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Journal of Applied Biotechnology
ISSN 2327-0640
2014, Vol. 2, No. 1

Efficient Expression of Recombinant L-phenylalanine Ammonia-lyase From *Rhodosporidium toruloides* using *Escherichia coli*

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Journal of Applied Biotechnology
ISSN 2327-0640
2014, Vol. 2, No. 1

Received: February 1, 2014 Accepted: February 27, 2014 Published: March 4, 2014

Doi: 10.5296/jab.v2i1.5235 URL: http://dx.doi.org/10.5296/jab.v2i1.5235

Abstract

We explored simple and cost effective method to obtain recombinant PAL of *Rhodosporidium toruloides* using *E. coli* expression system. *E. coli* strain BL21 (DE3) was transformed by pET28 plasmid containing L-phenylalanine ammonia-lyase (PAL) gene from *Rhodosporidium toruloides* and cultured in different conditions. To assess the physiological activity of microbial cells, we studied the growth kinetics in a series of batch fermentation and changes in its activity after the induction. The kinetics of the process of cultivation of recombinant *E. coli* strain was studied by the Ludeking-Paire model. A growth and induction regimen was established for use in bioreactor which results in the accumulation of 1,9 g l^{-1} of recombinant PAL. The point of maximum activity of the PAL produced was determined at 38.0-38.5 h after induction. The optimum concentration of inducer (IPTG) was 2 mM, we recommend to add the inducer at OD540=1.3. This simple and cost effective production process could be recruited for large scale production of PAL.

Keyword: Kinetics of microorganism growth, Microbial biomass, Specific growth rate, L-phenylalanine ammonia-lyase, Kinetic model

1. Introduction

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) catalyzes the conversion of L-phenylalanine to trans-cinnamic acid and ammonia. In higher plants, PAL catalyzes the first step in the phenylpropanoid pathway of biosynthesis of multiple phenylpropanoids, such as lignins and flavonoids (Hanson, 1981). In the past two decades, PAL has gained considerable significance in several clinical, industrial and biotechnological applications. PAL has been investigated as a potential medicine for the substitution therapy for phenylketonuria and for preparing of low phenyalalnine food (Sarkissian, 1999; Sarkissian, 2005). PAL substantially inhibited cancer cell growth *in vitro*, and produced cures in mice bearing lymphoblastic leukemia (Abell, 1973; Babich, 2013). In addition, PAL also can be used for the commercial production of L-phenylalanine (Evans, 1987).

In recent years, several methods have been investigated for biotechnological production of PAL (Hanson, 1981; Kane, 1985; Evans, 1987; Sarkissian, 1999; Sarkissian, 2005; MacDonald, 2007). There are a lot of articles describing the obtaining of PAL using raw plant materials such as tobacco, parsley, strawberries, beans, alfalfa, potatoes, mustards, but only a few microbial sources of the enzyme have been investigated (MacDonald, 2007; Kovacs, 2014). Various recombinant strains and different raw materials as well as different purification techniques have been commonly used (Rogers, 1978; Venkatesh, 1993; Nandasana, 2008). The use of yeast *Pichia pastoris* (Hsieh, 2011) and bacterial *E. coli* (Hsieh, 2010; Jaliani, 2013; Ma, 2013; Zhu, 2013) recombinant strains have been reported recently.

PAL purification usually requires multiple steps to obtain a homogenous protein of high purity (Goldson-Barnaby, 2013). However, PALs are relatively instable and new approaches are needed to simplify the expression and procedure of purification. One of the most important issue is the productivity of recombinant strain and the concentration of PAL in crude extract. The aim of this study was to estimate basic parameter values of the fermentation process in order to elaborate efficient expression of L-phenylalanine ammonia-lyase from *Rhodosporidium toruloides* in *E. coli* cells.

2. Materials and methods

2.1 Reagents

IPTG (“ALMABION Llc”, Russia), T4 DNA ligase, DNA polymerase Taq u pfu, Taq-polymerase buffer, pfu-polymerase buffer, 1 kb markers, deoxyribonucleoside triphosphates, (“SibEnzyme”, Russia), kanamycin, chloramphenicol, tris, phenylalanine, tryptone, yeast extract, NaCl (“Gibco BRL”, USA).

2.2 Bacterial Strains

Rozetta (DE3) (*E. coli* B F- ompThsdSB (rB- mB-) (DE3) gal dcmpRARE (CamR)) strain was received from “Stratagene” (USA).

2.3PAL Cloning

The *pal* gene from *Rhodosporidium toruloides* was amplified by PCR with the use of following primers: *Pal9-Hind* — 5'-CAACAAGCTTCGCCAGCATTTTCAG, *M13rev* —

5'-AGCGGATAACAATTTCACACAGGA ("Evrogen", Russia), purified from agarose gel ("Bio-Rad", PITTA) and placed with endonucleases HindIII and NcoI ("Sibenzyme", Russia) on the pET28a vector ("Novagen", USA) (Van den Berg, 2011). The gene was fused with His6-Tag at C-terminus. The resulting recombinant plasmid pETPAL28a was used for transformation the *E. coli* cells. Manipulations with DNA were performed using standard methods (Rogers 1978). Cycle sequencing was testedwith BigDye® Terminator v 3.1 Cycle Sequencing Kit.

2.4 Expression and Purification of PAL

Cells were grown in LB-broth supplemented with kanamycine (40 μg/ml) and chloramphenicol (20 μg/ml) in 5 l bioreactor (Biostat A plus MO, Sartorius, Germany), 23 °C to an optical density at 590 nm (OD_{590}) 0.7, and then was induced by 1 mM IPTG at 23 °C for 24 h. After incubation the cells were harvested by centrifugation for 15 min at 5000 g, suspended in phosphate buffer (pH 8.0) and sonicated for 6 times (20 s pulse on, 60 s pulse off).

We added into cell-free extract ammonium sulphate up to 25% saturation. The mixture was left for 15 min at 4 °C, followed by centrifugation at 10,000 rpm for 30 min. Then ammonium sulphate was added up to 50% saturation followed by second cycle of cold exposure and centrifugation at the conditions described above. The precipitate was dissolved in 55% ammonium sulphate for EDTA eliminating, centrifuged and then resolved in buffer A (150 mM phosphate buffer pH 8.0 containing 10 mM imidazole). The insoluble proteins were separated by centrifugation (10,000 rpm, 20 min).

Soluble fraction was applied on to a Ni^{2+}-NTA-agarose column (7 ml) equilibrated with buffer A. After washing with buffer A (21 ml) and 150 mM phosphate buffer pH 8.0 containing 20 mM imidazole (21 ml) the protein was eluted by application of 150 mM phosphate buffer pH 8.0 containing 250 mM imidazole (21 ml). This purification step was carried out at 15 °C. We added in samples $(NH_4)_2SO_4$ up to 0.4 M concentration and centrifuged for 15 min at 15,000 rpm.

For hydrophobic chromatography we used FPLC-chromatograph with Pharmacia LKB-UV-MII detector at 280 nm ("Pharmacia", Sweden) using Protein PAK Glass HIK column (7 × 10 cm) with phenyl-TSK-5PW ("Nihon Waters Ltd.", Japan). The received enzyme solution was loaded onto a phenyl-TSK-5PW-column equilibrated with 50 mM phosphate buffer and 0.8 $(NH_4)_2SO_4$, pH 8.2. The active fractions were eluted with $(NH_4)_2SO_4$ in a linear gradient (0.8-0.0 M) in 50 mM phosphate buffer and collected. Protein concentration was determined spectrophotometrically at 280 nm.

2.5 Enzyme Assay

Activity of PAL was measured spectrophotometrically according to standard method (Sigma). Formation of trans-cinnamic acid was monitored at 270 nm with UV-2401 PC spectrophotometer ("Shimadzu", Japan). Assay was started by the addition of 40 μL sample containing PAL with specific activity 0.025–0.125 U/ml into 0.5 mL 0.2 MTris-HCl buffer, pH 8.5 containing 0.04 mL (0.05 M) L-Phe and 0.42 ml deionized water MilliQ ("Millipore", France). The formation of trans-cinnamic acid was monitored at 270 nm at 30 °C, and

activity was calculated according to the formula: U/ml = [(ΔOD_{270}/min, PAL sample – ΔOD_{270}/min, control sample) × $V_{mixture}$ × f] / 19.73 × V_{sample}, where $V_{mixture}$ — volume of mixture, ml; f —coefficient of PAL dilution; 19.73 — millimolar extinction value of trans-cinnamic acid at 270 nm;V_{sample} — volume of sample, ml. One U was determined as the amount of enzyme catalyzing the conversion of 1 μmol of L-Phe per min at 30 °C and pH 8.5. We defined the specific activity as the number of U per milligram of protein.

2.6 Bacterial Culture Growth Control

E. coli/pETPAL28a strain was cultured in liquid media containing tryptone 10 g/l, yeast extract 5 g/l and NaCl 10 g/l. We used a spectrophotometer UV-1800 (“Shimadzu”, Japan) to monitor growth of bacterial cultures by measuring the optical density at 540 nm (OD540). Growth curves were constructed based on the differences in the OD values obtained.

The parameters of growth of liquid cultures (growth phases, growth rate, productivity, etc) were determined, and the Ludeking-Paire model was used to determine the efficiency of the fermentation process with particular attention to biomass production rate as well as the rate of PAL formation (1), (2).

$$\frac{dX}{dt} = \mu X\,, \tag{1}$$

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X\,, \tag{2}$$

where $X = X(t)$ — biomass concentration, $P = P(t)$ — synthesis product concentration, α, β, μ — process parameters, t — time.

The solution (1), the initial condition $X(0) = X_0$, is given by

$$X(t) = X_0 \cdot e^{\mu \cdot t}\,, \tag{3}$$

if to express the constant μ from the last equation, then at $t > 0$,

$$\mu = \frac{\ln(X(t)/x_0)}{t}\,, \tag{4}$$

where μ — specific rate of biomass in each fixed moment of time t.

It is known that the amount of product formed is directly proportional to the formed biomass, i.e.

$$P(t) = \gamma \cdot X(t)\,, \tag{5}$$

where γ — yield of the product, referred to the resulting biomass. Dependence found in the study

$$\mu_1 = \gamma \cdot \mu\,. \tag{6}$$

where μ_1 — specific rate of fermentation product, then formulas (1), (2), (5) and (6) imply

that

$$\mu_1 = (\alpha\mu + \beta)/\gamma . \quad (7)$$

Specific productivity of biomass and protein per unit of time was defined as the increase of the index per unit of time:

$$Q = \frac{\Delta X(t_i)}{\Delta t_i} = \frac{X(t_i) - X(t_{i-1})}{t_i - t_{i-1}}, \quad (8)$$

$$q = \frac{\Delta P(t_i)}{\Delta t_i} = \frac{P(t_i) - P(t_{i-1})}{t_i - t_{i-1}}, \quad (9)$$

where $\Delta X(t_i)$, $\Delta P(t_i)$, Δt_i — increment of the function $X(t)$, $P(t)$, of the argument t in the interval (t_{i-1}, t_i), $i = 1,2,3,...$

3. Results

3.1. Cloning, Expression and Purification of PAL

The sequence of the *pal* gene consisted of 2148 bp was 100% identical to native gene from *Rhodosporidium toruloides*. Created recombinant strain showed stable protein production for 12 passages. SDS-PAGE electrophoresis indicated the presence of one major band with the molecular weight of 78.4 kDa (Figure 1), in agreement with the calculated mass of PAL 6×His.

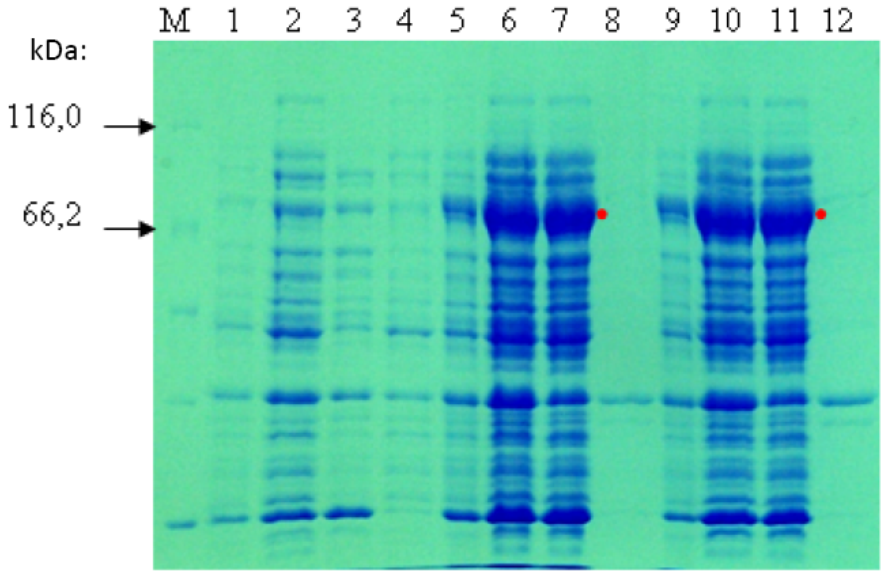

Figure 1. Expression of PAL in *E. coli*: M — molecular markers ("Fermentas", #SM0431); Tracks 1–4 — proteins from BL21(DE3) pLysE CodonPlus RP/pET28a; Tracks 5–8 — proteins from pETPAL28a bearing strain BL21(DE3)pLysECodonPlus RP/pETPAL28a; Tracks 9–12 — proteins from pETPAL28a bearing strain Rozetta (DE3)/pETPAL28a; Tracks 1, 5, 9 — cellular proteins before induction; 2, 6, 10 — cellular proteins after induction, 24 h; Tracks 3, 7, 11 — cleared lysate after induction, 24 h; Tracks 4, 8, 12 — insoluble fraction of proteins after induction, 24 h.

3.2 Analysis of Biomass, Protein Concentration and Activity of PAL During the Process of Fermentation

To assess the physiological activity, we studied the growth curves of *E. coli* BL21 (DE3) / pETPAL28) strain without any induction and after induction. The studies were conducted to estimate the maximum biomass production. The simulation of the cultivation process was performed. Figures 2 and 3 show the typical profiles of biomass production and protein without induction, and after addition of inducer, respectively.

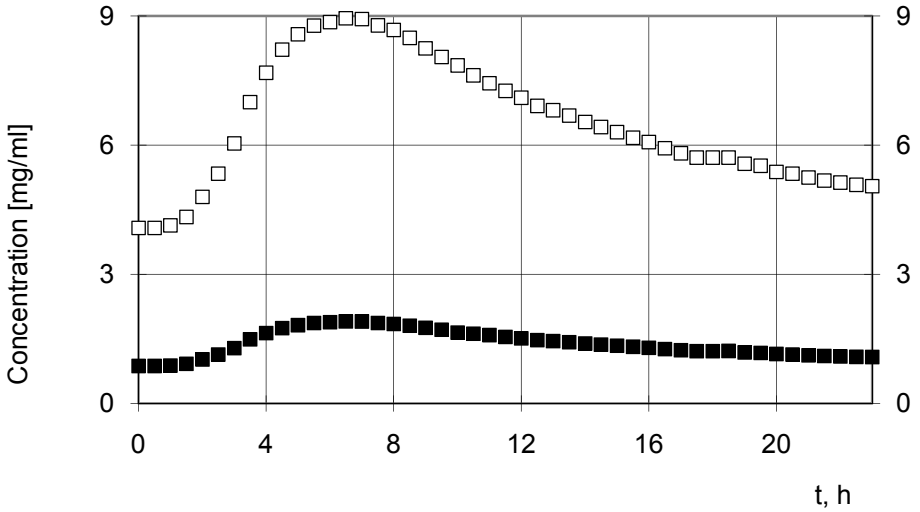

Figure 2. Dependence of the concentration of biomass and protein on the time without induction (■ — biomass accumulation, □ — protein accumulation)

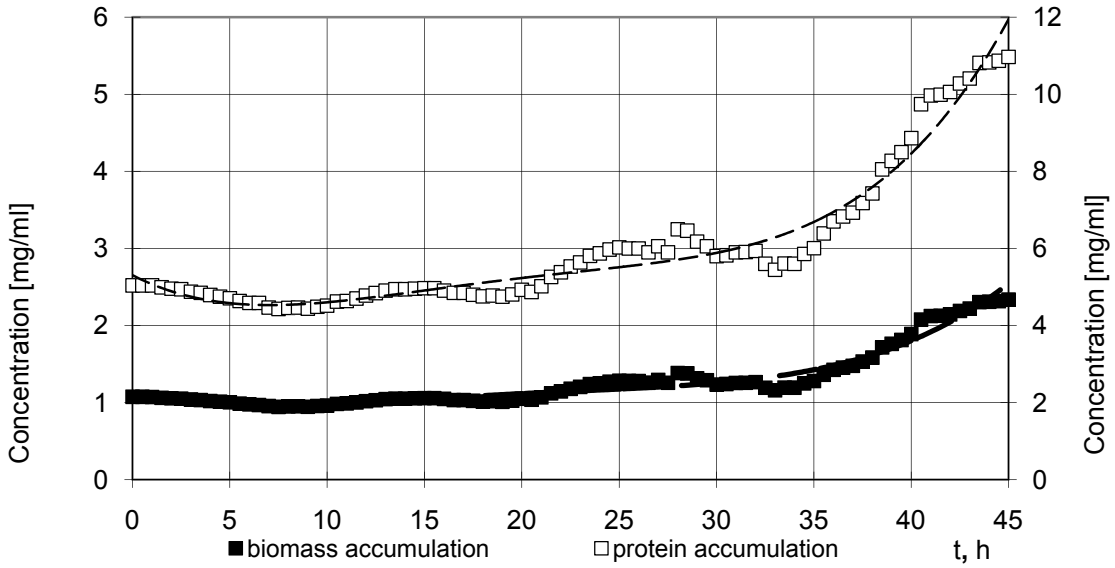

Figure 3. Dependence of the concentration of biomass and protein on time since the induction (■ — biomass accumulation, □ — protein accumulation)

Curve of the biomass accumulation shows the traditional behavior. There are four phases of growth: initial (for about 2 h), exponential (from 2 to 6 h), stable (from 6 to 8 h), dying (from 8 h onwards). Biomass and protein showed enrichment of concentration after 2 h of fermentation. By the end of the second phase biomass enrichment stopped, while the protein was still being accumulated for approximately 1.5 h.

Accumulation of PAL in the early stages leads to the inhibition of growth and metabolism of cells. The phase of negative acceleration of growth was registered up to 21 h. Then, as shown in Figure 3, there is a gradual increase in biomass and protein. In the first period after

induction the highest production rate of PAL was observed at 25 h, the highest rate of biomass accumulation — at 21.5 h. Then we identified a small decline up to 34.0 h followed by significant increase. Starting at 35 h we observed a constant increase with the greatest acceleration of PAL production up to 42 hours.

The final period of the process (34–42.5 h after induction) is of the greatest interest (Figure 4). If we assume that the interval 34–42 h after the induction corresponds to the exponential phase, it can be described by the relation of the form (1) with the initial conditions $X(34) = X_0$ (the error does not exceed 3%), parameter $\mu = 0{,}075$ 1/h — average specific rate of biomass, calculated by formula (4) in the interval $[t_0, t]$, $t_0 = 34$, $t \in (34, 42]$.

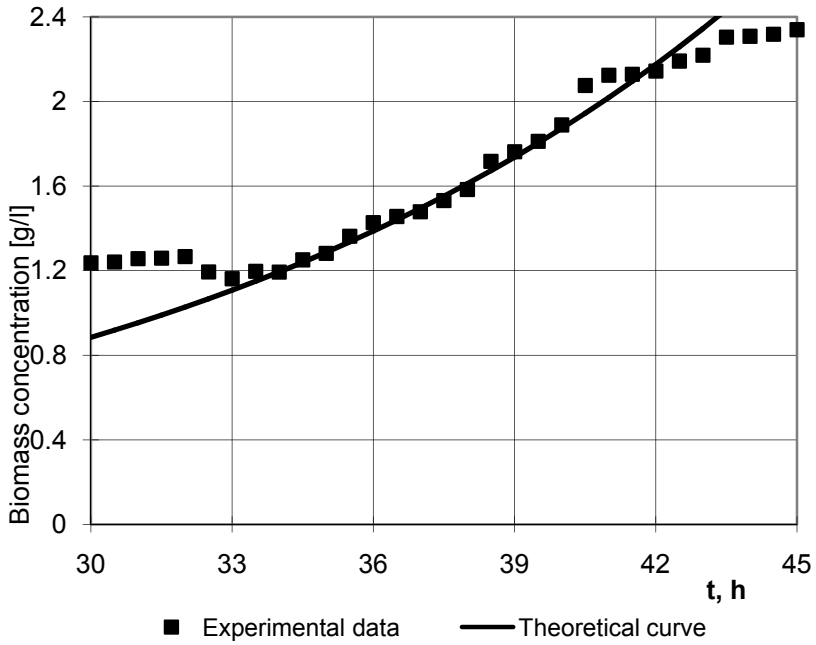

Figure 4. Biomass concentration dependence of the time since the induction (■ — experimental data, □ — theoretical curve)

The basic rate characteristics of the biomass and product accumulation for this period were calculated by the formulas (4), (8), (9). According to calculations, the highest increase in biomass corresponds to 44.0 h (then comes decrease), in the protein — 45.5 h.

Since both kinetic curves of the biomass and protein accumulation exhibit the same behavior (Figures 2, 3), then the further duration of the process will lead to a decrease of the enzyme. Therefore we limited the duration of fermentation up to 46 h. According to the calculations, the highest average specific capacity of the biomass was observed at 44 h. It happens due to the cycling growth and to the fact that the production of PAL requires a certain period of accumulation of energy after cell division.

However, not only the amount of the produced protein is an important indicator, but also the activity of the enzyme is, which in this case is reflected in the rate of accumulation of

trans-cinnamic acid. Thus, in order to clarify the behavior of the activity of PAL we measured the formation of trans-cinnamic acid from 37.0 h after induction till the end of the process. The activity measurements were performed every 30 min for 8 hours. Samples taken for analysis were previously sonicated. The activity assay confirmed the curves of the accumulation of the protein described above. The highest activity of the enzyme was marked at 37.5–38.5 h after the induction. From 39.0 to 42.5 h we observe a decline of activity. In the final period of the process, there was an inverse dependence between the accumulation of the protein, biomass and enzyme activity: the concentration of biomass and protein reached maximum whereas its activity was decreased. Therefore, the period, when the highest activity of the enzyme was reached, was 38.0–38.5 h after induction.

3.3 Dependence of Efficiency of Fermentation on Time of Induction and Inducer Concentration

We studied the activity of PAL after induction with IPTG with the range of concentrations from 0.84 to 21 mM. It was shown (Table 1) that the concentration of inducer within studied levels did not produce a significant effect on fermentation process. Moreover, one could prefer the lower concentrations of inducer due to slight increase of PAL activity.

Table 1. *Dependence of efficiency of fermentation on time of induction and inducer concentration*

OD540	Inducer concentration, мМ	Activity, U/ml		OD540	
		After 2 h	After 5 h	After 2 h	After 5 h
1.3	0.84	1.3	1.6	1.3	2.0
	2	1.8	2.2	1.3	2.0
	4.2	1.1	1.4	1.3	2.0
	8.4	1.1	1.4	1.3	2.0
	21	1.1	1.2	1.3	2.0
0.5	0.84	1.4	1.4	1.5	1.6
	4.2	1.3	1.4	1.4	1.5
	8.4	1.2	1.2	1.5	1.6
2.0	0.84	1.8	1.8	2.6	2.6
	8.4	1.8	1.8	2.5	2.5

4. Conclusion

During a few past decades a lot of PALs have been isolated from different sources, and lots of

recombinant strains have been created to produce enzymes with a high yield. Dozens of new methods have been used, the goals of which was to increase the productivity of strains and simplify the purification protocol. On the one hand, the purification of PAL usually requires several complex steps (Goldson-Barnaby, 2013). On the other hand, due to the PALs relative instability, a rapid purification scheme is desirable. Two typical approaches have been used for this purpose: creation of recombinant strains carrying tags with specific affinity and use strains with high concentration of target protein in crude extract. For example, the use of recombinant *E. coli* TOP10 bearing plasmid containing *Rubrobacter xylanophilus* gene and N-terminal His6-affinity tag have been reported (Kovacs, 2014). Goldson-Barnaby and Scaman (2013) investigated the purification by acid precipitation, aqueous two-phase partitioning, and anion exchange chromatography with an approximate yield of 20% and 50-fold purification from the HiTrap Q-Sepharose column. Reported two-step purification procedure was more efficient and rapid in comparison with the four-step procedure described by Vanelly (2007), which produced an overall yield of 6%. The efficiency of expression of PAL seems to be extremely low, if compared with other recombinant enzymes, such as L-asparaginases, where overall yield more than 40% is quite typical (Khushoo, 2004; Pokrovskaya, 2012).

Taking into consideration all these issues, we studied the kinetics of growth of recombinant strain producing PAL (Babich, 2013) as well as the yield of the protein in order to explore its productivity and potential for use in the synthesis of PAL and hydroxycinnamic acid for clinical and industrial application. We explored a simple and cost effective method to obtain recombinant PAL of *Rhodosporidium toruloides* using *E. coli* strain BL21 (DE3) expression system. As well as studies mentioned above the gene of PAL was fused with His6-Tag at C-terminus for separation from the bulk of other bacterial proteins using metal chelate affinity chromatography. Our studies primarily focused on estimation of basic parameter values of the fermentation process in order to elaborate efficient expression of PAL in *E. coli* cells. Studies showed that the addition of an inducer to the medium led to the increased productivity of biomass (approximately 1.9 times) and protein (approximately 1.2 times). A simple Ludeking-Paire model was used to describe the process. This basic model (Luedeking 1959)is commonly *used for* the description of the kinetics of complex biotechnological process, including restricted and modified processes. The kinetic curves of growth allowed to define the critical points of growth, reflecting the crucial moments in the development of the microbial population in the medium after the inducer adding: the transition of the increasing rate of growth into the decreasing (the inflection point of growth), the maximum acceleration of the growth phase and the negative acceleration phase.

The relationship between the accumulation of the protein, biomass and enzyme activity was determined, and the point of maximum activity of the produced enzyme was identified. The point of maximum activity of the PAL produced was determined as 38.0–38.5 h after induction. The optimum concentration of inducer (IPTG) was 2 mM. This simple and cost effective production process could be recruited for large-scale production of PAL.

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