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EXPRESSION OF RECOMBINANT L-PHENYLALANINE AMMONIA-LYASE IN ESCHERICHIA COLI

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Abstract: The *pal* gene coding for L-phenylalanine ammonia-lyase of *Rhodosporidium toruloides* (GenBank entry no. X12702.1) with optimized sequence was cloned into an expressing vector pET28a. Three parameters of expression (inductor type, duration, and temperature of induction) were optimized, which resulted in a strain producing recombinant L-phenylalanine ammonia-lyase with the maximal productivity, that is, 35 ± 1% to total cell protein, upon utilization of 0.2% lactose (according to Studier) induction during 18 h at 37°C. The recombinant L-phenylalanine ammonia-lyase was found to be insoluble by 99%. Solubility of the protein did not improve upon utilization of 1 mM IPTG as an inductor instead of 0.2% lactose, or upon bacterium cultivation at various temperatures, that is 25°C and 37°C.

Keywords: L-phenylalanine ammonia-lyase, cloning, expression, recombinant protein, induction, L-phenylalanine, phenylketonuria

INTRODUCTION

L-Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the reaction of reversible deamination of L-phenylalanine to *trans*-cinnamic acid and ammonia [1]. PAL is the key enzyme of phenylpropanoids metabolism in plants and fungi, where it is involved in biosynthesis of secondary metabolites (flavonoids, furanocoumarins, and components of cell wall) and exists in multiple isoforms [2]. First three-dimensional structure of PAL from the yeast species *Rhodosporidium toruloides* has been determined at 2.1 A resolution. Molecular weight of PAL is 76880 Da. Molecule of the enzyme contains 716 amino acid residues. Typically, optimal pH values for PAL are in the range of 8.2 to 9.0. Optimal temperature lies within the range of 35 to 55°C in function of the enzyme source [3].

Enzyme isolation is of interest for application as a therapeutic agent in treatment of phenylketonuria; it may be used directly as a drug in phenylketonuria therapy or in production of phenylalanine-free food [1, 2]. In addition to medicinal applications, PAL may be used in biotechnology to produce L-phenylalanine from *trans*-cinnamic acid [3].

Application of *Escherichia coli* strain producing recombinant L-phenylalanine ammonia-lyase as a source of the enzyme in industry seems promising [4, 5].

Escherichia coli is one of the most efficient and simple ways of large-scale production of recombinant proteins in view of the well-studied genetics of the microorganism, availability of convenient expression vector systems and host strains, simple use, low price, and high levels of target gene expression reaching 40–45% to the total cell protein [5, 6].

The aims of the present work were cloning of L-phenylalanine ammonia-lyase gene and its expression in *E. coli* cells, as well as characterization of the expression product.

MATERIALS AND METHODS

Reagents. Acrylamide, *N',N'*-methylene-bisacrylamide, sodium dodecylsulfate (SDS), bromophenol blue, glycogen, glycerol, 2-mercaptoethanol, ammonium persulfate, Tween 20, Triton X-100, Tris(hydroxymethyl)aminomethane (Tris), *N,N,N',N'*-tetramethylethylenediamine (TEMED), ethylenediaminetetraacetic acid (EDTA), and glucose from Serva (Germany); agarose, ethidium bromide, bovine serum albumin (BSA), deoxyribonucleoside 5'-triphosphates, mineral oil, protease K, isopropyl β-D-1-thiogalactopyranoside (IPTG), and lysozyme from Sigma (United States); yeast extract, bacto-tryptone, and agar from Dafco (United Kingdom). Phenol, lysozyme, chloroform, ethanol, acids, alkalis, and salts (analytically and chemically pure grades) from Reakhim (Russia); LB medium from Gibco BRL (United States); kanamycin sulfate from Sintez (Kurgan, Russia); restriction endonucleases NcoI and HindIII, T3 DNA ligase, Pfu-pol, and Taq-pol from Sibenzim (Russia).

Bacterial strains. Cells of *E. coli* strain BL21[DE3]Star (Invitrogen, United States) of F- ompT hsdSB (rB-mB-) gal dcm rne131 (DE3) phenotype

containing λDe3 lyzogene and rne131 mutation in the genome were used for the target protein expression. Mutant gene rne (rne131) codes for a shortened RNAase E form, which decreases intracellular degradation of mRNA leading to increase in its enzymatic stability.

Plasmid DNA. Vector pET28a, containing promoter for T7 phage polymerase, lac-operon, ribosomal complex binding site (RBS), starting codon for translation of the cloned fragments, and a polyhistidine-tag fragment within the reading frame, was used for expression in *E. coli* cells. Any nucleotide sequence cloned in the vector is expressed as a protein fused with polyhistidine for convenience of further purification by immobilized metal affinity chromatography.

Gene synthesis was performed in such a way that it would contain restriction sites NcoI and HindIII for amplification and further insertion into the gene fragment of polylinker pET28a.

Amplification of the pal gene was performed by the method of polymerase chain reaction (PCR). Oligonucleotide primers were designed using the OLIGO (version 3.3) software taking into account the data on primary structure of the *pal* gene. To amplify the coding region of the *pal* gene from *R. toruloides*, sequence from GenBank database (X12702.1) was used as a template. At their 5'-ends, primers contained additional sequences incorporating restriction sites NcoI in case of the forward primer and HindIII, in case of the reverse one, in order to amplify the gene structural region and insert it into a polylinker of the pET28a expressing vector at relevant sites. Reverse primer was constructed so that the amplicon would not contain a stop codon and joining of the reading frames of the gene and His_6 sequences would be ensured.

Polymerase chain reaction was performed in 20–50 ?L solution prepared on the basis of ten-fold buffer for Taq polymerase containing deoxynucleoside triphosphate, 200 ?M each, 0.5 ?M of each primer, 2 mM $MgSO_4$, 10 ng template, 2 units Taq DNA polymerase, and 0.1 units Pfu DNA polymerase. Temperature of annealing of oligonucleotides was calculated according to an empiric formula $T_m = 67.5 + 34[\%\ GC] - 395/n$, where %GC = (G + C)/n, n is the number of nucleotides. Analysis of PCR products was performed by electrophoresis in 1% agarose gel.

Sanger sequencing was performed on an ABI3730xl (Applied Biosystems, United States) equipment using BigDye® Terminator v3.1 Cycle Sequencing Kit according to the manufacturers' protocols.

DNA hydrolysis by restriction endonucleases NcoI and HindIII was performed in buffer solutions under optimal conditions of incubation medium recommended for each of the enzymes by the manufacturers. Completion of hydrolysis was controlled by electrophoresis in agarose gel. Reaction mixture was purified from the reaction products using the QuickClean kit.

Isolation of DNA fragments from agarose gel. Samples of DNA were separated by electrophoresis in Tris–acetate buffer in a 0.7–0.8% agarose gel (Bio-Rad, United States) containing 0.3 ?g/mL ethidium bromide and analyzed by fluorescence under ultraviolet light at 254 nm. Gel pieces containing fragments of interest were cut out and transferred into microcentrifuge tubes, then DNA fragments were eluted from the gel using the "Isolation of DNA from agarose gels" kit (Boeringer Mannheim, Germany). Sodium perchlorate was added to the tubes in the amount of 400 ?L per 100 mg weight of the cut out gel. The mixture was heated to 65°C, then agarose was dissolved in salt buffer. Glass milk microbeads were introduced into the suspension at the amount of 20 μL per 100 mg of gel weight. In the salt solution, DNA contained in the gel adsorbed on the surface of the microbeads. They were washed (consecutive precipitation–resuspension) with the same salt solution once and with 70% ethanol, two times. DNA was desorbed from the beads by resuspension in TE buffer (10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA) in the amount of 50 ?L per 100 mg gel weight.

Ligation. Products of hydrolysis of the vector DNA obtained as described above and the *pal* gene amplicon were ligated by phage T4 DNA ligase. Concentrations of the vector and gene in the reaction mixture were 5 ng/mL each. Concentration of phage T4 DNA ligase was 5 units/?L. Reaction was performed at 15°C during 24 h.

Preparation of competent E. coli cells for transformation. To prepare competent cells for the following transformation by electroporation, individual colony was grown on LB agar and placed into 5 mL LB medium. Cells were grown during night at 37°C and constant stirring (250 rpm). Two milliliter of the night culture were placed into 200 mL LB medium. Cells were grown at 37°C at constant stirring (250 rpm) till the optical density at 600 nm reached 0.6, then they were sedimented by centrifugation during 10 min at 4000 *g* at 4°C. Cells were washed in deionized water in the initial volume followed by centrifugation. The procedure of washing was preformed three times. After washing, cell sediment was resuspended in small volume of deionized water and centrifuged during 30 s at 5000 rpm in a microcentrifuge. Three volumes (of the cell sediment volume) of 15% glycerol solution were added to the sediment, it was resuspended and quickly frozen in liquid nitrogen. Cells ready for transformation were stored at –70°C.

Transformation of E. coli cells. Transformation of competent cells was performed by electroporation. Plasmid DNA (2 ?L) at concentration of 0.3–1.0 ng/?L was added to 12 ?L of competent cells and mixed; electroporation was performed in a GVI-1 generator of high-voltage impulses in sterile cells under electrical impulse strength of 10 kV/cm and duration of 4 ms. After transformation, cells were put in 1 mL SOC medium (2% bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and incubated for 40 min at 37°C. After incubation 10–250 ?L cell suspension were inoculated into a selective LB medium containing kanamycin (25 ?g/mL) to select the recombinant clones.

Induction of gene expression with IPTG. Induction of expression of genes coding for the recombinant enzyme in producer strain was performed using IPTG at final concentration of 1 mM. For this purpose, a single producer strain colony was inoculated onto standard liquid medium LB containing kanamycin at concentration of 25 ?g/mL and 1% glucose and

fermented at 37°C in a rotor-type temperature-controlled shaker overnight at 250 rpm. Then, after optical density at 600 nm was measured, the culture was diluted with the LB liquid medium containing kanamycin at concentration of 25 ?g/mL to the optical density of 0.1 OU and fermented during 2–3 h at 37°C to the optical density of 0.6–0.8 OU. Then, the culture was divided into two equal parts: IPTG was added to one of the parts to the final concentration of 1 mM and it was fermented during 5 h at temperature of 25°C or 37°C, cell aliquots collected for analysis at certain time intervals. The aliquots were stored at –20°C.

Autoinduction of expression with 0.2% lactose. To induce autoinduction of expression according to Studier [7], modified PYP-5052 medium, containing 1% peptone, 0.5% yeast extract, 50 mM Na_2HPO_4, 50 mM K_2HPO_4, 25 mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.5% glycerol, 0.05% glucose, and 0.2% lactose, was used.

A single colony of producer strain was inoculated into PYP-5052 medium containing 25 ?g/mL kanamycin. After that, the colony was fermented at 25°C or 37°C in a temperature-controlled rotor-type shaker at 250 rpm during 32, or 18 h, or till no significant change in optical density at 600 nm occurred per 1 h. Then, an aliquot of cells was collected for analysis. Aliquots were stored at –20°C.

Polyacrylamide gel electrophoresis (PAGE). Electrophoresis of cell lysates and proteins was performed according to disk-electrophoresis procedure in 10% PAGE under denaturing conditions according to Laemmli.

Destruction of bacterial cells under native conditions. Bacterial cells were destroyed under native conditions using ultrasonic treatment. Wet cell sediment obtained from 300 ?L culture medium were resuspended in 30 mL buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0) and sonicated for 10 min at the amplitude of 60%, sonication duration 30 s, pause of 30 s, and a working temperature of 4°C. Destruction was controlled by inoculation of cells after sonication on a standard agarized LB medium containing kanamycin at concentration of 25 ?g/mL. After sonication, cell lysate was centrifuged during 20 min at 15000 *g*, and the precipitate and sediment were used for analysis of p17 protein localization.

Computer methods of data analysis. Analyses of nucleotide and amino acid sequences were performed using a Lasergene v.7.1.0 (DNAStar, United States) and BioEdit v.5.0.9 software packages.

Search for homologous sequences was performed using the BLAST2 (http://www.wbi.ac.uk/blastall/) software. Comparison of amino acid sequences was performed using a ClustalW1.8 (http://www.ebi.ac.uk/clustalw/index.html) software.

RESULTS AND DISCUSSION

Cloning of the pal gene in E. coli cells. Due to the difficulties in genome organization of the *pal* gene (6 introns), it was synthesized using the sequence of the *pal* gene isolated from *R. toruloides* (GenBank: X12702.1). The gene was treated by restriction endonucleases NcoI and HindIII to obtain sticky ends.

Expression vector pET28a designed for expression of recombinant proteins in *E. coli* and containing kanamycin resistance gene was chosen for cloning. In addition to that, the vector contains sequence coding for His-Tag end near the polylinker, which considerably simplifies chromatography on a Ni-containing carrier. To prepare for cloning, the vector was treated with restriction endonucleases NcoI and HindIII and purified from the reaction products with the QuickClean kit. After hydrolysis with restriction endonucleases, the vector gained sticky ends complementary to the *pal* gene.

Preparation of the PAL protein producer strain. Cells of *E. coli* strain BL21[DE3]Star containing the gene coding for T7 phage polymerase under the control of an inducible bacterial promoter in their genome were transformed with pET28a DNA containing the *pal* gene coding for L-phenylalanine ammonia-lyase from *Rhodosporidium toruloides* (GenBank: X12702.1) by electroporation. The strain was chosen because it contains the DE3 lysogen carrying a gene coding for phage T7 polymerase under the control of the lacUV5 inducible promoter necessary for the expression of the gene cloned in the pET28a plasmid. Besides, these cells do not contain protease lon and carry a mutation in the gene coding for the outer membrane protease OmpT.

The absence of these two proteases decreases degradation of heterologous proteins. Also, the strain carries a mutant rne gene coding for a truncated RNAase, which should lead to increase in the stability of mRNA in cell due to decrease in its enzymatic degradation [8, 9].

These manipulations resulted in the *E. coli* strain BL21[DE3]Star pET28aPAL producing the L-phenylalanine ammonia-lyase of *Rhodosporidium toruloides*, which we will call BL21PAL further on.

Biosynthesis of the PAL recombinant protein by E. coli strain BL21PAL after induction of expression with 1 mM IPTG. To culture the obtained PAL protein producer strain, standard agarized LB medium containing kanamycin at concentration of 25 ?g/mL and 1% glucose was used. The level of the enzyme expression was optimized in function of time and temperature of induction. Fermentation was performed in 2-L flasks in 250 mL medium containing kanamycin at concentration of 25 ?g/mL in a temperature-controlled rotor-type shaker at 37°C and 250 rpm. IPTG at concentration of 1 mM was used as an inductor. Expression induction was performed when optical density of cell culture at 600 nm reached 0.6–0.8 OU. Then, after a certain period of time (from 15 min to 1 h) aliquots of the cell culture were collected. Cell culture without induction was used as control. Without the induction, cells grow according to a logarithmic law: linear growth is observed till the optical density of 0.6–0.8 OU, then exponential growth occurs to 3.8–4.2 OU; after that the growth curve reaches a plateau of the stationary growth phase. After the addition of the inductor (1 mM IPTG) to cell culture cells grow more slowly, optical density of the culture grows from 0.6 to 1.5 OU within 4 h, however, later on exponential growth phase with late transfer to a stationary growth phase occurs. When studying the dynamics of biosynthesis of the PAL recombinant protein by *E. coli*

cells after expression induction with 1 mM IPTG, maximal expression of the *pal* gene in *E. coli* strain BL21PAL was noted after 3 h of fermentation. According to densitometry analysis, protein yield was 32% to the total protein (Fig. 1). When expression was induced at 25°C, longer (9 h) transfer of culture cells to exponential growth phase occurred.

Maximal level of expression (32%) at all temperatures was the same. Without the induction of expression, undeliberate synthesis of recombinant protein, or "T7 promoter leakage", often observed in this expression system [10], occurred.

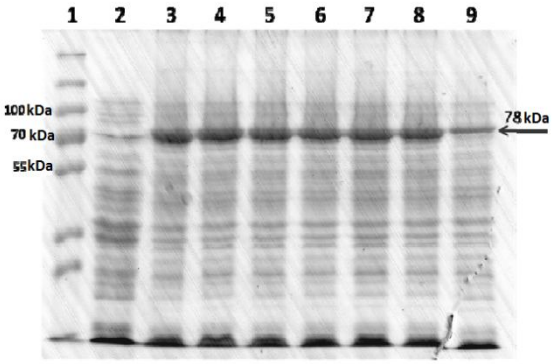

Fig. 1. Analysis of *E. coli* strain BL21PAL cell lysates after induction of expression under various condition:
1, molecular weight marker "PageRuler™ Prestained Protein Ladder", Fermentas; 2, negative control (lysate of producer strain cells without the addition of an inductor); 3, *E. coli* strain BL21PAL cell lysate after induction of expression with 0.2% lactose according to Studier at 25°C during 32 h; 4, *E. coli* strain BL21PAL cell lysate after induction of expression with 0.2% lactose according to Studier at 37°C during 18 h; 5, *E. coli* strain BL21PAL cell lysate after induction of expression with 1 mM IPTG at 37°C during 3 h; 6, *E. coli* strain BL21PAL cell lysate after induction of expression with 1 mM IPTG at 37°C during 5 h; 7, *E. coli* strain BL21PAL cell lysate after induction of expression with 1 mM IPTG at 25°C during 5 h; and 8, *E. coli* strain BL21PAL cell lysate after induction of expression with 1 mM IPTG at 25°C during 8 h.

Biosynthesis of the PAL recombinant protein by E. coli strain BL21PAL after autoinduction of expression with 0.2% lactose according to Studier. Fermentation was performed in 2-L flasks in 250 mL PYP-5025 medium for autoinduction containing kanamycin at concentration of 100 ?g/mL in a temperature-controlled rotor-type shaker at 250 rpm during 19 h at temperature of 37°C and during 32 h at temperature of 25°C.

Average optical density (OD600) of the E. coli strain BL21PAL cell culture was the same at different temperatures of cultivation and was 7 OU. After PAGE of E.coli BL21PAL cell lysates with autoinduction of expression with 0.2% lactose, PAL protein content in E. coli cells was determined by densitometry of the obtained electrophoregrams using the TotalLab software. The results of the densitometry analysis showed that, in E. coli cells of BL21PAL, PAL protein accumulated in the amount of 35% to the total cell protein at fermentation temperature of 37°C and 33%, at temperature of 25°C (Figs. 2 and 3).

The method of expression induction was chosen as the simplest efficient and cheap alternative to classic induction using IPTG in expression systems based on lactose operon. When autoinduction is used, there is no need in the following cell culture optical density monitoring or addition of an inductor.

From the moment of the colony inoculation into an autoinduction medium to the collection of bacteria biomass with OD600 = 7–15 with synthesized target proteins, 15–20 h pass.

The phenomenon of expression autoinduction is based on the mechanisms that bacteria utilize to regulate consumption of carbon and energy source from the nutrinet medium. If there is glucose in the medium, catabolic repression and exclusion of the inductor prevent lactose consumption by lac-permease, product of the lacY gene [11, 12].

When glucose resources are exhausted, lac-permease starts to consume lactose, and β-galactosidase inside bacterial cells turns lactose into a natural inductor, allolactose [6].

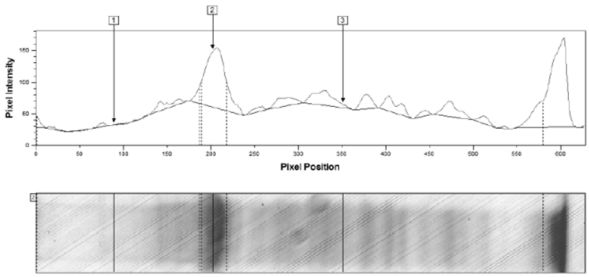

Fig. 2. Densitogram of *E.coli* strain BL21PAL cell lysate after expression induction with 0.2% lactose according to Studier at 25°C during 32 h.

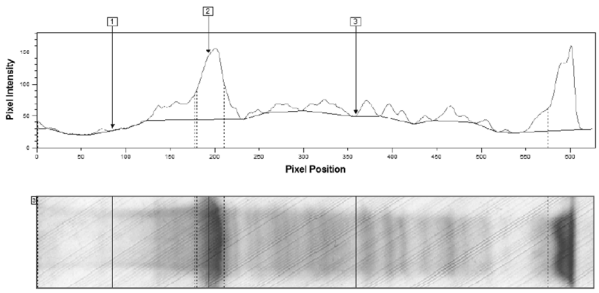

Fig. 3. Densitogram of *E.coli* strain BL21PAL cell lysate after expression induction with 0.2% lactose according to Studier at 37°C during 18 h.

Utilization of a substance not associated with induction and lactose operon (for example, glycerol) as a source of carbon and energy allows to almost double the yield of the target protein by comparison with the equivalent amounts of lactose as a primary energy source. This is because T7 RNA polymerase is so much active that induction may direct most of cellular transcription and translation toward production of target protein [13], which may overlap with the ability to

serves as an efficient source of carbon and energy. The presence of 0.05% glucose in the autoinduction medium accelerates the process of bacterial cell growth at initial stages and simultaneously blocks induction with 0.2% lactose present in the medium, while 0.5% glycerol, also present in the medium, is an efficient source of carbon and energy.

The maximal level of expression of the PAL recombinant protein in *E. coli* strain BL21PAL cells was 35% upon utilization of autoinduction medium in contrast to the maximal level of 32% upon induction with IPTG. Increase in the maximal level of expression upon autoinduction with 0.2% lactose was 7–8%, if compared to induction with IPTG. The data evidence that utilization of a simpler and cheaper autoinduction for synthesis of recombinant protein p17 leads to similar results obtained upon utilization of 1 mM IPTG as inductor, while bacterial biomass and, thus, protein yield, increased 6–7 times.

Solubility of the PAL recombinant protein upon its synthesis in E. coli strain BL21PAL was determined by disc-electrophoresis of *E. coli* cells destroyed upon induction of expression with 0.2% lactose during 18 h at 37°C according to Studier in PAGE. Both sediment and supernatant formed upon sedimentation of cell debris were analyzed.

It was demonstrated that 99% of PAL recombinant protein synthesized in *E. coli* strain BL21PAL cells upon expression induction under optimal conditions is in the sediment formed upon sedimentation of cell debris and probably only the insignificant amount (1%) is in the supernatant (Fig. 4).

Utilization of 1 mM IPTG instead of 0.2% lactose as an inductor did not change protein solubility, neither did bacteria cultivation at different temperatures, 25°C or 37°C.

Accumulation of PAL protein in cells of producer strain in insoluble form indirectly evidences its non-native conformation (tertiary structure).

The data on solubility do not agree with the literature data, which demonstrate that utilization of different techniques to express the *pal* gene coding for L-phenylalanine ammonia-lyase of *Rhodosporidium toruloides* (GenBank: X12702.1) in *E. coli* cells results in a soluble target protein. The probable reason for accumulation of PAL in cells of the producer strain in insoluble form is utilization of the gene with modified codon sequence for expression. As a result of codon composition optimization, regions necessary for ribosome slow-down at the boarders of domain folding could be impaired, which could have led to start of new domain translation when folding of the previous one was not complete, which, in turn, could lead to formation of insoluble protein aggregates.

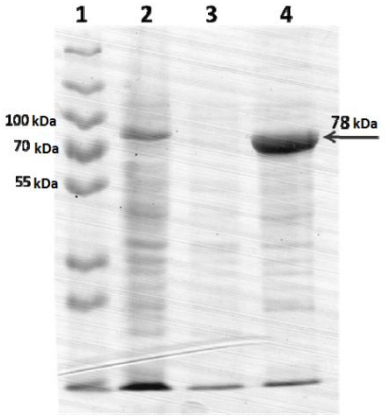

Fig. 4. Determination of PAL recombinant protein solubility upon its synthesis in *E. coli* strain BL21PAL cells: 1, molecular weight marker "PageRiler™ Prestained Protein Ladder" (Fermentas); 2, lysate of *E. coli* strain BL21PAL cells destroyed by sonication; 3, supernatant formed upon centrifugation of the lysate of *E. coli* strain BL21PAL cells destroyed by sonication; and 4, sediment formed upon centrifugation of the lysate of *E. coli* strain BL21PAL cells destroyed by sonication.

Therefore, *E. coli* strain producing L-phenylalanine ammonia-lyase of *Rhodosporidium toruloides* was obtained. Optimization of expression resulted in the maximum level of expression of 35% upon induction with 0.2% lactose according to Studier during 18 h at 37°C. When expressed in *E. coli* cells, the recombinant L-phenylalanine ammonia-lyase was insoluble (formed inclusion bodies) under all conditions studied.

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