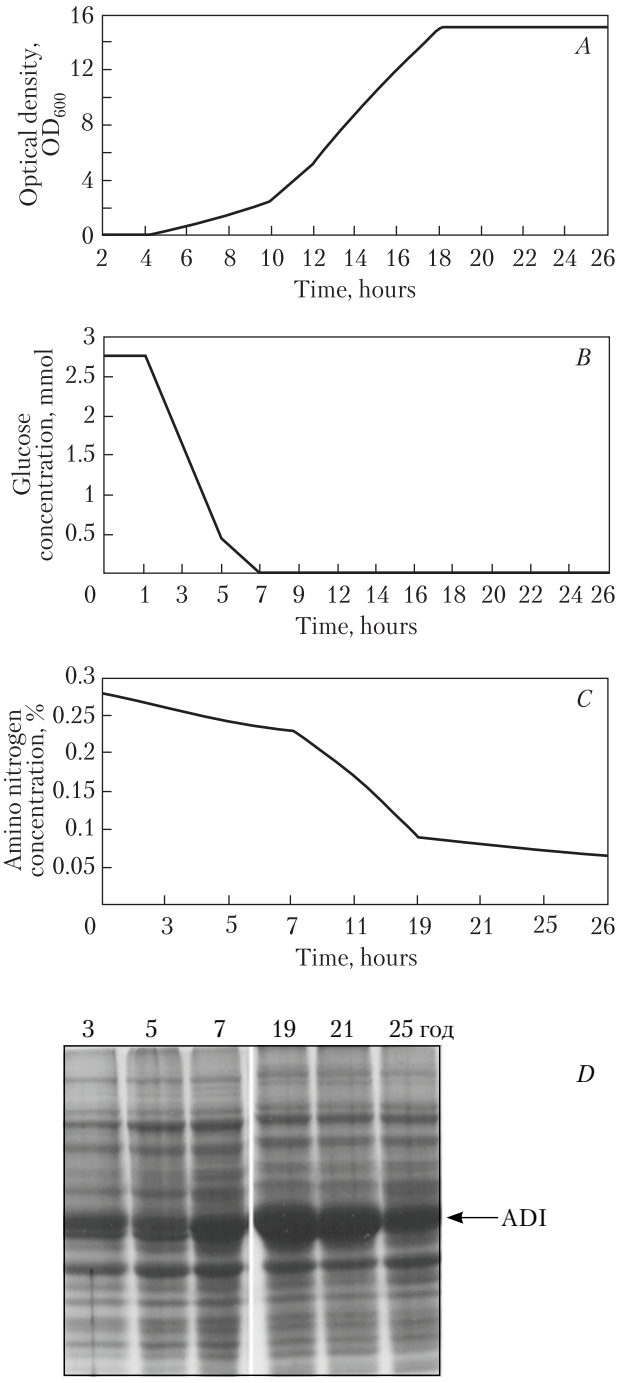

Fig. 4. Growth of producer strain and ADI production in laboratory fermenter (1 l): *A*) optical density of culture at a wavelength of 600 nm; *B*) concentration of glucose; *C*) concentration of amino nitrogen; *D*) electrophoregram of cell-free producer extracts (ADI position is indicated with arrow)

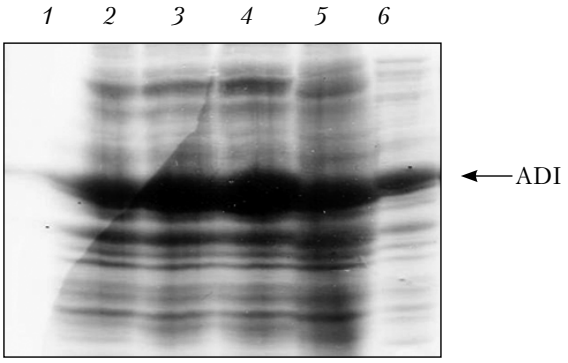

Fig. 5. Electrophoregram of cell-free ADI producer extracts obtained by *Enzyme*, PJSC: *1*; *6* — inoculate; *2* — 12th hour of fermentation; *3* — 14th hour of fermentation; *4* — 16th hour of fermentation; 5 — the same cells were kept before extraction at T = 15 °C for 5—6 hours (ADI position is indicated with arrow)

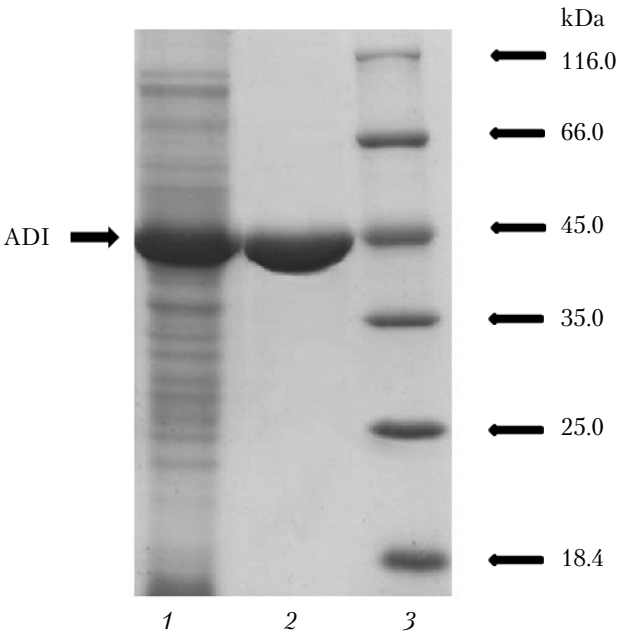

Fig. 6. Electrophoregram of ADI samples before and after purification: *1* — total protein of producer induced cells; *2* — ADI sample after purification; *3* —markers of molecular mass chromatography

from the previous medium. This greatly improves the sterility of manipulation and facilitates the scaling of fermentation process.

As a result of the optimization of medium and conditions of culturing the producer strain an increased cell biomass yield (12—14 g/l) and a high level of ADI expression (about 25% of total protein) have been obtained. Given the cost of reagents, the cost of 1 liter of used mineral medium with lactose is assumed to be 3 times less than that of LB medium with IPTG inducer. The results obtained at these stages were used as a framework for the scaling of producer biomass growth.

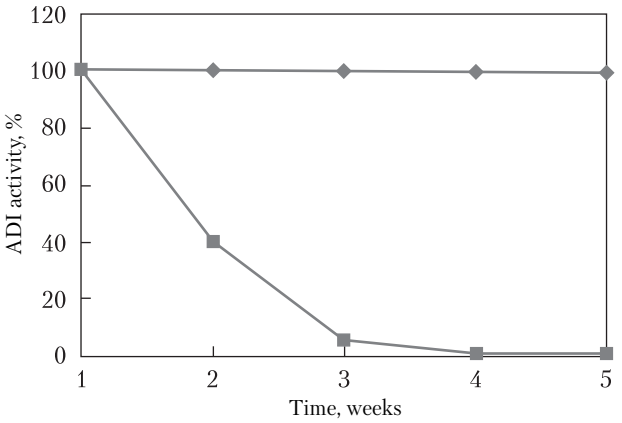

Fig. 7. Dynamics of activity of ADI sample during its storage at T = 4 °C: ■ — with 1 M NaCl added; ◆ — without NaCl

Optimization of Conditions for Culturing the ADI Producer Strain in Bioreactors

At the next stage of research we tested the selected conditions by cultivating producer strain in laboratory fermenter having a volume of 1 liter. The volume of medium in the fermenter was 0.6 liters. The initial concentration of cells (inoculation) was A_{600} = 0.01. The cultivation temperature was 32 °C, the frequency of mixing was 600 rev./min., and the concentration of glycerol accounted for 1.25%.

Under the above conditions, the optimal aeration rate was 0.7 l/min. The results of one of standard experiments are showed in Fig. 4. One can see that glucose was completely utilized at the beginning of the exponential growth phase (Fig. 4, B). The use of fermenter allowed the researchers to achieve a greater producer biomass yield, as well as a higher growth rate in comparison with flasks (Fig. 4, A). As a result, the stationary phase was

reached after 17—19 hours of incubation. Maximum ADI production was observed with this period of growth (Fig. 4, D). Transition of culture to the stationary phase coincided with a significant decrease in concentration of available amine nitrogen in the medium (Fig. 4, B).

Scaling of ADI Producer Growth in Industrial Environments

The process was scaled further on the equipment of *Enzyme*, PJSC (Ladyzhin, Vinnytsia Oblast). The capacity for growth and ADI induced synthesis was checked during the growth in bioreactor having a volume of 100 liters. The initial volume of medium was 70 liters.

The conditions of growing in the 100-liter bioreactor were similar to the conditions of fermentation in 1-liter bioreactor at the laboratory. The duration of fermentation was 16—20 hours (Fig. 5). As one can see from the figure, the maximum level of ADI production was observed on the 16th hour of incubation. This time corresponded to the beginning of transition of culture to the stationary phase. The grown cells were pelleted and frozen. In further experiments, the micro-bodies of inclusions containing ADI were received from these cells; the micro-bodies were solubilized, denatured and renatured as described in [16]. The received ADI samples were purified by the ion exchange chromatography on Q-sepharose and the hydrophobic chromatography on phenylsepharose as described in [16]. The enzyme sample was concentrated by ultrafiltration on membrane *VIVASPIN TURBO*-15-10000 (*Sartorius*) and stored at $T = 4$ °C.

Thus, the highly purified ADI samples with a specific activity of 30—34 U/mg (Fig. 6) have been obtained. These properties correspond to the best examples described in the literature [8, 10, 11, and 12]. The enzyme yield did not exceed 35—40% of the total activity obtained at the stage of renaturation. The ADI samples were stored at T = 4 °C, in 20 mmol of potassium-sodium phosphate buffer with 1 mol of NaCl.

As described earlier, the enzyme is stable under these conditions. As NaCl was removed from the buffer the enzymatic activity was lost rapidly (Fig. 7).

CONCLUSIONS

Highly productive clones of recombinant *E. coli* strain which produces ADI have been obtained. The designed producer has been showed not to lose the capacity for ADI overexpression during storage for 50 days at T = 4 °C in LB medium with 0.2% arginine and 0.5% glucose. The mineral environment and conditions for enzyme expression induction using a cheap inductor, lactose, have been optimized. An increased producer strain biomass yield (12—14 g/l) and high level of ADI expression (about 20—25% of total protein) has been obtained.

The conditions for culturing producer strain in bioreactors of various volumes have been optimized. Highly purified long-storage ADI preparations which preserve their enzymatic activities have been obtained and presented.

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РАЗРАБОТКА ТЕХНОЛОГИИ КУЛЬТИВИРОВАНИЯ РЕКОМБИНАНТНОГО ШТАММА-ПРОДУЦЕНТА *ESCHERICHIA COLI* С ЦЕЛЬЮ ПОЛУЧЕНИЯ АРГИНИНДЕЗИМИНАЗЫ *MYCOPLASMA HOMINIS*

Сконструирован рекомбинантный штамм кишечной палочки *Escherichia coli*, продуцирующий аргининдезиминазу бактерий *Mycoplasma hominis*. Подобраны условия для стабилизации высокопродуктивных клонов штамма-продуцента фермента. Проведено оптимизацию условий культивирования штамма-продуцента в биореакторах различного объема. Получены высокоочищенные препараты аргининдезиминазы и подобраны условия их длительного хранения.

Ключевые слова: аргининдезиминаза, штамм-продуцент, бактерии *Escherichia coli*, *Mycoplasma hominis*.

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РОЗРОБКА ТЕХНОЛОГІЇ КУЛЬТИВУВАННЯ РЕКОМБІНАНТНОГО ШТАМУ-ПРОДУЦЕНТА *ESCHERICHIA COLI* З МЕТОЮ ОТРИМАННЯ АРГІНІНДЕЗІМІНАЗИ *MYCOPLASMA HOMINIS*

Сконструйовано рекомбінантний штам кишкової палички Escherichia coli, що продукує аргініндезіміназу бактерій Mycoplasma hominis. Підібрано умови для стабілізації високопродуктивних клонів штаму-продуцента фермента. Проведено оптимізацію умов культивування штаму-продуцента в біореакторах різного об'єму. Отримано високоочищені препарати аргініндезімінази та підібрано умови їх довготривалого зберігання.

Ключові слова: аргініндезіміназа, штам-продуцент, бактерії *Escherichia coli*, *Mycoplasma hominis*.

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