**SECTION 11. Biology. Ecology. Veterinary.**

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# DESIGNING OF L-PHENYLALANINE-AMMONIA-LYASE STRAIN-PRODUCER ON THE BASIS OF CELLS *ESCHERICHIA COLI*

*The purpose of this study is to design of L-phenylalanine-ammonia-lyase strain-producer on the basis of cells Escherichia coli.*

Keywords: enzyme, phenylalanine, genes.

**Introduction.**

L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the reversible deamination reaction of amino acid L-phenylalanine to trans-cinnamic acid and ammonia [1, p. 22]. The enzyme is of interest as a therapeutic agent for the treatment of phenylketonuria, it can be used for direct treatment of phenylketonuria and for the production of full value food products that does not contain phenylalanine [2, p. 2340; 3, p. 417].

Earlier attempts were made to clone the L-phenylalanine ammonia-lyase gene pal in the cells of E. coli; but they can not be considered successful, since the level of expression of this gene did not exceed 20-25% [4, p. 208-210; 5, p. 190-192; 6, p.316-318], which prevents the use of a strain on an industrial scale.

The purpose of this study is to design of L-phenylalanine-ammonia-lyase strain-producer on the basis of cells *Escherichia coli*.

**Materials and methods.**

**Reagents.**

Acrylamide, N'N'-methylene-bisacrylamide, sodium dodecyl sulfate (SDS-Na), bromophenol blue, glycogen, glycerol, 2-mercaptoethanol, ammonium persulfate, Tween 20, Triton X-100, hydroksymetylaminometan (Tris), N,N,N',N' - tetramethylethylenediamine (TEMED), ethylenediaminetetraacetic acid (EDTA), glucose, agarose, ethidium bromide, bovine serum albumin (BSA), deoxyribonucleosides 5'-triphosphates, mineral oil, proteinase K, isopropyl-β-D-1-thiogalaktopiranozid (IPTG), lysozyme, yeast extract, Bacto-tryptone, agar, phenol, lysozyme, chloroform, ethanol, acids, alkalis, salts, LB-Wednesday, kanamycin sulfate, restriction endonucleases NcoI and HindIII, T4 DNA ligase, Pfu-pol, Taq-pol.

**The bacterial strains.**

For expression were selected strains BL21 (DE3) / pLysECodonPlus RP and Rozetta (DE3) (Stratagene).

**Plasmid vector.**

For protein expression in cells of *E. coli* vector pET28a was used. The synthesis of the gene was carried out according to the procedure described in [7, p. 107-118, 205-224] on an automated sequencer according to the sequence of the gene ABI3730xl *pal*, isolated from *Rhodosporidium toruloides*. The synthesis was carried out in such a way that it contained at their ends restriction sites NcoI and HindIII and was used for amplification and subsequent insertions into the polylinker region of the gene pET28a.

**Methods.**

Amplification of *pal* gene was performed using polymerase chain reaction (PCR). Development of oligonucleotide primers for amplification of the gene was performed using the program OLIGO (version 3.3), taking into account data on the primary structure of the gene *pal*. As a template for amplification of the coding region of the gene *pal* from *R. toruloides* used the sequence of the gene from the databases of GenBank (X12702.1). The primers contained 5'-ends of the additional sequences comprising restriction sites for NcoI forward primer and HindIII for the reverse, and were designed for amplification and subsequent insertions of the structural gene into expression vector pET28a polylinker at appropriate sites. The reverse primer was designed so that the resulting amplicon did not contain a stop codon, and protect the docking reading frames and gene sequences of His6.

Polymerase chain reaction was performed in 20-50 ml of the solution prepared by tenfold buffer for Taq-polymerase, which contained 200 mM of each deoxynucleoside triphosphates, 0,5 mM primer, 2 mM $MgSO_4$, 10 ng of the matrix, 2 units of Taq DNA polymerase and 0,1 units of Pfu DNA polymerase. Analysis of PCR products was performed by electrophoresis in 1% agarose gel.

Sequencing was carried out by the method of Sanger according to the manufacturer's protocol automatic sequencer ABI3730xl company Applied Biosystems (USA), using kits for sequencing BigDye ® Terminator v3.1 Cycle Sequencing Kit.

Preparation of competent cells of *E. coli* and their transformation with plasmid DNA was performed using standard techniques [7, p. 241-244].

Electrophoresis of cell lysates and proteins were performed using the disc-electrophoresis in 10% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions by reducing Lemmli.

Protein concentration was determined spectrophotometrically at 280nm.

Determination of activity of the protein carried out on spectrophotometer «DU 800» (Beckman Coulter).

**Results and discussion.**

**Cloning of the gene *pal* in cells of *E. coli*.** Due to the complexity of the genomic organization of the gene *pal* (six introns) was synthesized according to the sequence of the gene *pal*, isolated from *Rhodosporidium toruloides* (GenBank: X12702.1). The gene was treated with restriction endonucleases NcoI and HindIII to produce sticky ends.

To clone was selected expression vector pET28a, designed for expression of recombinant proteins in *E. coli* and containing in the structure gene for resistance to kanamycin. In addition, near the polylinker vector contains a sequence encoding a His-Tag an end, which greatly facilitates chromatography on Ni-containing media. In preparation for the cloning vector was treated with restriction endonucleases NcoI and HindIII and purified from the reaction products using a set QuickClean. After hydrolysis by restriction endonucleases the vector appeared sticky ends complementary ends of the gene *pal*.

**Expression of *pal* gene *Rhodosporidium toruloides* in cells of *E. coli*.** In terms of induction in cells of *E. coli* BL21 (DE3) / pLysECodonPlus RP and Rozetta (DE3), transformed with plasmid pETPAL-28a gene L-phenylalanine ammonia-lyase *Rhodosporidium toruloides*, the accumulation of the protein with a molecular weight close to that calculated for the PAL-6xHis, which is equal to 78,4 kDa (Fig.1). In the cells of the control strain that does not contain the gene *pal*, this protein is absent. This allows to conclude about high expression of the target gene. In addition, analysis of cell fractions suggests that the protein is almost entirely in soluble form.

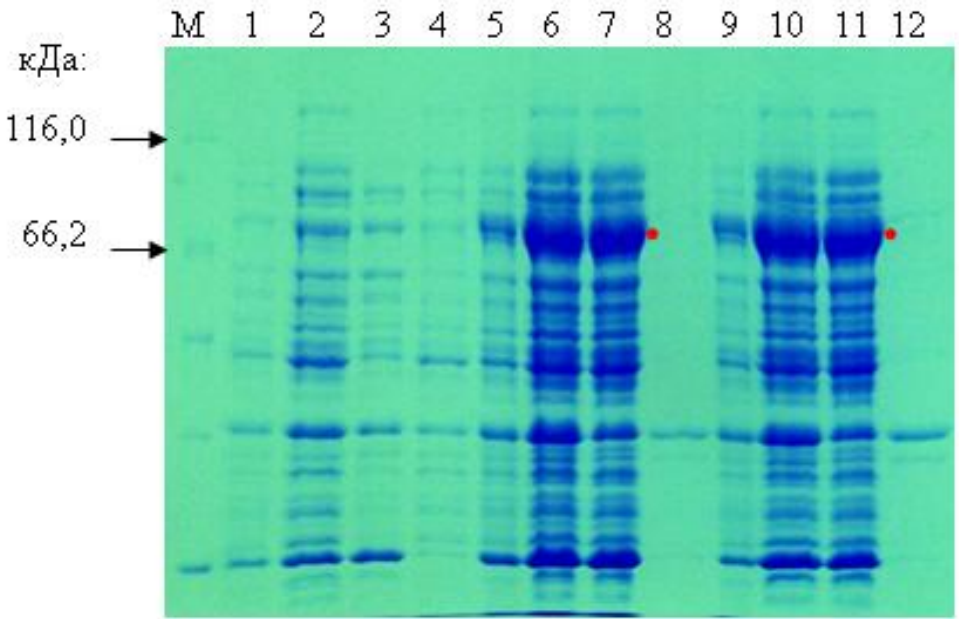


**Figure 1. The expression of PAL in *E. coli*. M - molecular weight marker;**
**1–4 - BL21(DE3) pLysE CodonPlus RP/pET-28a:**
**1 - total cellular protein before induction,**
**2 – total cellular protein 24 h after induction,**
**3 – clarified lysate of cells 24 h after induction,**

**4 – insoluble fraction of protein 24 h after induction;**
**5–8 - BL21(DE3) pLysE CodonPlus RP/pETPAL-28a:**
**5 - total cellular protein before induction,**
**6 – total cellular protein 24 h after induction,**
**7 – clarified lysate of cells 24 h after induction,**
**8 – insoluble fraction of protein 24 h after induction;**
**9–12 - Rozetta (DE3)/pETPAL-28a:**
**9 - total cellular protein before induction,**
**10 – total cellular protein 24 h after induction,**
**11 – clarified lysate of cells 24 h after induction,**
**12 – insoluble fraction of protein 24 h after induction**

Protein concentration was determined spectrophotometrically at $A_{280}$. As a result of the measurement of protein concentration in the product was 13 mg/ml. Based on this fact we can conclude that the yield of the target protein in one-step purification of approximately 130mg/l, activity of 3,3 U/mg.

**Conclusion.**

Producer strains of recombinant protein L-phenylalanine ammonia-lyase with a maximum productivity of 40±1% of total cellular protein were obtained. The yield of target protein in one-step purification is approximately 130 mg/l with a specific activity of 3,3 U/mg.

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