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Systematic engineering of the rate-limiting step of β-alanine biosynthesis in *Escherichia coli*

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ABSTRACT

Background: Large amounts of β-alanine are required in fine chemical and pharmaceutical synthesis and other fields. Profitable and green methods are required for the industrial production of β-alanine.
Results: Replacing endogenous *panD* of *Escherichia coli* with heterologous C*gpanD* from *Corynebacterium glutamicum* enabled β-alanine synthesis of 0.67 g/L by strain B0016-082BB. Overexpressing C*gpanD* on both plasmids and chromosomes to enhance the rate-limiting step improved the β-alanine titer to 4.25 g/L in strain B0016-083BB/pPL451-*panD* with a slighter metabolic burden. Growth factors were introduced by addition of yeast extract, and 6.65 g/L of β-alanine was synthesized by strain B0016-083BB/pPL451-*panD* in the M9-3Y medium.
Conclusions: Enhancement of the rate-limiting steps in the β-alanine biosynthetic pathway, recruitment of the temperature-sensitive inducible p_L promoter, and optimization of the fermentation process could efficiently increase β-alanine production in *E. coli*.
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1. Introduction

As the sole natural β-amino acid, β-alanine plays important roles in fine chemical and pharmaceutical synthesis and in food and animal feed additives. A large amount of β-alanine is required in industrial production. Currently, β-alanine is mainly prepared by chemical synthesis by converting a mixture of acrylonitrile and ammonia under heat and pressure conditions [1]. This method requires special equipment, and the conversion process consumes much energy, which calls for a high production cost and is not environmentally friendly [2]. More profitable and greener methods are required for the industrial production of β-alanine.

Bioengineering technology using renewable resources to prepare chemicals through microbial fermentation is a more energy-saving and environmentally friendly method. A wide range of industrial products, ranging from biofuel compounds to complex natural products, have been successfully synthesized by metabolically engineered microorganisms [3]. *Escherichia coli* has been widely used as a cell factory owing to its well-characterized genetic background [4], easy genetic manipulation, and high growth rate [5]. Large amounts of industrial products initially generated by plants, animals, or chemicals such as artemisinin [6], docosahexenoic acid [7], and polylactic acid [8] have been synthesized by engineered *E. coli*. Several studies have been conducted on the biosynthesis of β-alanine [2,9,10]. To make the process of β-alanine production easier and simpler for industrial production, synthetic biology has been applied to develop recombinant *E. coli* for β-alanine production.

To accumulate the desired metabolites efficiently, the intrinsic metabolic pathways must be manipulated rationally. Enhancing the rate-limiting steps by improving gene expression in microbial cell factories has been proven to be an efficient strategy [11]. In general, desired gene(s) are mostly expressed through plasmid expression systems due to their high efficiency and ease of manipulation. However, metabolic burden and structural instability are the main issues when overexpressing large genes, especially when multiple copies are produced [12,13]. For instance, Lee engineered the CWF4NA2 strain harboring two plasmids and achieved a high yield of β-alanine; however, a heavy metabolic burden due to the dual plasmids led to impaired cell growth [14]. These problems could be avoided if the target gene(s) are expressed on the chromosome. Nevertheless, the disadvantages of gene expression on

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chromosomes include low copy number and difficult operation. To produce β-alanine with a lower metabolic burden and simplified operation, the rate-limiting gene of *panD* in the synthetic pathway of β-alanine was overexpressed on both plasmids and chromosomes (Fig. 1a).

While producing toxic products such as β-alanine, the entire fermentation process is normally separated into two stages: the cell growth phase and the product accumulation phase [15,16,17]. Inducible promoters have been used to trigger an OFF to ON state for product accumulation [18]. The T7 promoter is the most popular inducible promoter and can be induced by IPTG or lactose. However, with the addition of IPTG or lactose, the cost of production increases significantly and may impair application in the food and medicine fields as well as affect the growth of bacteria. The temperature-sensitive p_L promoter, which triggers an OFF state at 33°C to abolish gene expression and triggers an ON state at 42°C to express target genes efficiently (Fig. 1b), can be used to separate the entire fermentation process to maintain

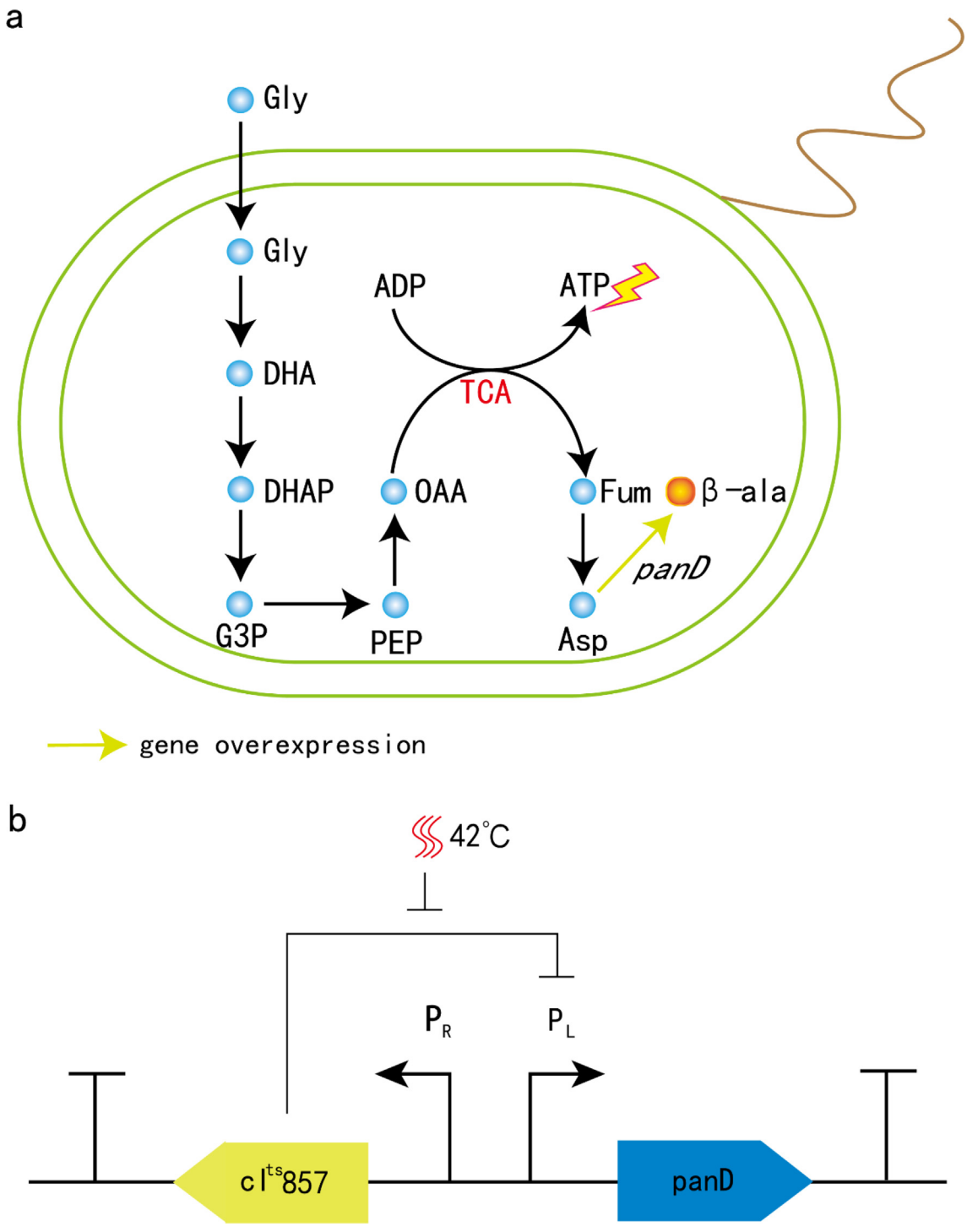

Fig. 1. The metabolic pathway designed for the production of β-alanine. (a) The metabolic pathway from glycerol to β-alanine. Gly: glycerol, DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, G3P: glyceraldehyde-3-P, PEP: phosphoenolpyruvic acid, OAA: oxaloacetic acid, Fum: fumaric acid, Asp: aspartic acid, β-ala: β-alanine. (b) The temperature-sensitive inducible system was used to overexpress the Cg*panD* gene. The expression of the Cg*panD* gene can be initiated at 42°C.

microbial growth and metabolite synthesis phases without any addition of toxic reagents.

In this study, *E. coli* was used for the production of β-alanine. Expression of heterologous C*gpanD* from *Corynebacterium glutamicum* with higher activity [14] was conducted on both plasmids and chromosomes to enhance the main rate-limiting step in the biosynthesis of β-alanine. The temperature-sensitive p_L promoter was used to regulate the expression of the *panD* gene to separate the fermentation process into the microbial growth and product accumulation phases.

2. Materials and methods

2.1. Strains and plasmids

The genotypes of the microbial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain B0016-080BB (Δ*ackA-pta* Δ*pflB* Δ*adhE* Δ*frdA* Δ*ldhA* Δ*lysC* Δ*panC* Δ*ptsG*) was reported previously [19]. Strain B0016-082BB (B0016-080BB, *panD*:: C*gpanD*), which contains the *panD* gene from *C. glutamicum* (CgpanD), was constructed in this study. Strain B0016-083BB (B0016-080BB, *panD*::*kan-ci*^{ts}857-p_R-p_L-C*gpanD*), which contains the *panD* gene from *C. glutamicum* and the cassette of *km-ci*^{ts}857-p_R-p_L that ensures that the strain can be induced efficiently by temperature changes, was constructed in this study.

The method of Red-mediated recombination was used to manipulate chromosome [20]. To construct B0016-082BB, the km-C*gpanD* cassette, which encodes a kanamycin resistance selectable marker and the C*gpanD* gene, was cloned to replace the endogenous *panD* gene in B0016-080BB. The *kan* fragment was amplified from pKD13 using EcoRI-pkd13F and EcoRI-pkd13R and yielded a 1721-bp fragment; this fragment was then cloned onto the plasmid of pPL451-*panD*, resulting in a new plasmid, pPL451-*panD-kan,* which contained the cassette of km-C*gpanD*. The km-C*gpanD* cassette was then amplified from pPL451-*panD-kan* using panD-pkd13F and panD-pkd13R and was then electroporated into strain B0016-080BB harboring the Red recombinase plasmid pKD46. The presence of the *pan*D::*kan*-C*gpanD* mutation was confirmed by performing colony PCR with the primers YpanDF and YpanDR, whose amplification yielded a 561-bp fragment from the wild-type *panD* gene and a 1901-bp fragment from the *pan*D::*kan*-C*gpanD* mutation. The pCP20 plasmid was then electroporated into the *pan*D::*kan*-C*gpanD* mutation, which was cultured at 42°C and confirmed to eliminate the Red recombinase plasmid pKD46 to dismiss the *kan* fragment, resulting in the B0016-082BB strain (*pan*D::C*gpanD*). The *pan*D::C*gpanD* mutation was confirmed by performing colony PCR with the primers YpanDF and YpanDR, whose amplification yielded a 1901-bp fragment from the *pan*D::*kan*-C*gpanD* mutation and a 679-bp fragment from the *pan*D::C*gpanD* mutation. The construction of B0016-083BB was accomplished under similar manipulations with the primers panD-pkd13F and panD- CI857R; this was followed by the amplification of the cassette of *kan-ci*^{ts}857-*pR-pL-*C*gpanD* from the plasmid pPL451-*panD-kan*, and the *kan-ci*^{ts}857-*pR-pL-*C*gpanD* cassette was then electroporated into strain B0016-080BB harboring the Red recombinase plasmid pKD46, resulting in the mutation of B0016-083BB (*pan*D:: *ci*^{ts}857-*pR-pL-*C*gpanD*). All mutations were confirmed by performing colony PCR with the primers YpanDF and YpanDR, whose amplification yielded a 561-bp fragment from the wild-type *panD* gene, a 3208-bp fragment from the *pan*D::*kan-ci*^{ts}857-*pR-pL-*C*gpanD* mutation and a 19869-bp fragment from the *pan*D:: *ci*^{ts}857-*pR-pL-*C*gpanD* mutation [21]. All the primers are listed in Table 2.

2.2. Media and culture conditions

E. coli was cultured on Luria-Bertani (LB) agar plates (2% agar powder, w/v) or LB liquid broth for active culture, gene manipulation, and plasmid construction. Seed cultures were performed in 250 mL flasks containing 50 mL of medium at 37°C in a rotary shaker at 200 rpm overnight. The seed medium was centrifuged at 12,000 rpm at 4°C for 2 min and suspended in M9 medium. The cell suspension was inoculated into M9, M9-0.5Y, or M9-3Y medium to reach an initial *OD*600 of 0.05. Cultures were initially incubated at 33°C until the *OD*600 reached 2.5, and the temperature was then changed to 42°C to induce the expression of the *panD* gene. Glycerol (50%, w/v) at 500 μL was added to the flasks every 6 h. The pH was maintained at 7 by feeding the culture with 100 g/L $NaHCO_3$. Fermentation ceased at 42 h since induction. All the experiments were performed in triplicates for the analysis of mean values and standard deviations.

M9 medium contained (per L) 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 13.21 g of $(NH_4)_2SO_4$, 5 g glycerol and 5 mL of trace metal solution [22]. The M9-0.5Y and M9-3Y media were prepared by supplementing M9 with 0.5 g/L and 3 g/L yeast extract, respectively.

2.3. Measurement of β-alanine

The concentration of β-alanine in the culture broth was analyzed by high-performance liquid chromatography (HPLC) with the o-phthaldialdehyde derivatization (opa) method [23]. Samples were centrifuged and filtered through a 0.22-μm membrane filter, derivatized with opa, and analyzed using the precolumn derivatization of an Agilent HPLC system (Agilent 1200, USA) fixed with DAD detectors and equipped with an XBridge C18 column (4.6 × 250 mm, Waters) [24].

Table 1
E. coli strains and plasmids used in this study.

Strains	Relevant characteristics	Source or reference
B0016-080BB	B0016 Δ*ackA-pta* Δ*pflB* Δ*adhE* Δ*frdA* Δ*ldhA* Δ*lysC* Δ*panC* Δ*ptsG*	[19]
B0016-080BB/pPL451-*panD*	B0016-080BB with pPL451-*panD* plasmid	[19]
B0016-082BB	B0016-080BB, *panD*:: C*gpanD*	This study
B0016-083BB	B0016-082BB, *panD*::*kan-ci*^{ts}857-p_R-p_L-C*gpanD*	This study
B0016-083BB/pPL451-*panD*	B0016-083BB with pPL451-*panD* plasmid	This study
Plasmids		
pPL451-*panD*	Amp^r, panD from *Corynebacterium glutamicum*	[19]
pPL451-*panD-kan*	bla kan, panD:: C*gpanD*	This study
pKD46	Amp^r, γβexo (red recombinase), Temperature-conditional replicon	CGSC[a] [33]
pKD13	Amp^r, Kan^r, FRT-Kan-FRT cassette	CGSC[a] [33]
pCP20	Amp^r, FLP, Temperature-conditional replicon	CGSC[a] [33]

[a] Genetic Stock Center, Yale University.

Table 2
Primers used in this study.

Primers	Sequence (5′-3′) restriction italic/underlined	Restriction sites
EcoRI-pkd13F	TTT*GAATTC*[a]GTGTAGGCTGGAGCTGCTTC	*Eco*RI[a]
EcoRI-pkd13R	TTT*GAATTC*[a]ATTCCGGGGATCCGTCGACC	*Eco*RI[a]
panD-pkd13F	AAAGCAGGCATCGCCTGCTTCGTTAACGACAGGGTA GAAAGGTAGAAGTTATGCTGCGCACCATCCTCGG	
panD-pkd13R	TTGTTATCCCTGCGGCTGGTTACTCACCAGCCGCGACATCGTCTCCAGCG	
PanD-CI857R	TGGTGAGTAACCAGCCGCAGGGATAACAATCAAGCAACCTGTACCGGAATGCGGTAAGTCGCATAAAAACCATTCTTC	
YpanDF	TCTTAAGAACGGATTCGCTGGAGACGAT	
YpanDR	AGTGTCTACTCATCTGACGGCATTTGCG	

[a] Restriction sites with corresponding restriction enzymes.

3. Results and discussion

3.1. Gaining the ability of β-alanine biosynthesis

The reaction from L-aspartate to β-alanine catalyzed by the *panD* gene is reported to be a rate-limiting step in the β-alanine biosynthesis pathway. Enhancing this rate-limiting step may promote greater metabolic flux into the β-alanine biosynthesis pathway rather than the byproduct producing pathways [10]. In this study, heterologous *panD* from *C. glutamicum* (Cg*panD*) was used to enhance the rate-limiting step. While endogenous *panD* was replaced by heterologous Cg*panD*, 0.67 g/L β-alanine was synthesized by B0016-082BB, which indicated that the enzyme activity of L-aspartate-a-decarboxylase encoded by Cg*panD* was much higher than that encoded by heterologous *panD*. To investigate whether heterologous Cg*panD* overexpression on the plasmid could also enhance the rate-limiting step in the β-alanine synthetic pathway, B0016-080BB harboring the pPL451-*panD* plasmid (B0016-080BB/pPL451-*panD*) was applied, and the production of β-alanine was 3.94 g/L. From these findings, we can conclude that overexpressing Cg*panD* on both plasmids and chromosomes can enhance the rate-limiting step to improve the β-alanine titer. Furthermore, comparing different overexpressing methods, higher production of β-alanine was obtained owing to the higher copy numbers of plasmids [5].

As β-alanine is not normally produced at a high concentration in *E. coli*, it was predicted that a high concentration of β-alanine might be toxic to cells [25]. The tolerance of *E. coli* to β-alanine was measured in this study. β-Alanine was added to the fermentation medium at different concentrations, and the cell density was detected every 2 h. As shown in Fig. 2b, 0.1 g/L β-alanine slightly enhanced cell growth. With the increase in β-alanine concentration, the growth rate of *E. coli* slowed down gradually. β-Alanine concentrations higher than 0.5 g/L inhibited cell growth. Therefore, a two-stage fermentation strategy that produces β-alanine after achieving high cell density is essential to avoid cell growth inhibition.

3.2. Optimization of culture conditions for thermoswitched β-alanine production

A β-alanine-producing strain B0016-080BB/pPL451-*panD* with overexpression of the Cg*panD* gene (derived from *C. glutamicum* under the initiation of the P_L promoter on plasmid) was applied to optimize the fermentation condition. The temperature-sensitive p_L promoter could separate the fermentation process into a cell growth phase at 33°C and a β-alanine production phase at 42°C. The rotation rate was optimized to investigate the effect of dissolved oxygen on cell growth and β-alanine production. Without any guiding data, experiments were conducted for 48 h, and temperature was increased from 33°C to 42°C when the *OD*600 reached 2. As shown in Fig. 3a, the maximum production of β-alanine was achieved at the rotation rate of 200, which indicated that the process of β-alanine synthesis requires sufficient dissolved oxygen (DO). We speculated that two reasons might account for this phenomenon. The first reason is that the concentration of DO is a key factor that determines the growth of engineered microbes [26] and the activities of heterologous enzymes, and sufficient DO can enhance the activities of many enzymes, especially for these oxygen-dependent ones [27], which can activate many metabolic pathways downstream that not only enhance the substrate consumption rate but also supply much more important intermediary metabolites, such as pyruvate and asparagic acid (Fig. 1a), which are essential for cell growth and the downstream metabolic pathways. The second reason is that many toxic metabolic byproducts such as methane acid and acetic acid may be synthesized under the condition of low DO. Under the conditions of sufficient DO, the synthesis of such toxic metabolic byproducts

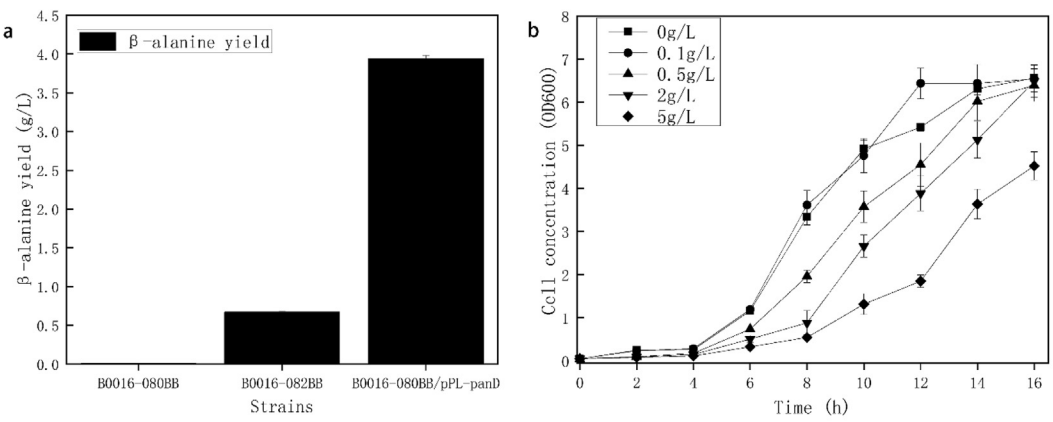

Fig. 2. Effect of Cg*panD* on the production of β-alanine (a) and the effect of β-alanine concentration on cell growth (b).

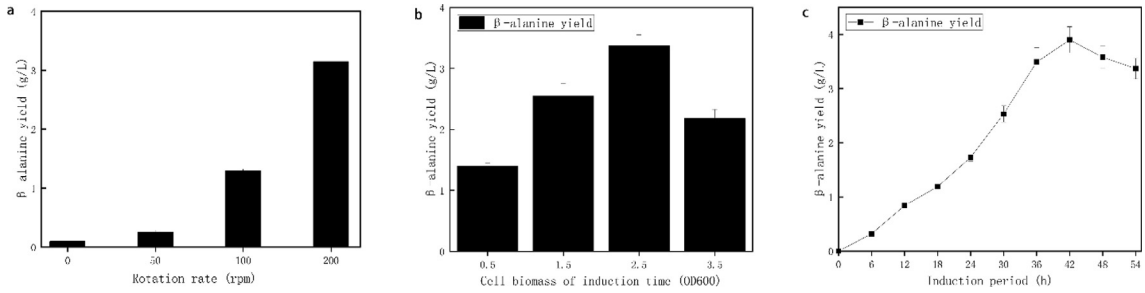

Fig. 3. Optimization of culture conditions with B0016-080BB/pPL451-*panD*. (a) Optimization of the rotation rate. (b) Optimization of the thermoinduction time for C*gpanD* overexpression. (c) Optimization of the fermentation period.

would decrease significantly, which may decrease the effects on growth [28].

Using the two-phase fermentation strategy to generate β-alanine, experiments were conducted for 48 h since induction to optimize the thermoinduction time for *panD* overexpression. As shown in Fig. 3b, the highest production of β-alanine was achieved when the expression of *panD* was induced at an *OD*600 of 2.5. Higher or lower cell density led to decreased β-alanine production. We speculated that expressing *panD* at lower cell biomass would compete with cell growth for endogenous metabolite precursors [29], which would cause less cell biomass accumulation followed by decreased β-alanine production. Conversely, if *panD* expression was induced at higher cell biomass, the cellular activity may not be sufficient for efficient β-alanine production in the latter phase of the life cycle. Therefore, an optimal induction time for *panD* expression is essential.

Experiments were also performed to investigate the optimal induction period. As shown in Fig. 3c, the highest β-alanine production was attained at 42 h after induction, and the parameters decreased after 42 h. This may be because toxic metabolites accumulate *in vivo*, leading to bacterial lysis with the degradation of β-alanine [30,31]. Thus, the optimal induction period was determined to be 42 h.

3.3. Enhancement of the rate-limiting synthetic pathway of β-alanine production

The main rate-limiting gene in the synthetic pathway of β-alanine is *panD* [10]. To relieve the metabolic block and drive the metabolic flux toward β-alanine biosynthesis, C*gpanD* from *C. glutamicum* was overexpressed on both plasmid and the *E. coli* chromosome.

As shown in Fig. 2a, β-alanine was hardly generated by B0016-080BB. After replacing endogenous *panD* with C*gpanD*, β-alanine production in strain B0016-082BB was improved to 0.67 g/L, indicating the significance of C*gpanD* for β-alanine production. When *CgpanD* was overexpressed on the plasmid, the production of β-alanine in strain B0016-080BB/pPL451-*panD* was significantly improved to 3.94 g/L at 54 h (Fig. 4b). Therefore, increasing the copy number of the C*gpanD* gene could be an effective approach

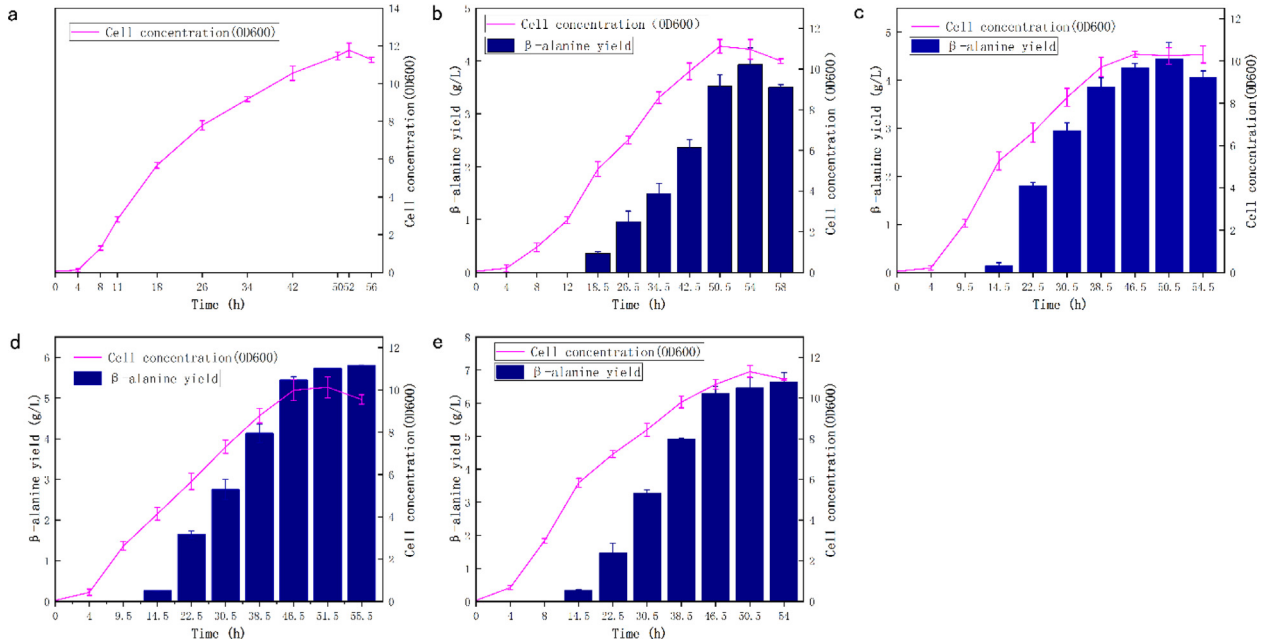

Fig. 4. Effects of C*gpanD* gene overexpression and yeast extract on β-alanine production and cell growth. (a) With the original *panD* of *E. coli*, no β-alanine was synthesized by B0016-080BB. (b) The rate-limiting gene of *panD* was overexpressed on the plasmid in strain B0016-080BB/pPL451-*panD*. (c) The rate-limiting gene of *panD* was overexpressed on both plasmids and chromosomes by strain B0016-083BB/*pPL451-panD*. (d) Incorporation of yeast extract in M9-0.5Y medium enhanced β-alanine synthesis by strain B0016-083BB/pPL451-*panD*. (e) Incorporation of yeast extract in M9-3Y medium enhanced β-alanine synthesis and cell growth by strain B0016-083BB/pPL451-*panD*.

to improve β-alanine production. However, two identical DNA fragments in one plasmid may lead to plasmid instability. Moreover, a large plasmid may also lead to a heavy metabolic burden. As a result, increasing the gene copy number on the *E. coli* chromosome was investigated. As shown in Fig. 4c, β-alanine production was further increased to 4.25 g/L at 46.5 h by strain B0016-083BB/pPL451-*panD*. A less amount of aspartic acid, the immediate precursor of β-alanine, was detected in strain B0016-083BB/pPL451-*panD* (data not shown), implying that the rate-limiting step in the β-alanine synthetic pathway was relieved. Biomass accumulations did not alter significantly among the above strains (Fig. 4a,b,c), which indicates that only a slight metabolic burden was generated by overexpressing Cg*panD* on both the plasmid and *E. coli* chromosome.

To enhance the growth of *E. coli* and β-alanine production, yeast extract was applied to provide growth factors [32] in the fermentation process of B0016-083BB/pPL451-*panD*. As shown in Fig. 4d, the β-alanine titer was improved to 5.80 g/L at 55.5 h by strain B0016-083BB/pPL451-*panD* when M9-0.5Y medium was used in the fermentation process. Furthermore, β-alanine production was increased to 6.65 g/L at 54 h by strain B0016-083BB/pPL451-*panD* in M9-3Y medium, with a significant enhancement in cell growth (Fig. 4e). We concluded that yeast extract contains growth factors that can effectively enhance the production of β-alanine.

4. Conclusions

The fermentation process could be separated into cell growth and β-alanine production phases with the application of a thermoinducible promoter to reduce the toxic effect of β-alanine on cell growth. The metabolic block for β-alanine production could be effectively relieved by overexpressing Cg*panD* on both plasmid and *E. coli* chromosome. Growth factors contained in yeast extract are essential for cell growth and β-alanine production. With the optimization of the fermentation process, the maximum β-alanine production reached 6.65 g/L.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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