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Enhancement of L-phenylalanine production by engineered *Escherichia coli* using phased exponential L-tyrosine feeding combined with nitrogen source optimization

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Nitrogen source optimization combined with phased exponential L-tyrosine feeding was employed to enhance L-phenylalanine production by a tyrosine-auxotroph strain, *Escherichia coli* YP1617. The absence of $(NH_4)_2SO_4$, the use of corn steep powder and yeast extract as composite organic nitrogen source were more suitable for cell growth and L-phenylalanine production. Moreover, the optimal initial L-tyrosine level was 0.3 g L^{-1} and exponential L-tyrosine feeding slightly improved L-phenylalanine production. Nerveless, L-phenylalanine production was greatly enhanced by a strategy of phased exponential L-tyrosine feeding, where exponential feeding was started at the set specific growth rate of 0.08, 0.05, and 0.02 h^{-1} after 12, 32, and 52 h, respectively. Compared with exponential L-tyrosine feeding at the set specific growth rate of 0.08 h^{-1}, the developed strategy obtained a 15.33% increase in L-phenylalanine production (L-phenylalanine of 56.20 g L^{-1}) and a 45.28% decrease in L-tyrosine supplementation.

[**Key words:** L-Phenylalanine; Engineered *Escherichia coli*; Nitrogen source; L-Tyrosine feeding; Fed-batch]

L-Phenylalanine (L-Phe) is one of the most important industrially produced amino acids, which is used as feed and food additives, flavor enhancer, infusion fluid, nutraceutical, and intermediate for the synthesis of pharmaceuticals (1). Notably, L-Phe is employed in the production of a sweetener known as aspartame, which has a steadily increasing demand all over the world (2). Currently, L-Phe is mainly produced by enzymatic or microbial processes. In contrast to the enzymatic production of a racemic mixture of D- and L-Phe, microbial production of the pure enantiomer by fermentation is beneficial for the optimization of the aspartame production process (3). Therefore, many researchers have been trying to enhance the microbial fermentative production of L-Phe by improving the producer strain(s) and fermentation process (4).

Among the various microorganisms used in the fermentative production of L-Phe, such as *Brevibacterium lactofermentum* (5), *Corynebacterium glutamicum* (6), *Bacillus subtilis* (7), recombinant *Escherichia coli* has attracted immense interest (8), owing to its advantages of high growth rate and well-known physiological characteristics. Many studies have been carried out on metabolic engineering (9) and fermentation process control using recombinant *E. coli* (10). However, only a relatively few studies have examined medium optimization for L-Phe production, especially optimization of nitrogen source, which is important for amino acid production (11,12). Moreover, overfeeding of L-tyrosine (L-Tyr) by the L-Phe-producing strain, usually obtained from L-Tyr auxotrophic strain, might result in decreased yields owing to overgrowth of the cells (13) and L-Tyr limitation that strongly reduces the microbial growth rate (14). Therefore, control of L-Tyr, which is regarded as a distinctive nitrogen source, is important for L-Phe production. Extensive research has been conducted to achieve optimal control of L-Tyr (8,15,16). For instance, Takagi et al. (15) suggested controlling L-Tyr feeding according to the predicted optimum set of specific L-Tyr consumption rates. However, the specific L-Tyr consumption rate is an unknown a priori value and may change from one experiment to another. Comparatively, the exponential L-Tyr feeding mode is more favorable for enhanced L-Phe production, which allows the cells to grow at a constant specific growth rate (17). However, it is known that cells experience a changing environment in fed-batch culture and exhibit different physiological characteristics (18). Thus, the exponential L-Tyr feeding mode with a constant specific growth rate may not be optimal for L-Phe production. In the present study, by using an L-Tyr auxotrophic strain, the optimal nitrogen sources, including ammonium sulfate concentration, organic nitrogen sources, and initial L-tyrosine concentration were investigated, and a novel phased exponential L-Tyr feeding method was developed.

MATERIALS AND METHODS

Microorganism The microorganism used in this work was an L-Tyr auxotrophic *E. coli* YP1617 (China Center for Culture Collection Center, CCTCC No: M 2013320). The genes *aroF* and *pheA* were cloned in the pACYC177 plasmid, transformed into *E. coli* YP1617. The vector also possesses a kanamycin resistance gene and gene expression of carrying *aroF* and *pheA* is constitutive.

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Precultivation The stock culture was maintained at −80°C in Luria–Bertani (LB) medium with 15% glycerol and 40 mg L^{-1} kanamycin sulfate. The inoculum medium was LB medium with 40 mg L^{-1} kanamycin sulfate. A total of 2 ml of the stock culture was inoculated into a 500-ml shake flask with 50 ml of inoculum medium, and the flask was incubated for about 12 h at 37°C on a rotary shaker at 200 rpm.

Cultivation The initial fermentation medium contained (g L^{-1}): glucose, 20; $MgSO_4$, 3; KH_2PO_4, 3; NaCl, 1; $(NH_4)_2SO_4$, 5; $CaCl_2 \cdot 2H_2O$, 0.015; $FeSO_4 \cdot 7H_2O$, 0.1125; sodium citrate, 1; yeast extract, 10; L-Tyr, 0.3; thiamine, 0.075; kanamycin sulfate, 0.04; and trace element solution (TES), 1.5 ml L^{-1}. The TES was composed of (g L^{-1}): $Al_2(SO_4)_3 \cdot 18H_2O$, 2; $CoSO_4 \cdot 7H_2O$, 0.75; $CuSO_4 \cdot 5H_2O$, 2.5; H_3BO_3, 0.5; $MnSO_4 \cdot 7H_2O$, 24; $Na_2MoO_4 \cdot 2H_2O$, 3; $NiSO_4 \cdot 6H_2O$, 2.5; and $ZnSO_4 \cdot 7H_2O$, 15. In the experiments of optimization, $(NH_4)_2SO_4$ concentration, L-Tyr concentration or the type of organic nitrogen source varied as a parameter while the levels of others were kept constant as previously described, respectively. In the experiments on phased exponential L-Tyr feeding, $(NH_4)_2SO_4$ concentration, initial L-Tyr concentration or the type of organic nitrogen source were used the optimums.

The fed-batch fermentations were performed in a 1.4-L benchtop bioreactor (Infors AG, Bottmingen, Switzerland) with an initial volume of 0.5 L of the fermentation medium and 10% inoculum. During the entire fermentation process, the pH was controlled at 7.0 with the addition of 25% ammonia water and the temperature was maintained at 37°C. The aeration rate was maintained at 0.7 vvm and the dissolved oxygen (DO) level was retained at around 10% by adjusting the agitation speed (200–800 rpm). The glucose concentration was maintained below 5 g L^{-1} by feeding 700 g L^{-1} glucose, and L-Tyr was fed at a constant rate of 0.003 g L-tyr h^{-1} after 12 h.

In the experiments on exponential L-Tyr feeding, L-Tyr was fed into a computer-controlled pump at a set point of specific growth rate (μ_{set} = 0.08, 0.05, and 0.02 h^{-1}). The feeding rate was changed every 1 h and predetermined using a mass balance equation (Eq. 1). Finally, phased exponential L-Tyr feeding method was employed, along with optimum nitrogen source. L-Tyr feeding was divided into three stages: exponential L-Tyr feeding at μ_{set} of 0.08, 0.05, and 0.02 h^{-1} after 12, 32, and 52 h, respectively. The L-Tyr feeding rate was also measured by using Eq. 1:

$$F = \frac{\mu_{set} X_0 V_0 \exp(\mu_{set} t)}{Y S_0} \quad (1)$$

where F is the L-Tyr feeding rate (L h^{-1}), μ_{set} is the set point of specific growth rate (h^{-1}), X_0 and V_0 are the initial cell concentration (g L^{-1}) and culture volume (L) before L-Tyr feeding, respectively, t is the cultivation time after exponential L-Tyr feeding (h), Y is the yield coefficient (g DCW g^{-1} L-Tyr) computed from batch culture, and S_0 is the L-Tyr concentration (g L^{-1}) in the feeding solution.

Analytical methods The cell optical density at 600 nm (OD_{600}) was measured by using a spectrumlab 752s (Shanghai Lengguang Technology Co. Ltd., China) after appropriate dilution. The dry cell weight (DCW) was computed according to a predetermined calibration, where one unit of OD_{600} represented 0.41 g L^{-1} DCW. Glucose concentration was determined by using an SBA-40C biosensor analyzer (Biology Institute of Shandong Province Academy of Sciences, Jinan, China). The contents of amino acids (L-Phe and L-Tyr) were analyzed by Agilent 1290 Infinity LC System (Agilent, USA) using a column (prevail C18 5 μ, 250 × 4.6 mm, Grace, USA) with a UV detector (1290 Infinity Diode Array Detector, Agilent) at 210 nm. As the mobile phase, 5% pure acetonitrile in 95% 20 mM $Na_2HPO_4 \cdot 12H_2O$ (pH adjusted to 6.0 by H_3PO_4) was used at a flow rate of 1 ml min^{-1}, and the column temperature was 30°C. Furthermore, the acetic acid content was analyzed by Agilent 1290 Infinity LC System (Agilent) using a column (prevail organic acid 5 μ, 250 × 4.6 mm, Grace, USA) with a UV detector (1290 Infinity Diode Array Detector, Agilent) at 215 nm. As the mobile phase, 25 mM KH_2PO_4 (pH adjusted to 2.5 by H_3PO_4) was used at a flow rate of 1 ml min^{-1}, and the column temperature was 25°C.

The L-Phe yield on glucose ($Y_{phe/Glc}$) was defined as the final amount of Phe produced by consuming 1 g of glucose. The L-Phe yield on cell ($Y_{phe/cell}$) was defined as the final amount of L-Phe produced from 1 g of dry cells. The L-Phe productivity was calculated as the final L-Phe concentration divided by production time. The specific growth rate (μ) was the increase in the unit cell mass in each unit of cultivation time. The specific L-Phe production rate (π_{phe}) was the amount of L-Phe produced by the unit cell mass in each unit of cultivation time.

RESULTS AND DISCUSSION

Effect of $(NH_4)_2SO_4$ concentration on L-Phe production As an inorganic nitrogen source, $(NH_4)_2SO_4$ has been widely used in amino acid fermentation. $(NH_4)_2SO_4$ contains the nitrogen for cell growth and amino acid production, but excessive amount of ammonia may lead to cell growth inhibition, especially in *E. coli* (19). However, so far, $(NH_4)_2SO_4$ has been used as a nitrogen source in all L-Phe fermentation. In the present study, the effect of initial $(NH_4)_2SO_4$ concentration on L-Phe production was evaluated. As shown in Table 1, L-Phe concentration, DCW, $Y_{phe/Glc}$, $Y_{phe/cell}$ and L-Phe productivity decreased with increasing initial $(NH_4)_2SO_4$ concentration and they decreased heavily above 10 g L^{-1} $(NH_4)_2SO_4$. Compared with the absence of $(NH_4)_2SO_4$ in fermentation medium, adding 10 g L^{-1} $(NH_4)_2SO_4$ led to a 19.71% decrease in DCW and a 56.57% decrease in L-Phe concentration. These results indicated that high concentrations of initial $(NH_4)_2SO_4$ (>10 g L^{-1}) showed a negative effect on cell growth and L-Phe production, according with the earlier study (19). Due to the gradual feeding of ammonia water in fermentation process, the slowly added ammonia would not inhibit cell growth but supply the nitrogen for cell growth and L-Phe production. Furthermore, the increase in $(NH_4)_2SO_4$ concentration from 0 to 20 g L^{-1} led to the accumulation of byproduct acetic acid in the broth, which could restrain the cell growth and reduce the production of amino acids (20). Compared with adding 5 g L^{-1} $(NH_4)_2SO_4$ in fermentation medium, the absence of $(NH_4)_2SO_4$ brought out a 47.48% decrease in acetic acid concentration, a 2.24% increase in DCW and a 7.95% increase in L-Phe concentration. Thus, addition of $(NH_4)_2SO_4$ to the initial fermentation medium was not favorable for enhanced L-Phe production.

Utilization of different organic nitrogen sources for L-Phe production Organic nitrogen source is rich in important ingredients for amino acid production, including amino acids, oligopeptides, vitamins, nucleotides, and significant amounts of inorganic and organic phosphates (13). So far, expensive yeast extract and tryptone have been mainly used as the organic nitrogen source for L-Phe production. Various types of cheap organic nitrogen sources were used in the following experiments, where each organic nitrogen source was added to achieve a total nitrogen concentration equivalent to 10 g L^{-1} yeast extract. Meanwhile, about 130 g/L glucose was consumed in each 72 h fermentation process.

In the present study, yeast extract, corn steep powder, cottonseed powder, soybean powder, and tryptone were investigated for L-Phe production. As shown in Fig. 1, it was obvious that *E. coli*

TABLE 1. Results and fermentation parameters of the L-Phe fermentation by *E. coli* YP1617 at varying $(NH_4)_2SO_4$ concentration from 0 to 30 g/L.

Fermentation parameter	Fed-batch culture with varying initial $(NH_4)_2SO_4$ concentration (g/L)				
	0	5	10	20	30
Glucose concentration (g/L)	130 ± 0.01	130 ± 0.58	130 ± 0.03	94 ± 0.41	84 ± 8.19
Cultivation time (h)	72	72	72	72	72
DCW (g/L)	44.69 ± 0.81	43.71 ± 0.87	35.88 ± 1.51	29.23 ± 0.29	28.11 ± 0.32
L-Phe (g/L)	25.79 ± 1.66	23.89 ± 0.86	11.20 ± 0.17	1.97 ± 0.09	0.67 ± 0.007
Acetic acid (g/l)	2.19 ± 1.23	4.17 ± 0.54	4.67 ± 0.16	5.98 ± 1.09	1.25 ± 0.49
$Y_{phe/Glc}$ (g/g)	0.198 ± 0.013	0.183 ± 0.006	0.086 ± 0.001	0.021 ± 0.001	0.008 ± 0.001
$Y_{phe/cell}$ (g/g)	0.577 ± 0.048	0.547 ± 0.009	0.312 ± 0.008	0.067 ± 0.003	0.024 ± 0.00002
L-Phe productivity [g/(L*h)]	0.358 ± 0.023	0.332 ± 0.012	0.156 ± 0.002	0.027 ± 0.001	0.009 ± 0.0001

Except $(NH_4)_2SO_4$ concentration, these fed-batch culture fermentations are experimented under the same condition. Each value is an average of three parallel replicates and is represented as ±mean standard deviation.