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Optimal culture condition for the production of phenyalanine ammonia lyase from *E. coli*

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Abstract−The effects of culturing conditions on phenylalanine ammonia lyase production by a recombinant *E. coli* strain were investigated by using a controlled fed-batch fermentation system. In a 5 L fermentor, the optimal composition of the batch medium was 2% glucose, 1% yeast extract, 0.7% K_2HPO_4, 0.8% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$. The optimal feed glucose solution was 50%. Glucose concentration and pH of the culture broth were maintained at about 2.0 g/L and 7.0 during the fed-batch phase, respectively. Following 24-h cultivation, 0.2 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) was added and temperature was shifted from 37 °C to 42 °C to induce *pal* gene expression. Under optimal conditions, a high productivity of 300 U/g could be achieved after 48 h culture, and a cell density of OD_{600} about 82 was obtained at 52 h culture at 500 r/m stirrer speed and 1 vvm, respectively.

Key words: Activity, Culturing Conditions, Fed-batch, Phenylalanine Ammonia Lyase, Recombinant *Escherichia coli*

INTRODUCTION

Phenylalanine ammonia lyase (E.C.4.3.1.5-PAL) is widely distributed in higher plants, some fungi, yeasts and *Streptomyces* [1-4]. It has been used chiefly in the manufacture of L-phenylalanine by reversing the enzyme reaction with high concentration of trans-cinnamic acids and ammonia at an elevated pH. There is a great demand for the production of L-phenylalanine, since it is one of the two precursors required for the synthesis of the artificial sweetener aspartame. The enzyme is therefore of particular interest to researchers in the biotechnological industry. At present, the industrial-scale production of PAL mainly utilizes the genus *Rhodotorula* [3]. The levels of enzyme in these wild-type strains are relatively low; thus, the production of L-phenylalanine from trans-cinnamic acids was of limited success. A recombinant strain capable of producing a large amount of PAL is therefore highly desirable in order to improve the production of L-phenylalanine from trans-cinnamic acids. Although many efforts have been made to construct recombinant strains with high PAL activity [5-7], few results have been reported that the effects of culturing conditions on production of PAL in recombinant *E. coli*. A recombinant *E. coli* strain producing a significant amount of PAL was constructed in our earlier report [7]. This recombinant *E. coli* strain harbors the *pal* gene from *Rhodosporidium toruloides* (*R. toruloides*) under the control of combined promoter *tac* and P_LP_R. In this study, we investigated the optimal culture conditions for PAL production in shaker flasks or fermentor under batch and fed-batch operations. Furthermore, we report a fed-batch procedure that allows high cell density culture and leads to high-level expression of PAL.

MATERIALS AND METHODS

1. Chemicals

Isopropyl-β-D-thiogalactopyranoside (IPTG) and Coomassie brilliant blue R-250 were purchased from Sigma Chemical Co. (St Louis, MO, USA). All of other chemicals were of analytical grade and were purchased from commercial suppliers.

2. Microorganism and Media

The recombinant expression plasmid pBV-PAL (Amp^r, containing combined promoter *tac* and P_LP_R) was constructed according to the report [7]. The expression of PAL was controlled by combined promoter *tac* and P_LP_R. The plasmid pBV-PAL was transformed in *E. coli* JM109. The strain was maintained as 40% (V/V) glycerol stock at −80 °C in Luria-Bertani (LB) medium. The recombinant *E. coli* JM109 was used throughout this work. LB medium [8] was employed for the propagation of *E. coli* transformants under the uninduced state. When necessary, ampicillin was added to the medium at concentrations of 100 μg/mL. Modified LB (MLB) medium was LB medium supplemented with the following per liter: 20 g glucose, 0.1 g ampicillin, 10 g yeast extract and 10 g NaCl. Semi-synthetic production (SSP) medium contained the following per liter: 20 g glucose, 10 g yeast extract, 7 g K_2HPO_4, 8 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7H_2O$ and 0.1 g ampicillin. A synthetic medium (SM) modified from the previous report [9] contained the following per liter: glucose 20 g, 13 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$. Trace elements solution (per liter) was comprised of 8.4 mg EDTA, 2.5 mg $CoCl_2 \cdot 6H_2O$, 15 mg $MnCl_2 \cdot 4H_2O$, 1.5 mg $CuCl_2 \cdot 4H_2O$, 3 mg H_3BO_4, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 13 mg $Zn(CH_3COO)_2 \cdot 2H_2O$. Sterilization was performed at 121 °C for 20 min. Glucose was autoclaved separately from the rest of the medium and added into the medium prior to inoculation. Ampicillin was sterilized by filtration.

3. Culture Conditions

1 mL frozen stock culture was added to LB medium (50 mL working volume in 500 mL Erlenmeyer flask) with ampicillin at a concentration of 100 μg/mL at 37 °C and 200 r/m for 12 h. Subsequently, one further preculture (50 mL LB medium with ampicillin at a concentration of 100 μg/mL in a 500 mL Erlenmeyer flask) was inoculated with the first preculture (2% V/V) and incubated on a rotary

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shaker at 37 ºC and 200 r/m for 12 h [10]. For shaker-flask culture, 10 mL of inoculum culture was added to 90 mL fermentation medium in a 500 mL Erlenmeyer flask. The cultures were incubated at 37 ºC in a rotary set at 200 r/m until optical density (OD_{600}) reached 0.6; at this point, 0.2 mmol/L IPTG was added and the cells were cultured at 42 ºC for 4 h for induction of the PAL. For larger-scale experiments, fed-batch was performed in a classical 5 L jar fermentor (BIOF-2000, Beauty Corp., Shanghai, China). Antifoam 289 (Sigma A-5551) was automatically added as necessary to control foaming in the fermentor. 30 mL of inoculum culture was added to 3 L of fermentation medium. Initial batch culture conditions were as follows: initial culture volume, 3 L; temperature, 37 ºC; air flow rate, 1.0 vvm; stirrer speed, 500 r/m; dissolved O_2(DO) was automatically maintained at 30% by controlling the stirrer speed and aeration rate. The pH was maintained at 6.8-7.0 by addition of aqueous ammonium (12.5% V/V) and solution of 500 g/L glucose. When the pH exceeded the set high limit of 7.0, 500 g/L glucose solution was automatically added so that the glucose concentration in the culture broth was increased while the pH was decreased. Fed-batch culture was performed in two distinct stages. The initial stage was unlimited batch growth, which lasted approximately 10-16 h and allowed consumption of the initial glucose (20 g/L). Once the glucose fell below 2 g/L, the fed-batch phase of the fermentation was initiated. When the pH was decreased below the lower limit of 6.8, aqueous ammonium (12.5% V/V) was automatically added to recover pH as well as supply a nitrogen source. The time points of feeding and harvesting are reported in the Results section. For induction of the PAL, 0.2 mmol/L IPTG was added and the cells were cultured at 42 ºC for 6 h.

4. Analytical Methods

PAL activity of recombinant *E. coli* JM109 whole cells was determined by a modification of the procedure of Orndorff et al. [11]. First, cells were recovered from the culture by centrifugation. The collected cells were washed by suspending them in a 0.85% sodium chloride solution and were recovered by centrifugation. The washed cells were then suspended in 25 mmol/L Tris-HCl buffer solution (pH 8.8). The suspension was added to an enzymatic reaction medium consisting of a 25 mmol/L Tris-HCl buffer solution (pH 8.8) supplemented with 25 mmol/L L-phenylalanine. The resultant reaction medium was incubated at 30 ºC for 20 min. The reaction was terminated by addition of 1 mol/L HCl. After centrifugation, the rate of formation of trans-cinnamic acids was determined by measuring the increase in A_{280} with a 752 spectrophotometer (Shanghai precision and scientific instrument Co., China). One unit of PAL activity was defined as the amount of enzyme required to convert one umole of L-phenylalanine to trans-cinnamic acids per min. PAL activity (specific activity) is expressed as units of enzyme per gram (dry cell weight) of cells. Dry cell weight (DCW) was measured by a modification of the procedure of Jung et al. [12]. DCW were estimated by using a calibration curve obtained from the relationship between the OD at 600 nm and the DCW. Higher OD samples were diluted suitably to have an absorbance at the range of 0.1-0.6. The cell pellets which resulted from the centrifugation were washed with distilled water and dried to constant weight at 80 ºC. One OD_{600} unit corresponds to 0.46±0.05 g DCW/L. Glucose concentrations were measured with a glucose kit (Shanghai Institute of Biological Products, China). Expression level of recombinant protein was determined by 10% sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized by straining with Coomassie Brilliant Blue R250 (Sigma) and quantified by densitometric analysis of strained bands on the gels with Gene Genius Bio Imaging System (Gene genius, SYNGENE Co., USA).

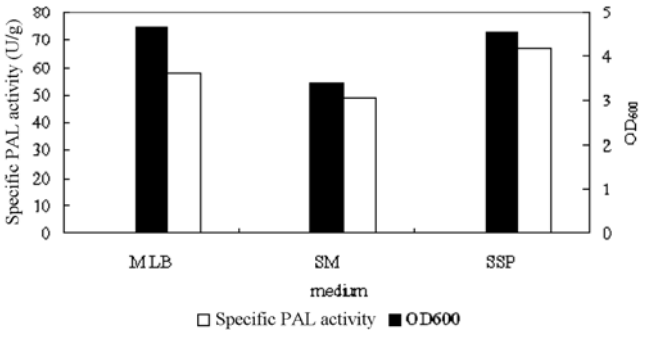

Fig. 1. Effects of medium on cell growth and PAL production.

RESULTS

1. Effects of Medium Composition on Cell Growth and PAL Production

MLB medium, SM and SSP medium were used to evaluate the influence of medium composition on cell growth and PAL production in a 500 mL Erlenmeyer flask. The results show that high cell density (OD_{600}) and PAL activity were achieved in MLB medium and SSP medium (Fig. 1). However, there was low cell density (OD_{600}) and PAL activity in the SM medium. Moreover, PAL activity in SSP medium was the higher than that in MLB medium, and reached 65.32 U/g. Thus, the SSP medium was selected as the fermentation medium because of its simple composition and high effectiveness. Effects of yeast extract on cell growth and PAL production are given in Table 1. The results show that the presence of yeast extract significantly increased cell density (OD_{600}) and PAL activity. The cell density (OD_{600}) increased with increasing concentrations of yeast extract up to 12.5 g/L. However, PAL activity decreased when concentrations of yeast extract exceeded 10 g/L. Moreover, the maximal PAL enzyme activity was obtained as the concentration of yeast extract was 10 g/L, to reach 65.52 U/g. Other components were tested but no significant effects were observed, such as different concentration of K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, and $MgSO_4 \cdot 7H_2O$ (data not shown).

2. Effects of IPTG Concentration and Temperature on PAL Activity

Shake flask experiments were conducted to evaluate the influ-

Table 1. Effects of yeast extract on cell growth and PAL production

Yeast extract (g/L)	OD_{600}	Specific PAL activity (U/g)
0	2.45	34.63
5	3.35	40.81
7.5	4.25	48.74
10	4.8	65.52
12.5	4.92	64.89
15	4.87	64.35

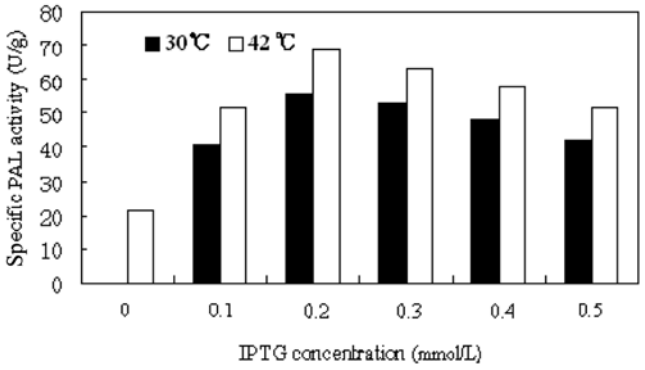

Fig. 2. Effects of IPTG concentration and induction temperature on PAL activity. *E. coli* (pBV-PAL) was grown in SSP medium in shaker flaska at 37 ºC under 200 r/m. After 12 h cultivation, different amounts of IPTG were added and the flasks were transfered to another tow shaker set at 30 ºC and 42 ºC under 200 r/m and cultured for 6 h PAL activity was determined as described in Material and Methods.

ence of IPTG concentration and temperature on induction of PAL production in *E. coli* (containing pBV-PAL) (Fig. 2). Fig. 2 shows that the highest PAL activity was obtained by induction of the combination of 42 ºC and 0.2 mmol/L IPTG. PAL activity was improved over 3.3-fold and 1.2-fold as compared with those of 42 ºC and the combination of 30 ºC and 0.2 mmol/L IPTG, respectively, to reach 69.8 U/g. It was indicated that the presence of IPTG significantly increased PAL activity. But PAL activity decreased when concentrations of IPTG exceeded 0.2 mmol/L. At the same time, the results also show that higher temperature had a remarkable effect on induction of the PAL; even in the absence of IPTG, some amount of PAL was expressed at 42 ºC. Thus, PAL activity by induction of 42 ºC was higher than that of 30 ºC.

3. Effects of Induction Time on PAL Activity

Effects of induction time on PAL activity were investigated by shake flask experiments. A typical time course of the cultivation of *E. coli* JM109 (pBV-PAL) is shown by shake flask experiments in Fig. 3. Moreover, effects of induction time on PAL activity are also shown in Fig. 4. The results indicate that the *pal* gene could be efficiently induced with 0.2 mmol/L IPTG in the mid-exponential phase

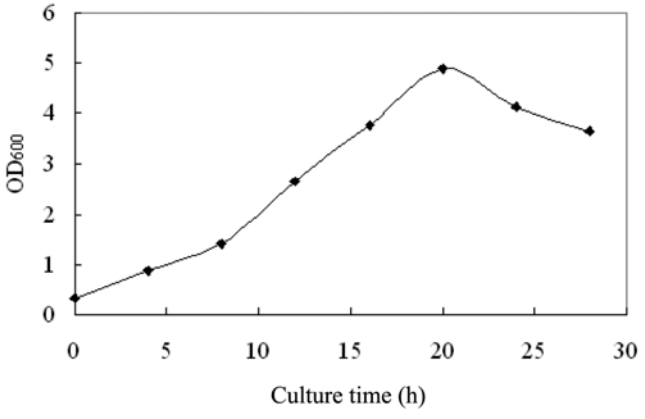

Fig. 3. A typical time course of the cultivation of *E. coli* JM109 (pBV-PAL) by shake flask experiments.

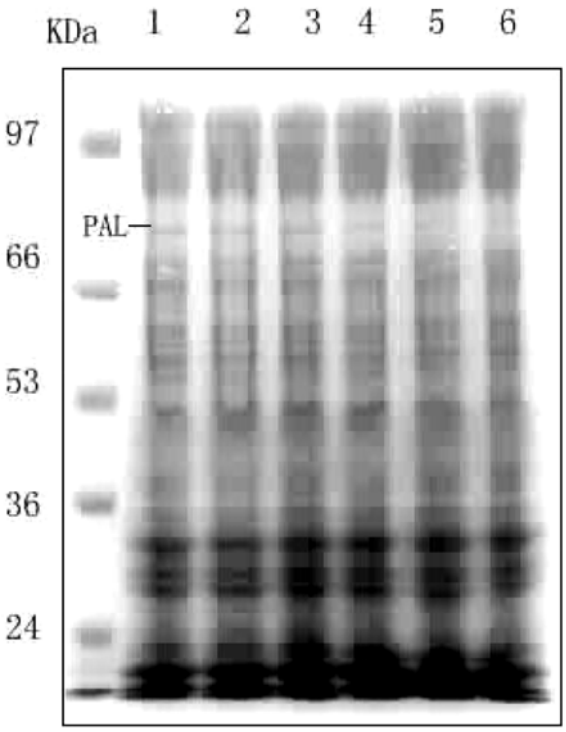

Fig. 4. 10% SDS-PAGE analysis of expression levels induced at different time. Cells were grown in SSP medium in shake flask at 37 ºC under 200 r/m. 0.2 mmol/L IPTG was added after 4 h, 8 h, 12 h, 16 h, 20 h and 24 h cultivation, respectively, at the same time, the tlask also respectively was transterred immediately to another shaker at 42 ºC lane 1, 4 h, lane 2, 8 h, lane 3, 12 h, lane 4, 16 h, lane 5, 20 h, lane 6, 24 h.

of cell growth (at around 12 h). The maximal PAL activity was obtained when induced at 12 h following inoculation. PAL activity was about 1.3-fold and 10-fold as compared with that of 4 h (the fore-exponential phase of growth) and 24 h (the late log phase of growth), respectively. Little PAL activity was detected when cells were induced at the late log phase of growth (at 24 h). To deter-

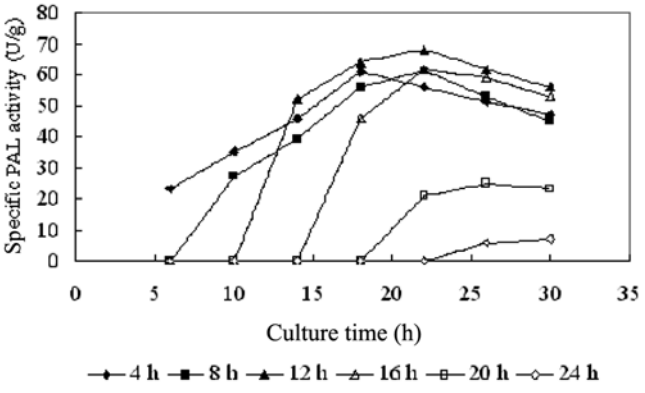

Fig. 5. Effects of induction time on PAL activity. Cells were grown in SSP medium at 37 ºC and 200 r/m. After 4 h, 8 h, 12 h, 20 h and 24 h cultivation. 0.2 mmol/L IPTG was added, and the flask was transferred to another shaker set at 42 ºC under 200 r/m and cultured for 5 h. PAL activity was determined as described in Materials and Methods.

mine whether PAL expression-levels in *E. coli* JM109 (pBV-PAL) were changed due to effects of different induction time, SDS-PAGE was used to observe effects of different induction time on the expression levels of PAL. As shown in Fig. 5, the maximal PAL expression level was about 8.8% of total cellular protein when induced at 12 h. However, the PAL expression level was about 4.2% and 0.8% of total cellular protein when induced at 4 h and 24 h, respectively. It indicated that the effect of induction in the mid-exponential phase of cell growth (at around 12 h) was better than any other induction time.

4. Fed-batch and PAL Production

Based on the results described above, optimal conditions for PAL production of *E. coli* JM109 (pBV-PAL) in a 5 L jar fermentor were as follows. The optimal initial batch medium was SSP medium; culture volume, 3 L; temperature, 37 ºC; air flow rate, 1.0 vvm; stirrer speed, 500 r/m; dissolved O_2(DO) was automatically maintained at 30% by controlling the stirrer speed and aeration rate. The pH was maintained at 6.8-7.0 by addition of aqueous ammonium (12.5% V/V) and a solution of 500 g/L glucose. As described in Materials and Methods, following 24 h cultivation, which was about the mid-exponential phase of cell growth, 0.2 mmol/L IPTG was added and the temperature was shifted from 37 ºC to 42 ºC. Under these conditions, a typical time course of the cultivation of *E. coli* JM109 (pBV-PAL) in a 5 L jar fermentor is shown in Fig. 6. The highest specific PAL activity of about 300 U/g was attained within 42 h and the activity remained relatively high even at 56 h. The maximum cell concentration reached around OD_{600}=82 with 52 h. During the first 10 h of culture, a high specific growth rate of 0.498/h was obtained (data not shown). The glucose was consumed rapidly. The beginning of this second phase coincided with the onset of initial glucose depletion. Glucose was fed at this stage to maintain the maximum cell concentration for intracellular synthesis. To avoid excessive feeding of glucose, which is known to cause acetate accumulation [13-15], the feeding solution was added only when the glucose concentration decreased to a certain level, as indicated by an increase in pH.

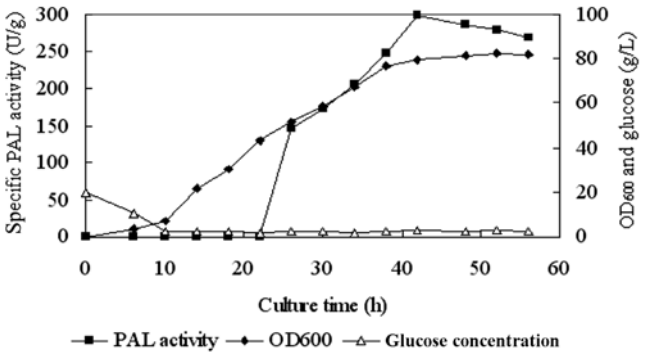

Fig. 6. Profile of PAL production by fed-batch hogh cell density culture of *E. coli* (pBV-PAL). The composition (per L) of the initial fermentation medium was 20 g glucose, 10 g yeast extract, 7 g K_2HPO_4, 8 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7H_2O$ and 0.1 g ampicillin, and that of the feeding solution was: 500 g glucose, 300 mL 12.5% ammonia water. The time courses of biomass OD600, glucose concentration and PAL specific activity were shown. Agitation speed and acretion rate were kept constant at 500 r/m and 1 vvm, respectively. The time of IPTG addition, followed immediately by temperature shift 37 to 42 ºC, was indicated by the arrow.

DISCUSSION

The previous reports showed that the commercial source of PAL was mainly obtained from wild-type *Rhodotorula* [16,17]. PAL specific activities from wild-type *R. toruloides* were in the range of 10-15 U/g (DCW) [18,19]. Furthermore, the levels of PAL enzyme in these strains are relatively low. To improve PAL activity and production, the entire uninterrupted *pal* gene from *R. toruloides* was inserted into the *E. coli* expression vector pKK223-3. *E. coli* cells containing this vector synthesized a protein of pal-like activity [5]. The *pal* gene from *R. toruloides* was expressed in *Saccharomyces cerevisiae* (*S. cerevisiae*) and *E. coli* by using a bifunctional expression system. PAL expression levels were approx 100-fold higher than those of Orum et al. [6]. However, the yields of recombinant enzyme obtained were disappointingly low. Thus, in terms of large-scale production of PAL for industrial and medical uses, PAL activity in recombinant *E. coli* needs to be improved substantially. In our earlier reports, the *pal* gene of *R. toruloides* was highly expressed in *E. coli* JM109 (pBV-PAL); specific PAL activity reached 35 U/g [7]. However, cell density and PAL activity were still low for large-scale production. In this study, the optimal culture conditions to allow the production of significant amount of PAL were investigated. A glucose-controlled strategy was used to improve recombinant PAL activity and cell density. The application of these modes of fed-batch cultivation resulted in a 5 to 6-fold (about 300 U/g) increase in PAL activity compared to shake flask cultivation (about 60 U/g). The enzyme production process can be divided into two phases (Fig. 6). A rapid enzyme production phase positively correlated to the late exponential phase of growth, which was followed by a slower phase of production resulting from a slower growth. However, PAL activity continued to decrease after the stationary phase was reached. Under simple culture conditions, a satisfactory PAL activity could be obtained. PAL activity reached 300 U/g; the maximal cell density (OD_{600}) was 82. For industrial applications, high cell density cultivation (HCDC) is required to improve microbial biomass and product formation substantially. And Fed-batch operation of fermentation process has been receiving a great deal of interest as it offers the possibility to control a substrate concentration at a desired condition [14]. In particular, there is important significance in the manufacture of L-phenylalanine by reversing the PAL enzyme reaction with high concentration of trans-cinnamic acids and ammonia at an elevated pH.

To the best of our knowledge, this is the first report in which a glucose-controlled strategy was used to efficiently improve PAL activity in recombinant *E. coli* by optimizing culture conditions, as much as five-fold increase as compared to the state of the art. These approaches for manipulating recombinant *E. coli* in an effort to create a rich enzyme source would therefore serve as a biotechnologically important protocol for production of L-phenylalanine.

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