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# Influence of Controlled- and Uncontrolled-pH Operations on Recombinant Phenylalanine Ammonia Lyase Production in *Escherichia coli*

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**Abstract** Effects of controlled- and uncontrolled-pH operations on phenylalanine ammonia lyase (PAL) production by a recombinant *Escherichia coli* strain were investigated at uncontrolled-pH ($pH_{UC}$) and controlled-pH ($pH_C$) of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 in bioreactor systems. The results showed that the recombinant PAL activity was improved significantly by controlled pH strategy. Among the $pH_C$ operations, the highest PAL activities were obtained under $pH_C$ 7.5 strategy where cell mass ($OD_{600\ nm}$) and PAL activity was 1.3 and 1.8 fold higher than those of $pH_{UC}$, respectively. The maximum PAL activity reached 123 U/g. The $pH_C$ 7.5 strategy made recombinant plasmid more stable and therefore allowed easier expression of PAL recombinant plasmid, which increased PAL production. It was indicated that the new approach (controlled-pH strategy) obtained in this work possessed a high potential for the industrial production of PAL, especially in the biosynthesis of L-phenylalanine.

**Keywords:** enzyme activity, recombinant *Escherichia coli*, controlled-pH strategy, phenylalanine ammonia lyase, fermentation

## Introduction

L-Phenylalanine is important as an essential amino acid for human nutrition and is widely used in pharmaceutical and food industries. There is a great demand for the production of L-phenylalanine, since it is one of the two precursors required for the synthesis of artificial sweetener aspartame. L-Phenylalanine can be produced by several chemical and biochemical processes; however, the advantage of stereospecific synthesis makes the enzymatic production favorable. Among the enzymatic processes, either with whole cells or isolated enzymes-bioconversion of *trans*-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL, EC.4.3.1.5) is one of the major routes in the production of L-phenylalanine. 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. The commercial source of enzyme has been mainly obtained from the genus *Rhodosporidium* (4). The production of L-phenylalanine from *trans*-cinnamic acids was of limited success, partly because of the relatively low specific activity and instability of PAL during the bioconversion. 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. Some efforts have been made to construct recombinant strains with high PAL activity (5,6), and a recombinant *Escherichia coli* strain producing a significant amount of PAL has been constructed in our earlier report (7). This recombinant *E. coli* strain harbors the *pal* gene from *Rhodosporidium toruloides* under the control of combined promoter *tac* and $P_LP_R$, moreover, the effects of medium composition and isopropyl-β-D-thiogalactopyranoside (IPTG) for PAL production were investigated (8). Although some studies reported that effects of pH value for the production of the other biomolecules by using *E. coli* as a host microorganism (9,10), there is no detailed investigation in the literature on the effects of pH on any biomolecule production by the host *E. coli*. In particular, there is no work in the literature reporting the effects of pH value on PAL production in recombinant *E. coli*. Thus, the objective of the present work was to amply study influence of controlled-pH ($pH_C$) and uncontrolled-pH ($pH_{UC}$) operations on recombinant PAL production in *E. coli*.

## Materials and Methods

**Chemicals** IPTG, Antifoam 289 and Coomassie brilliant blue R-250 was purchased from Sigma-Aldrich (St. Louis, MO, USA). All of other chemicals were of analytical grade and were purchased from local commercial suppliers.

**Microorganism and media** The recombinant expression plasmid pBV-PAL ($Amp^r$, containing combined promoter *tac* and $P_LP_R$) was constructed according to the previous

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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. LB medium was employed for the propagation of *E. coli* transformants under the uninduced state. When necessary, ampicillin was added to the medium at a concentration of 100 μg/mL. The recombinant *E. coli* JM109 was used throughout this work. A fermentation medium modified from the previous report (11) contained the following: glucose 20 g, 13 g $KH_2PO_4$, 4 g $(NH_4)_2HPO_4$, and 1.2 g $MgSO_4 \cdot 7H_2O$ in 1 L solution. Trace elements solution comprises of 8.4 mg ethylene-diaminetetraacetic acid (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 $Na_2MoO_4 \cdot 2H_2O$, and 13 mg $Zn(CH_3COO)_2 \cdot 2H_2O$ in 1 L solution at pH 7.0. Sterilization was performed at 121°C for 20 min. Glucose was autoclaved separately from the rest of medium and added into the medium prior to inoculation. Ampicillin was sterilized by filtration.

**Culture conditions** One mL of 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 30°C and 200 rpm for 12 hr. Subsequently, 1 mL of the first preculture was inoculated to a fresh medium (50 mL LB medium with ampicillin at a concentration of 100 μg/mL in a 500-mL Erlenmeyer flask) and incubated on a rotary shaker at 30°C and 200 rpm for 12 hr (12). For shaker-flask culture experiment, 10 mL of inoculum culture was added to 90 mL fermentation medium in a 500-mL Erlenmeyer flask on a bioreactor rotary set possessing on-line detection and control system for pH value (SHpH; GuoQiang Corp., Shanghai, China). The pH of fermentation medium was maintained respectively at 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 by addition of aqueous ammonium (12.5%, v/v) and 5 M $H_3PO_4$. The culture were incubated at 30°C in a rotary set at 200 rpm for 24 hr until optical density ($OD_{600\ nm}$) reached 0.6, at this point, 0.2 mM IPTG (4 mL) was added and the cells were cultured at 42°C for 4 hr for induction of the PAL. For batch-bioreactor experiments, 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. Thirty 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, 30°C; air flow rate, 1.0 vvm; stirrer speed, 500 rpm; dissolved $O_2$ (DO) was automatically maintained at 30% by controlling the stirrer speed and aeration rate. The pH was maintained at 7.5 by addition of aqueous ammonium (12.5%, v/v) and 5 M $H_3PO_4$. When the pH exceeded 7.5, 5 M $H_3PO_4$ was automatically added while the pH was decreased. When the pH was decreased below 7.5, aqueous ammonium (12.5%, v/v) was automatically added to recover pH as well as supply a nitrogen source. For induction of the PAL, 0.2 mM IPTG (10 mL) was added and the cells were cultured at 42°C for 6 hr. Uncontrolled-pH ($pH_{UC}$) operation was regarded as the control.

**Analytical methods** PAL activity of recombinant *E. coli* JM109 was determined by a modification of the procedure of Orndorff *et al.* (13). First, cells were recovered from the culture by centrifugation. The harvested cells were permeabilized by treatment with 0.3 g/L cetyl trimethyl ammonium bromide (CTAB) at 30°C for 30 min. The collected cells were washed by suspending them in a 0.85% NaCl solution and were recovered by centrifugation. The washed cells were then suspended in 25 mM Tris-HCl buffer solution (pH 8.8). The suspension was added to an enzymatic reaction medium comprising a 25 mM Tris-HCl buffer solution (pH 8.8) supplemented with 25 mM L-phenylalanine, and incubated at 30°C for 20 min. The reaction was terminated by addition of 1 M HCl (0.2 mL). After centrifugation, the rate of formation of *trans*-cinnamic acids was determined by measuring the increase in $Abs_{280\ nm}$ with a 752 spectrophotometer (Shanghai Precision and Scientific Instrument Co., Shanghai, China). One unit of PAL activity was defined as the amount of enzyme required to convert 1 μmol of L-phenylalanine to *trans*-cinnamic acids/min. PAL activity (specific activity) is expressed as units of enzyme/g (dry cell weight) of cells. Dry cell weight (DCW) was measured by a modification of the procedure of Song *et al.* (14). DCW were estimated using a calibration curve obtained from the relationship between the optical density (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 was washed with distilled water and dried to constant weight at 80°C. One $OD_{600}$ unit corresponds to 0.46±0.05 g DCW/L. Glucose concentrations were measured using a glucose kit (Shanghai Institute of Biological Products, Shanghai, China). Expression level of recombinant protein was determined by 10% sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized by staining with Coomassie brilliant blue R250 and quantified by densitometric analysis of stained bands on the gels with Bio Imaging System (GeneGenius; Syngene Co., Boston, MA, USA). Stability of the recombinant plasmid was determined by following methods: First, plasmid-bearing *E. coli* were cultured in 500-mL Erlenmeyer flask containing 100 mL fermentation medium with ampicillin at a concentration of 100 μg/mL. Cultures were aptly diluted with 0.85% sterile sodium chloride solution. One tenth mL of diluted cultures was spreaded onto LB agar medium in the absence of ampicillin using a spreader, and incubated at 30°C for 24 hr. Then, 100 colonies of *E. coli* were picked and incubated onto LB agar medium with ampicillin at a concentration of 100 mg/mL. The percentage of plasmid-bearing *E. coli* cells in the population was determined by using the ratio colonies appearing on LB agar medium with ampicillin/total number of colonies (100 colonies).

## Results and Discussion

Previous reports showed that the commercial source of PAL was mainly obtained from wild-type *Rhodotorula* (2,13). However, the levels of PAL enzyme in these strains are relatively low. In order to improve PAL activity and production, the entire uninterrupted *pal* gene from *Rhodotorula 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* and *E. coli* by using a bifunctional expression system. PAL expression levels were approximately 100 fold higher than those by Orum and Rasmussen (5). 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* need to be improved substantially. In our earlier reports, the *pal* gene of *R. toruloides* was highly expressed in *E. coli* JM109 (pBV-PAL), and specific PAL activity reached 35 U/g (7). Moreover, activity of phenylalanine ammonia lyase in permeabilized recombinant *E. coli* was increased by RSM (15). However, there is no work in the literature reporting the effects of bioreactor operation parameter, pH on PAL production by recombinant *E. coli*. But the studies in the literature using *E. coli* as a host microorganism for the production of the other biomolecules differ in the pH value they utilize (9,10). In this study, influences of $pH_C$ and $pH_{UC}$ operations on recombinant PAL production in *E. coli* were investigated.

**Effects of initial pH of fermentation medium on PAL activity and cell mass ($OD_{600\ nm}$)** Shaker-flask experiments were conducted to evaluate the influence of initial pH of fermentation medium on PAL production in *E. coli*. The results were shown in Fig. 1. With increasing pH, PAL activity significantly increased. In contrast, with increasing pH, cell mass ($OD_{600\ nm}$) was not significantly increased. The highest PAL activity was obtained when initial pH of fermentation medium was 7.5, reached 42.56 U/g. Moreover, with increasing initial pH of fermentation medium, final pH of fermentation medium increased. The above results showed that PAL activity was significantly influenced by initial pH of fermentation medium.

**Comparison of recombinant PAL activity and cell mass between $pH_C$ and $pH_{UC}$ operation** Comparison of recombinant PAL activity and cell mass between $pH_C$ and $pH_{UC}$ operations were shown in Fig. 2. The results showed that applied pH strategy was effective to enhance PAL activity significantly. Moreover, under $pH_C$ 7.5 strategy, cell mass ($OD_{600\ nm}$) was 1.8 fold higher than that of the control (Uncontrolled-pH operation). The maximum PAL activity was obtained when the pH was maintained at 7.5 by addition of aqueous ammonium (12.5%, v/v) and 5 M $H_3PO_4$ during whole fermentation. The recombinant PAL activity was above 2 fold in comparison to the control, reached 46 U/g. In particular, under $pH_C$ 7.5 strategy, final pH of fermentation broth was significantly higher than that of the control.

**Effects of $pH_C$ operation on PAL activity and cell mass ($OD_{600\ nm}$) for shaker-flask culture experiment** To obtain optimal $pH_C$ operation, for shaker-flask culture experiment, effects of $pH_C$ operation on PAL activity and cell mass ($OD_{600\ nm}$) were investigated. The results were shown in Fig. 3. The maximum PAL activity and cell mass was obtained when the pH was maintained at 7.5 by addition of aqueous ammonium (12.5%, v/v) and 5 mol/L $H_3PO_4$ during whole fermentation. In contrast, the PAL activity and cell mass decreased when the pH was maintained above 7.5 ($pH_C$ 8.0 and 8.5). The above results showed that $pH_C$ 7.5 probably would yield higher PAL production and cell formation compared to other control pH strategy ($pH_C$ 5.5, 6.0, 6.5, 7.0, 8.0, and 8.5). Ryan *et al.* (9) investigated the effect of pH on the growth of recombinant *E. coli* and β-lactamase production at 3 different $pH_C$ values. i.e., 7.0, 7.4, and 8.0. They reported that with the increase in pH, specific cell growth rate decreased; however, the highest β-lactamase production was obtained at pH 7.4. In contrast, in this study, with the increase in pH, PAL activity, and cell mass increased. However, the PAL activity and cell mass decreased when the pH was maintained above 7.5 ($pH_C$ 8.0 and 8.5) (Fig. 3). Moreover, the highest PAL production

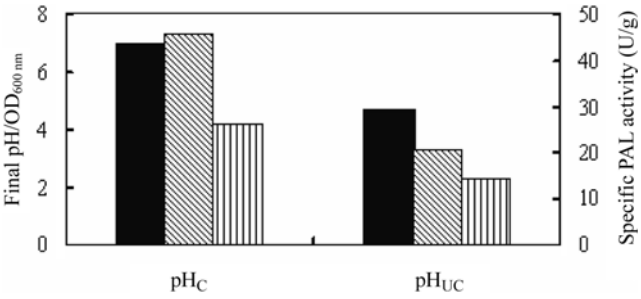


**Fig. 2. Comparison of recombinant PAL activity and cell mass between ($pH_C$) and uncontrolled-pH ($pH_{UC}$) operations.** Final pH (■), specific PAL activity (▧), $OD_{600\ nm}$ (▥). Control pH ($pH_C$), uncontrol pH ($pH_{UC}$).

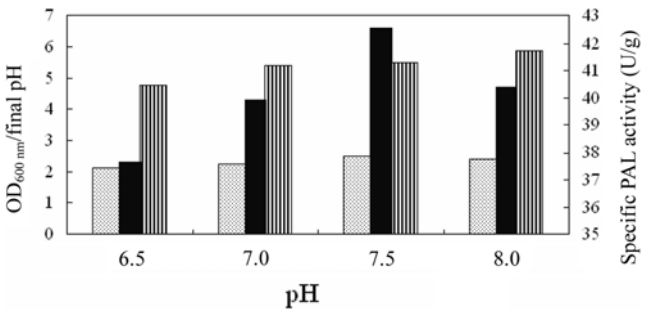


**Fig. 1. Effects of initial pH of fermentation medium on PAL activity and cell mass ($OD_{600\ nm}$).** $OD_{600\ nm}$(▨), specific PAL activity (■), final pH (▥).

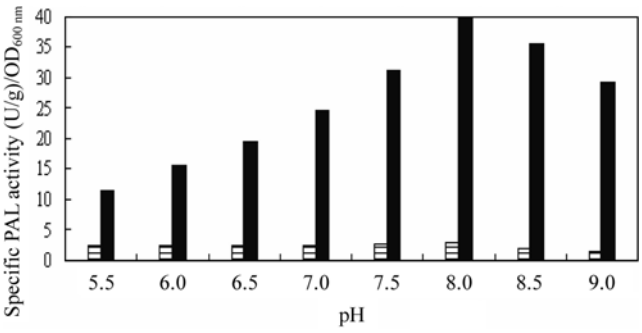


**Fig. 3. Effects of controlled-pH ($pH_C$) operation on PAL activity and cell mass ($OD_{600\ nm}$) for shaker-flask culture experiment.** $OD_{600\ nm}$ (▤), specific PAL activity (■).

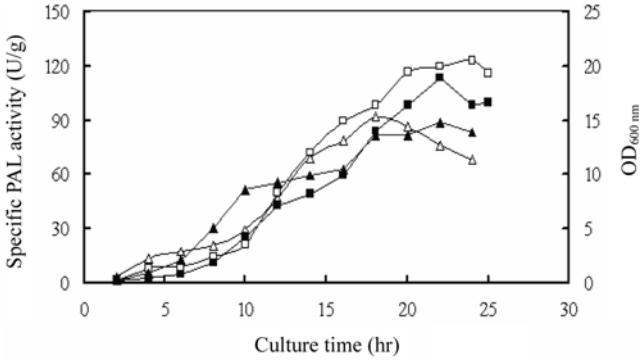


**Fig. 4. Effects of controlled-pH ($pH_C$) operation on PAL activity and cell mass ($OD_{600\ nm}$) for batch-bioreactor experiments.** Specific PAL activity ($pH_{UC}$, △), Specific PAL activity ($pH_C$ 7.5, □), $OD_{600\ nm}$ ($pH_{UC}$, ▲), $OD_{600\ nm}$ ($pH_C$ 7.5, ■).

was obtained at $pH_C$ 7.5. Our result differed with those of Ryan *et al*. (9).

**Effects of $pH_C$ operation on PAL activity and cell mass ($OD_{600\ nm}$) for batch-bioreactor experiments** Effects of $pH_C$ operation on PAL activity and cell mass ($OD_{600\ nm}$) for batch-bioreactor experiments were developed following shaker-flask culture experiment. The results were shown in Fig. 4. The results showed that applied pH strategy was effective to enhance significantly PAL activity and cell mass. Under $pH_C$ 7.5 strategy, cell mass ($OD_{600\ nm}$) and PAL activity was 1.3 and 1.8 fold higher than those of uncontrolled-pH, respectively. The maximum PAL activity reached 123 U/g. To determine whether PAL expression-levels in *E. coli* JM109 were changed due to effects of pH, SDS-PAGE was used to observe effects of $pH_C$ 7.5 and uncontrolled-pH on the expression levels of PAL. As shown in Fig. 5, The maximal PAL expression level was about 9.2% of total cellular protein at $pH_C$ 7.5. However, the PAL expression level was about 6.8% of total cellular protein at uncontrolled-pH. It was indicated that effects of $pH_C$ 7.5 on PAL expression level were better than that of uncontrolled-pH. Moreover, effect of pH on recombinant plasmid stability was investigated. The results indicated that the recombinant plasmid was stable at $pH_C$ 7.5. Ninety-six % colonies maintained the recombinant plasmid when *E. coli* cells were cultured 22 hr. In contrast, under uncontrolled-pH, only 82% colonies maintained the recombinant plasmid when *E. coli* cells were cultured 22 hr (Table 1). Calik *et al*. (16) investigated that influence of controlled- and uncontrolled-pH operations on recombinant benzaldehyde lyase (BAL) production by *E. coli*. Their results showed that the highest BAL activities were obtained when cell concentrations were high, and the lowest BAL activity was obtained if the least amount of cells were generated. They thought that BAL production could be enhanced by increasing the concentrations of BAL producing cells. In this study, we found that PAL activity enhanced with increasing cell mass (Fig. 3 and 4). And the highest PAL activities were obtained when cell concentrations were high (Fig. 3). These results accorded with those of Calik *et al*. (16). However, the highest amount of cell and enzyme was obtained at $pH_C$ 7.5. This

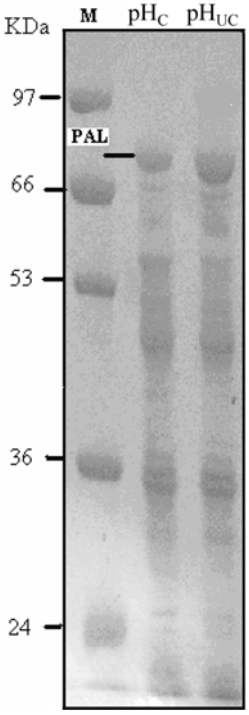


**Fig. 5. Comparison of expression levels of recombinant PAL between controlled- ($pH_C$) and uncontrolled-pH ($pH_{UC}$) operation using 10% SDS-PAGE.**

**Table 1. Effect of pH on recombinant plasmid stability** (%)

| Culture time (hr) | 12 | 16 | 18 | 20 | 22 |
|---|---|---|---|---|---|
| Controlled pH ($pH_C$ 7.5) | 100 | 100 | 98 | 98 | 96 |
| Uncontrolled pH ($pH_{UC}$) | 100 | 96 | 92 | 87 | 82 |

result differed with those of Calik *et al*. (16). On the other hand, under $pH_C$ 7.5 strategy, cell mass ($OD_{600\ nm}$) and PAL activity was 1.3 and 1.8 fold higher than those of $pH_{UC}$ 7.0, respectively. The maximum PAL activity reached 123 U/g. Moreover, there were distinct recombinant plasmid stability and expression levels of PAL in recombinant *E. coli* between $pH_C$ and $pH_{UC}$ (Fig. 5 and Table 1). The more likely explanation was that controlled-pH strategy made recombinant plasmid more stable and therefore allowed easier expression of PAL recombinant plasmid, which increased PAL production.

Since the phases of a bioprocess are dynamic and are the consequences of directed of the bioreaction network interacting strongly with the microenvironment of the cell, the influence of the operational variable pH on the overall bioreaction is indeed important and needs clarification in order to develop an operational strategy. In this study, we found that PAL activity enhanced with increasing cell mass under controlled-pH operations. The maximum PAL activity reached 123 U/g. So, for recombinant protein production by *E. coli*, the following pH strategy can be proposed. The initial pH of fermentation medium should be set to pH 7.5, and the pH of the fermentation medium can be let to decrease until pH 7.0. After that, pH of the medium should be controlled between 7.0-7.5, in order to have high mass and protein production.

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