J Ind Microbiol Biotechnol (2011) 38:1219–1227
DOI 10.1007/s10295-010-0900-9

ORIGINAL PAPER

Enhanced L-phenylalanine production by recombinant *Escherichia coli* BR-42 (pAP-B03) resistant to bacteriophage BP-1 via a two-stage feeding approach

Haiyan Zhou · Xianyan Liao · Long Liu · Tianwen Wang · Guocheng Du · Jian Chen

Received: 31 May 2010 / Accepted: 3 November 2010 / Published online: 21 November 2010

Abstract The L-phenylalanine (L-Phe) production by *Escherichia coli* WSH-Z06 (pAP-B03) was frequently prevented by bacteriophage BP-1 infestation. To cope with the bacteriophage BP-1 problem for an improved L-Phe production, one bacteriophage BP-1-resistant mutant, *E. coli* BR-42, was obtained from 416 mutant colonies of *E. coli* WSH-Z06 after *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) mutagenesis by selection for resistance to bacteriophage BP-1. The recombinant *E. coli* BR-42-carrying plasmid pAP-B03 had a high capacity in L-Phe production and a remarkable tolerance to 1×10^{10} pfu (plaque-forming unit)/ml bacteriophage stock. For an enhanced L-Phe production by *E. coli* BR-42 (pAP-B03), the effects of different feeding strategies including pH–stat, constant rate feeding, linear decreasing rate feeding, and exponential feeding on L-Phe production were investigated; and a two-stage feeding strategy, namely exponential feeding at $\mu_{set} = 0.18\ h^{-1}$ in the first 20 h and a following linear varying rate feeding with $F = (-0.55 \times t + 18.6)$ ml/h, was developed to improve L-Phe production. With this two-stage feeding approach, a maximum L-Phe titer of 57.63 g/l with a high L-Phe productivity (1.15 g/l/h) was achieved, which was 15% higher than the highest level (50 g/l) reported so far according to our knowledge. The recombinant *E. coli* BR-42 (pAP-B03) is a potential L-Phe over-producer in substantial prevention of bacteriophage BP-1 infestation compared to its parent strain WSH-Z06 (pAP-B03).

Keywords L-phenylalanine · Bacteriophage-resistant · Fed-batch culture · Glucose feeding

H. Zhou · L. Liu · T. Wang · G. Du · J. Chen
State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, China

H. Zhou · L. Liu · T. Wang (✉) · G. Du (✉) · J. Chen
School of Biotechnology, Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, China
e-mail: tianwenw@yahoo.cn

G. Du
e-mail: gcdu@jiangnan.edu.cn

X. Liao
School of Life Sciences, Shanghai University, Shanghai 200444, China

Introduction

Among the essential amino acids, L-phenylalanine (L-Phe) is known for its commercial value in pharmaceutical and food industries. Recently, its production has been stimulated by the great demand for the low-calorie sweetener aspartame (L-aspartyl-L-phenylalanine methyl ester) [17]. For the large-scale production of L-Phe, fermentation processes based on glucose-consuming L-Phe-producing mutants, especially metabolically engineered strains of *Corynebacterium glutamicum* or *E. coli*, seem to be economically superior to the alternatives such as the chemical synthesis from substrates (benzaldehyde, glycine, acetanhydride, etc.) and the chemo-enzymatic processes [10, 21].

In *E. coli*, the tight regulation on L-Phe biosynthesis is primarily through end-product feedback inhibition and/or repression in three successive enzymatic steps catalyzed by 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DS), chorismate mutase (CM), and prephenate dehydratase (PDT) in the biosynthetic pathway [21]. To achieve the over-production of L-Phe, researchers have adopted numerous metabolic engineering strategies in manipulating the metabolic pathways and dampening the control

architecture in the cells [2, 18, 28]. In 1990, Backman et al. [3] engineered *E. coli* for L-Phe production through over-expression of feedback-resistant DS (encoded by $aroF^{fbr}$ gene) and feedback-resistant CM and PDT (encoded by $pheA^{fbr}$gene). Combining an efficient fermentation process, they achieved a final L-Phe titer of 50 g/l and a yield on glucose of 0.25 mol/mol after 36 h. This has been the highest level reported so far, however, the genetic basis of the L-Phe over-production is unrevealed. In our previous work [29], a metabolically engineered strain, *E. coli* WSH-Z06 (pAP-B03), was constructed by introducing a recombinant plasmid pAP-B03 carrying a novel $pheA^{fbr}$ gene as well as a wild-type *aroF* gene [encoding L-tyrosine (L-Tyr)-sensitive DS]. This strain exhibited strong resistance to high-level L-Phe and good potential for over-production of L-Phe: 35.38 g/l L-Phe with a yield coefficient of 0.26 mol/mol glucose in pH–stat fed-batch culture within 58 h. A further improved titer of around 55 g/l at 50 h has been achieved via the optimization of fermentation conditions of L-Phe production by *E. coli* WSH-Z06 (pAP-B03) (unpublished data).

However, L-Phe production by the promising recombinant *E. coli* WSH-Z06 (pAP-B03) was frequently challenged by bacteriophage attacks in the earlier period during the induction phase, resulting in the retarded utilization of glucose due to cell lysis, and consequently the dramatically reduced L-Phe production. Control of phage problems in the commercial arena requires preventing phage contamination by employing strains that are resistant to phage infection, and minimizing opportunities for the appearance of new virulent phages [12]. In this work, we isolated one mutant host strain *E. coli* BR-42, which were resistant to bacteriophage BP-1 isolated from the contaminated fermentation broth of L-Phe production by *E. coli* WSH-Z06 (pAP-B03) via *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) treatment of *E. coli* WSH-Z06. Based on this resistant host, a recombinant L-Phe-producing strain *E. coli* BR-42 (pAP-B03) was obtained.

Various fermentation strategies and techniques have been developed to improve L-Phe production, such as medium optimization [5], oxygen supply rate control [23], substrate feeding rate control [8, 25], temperature control [16, 22], on-line separation [7, 20], and non-growing culture [5, 27]. Fed-batch cultivation is a fundamental method for industrial L-Phe production, during which the substrate feeding rate is a key factor for *E. coli* cultivation due to the fact that some inhibitory metabolites, particularly acetic acid, can be excreted if an inappropriate feeding strategy is adopted [14]. The prevention of acetic acid excretion was achieved by controlling the glucose feeding rate lower than a critical glucose uptake rate and the L-Phe production reached 46 g/l [14]. However, the critical specific glucose uptake rate calculated from an empirical relationship does not represent the actual value in each case. To provide a suitable nutrient level, Gerigk et al. [6] developed a process for L-Phe production by recombinant *E. coli* F4/pJF119EH $aroF^{wt}$ $pheA^{fbr}$ $aroL^{wt}$ concerning the online control of glucose and L-Tyr in fed-batch cultivation. As reported, an optimum glucose concentration of 5 g/l was identified and a higher L-Phe titer (34 g/l) was achieved. As is known, the online control of glucose concentration requires a sterile sample loop and glucose analyzer, which is not considered robust enough for industry, even though it has been used successfully in academic settings [11].

In this work, with the bacteriophage-resistant mutant, recombinant *E. coli* BR-42 (pAP-B03), different glucose feeding strategies were examined for an improved L-Phe production, such as pH–stat, constant rate feeding, linear decreasing rate feeding, and exponential feeding. Finally, a two-stage feeding, namely the exponential feeding in the first 20 h, and a following linear decreasing rate feeding was proposed based on the changes in specific growth rate and cell physiology. In order to achieve high L-Phe productivity, the effect of cell concentration at the beginning of the induction on L-Phe production was also investigated for a suitable induction in fed-batch cultures of recombinant *E. coli* BR-42 (pAP-B03).

Materials and methods

Bacterial strain, plasmid, and bacteriophage

L-Tyr auxotrophic *E. coli* K12 WSH-Z06, a host for L-Phe production in our previous work, was used as parent strain for isolation of bacteriophage BP-1-resistant mutants after NTG mutagenesis.

Plasmid pAP-B03 with *E. coli* genes *aroF* (encoding L-Tyr-sensitive DS) and $pheA^{fbr}$ (encoding L-Phe feedback-resistant CM-PDT) under the control of the temperature-inducible phage lambda promoters (pR and pL), respectively, was constructed in our previous work [29]. Kanamycin (Kan) resistance was employed as a selection marker for plasmid-containing cells.

Bacteriophage BP-1 was isolated from the fermentation broth of L-Phe production by *E. coli* WSH-Z06 (pAP-B03) with the soft agar (0.7%) overlay method [1] and propagated with strain WSH-Z06. BP-1 was a temperate bacteriophage and a member of the lambda-like bacteriophage family. The particle of bacteriophage BP-1 was visualized with a transmission electron microscope (H-7000, Hitachi, Tokyo, Japan) to be tadpole-like with an isometric head about 45 nm in diameter and a tail about 125 nm long and 6–8 nm in thickness, with no visible tail fibers.

NTG Mutagenesis and isolation of bacteriophage BP-1-resistant mutants

E. coli WSH-Z06 cells were grown in LB medium until log phase, collected by centrifugation (8,000 rpm, 10 min at 4°C) and washed twice with 100 mM potassium phosphate buffer (pH 7.0). The cells were treated with different concentrations of NTG (0.2, 0.4, 0.6, and 0.8 mg/ml) for 30 min, collected, washed twice, resuspended in potassium phosphate buffer (100 mM, pH 7.0) and incubated in LB medium for 4–6 h to allow segregation and expression of mutations. Different dilutions were spread on an LB agar plate and cultured at 37°C for 24 h; single colonies with various sizes, colors, or shapes were transferred to the square grids of LB agar plates and incubated at the same conditions for the short-times stocks and detection of resistance to bacteriophage BP-1 in spotting method [19].

Each colony in grid-plates was transferred into and suspended in 1 ml of LB medium, and 100 μl of aliquots were spread on the previously prepared TY [19] agar plates, and 10 μl of 1×10^{10} pfu (plaque-forming unit)/ml phage stock was spotted into each of four quadrants of the plates containing *E. coli* WSH-Z06 mutant strains in the surface. After incubation at 37°C for 24–48 h, the plates were examined for the presence or absence of plaques in the lawn of confluent cell growth. The tested strains that grew well without any lysed areas on the surface of their lawn were considered to be bacteriophage BP-1-resistant and were selected for further examination of bacteriophage BP-1 resistance in soft agar (0.7%) overlay method [1] in the following procedure. The tested *E. coli* strains were inoculated into LB medium and cultured at 37°C until log phase. One hundred microliters of cell broth and 100 μl of different viable concentrations of phage stock (1×10^4, 1×10^7, and 1×10^{10} pfu/ml) were added to 8 ml of TY soft agar (0.7%, 40–50°C) and the mixture was poured onto the base layer of TY plates. The cultivation conditions and examination method of the results were the same as in the spotted method. Strains that showed strong resistance to bacteriophage BP-1 were selected for L-Phe production.

Media and cultivation conditions

E. coli cells were routinely grown in LB medium for 12 h at 37°C and 200 rpm on rotary shakers. Kan was used at 40 μg/ml as required.

Bacteriophage-involved experiments, including bacteriophage propagation, titer measurement, and the isolation of *E. coli* mutant resistant to bacteriophage were performed at 37°C and 200 rpm in TY medium [19]. The TY plate was added with 2% (w/v) and 0.7% agar for base layer and upper layer, respectively.

Production of L-Phe by recombinant *E. coli* was conducted in a fermentation medium slightly modified according to [7, 29] (g/l): 20 glucose, 5 $(NH_4)_2SO_4$, 3 KH_2PO_4, 3 $MgSO_4{\cdot}7H_2O$, 1 NaCl, 1.5 Na-Citrate, 0.015 $CaCl_2.2H_2O$, 0.1125 $FeSO_4.7H_2O$, 0.075 Thiamine-HCl, 0.4 L-Tyr, 3 yeast extract, 40 μg/ml Kan, and 1.5 ml/l trace elements solution (TES). TES contained (g/l): 2.0 $Al_2(SO_4)_3{\cdot}18H_2O$, 0.75 $CoSO_4{\cdot}7H_2O$, 2.5 $CuSO_4{\cdot}5H_2O$, 0.5 H_3BO_3, 24 $MnSO_4{\cdot}7H_2O$, 3.0 $Na_2MoO_4{\cdot}2H_2O$, 2.5 $NiSO_4{\cdot}6H_2O$, and 15 $ZnSO_4{\cdot}7H_2O$.

Shake flask fermentations were carried out in 500-ml Erlenmeyer flasks containing 50 ml fermentation medium inoculated with 10% (v/v) seed cultures at 33°C and 200 rpm on rotary shakers. Calcium carbonate (12 g/l) was added to adjust the pH of the medium. After 12 h and at an OD_{610} of 1.5–2.0, the cells were induced by up-shifting the temperature to 38°C. The fermentation lasted for 48 h.

Fed-batch cultures were performed in a 3-l jar fermentor (LiFlus GM BioTRON, Korea) with an initial broth volume of 1.35 l of fermentation medium. The batch culture was initiated by inoculating 150 ml pre-culture grown to mid-exponential phase (OD_{610} = 2–2.5) at 37°C and 200 rpm. The culture pH was adjusted and controlled at 6.8 ± 0.1 with 20% (v/v) ammonia solution. The temperature was kept at 33°C in the cell growth phase and 38°C in the induction phase. The dissolved oxygen (DO) level was maintained above 20% saturation by cascading the agitation speed (400–900 rpm) and aeration rate (1–4 vvm). When the initial glucose was depleted as indicated by a sharp increase of DO (approximately 12 h), fed-batch was started by feeding 700 g/l glucose in different modes as described below.

pH–stat feeding mode

Glucose was automatically added in pH–stat mode using a computer-controlled pump. It started as the pH was above the set-point of 6.8 and stopped as the pH was below 6.8 during the whole feeding period.

Constant rate feeding

Glucose was fed at a constant rate (F = 16 and 10 ml/h) into the fermentor.

Linear decreasing rate feeing mode

Glucose was fed at a linear decreasing rate [$F = (-0.55 \times t + 23)$ ml/h]. The feeding rates were changed every 1 h.

Exponential glucose feeding

Glucose was exponentially fed into the fermentor using a computer-controlled pump with two different specific growth rates ($\mu_{set} = 0.18$ and $0.12\ h^{-1}$). The feeding rates were changed every 30 min and predetermined using the following mass balance equation:

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

where F is the feeding rate (l/h), μ_{set} is the set specific growth rate (h^{-1}), X_0 and V_0 are the initial cell concentration (g/l) and culture volume (l) at beginning of glucose feeding, respectively. t is the cultivation time after the initiation of glucose exponential feeding (h). Y is the yield coefficient (g DCW/g glucose) and is estimated from batch culture, and S_0 is glucose concentration (g/l) in the feeding solution.

Two-stage feeding strategy

Glucose was supplied with $0.18\ h^{-1}$ of specific growth rate in exponential feeding mode before 20 h, followed by a linear decreasing feeding [$F = (-0.55 \times t + 18.6)$ ml/h] so as to avoid the excessive glucose in the medium.

For all fed-batch cultures, the glucose was fed until glucose was accumulated up to 10 g/l and the induction was started when cell concentration reached 9.5 g/l unless otherwise specified.

Analytical methods

The cell concentration was measured as the optical density at 610 nm (OD_{610}) with a spectrophotometer-722 (Third Analytical Instrument Factory, Shanghai, China) after an appropriate dilution, where one unit of OD_{610} was equivalent to 0.32 g/l dry cell weight (DCW) [29]. To determine DCW, 10 ml of fermentation broth in a pre-weighed centrifuge tube was centrifuged for 10 min at 10,000 rpm and 4°C. After the supernatant was poured off, the cell pellets were washed twice with distilled water. The tubes containing cell pellets were dried at 85°C to constant weight. For the measurement of glucose, amino acids, and acetic acid, 5 ml of fermentation broth was centrifuged at 10,000 rpm for 10 min. The supernatant was diluted and filtered through a membrane (pore size = 0.22 μm). Glucose concentration was determined by a glucose-glutamate analyzer SBA-40C (Biology Institute of Shandong Academy of Sciences, Jinan, China). Amino acids were automated precolumn derivatized by o-phthaldialdehyde (OPA) and were analyzed with an Agilent 1100 HPLC (Agilent, Palo Alto, USA) equipped with a reverse-phase column (Zorbax Eclipse-AAA) and UV detector at 338 nm according to the procedure established by Henderson et al. [9]. Acetic acid concentration was measured by HPLC (Agilent 1100 series, Santa Clara, CA) with a reverse phase column (Zorbax SB-Aq) with an injection volume of 5 μl. The mobile phase consisted of 0.1% phosphoric acid aqueous solution flowed at a rate of 1.0 ml/min. The column temperature was maintained at 30°C. The detection wavelength was 210 nm.

Statistical analysis

All the experiments were performed at least three times, and the results were expressed as the mean ± standard deviation (SD). Statistical analysis was performed with Student's t test. P values of < 0.01 were considered statistically significant.

Results and discussion

Isolation and fermentative performance of bacteriophage BP-1-resistant mutants

To isolate mutants resistant to bacteriophage BP-1, single colonies of WSH-Z06 after mutagenesis with four different concentrations of NTG (0.2, 0.4, 0.6, and 0.8 mg/ml) were tested for phage-resistance by the spotting method [19] and rechecked using a soft agar overlay method [1]. Of 416 isolates of *E. coli* WSH-Z06 tested, two strains exhibited strong resistance to BP-1, and no plaque was observed both in monolayer cell culture and under overlay conditions. These two isolates were selected and designated as BR-42 and BR-130. *E. coli* BR-42 and BR-130 were also resistant against other lambda bacteriophages from our laboratory collection. Both of them were unable to utilize maltose, which was probably due to the fact that genetic mutation rendering resistance to bacteriophage BP-1 was involved in maltose metabolism. As is well known, adsorption of bacteriophage lambda on *E. coli* cell receptor occurs through specific binding between the phage tail fiber protein and the outer membrane protein LamB, a component of the transport system for maltose, and *E. coli* with inactive LamB could prevent infection by lambda bacteriophages [4, 24]. It was speculated that resistance to BP-1 resulted from the alteration in outer membrane protein of *E. coli* BR-42 and BR-130 and the study on their defense mechanisms against BP-1 infection are currently in progress (unpublished data). However, mutations involved in the regulation of sugar metabolism may also present in bacteriophage BP-1-resistant mutants resulting from NTG treatment of *E. coli* WSH-Z06. This could drastically influence L-Phe production. Therefore, it is necessary to examine their capacity of glucose utilization, cell growth,

and L-Phe production, and select one ideal strain for L-Phe over-production.

As derivatives of *E. coli* K12 WSH-Z06, BR-42 and BR-130 were L-Tyr auxotrophic, resulting in decreased unwanted by-product L-Tyr and controllable biomass. *E. coli* BR-42 and BR-130 transformed with plasmid pAP-B03 were examined for their capacity of L-Phe production in shake flask fermentation. There was a decrease of 48% in the final DCW (3.67 ± 0.48 g/l) and 53% in L-Phe titer (2.11 ± 0.13 g/l) of BR-130 (pAP-B03), respectively, compared with a DCW of 7.05 ± 0.19 g/l and a L-Phe titer of 4.47 ± 0.20 g/l of the parent strain WSH-Z06 (pAP-B03), which implied a reduced metabolism possibly resulted from NTG mutagenesis. In addition, the L-Phe yield on glucose of *E. coli* BR-130 (pAP-B03) (0.16 ± 0.01 mol/mol) decreased by 33% compared to that of the parent strain (0.24 ± 0.01 mol/mol). By contrast, BR-42 (pAP-B03) showed no considerable differences in final DCW (7.18 ± 0.25 g/l) and L-Phe titer (4.24 ± 0.11 g/l) in comparison with the control. Moreover, the L-Phe yield on glucose in the case of *E. coli* BR-42 (pAP-B03) (0.24 ± 0.01 mol/mol) was comparable to its parent strain WSH-Z06 (pAP-B03).

In order to confirm the resistance of BR-42 (pAP-B03) to bacteriophage BP-1 during L-Phe production, a further examination in pH–stat fed-batch fermentation was carried out in a 3-l jar fermentor, in which approximately 1×10^{10} pfu/ml BP-1 suspension was supplemented at a ratio of 1:100 (v/v). Figure 1a shows that the parent strain WSH-Z06 (pAP-B03) could not grow in the presence of BP-1, and cell lysis occurred after 2 h of inoculation. Cell concentration was decreased to near zero at 14 h, leading to an irreversible cessation of fermentation. No L-Phe was accumulated in the broth. Figure 1b shows that BR-42 (pAP-B03) exhibited a strong resistance to BP-1, and its high capacity of L-Phe biosynthesis was unaffected. The maximum cell density and L-Phe titer reached 15.41 ± 0.62 g/l and 34.87 ± 1.71 g/l, respectively, which were comparable to those of WSH-Z06 (pAP-B03) in the absence of bacteriophage BP-1 [29]. These results suggested that phage-resistant recombinant *E. coli* BR-42 (pAP-B03) was a promising L-Phe-producer compared to its parent strain, and thus was applied in the fed-batch processes for improved L-Phe production in this work.

Effect of induction time point on L-Phe production by *E. coli* BR-42 (pAP-B03)

The temperature-inducible expression system of *E. coli* is extensively applied for producing various recombinant proteins and biochemical products due to ease in induction of expression by simply shifting the culture temperature.

To determine the optimal initiation of induction, pH–stat fed-batch cultures of *E. coli* BR-42 (pAP-B03) were carried out, in which the induction was initiated at a dry cell weight of 5.76 ± 0.16 (early exponential phase), 9.60 ± 0.31 (mid-exponential phase), and 13.44 ± 0.28 g/l (late exponential phase), respectively. Table 1 shows that after being induced at DCW of 13.44 ± 0.28 g/l, cell growth almost ceased throughout the post-induction process, leading to a reduced maximum cell concentration (14.23 ± 0.31 g/l), possibly due to the declined adaptability of cells at late exponential to circumstance alteration, resulting in significant changes in cellular physiology and metabolism in the post-induction phase. Compared to induction at 13.44 ± 0.28 g/l, cell concentration reached a maximum DCW of 15.68 ± 0.33 and 15.29 ± 0.38 g/l by induction at the cell densities of 5.76 ± 0.16 and 9.60 ± 0.31 g/l, respectively. Among the three cases, induction at DCW of 9.60 ± 0.31 g/l resulted in the highest L-Phe titer (42.75 ± 1.37 g/l) and highest productivity (0.778 ± 0.043 g/l/h). The L-Phe titer and productivity were 13% and 16% higher than those of induction at DCW of 5.76 ± 0.16 g/l, and 24% and 18% higher than those of induction at 13.44 ± 0.28 g/l, respectively.

These results indicated that L-Phe production by recombinant *E. coli* BR42 (pAP-B03) was significantly affected by induction time. Induction at mid-exponential phase was most favorable to L-Phe production.

Fed-batch cultures of *E. coli* BR-42(pAP-B03) for L-Phe production by various feeding approaches

During the fed-batch fermentation process, an appropriate feeding strategy is essential, because the starvation or the accumulation of the restrictive nutrient substance will lead to cell disruption or by-products accumulation. The pH–stat is a simple feedback control scheme that couples nutrient feeding with measurement of pH, based on the fact that pH rises due to excretion of ammonium ions when the principal carbon source is depleted [13, 15]. In this study, with pH–stat feeding mode, the residual glucose levels remained quite low (below 2 g/l) and no acetate accumulation was detected throughout the fed-batch process (Fig. 2). However, the pH did not respond immediately to the glucose starvation in pH–stat cultures, leading to a long lag time, which was reflected in the strenuous vibration of DO (Fig. 2). The repeated depletion of carbon source in lag period may induce considerable cell stress, and the existence of lag period inevitably required an increased fermentation time to reach the maximum L-Phe titer. As a consequence, the L-Phe productivity was undesirably reduced (Table 2).

Two runs of fed-batch fermentation were conducted with the constant feeding rates of 16 ml/h and 10 ml/h,

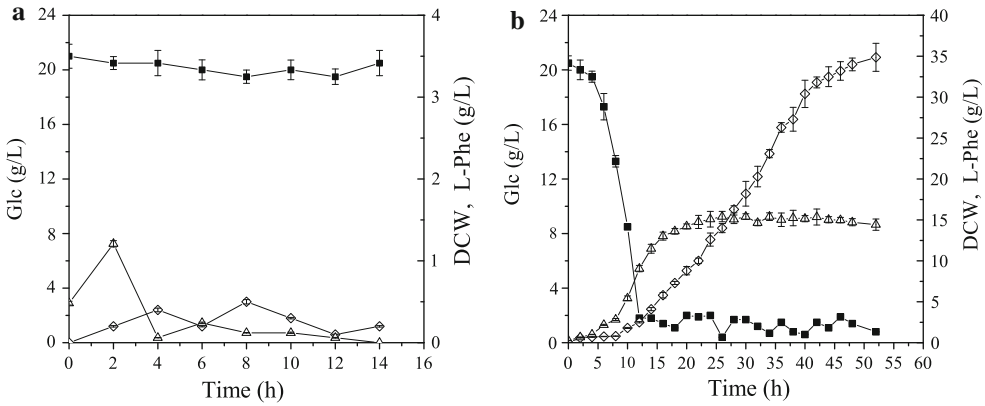

Fig. 1 L-Phe production by *E. coli* WSH-Z06 (pAP-B03) (**a**) and *E. coli* BR-42 (pAP-B03) (**b**) in the presence of bacteriophage BP-1. The bacteriophage BP-1 solution (1×10^{10} pfu/ml) was added into the 3-l jar fermentor at a ratio of 1:100 (v/v) at the beginning of fermentation process. Glucose (*filled square*) (Glc); DCW (*open triangle*); L-Phe (*open diamond*)

Table 1 Effect of induction point on cell growth and L-Phe production by recombinant *E. coli* BR-42 (pAP-B03) in pH–stat fed-batch cultures

Fermentation parameters	Induction point [DCW (g/l)]		
	5.76 ± 0.16	9.6 ± 0.31	13.44 ± 0.28
Glucose consumption (g/l)	172.4 ± 3.68	184.2 ± 4.04	165.3 ± 4.74
Culture time (h)	56 ± 1.73	55 ± 1.32	52 ± 1.32
Maximum DCW (g/l)	15.68 ± 0.33	15.29 ± 0.38	14.23 ± 0.31
Maximum L-Phe concentration (g/l)	37.72 ± 1.17	42.75 ± 1.37	34.35 ± 0.76
Cell productivity (g/l/h)	0.281 ± 0.014	0.278 ± 0.013	0.274 ± 0.011
L-Phe productivity (g/l/h)	0.674 ± 0.039	0.778 ± 0.043	0.661 ± 0.013
Cell yield on glucose (g/g)	0.091 ± 0.002	0.086 ± 0.003	0.086 ± 0.004
L-Phe yield on glucose (mol/mol)	0.238 ± 0.008	0.253 ± 0.01	0.227 ± 0.007

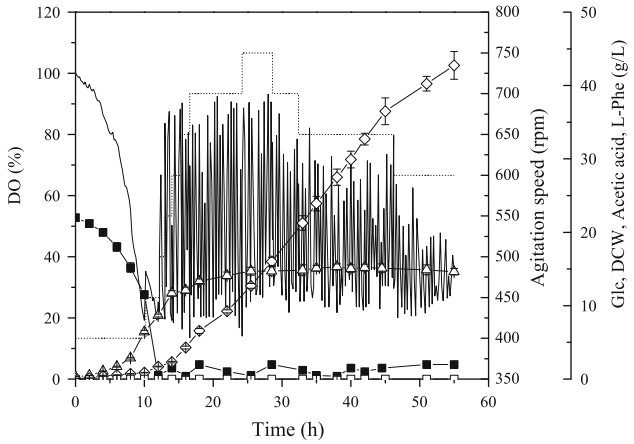

Fig. 2 L-Phe production by *E. coli* BR-42 (pAP-B03) in pH–stat fed-batch cultures. The agitation speed (*dashed line*) was set at 400 rpm at the initial of fermentation and was adjusted between 400 and 900 rpm to keep the DO (*solid line*) above 20% during the entire fermentation process. Glucose (*filled square*) (Glc); DCW (*open triangle*); L-Phe (*open diamond*); acetic acid (*open square*)

respectively. In the case of $F = 16$ ml/h, the glucose feeding was excessive after 30 h. The cell growth was inhibited with a decline in DCW and L-Phe concentration increased with a very low rate and reached a maximum value of 29.12 ± 1.20 g/l at 34 h, which may result from the substrate inhibition and acetic acid accumulation (Fig. 3a). For the constant rate of 10 ml/h, the glucose concentration in the culture medium was maintained near zero until 46 h and the formation of by-product acetic acid was undetectable during the underfeeding period, after which glucose accumulation and acetic acid excretion were observed (Fig. 3b). Compared to the case of $F = 16$ ml/h, glucose feeding at $F = 10$ ml/h resulted in a higher L-Phe titer (45.10 ± 0.94 g/l), but L-Phe productivity (0.835 ± 0.021 g/l/h) was slightly reduced due to a prolonged process time, which can be explained by the constant exposure to insufficient glucose supply in the first 46 h.

Although the L-Phe productivity in both cases of constant rate feeding ($F = 16$ ml/h and 10 ml/h) was slightly improved, with an increase of 10.03–7.33% compared to that of pH–stat, respectively, these feeding rates were not able to supply the necessary level of nutrients required for maintaining acceptable cell growth and L-Phe production. So, a linear decreasing feeding rate [$F = (-0.55 \times t + 23)$ ml/h] based on the analysis of constant rate feedings was proposed. As shown in Fig. 3c, glucose was well controlled between 0.75 and 10 g/l and the formation of by-product acetic acid was kept below 2 g/l. Table 2 shows that, the L-Phe production was significantly improved and the fermentation time was remarkably shortened, reaching

Table 2 Comparison of L-Phe production by *E. coli* BR-42 (pAP-B03) with different glucose feeding patterns

Parameters	pH–stat	Constant rate feeding		$F = (23-0.55 \times t)$ ml/h	Exponential feeding		Two-stage feeding
		$F = 16$ ml/h	$F = 10$ ml/h		$\mu_{set} = 0.12\ h^{-1}$	$\mu_{set} = 0.18\ h^{-1}$	
Glucose consumption (g/l)	184.27 ± 4.57	152.20 ± 3.99	202.83 ± 8.03	225.92 ± 9.29	89.19 ± 2.61	84.30 ± 3.50	237.47 ± 6.74
Culture time (h)	55 ± 1.5	34 ± 1.3	54 ± 1.3	52 ± 1.3	28 ± 1.0	24 ± 1.0	50 ± 1.0
Maximum DCW (g/l)	15.29 ± 0.31	15.62 ± 0.37	15.42 ± 0.34	15.58 ± 0.33	14.73 ± 0.44	15.43 ± 0.31	15.36 ± 0.33
Maximum L-Phe concentration (g/l)	42.75 ± 1.06	29.12 ± 1.20	45.10 ± 0.94	53.12 ± 1.31	20.26 ± 0.58	19.37 ± 0.54	57.63 ± 1.29
Maximum acetic acid concentration (g/l)	0.00 ± 0.00	3.20 ± 0.15	2.50 ± 0.11	1.60 ± 0.08	2.75 ± 0.13	3.25 ± 0.12	2.00 ± 0.07
Average specific glucose consumption rate (h^{-1})	0.279 ± 0.006	0.441 ± 0.010	0.312 ± 0.007	0.423 ± 0.012	0.312 ± 0.009	0.369 ± 0.008	0.370 ± 0.008
Average specific growth rate (h^{-1})	0.092 ± 0.030	0.127 ± 0.003	0.084 ± 0.002	0.089 ± 0.004	0.113 ± 0.003	0.142 ± 0.004	0.090 ± 0.003
Average specific L-Phe production rate (h^{-1})	0.070 ± 0.002	0.058 ± 0.003	0.072 ± 0.002	0.089 ± 0.003	0.075 ± 0.004	0.095 ± 0.003	0.100 ± 0.002
Cell productivity (g/l/h)	0.278 ± 0.013	0.459 ± 0.009	0.286 ± 0.008	0.300 ± 0.013	0.526 ± 0.027	0.643 ± 0.035	0.307 ± 0.008
L-Phe productivity (g/l/h)	0.778 ± 0.039	0.856 ± 0.019	0.835 ± 0.021	1.022 ± 0.052	0.724 ± 0.035	0.807 ± 0.035	1.153 ± 0.033
Cell yield on glucose (g/g)	0.083 ± 0.004	0.103 ± 0.005	0.076 ± 0.005	0.069 ± 0.002	0.165 ± 0.009	0.183 ± 0.008	0.065 ± 0.002
L-Phe yield on glucose (mol/mol)	0.253 ± 0.012	0.208 ± 0.011	0.242 ± 0.005	0.256 ± 0.013	0.248 ± 0.007	0.250 ± 0.006	0.264 ± 0.011

a maximum titer of 53.12 ± 1.31 g/l and a high productivity of 1.022 ± 0.052 g/l/h. The L-Phe titer and productivity were 1.82 and 1.19-fold of those in the case of $F = 16$ ml/h, and 1.18 and 1.22-fold of those in the case of $F = 10$ ml/h, respectively.

Exponential feeding strategy supplies nutrients into the fermentor based on a pre-determined feeding rate without considering the actual glucose concentration in the fermentor, assuming that cells will grow at a constant specific growth rate regardless of cell concentration and physiological conditions [26]. Based on the previous experimental data from various fed-batch cultures, it was observed that the specific growth rate reached a maximum value of 0.35 h^{-1} at approximately 8–10 h in the batch stage and decreased gradually to a very low value (near zero) at 20 h during the feeding stage (Data not shown). Accordingly, an exponential feeding strategy at two predetermined specific growth rates ($\mu_{set} = 0.12$ and 0.18 h^{-1}) was applied in fed-batch cultures of recombinant *E. coli* BR-42 (pAP-B03) for L-Phe production.

With $\mu_{set} = 0.12\ h^{-1}$, the highest L-Phe concentration (20.26 ± 0.58 g/l) was achieved at 28 h with a L-Phe productivity of 0.724 ± 0.035 g/l/h. Glucose was detected below 5 g/l until 24 h, after which accumulation in glucose was accompanied by the decrease in cell growth, L-Phe production, and the formation of acetic acid (Fig. 3d). Increasing the feeding rate at $\mu_{set} = 0.18\ h^{-1}$ can slightly enhance L-Phe production with a high L-Phe productivity (0.807 ± 0.035 g/l/h). However, a shortened period of L-Phe biosynthesis (24 h) greatly limited L-Phe titer (19.37 ± 0.54 g/l) due to a rapid accumulation of glucose in the medium after 20 h (Fig. 3e).

It seemed that simple exponential feeding was not suitable for the entire fed-batch culture of the recombinant *E. coli* BR-42 (pAP-B03) for L-Phe production in comparison to the linear decreasing feeding rate [$F = (-0.55 \times t + 23)$ ml/h. However, as shown in Table 2, an exponential feeding strategy at $\mu_{set} = 0.18\ h^{-1}$ resulted in a higher average specific L-Phe production rate (0.095 ± 0.003 h^{-1}). Thus a two-stage feeding strategy, namely exponential feeding at $\mu_{set} = 0.18\ h^{-1}$ before 20 h and a following linear varying rate feeding with $F = (-0.55 \times t + 18.6)$ ml/h, was developed. Figure 3f shows that glucose was constantly maintained at a low level (0.5–10 g/l) during the entire feeding process by this two-stage feeding strategy, with less than 2 g/l acetic acid formation at the late stage of fermentation process. The fermentation time was shortened to 50 h and a maximum L-Phe titer of 57.63 ± 1.29 g/l was achieved, resulting in a 1.11-fold higher L-Phe productivity and a 1.12-fold higher average specific L-Phe production rate than those of linear decreasing rate feeding with $F = (-0.55 \times t + 23)$ ml/h, respectively.

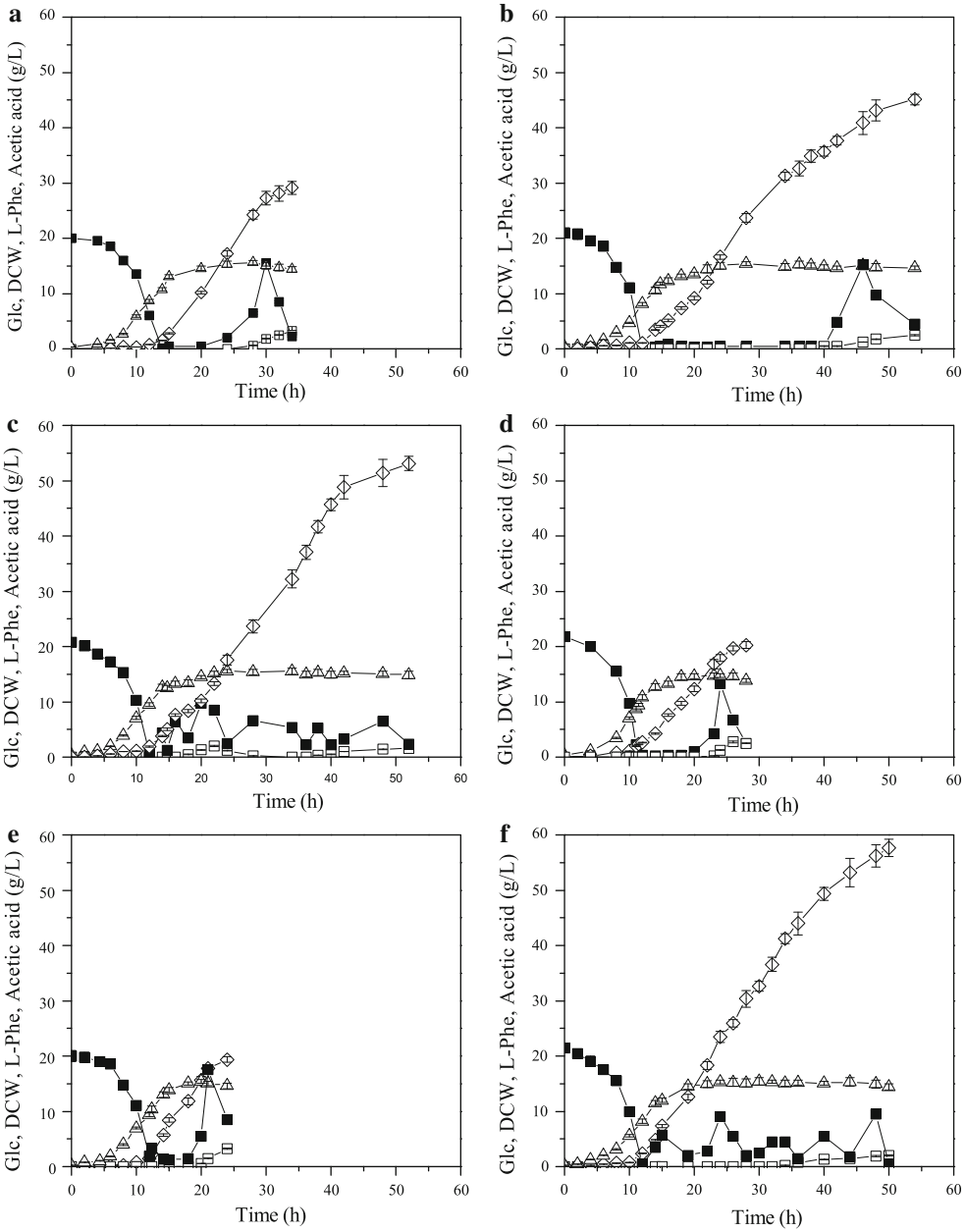

Fig. 3 L-Phe production by *E. coli* WSH-BR-42 (pAP-B03) in fed-batch cultures using different glucose feeding strategies. $F = 16$ ml/h (**a**); $F = 10$ ml/h (**b**), Linear decreasing feeding rate with $F = (-0.55 \times t + 23)$ ml/h (**c**), Exponential feeding at $\mu_{set} = 0.12\ h^{-1}$(**d**), Exponential feeding at $\mu_{set} = 0.18\ h^{-1}$(**e**), and two-stage feeding (**f**). Glucose (*filled square*) (Glc); DCW (*open triangle*); L-Phe (*open diamond*); acetic acid (*open square*)

Conclusions

In this work, a bacteriophage BP-1-resistant *E. coli* BR-42 was isolated. After transforming the L-Phe synthesis-related plasmids pAP-B03, *E. coli* BR-42 (pAP-B03) exhibited satisfactory performance in cell growth and L-Phe production in the presence of 1×10^{10} pfu/ml bacteriophage stocks. Various glucose feeding strategies were investigated to further improve L-Phe production by recombinant *E. coli* BR-42 (pAP-B03). A two-stage feeding approach, namely, exponential feeding at $\mu_{set} = 0.18\ h^{-1}$ in the first 20 h and a linear varying rate feeding with $F = (-0.55 \times t + 18.6)$ ml/h was an efficient method for enhanced L-Phe production, achieving the maximum L-Phe titer of 57.63 ± 1.29 g/l with a high productivity of 1.153 ± 0.033 g/l/h, which was the best result obtained so far. The recombinant *E. coli* BR-42 (pAP-B03) is a potential L-Phe over-producer in substantial prevention of bacteriophage BP-1 infestation compared to its parent strain WSH-Z06 (pAP-B03).

Acknowledgments Funding for this research was supported by a grant from National Science Fund for Distinguished Young Scholars (No. 20625619), the Key Program of National Natural Science Foundation of China (No. 20836003), the Key Project of Chinese National Programs for Fundamental Research and Development (973 program, No. 2007CB71403), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0532), the

Fundamental Research Funds for the Central Universities (No. JUSRP10917), the Fundamental Research Funds for the Central Universities (No. JUSRP30901), and 973 Project (2010CB535014).

References

1. Albert MJ, Bhuiyan NA, Rahman A, Ghosh AN, Hultenby K, Weintraub A, Nahar S, Kibriya AK, Ansaruzzaman M, Shimada T (1996) Phage specific for *Vibrio cholerae* O139 Bengal. J Clin Microbiol 34:1843–1845
2. Baez-Viveros JL, Osuna J, Hernandez-Chavez G, Soberon X, Bolivar F, Gosset G (2004) Metabolic engineering and protein directed evolution increase the yield of L-phenylalanine synthesized from glucose in *Escherichia coli*. Biotechnol Bioeng 87:516–524
3. Backman K, O'Connor MJ, Maruya A, Rudd E, McKay D, Balakrishnan R, Radjai M, DiPasquantonio V, Shoda D, Hatch R, Venkatasubramanian K (1990) Genetic engineering of metabolic pathways applied to the production of phenylalanine. Ann N Y Acad Sci 589:16–24
4. Esquinas-Rychen M, Erni B (2001) Facilitation of bacteriophage lambda DNA injection by inner membrane proteins of the bacterial phospho enol-pyruvate: carbohydrate phosphotransferase system (PTS). J Mol Microbiol Biotechnol 3:361–370
5. Forberg C, Haggstrom L (1987) Effects of cultural conditions on the production of phenylalanine from a plasmid-harboring *E. coli* strain. Appl Microbiol Biotechnol 26:136–140
6. Gerigk M, Bujnicki R, Ganpo-Nkwenkwa E, Bongaerts J, Sprenger G, Takors R (2002) Process control for enhanced L-phenylalanine production using different recombinant *Escherichia coli* strains. Biotechnol Bioeng 80:746–754
7. Gerigk MR, Maass D, Kreutzer A, Sprenger G, Bongaerts J, Wubbolts M, Takors R (2002) Enhanced pilot-scale fed-batch L-phenylalanine production with recombinant *Escherichia coli* by fully integrated reactive extraction. Bioprocess Biosyst Eng 25:43–52
8. Hwang SO, Gil GH, Cho YJ, Kang KR, Lee JH, Bae JC (1985) The fermentation process for L-phenylalanine production using an auxotrophic regulatory mutant of *Escherichia coli*. Appl Microbiol Biotechnol 22:108–113
9. Henderson JW, Ricker RD, Bidlingmeyer BA, Woodward C (2000) Rapid, accurate, sensitive, and reproducible HPLC analysis of amino acids. USA (Agilent App Note 5980-1193E): Agilent Technologies
10. Ikeda M (2006) Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering. Appl Microbiol Biotechnol 69:615–626
11. Johnston W, Cord-Ruwisch R, Cooney MJ (2002) Industrial control of recombinant *E. coli* fed-batch culture: new perspectives on traditional controlled variables. Bioprocess Biosyst Eng 25:111–120
12. Klaenhammer TR, Conkling MA, O'Sullivan D, Djordjevis G, Walker SA, Taylor CG (1998) Bacteriophage-triggered cell suicide systems and fermentation methods employing the same. US Patent 5792625
13. Kim BS, Lee SC, Lee SY, Chang YK, Chang HN (2004) High cell density fed-batch cultivation of *Escherichia coli* using exponential feeding combined with pH-stat. Bioprocess Biosyst Eng 26:147–150
14. Konstantinov KB, Nishio N, Yoshida T (1990) Glucose feeding strategy accounting for the decreasing oxidative capacity of recombinant *Escherichia coli* in fed-batch cultivation for phenylalanine production. J Ferment Bioeng 70:253–260
15. Lee J, Lee SY, Park S, Middelberg AP (1999) Control of fed-batch fermentations. Biotechnol Adv 17:29–48
16. Lee SB, Won CH, Park C, Lim BS (1994) A method for production of L-phenylalanine by recombinant *E. coli*. European Patent 0284185 B1
17. Leng Y, Zheng P, Sun ZH (2006) Continuous production of L-phenylalanine from phenylpyruvic acid and L-aspartic acid by immobilized recombinant *Escherichia coli* SW0209–52. Process Biochem 41:1669–1672
18. Ozcelik IS, Calik P, Calik G, Ozdamar TH (2004) Metabolic engineering of aromatic group amino acid pathway in *Bacillus subtilis* for L-phenylalanine production. Chem Eng Sci 59:5019–5026
19. Romig WR, Brodetsky AM (1961) Isolation and preliminary characterization of bacteriophages for *bacillus subtilis*. J Bacteriol 82:135–141
20. Ruffer N, Heidersdorf U, Kretzers I, Sprenger GA, Raeven L, Takors R (2004) Fully integrated L-phenylalanine separation and concentration using reactive-extraction with liquid-liquid centrifuges in a fed-batch process with *E. coli*. Bioprocess Biosyst Eng 26:239–248
21. Sprenger GA (2007) From scratch to value: engineering *Escherichia coli* wild type cells to the production of L-phenylalanine and other fine chemicals derived from chorismate. Appl Microbiol Biotechnol 75:739–749
22. Sugimoto S, Kato N, Kitaoka Y, Seki T, Yoshida T, Taguchi H (1990) Phenylalanine production by periodic induction of gene expression using a temperature-distributed dual fermenter system. J Ferment Bioeng 70:376–380
23. Shu CH, Liao CC (2002) Optimization of L-phenylalanine production of *Corynebacterium glutamicum* under product feedback inhibition by elevated oxygen transfer rate. Biotechnol Bioeng 77:131–141
24. Thirion JP, Hofnung M (1972) On some genetic aspects of phage lambda resistance in *E. coli* K12. Genetics 71:207–216
25. Takagi M, Nishio Y, Oh G, Yoshida T (1996) Control of L-phenylalanine production by dual feeding of glucose and L-tyrosine. Biotechnol Bioeng 52:653–660
26. Wong HH, Kim YC, Lee SY, Chang HN (1998) Effect of post-induction nutrient feeding strategies on the production of bioadhesive protein in *Escherichia coli*. Biotechnol Bioeng 60:271–276
27. Weikert C, Sauer U, Bailey JE (1998) Increased phenylalanine production by growing and nongrowing *Escherichia coli* strain CWML2. Biotechnol Prog 14:420–424
28. Yakandawala N, Romeo T, Friesen AD, Madhyastha S (2008) Metabolic engineering of *Escherichia coli* to enhance phenylalanine production. Appl Microbiol Biotechnol 78:283–291
29. Zhou HY, Liao XY, Wang TW, Du GC, Chen J (2010) Enhanced L-phenylalanine biosynthesis by co-expression of *pheA*(fbr) and *aroF*(wt). Bioresour Technol 101:4151–4156