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Expression of a *hemA* Gene from *Agrobacterium radiobacter* in a Rare Codon Optimizing *Escherichia coli* for Improving 5-aminolevulinate Production

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Abstract The 5-aminolevulinate (ALA) synthase gene (*hemA*) from *Agrobacterium radiobacter* zju-0121, which was cloned previously in our laboratory, contains several rare codons. To enhance the expression of this gene, *Escherichia coli* Rosetta(DE3), which is a rare codon optimizer strain, was picked out as the host to construct an efficient recombinant strain. Cell extracts of the recombinant *E. coli* were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under the appropriate conditions. The results indicated that the activity of ALA synthase expressed in Rosetta(DE3)/pET-28a(+)-*hemA* was about 20% higher than that in *E. coli* BL21(DE3). Then the effects of precursors (glycine and succinate) and glucose, which is an inhibitor for ALA dehydratase as well as the carbon sources for cell growth, on the production of 5-aminolevulinate were investigated. Based on an optimal fed-batch culture system described in our previous work, up to 6.5 g/l (50 mM) ALA was produced in a 15-l fermenter.

Keywords 5-aminolevulinate · *Agrobacterium radiobacter* · *Escherichia coli* Rosetta(DE3) · *hemA* · Rare codons

Introduction

5-Aminolevulinic acid or 5-aminolevulinate (ALA), a derivative of five-carbon amino acid, is a kind of photodynamic chemical. A drug, mainly consisted of ALA, as a photodynamic medicine for treating skin cancer was approved by the Food and Drug Administration, USA in 1999. Also, ALA as photosensitizer for photodynamic therapy is of potential use for other cancers [1, 2], oral errucous hyperplasia [3], and so on. And ALA has received wide attention as a selective and biodegradable herbicide or insecticide [4, 5] in agriculture.

Since the chemical synthesis of ALA is complicated with low yields and noxious byproducts due to the numerous reaction steps required [5], most of the studies have been

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focused on utilizing natural selected bacteria and algae, mutants of photosynthetic bacteria, and recombinant organisms to produce ALA.

ALA is synthesized biologically in cells by two kinds of distinguished metabolic pathways [6]: C_4 and C_5 pathways. As the C4 pathway involves only one gene (*hemA*) for ALA synthase, it is easier to construct a recombinant cell for ALA production. *Escherichia coli* is the most popular host cell for genetic engineering application. And mass production of ALA using recombinant *E. coli* has been studied by a few groups [7–12].

Agrobacterium radiobacter is a good producer of vitamin B_{12} [13]. The *hemA* gene from *A. radiobacter* ATCC 4718 was cloned and expressed in *E. coli* K12. In our previous work [9], a new *hemA* gene from *A. radiobacter* zju-0121 was cloned and expressed in *E. coli* BL21 (DE3). The sequence of this new gene showed 65.5% and 65.7% identity with the *hemA* genes from *Rhodopseudomonas sphaeroides* and *Bradyrhizobium japonicum*, respectively. And it showed only 92.6% homology with the *hemA* gene from *A. radiobacter* ATCC4718. This difference will possibly lead to a marked difference in the kinetics of the enzyme which required further investigation. However, the activity of ALA synthase was still low and ALA production was only 3.01 g/l under optimal fed-batch fermentation conditions [14]. In this situation, we are eager to develop a new strategy for high expression of ALA synthase and ALA production.

Several laboratories have shown that the yield of protein whose genes contain rare codons can be dramatically improved when the cognate tRNA is increased within the host [15–17]. And our previous work has shown that the adoption of rare codon optimizer strain, *E. coli* Rosetta (DE3) improved the expression of ALA synthase [12]. A complete compilation of codon usage of the sequences placed in the GenBank database can be found at http://www.kazusa.or.jp/codon/ [18] and *E. coli* displays frequencies of about 0.6% of rare codon CCC and 0.8% of rare codon GGA. Since there are five rare codons (CCC) and four rare codons (GGA) in the *hemA* gene (406 codons) from *A. radiobacter* (about 1.2% and 1.0% frequencies), it may be beneficial to apply a rare codon optimizer strain *E. coli* Rosetta(DE3), which is a derivative of BL21 (DE3) and contains a plasmid of pRARE (Cm^R) encoding rare codons including CCC and GGA.

The formation of ALA in cells is considered the rate limiting step for the biosynthesis of tetrapyrroles, and it is tightly regulated by feedback inhibition [19]. Thus, in natural microorganisms such as *A. radiobacter*, ALA production was very low. And it was reported that the addition of D-glucose [20] as a competitive inhibitor for ALA dehydratase could greatly improve the accumulation of ALA.

The aim of this work is to improve express of the *hemA* gene from *A. radiobacter* and increase ALA productivity via the adaptation of a rare codon optimizer strain and the optimization of cultivation conditions of the new recombinant *E. coli*.

Materials and Methods

Strain and Plasmid

The host strain applied in this work is *E. coli* Rosetta(DE3) (Novagen, Germany) [F^-ompT $hsdS_B(r_B^-m_B^-)$ gal dcm lacY1(DE3) pRARE(Cm^R)]. Recombinant pET-28a(+)-*hemA* (*hemA* from *A. radiobacter*) was constructed in our previous work [9]. And the expression plasmid pET-28a(+)-*hemA* was transformed into *E. coli* Rosetta(DE3) to obtain *E. coli* Rosetta(DE3)/pET-28a(+)-*hemA*.

Growth Conditions

The Luria–Bertani (LB) medium was used. And 30 mg/l kanamycin and 34 mg/l chloramphenicol for *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* were added in this medium.

Seed cultures were incubated for 8 h at 200 rpm and 37 °C in shake flasks containing 50 ml LB medium. The fermentation was conducted in a 15 l fermenter (Shanghai Guoqiang Bioengineering Equipment Co., Ltd., China). Nine liters of sterilized medium was added and 100-ml seed culture was inoculated to provide an initial optical density at 600 nm (OD600) of approximately 0.1. The fermenter was operated at 400 rpm and the air flow rate was regulated at 3 l/min. The pH value was controlled with 10% H_2SO_4 (*v*/*v*) or mixture of glycine and succinic acid. After cultivated for 2.0 h, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and culture temperature was lowered to 28 °C. And the feeding strategies of fed-batch fermentation were established in our previous work [21] with another recombinant *E. coli* for 5-aminolevulinate production.

Analysis

Cell density in the medium was represented by measuring OD_{600} (Ultrospec 3300 pro, Amersham Biosciences, Sweden). Succinate and glycine were analyzed by high-performance liquid chromatography as previously described [12].

ALA was determined using modified Ehrlich's reagent [22]. Specifically, 2 ml of sample or standard was mixed with 1 ml 1.0 M sodium acetate (pH 4.6) in a cuvette, and 0.5 ml acetylacetone (2,4-pentanedione) was added to each cuvette. Then the mixtures were heated in a water bath at 100 °C for 15 min. After cooling for 15 min, 2 ml of the reaction mixture and 2 ml freshly prepared modified Ehrlich's reagent [22] were mixed together. The absorbance at 554 nm was measured at room temperature 30 min later.

Enzyme Assay

Enzyme assay process was according to our previous work [12]. The ALA synthase activity in the supernatant was measured [22]. One unit of ALA synthase activity was defined as the amount of enzyme needed to produce 1 nmol ALA in 1 min. Protein was measured using a Pierce BCA Protein Assay Kit (Sigma, St. Louis, MO, USA).

The proteins of cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 12% (*w*/*v*) separation slab gel was prepared by the method [23]. Proteins were stained with Coomassie brilliant blue G. The images of gels were scanned by GEL-DOC 2000 gel documentation system (Bio-Rad, USA) and analyzed by Quantity One software, Version 4.4.0 (Bio-Rad).

Results

Effects of Induction Time, Culture Temperature after Induction, and IPTG Concentration on ALA Synthase

IPTG induction time was optimized for *E. coli* Rosetta(DE3)/pET-28a(+)-*hemA* and the results were summarized in Fig. 1. In all cases, 1.0 mM IPTG was added to induce the expression of ALA synthase, and the cultivation temperature was lowered from 37 °C to

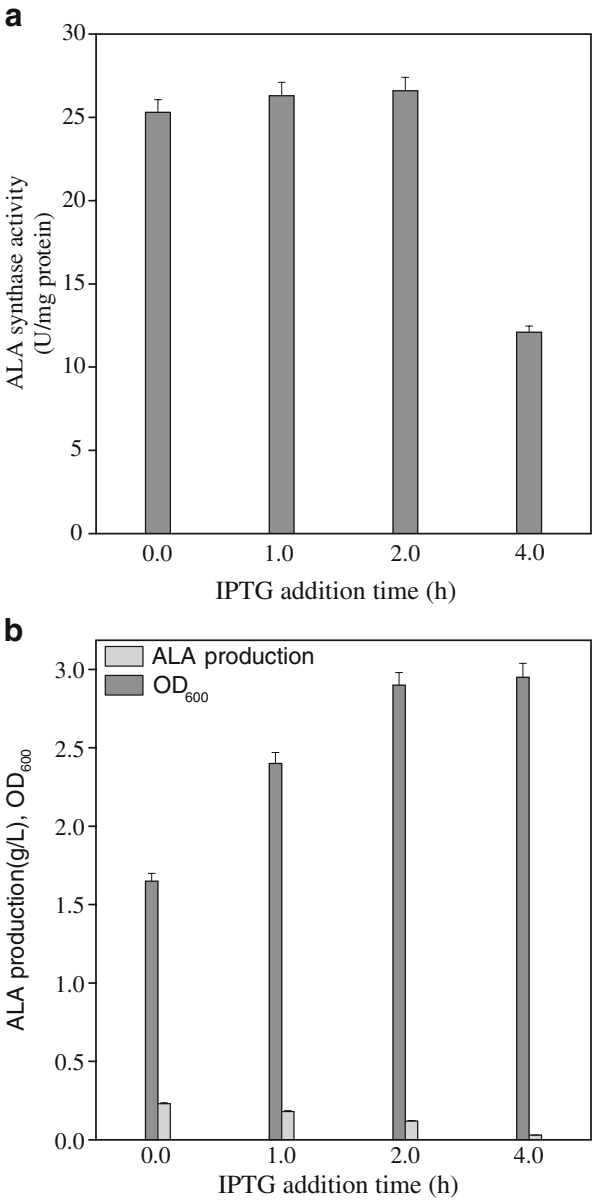

Fig. 1 Effect of IPTG addition time on ALA synthase activity in *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* (**a**). Effect of IPTG addition time on growth and ALA production of *E. coli* Rosetta (DE3)/pET28a(+)-*hemA* (**b**)

28 °C after induction. The cells were harvested after cultivation for 8.0 h. The highest ALA synthase activity appeared when IPTG was added at the early exponential phase when the cells were cultivated for 2.0 h and OD_{600} was approximately 1.0. Figure 1b showed that ALA production was low (under 0.25 g/l) in all these conditions though the highest ALA production was achieved when IPTG was added initially. The results indicated that it was suitable to add IPTG at 2.0 h after inoculation to obtain high ALA synthase activity and good cell growth though ALA production was not the highest one.

The effect of IPTG concentration on the ALA synthase activity was studied and the results are shown in Fig. 2a. IPTG was added after culture was carried out for 2.0 h and cultivated at 28 °C. It was obvious that without IPTG induction, ALA synthase activity was less than 2 U/mg protein (Fig. 2a). The highest ALA synthase activity was 50.6 U/mg protein with 0.05 mM IPTG. Figure 2b indicated that ALA production was still low in all the conditions and it was incredible to get the right IPTG concentration compared with ALA production.

The effects of culture temperature after IPTG induction on the ALA synthase activity were studied at 22, 28, and 37 °C. ALA synthase activity was 33.2, 45.5 and 19.1 U/mg protein, respectively. The results indicated that 28 °C as cultivation temperature after IPTG induction was favorable for higher expression of ALA synthase.

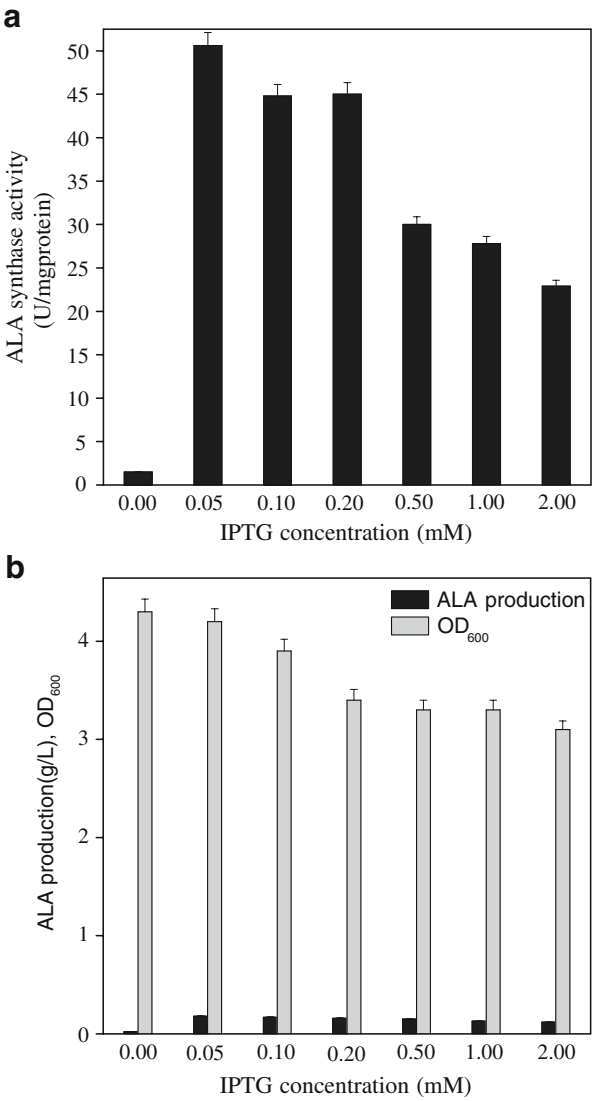

Fig. 2 Effect of IPTG concentration on ALA synthase activity in *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* (**a**). Effect of IPTG concentration on growth and ALA production of *E. coli* Rosetta (DE3)/pET28a(+)-*hemA* (**b**)

Comparison of ALA Synthase Expression in the Recombinant *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* and BL21(DE3)/pET28a(+)-*hemA*

Based on the above results, the optimal conditions for ALA synthase expression in the recombinant *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* were as follows: 0.05 mM IPTG was added at 2.0 h and culture temperature was adjusted to 28 °C from 37 °C after induction. Conditions for ALA synthase expression in the recombinant *E. coli* BL21(DE3)/pET28a (+)-*hemA* were from our previous work [9]. Figure 3 showed the SDS-PAGE of ALA synthase in the two recombinants. The soluble expressions of ALA synthase were both about 55% (*m*/*m*) in the two strains while the insoluble expression of ALA synthase was lower in the *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* than in the *E. coli* BL21(DE3)/pET28a (+)-*hemA*. ALA synthase activities were 35.3 U/mg protein and 29.1 U/mg protein, respectively in the *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* and *E. coli* BL21(DE3)/pET28a (+)-*hemA* under the appropriate conditions.

Effects of Precursors and Glucose on the ALA Yield

According to the above experiments, in the absence of precursors (glycine and succinic acid) in the LB medium, ALA production was quite low (less than 0.25 g/l). In this situation, no significant difference in ALA yield could be found when changing the cultivation conditions.

For the study of glucose concentration, 1 g/l glycine and 3 g/l succinic acid were added in the medium, the effects of initial glucose concentration on the ALA yield were examined, and the results are shown in Fig. 4. It was obvious that an optimal initial concentration of glucose existed for ALA production. When initial glucose concentration was 2 g/l, the highest ALA concentration in the final fermentation broth reached 0.93 g/l.

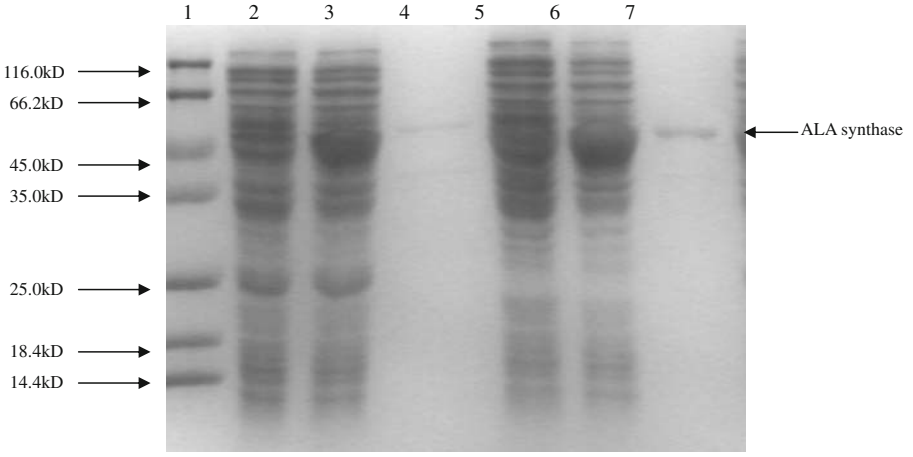

Fig. 3 Expression of foreign ALA synthase in the recombinant *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* and BL21(DE3)/pET28a(+)-*hemA*. *Lane 1*: marker. *Lane 2*: soluble proteins of *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* without induction. *Lane 3*: soluble proteins of *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* which was induced under the optimal conditions. *Lane 4*: insoluble proteins of *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* which was induced under the optimal conditions. *Lane 5*: soluble proteins of *E. coli* BL21(DE3)/pET28a(+)-*hemA* without induction. *Lane 6*: soluble proteins of *E. coli* BL21(DE3)/pET28a(+)-*hemA* which was induced under the optimal conditions. *Lane 7*: insoluble proteins of *E. coli* BL21(DE3)/pET28a(+)-*hemA* which was induced under the optimal conditions

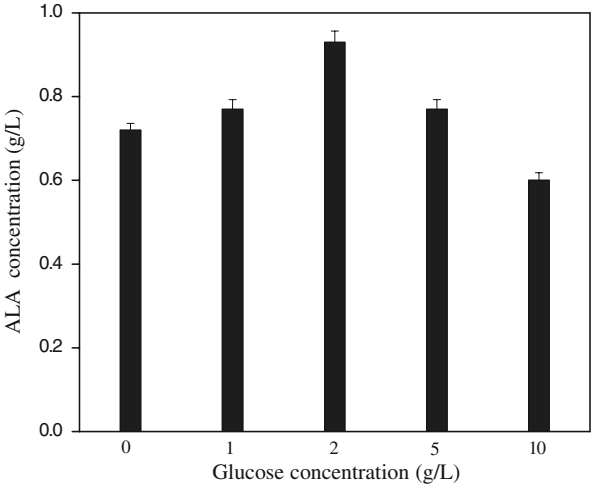

Fig. 4 Effect of initial glucose concentration on ALA production in *E. coli* Rosetta(DE3)/pET28a (+)-*hemA*

Since high concentration of glycine was harmful to cell growth, effects of initial glycine concentration on growth of Rosetta(DE3)/pET28a(+)-*hemA* were studied and the results were shown in Fig. 5. It indicated that, when glycine concentration was achieved to 5 g/l, cell growth was severely inhibited. And 1 g/l and 2 g/l initial glycine concentration led to a similar cell growth. The two concentrations of initial glycine might be suitable for further study. Then the effects of initial succinic acid concentration on ALA production were summarized in Fig. 6. And 9 g/l initial succinic acid concentration resulted in the highest ALA production.

Considering that there probably were some interactions between glycine and succinic acid for ALA production, the effects of precursors, glycine and succinic acid, and their ratio on the ALA production are shown in Fig. 7. In all the experiments, the initial glucose concentration in the medium was kept at 2 g/l.

The results indicated that the addition of both precursors was necessary for high ALA yield. It was obvious that the concentration of glycine and succinic acid in the medium was very important on ALA yield. When glycine and succinic acid concentrations were

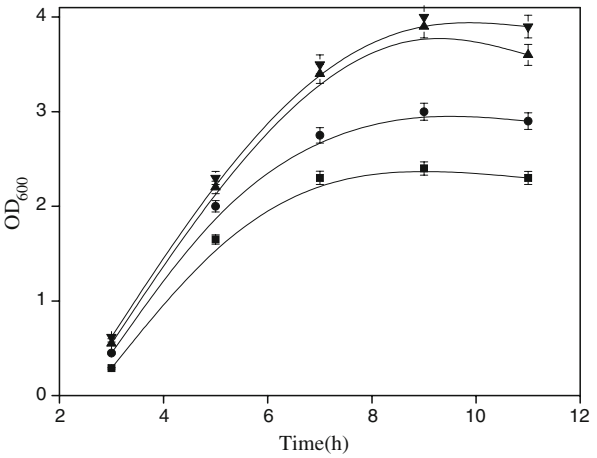

Fig. 5 Effect of initial glycine concentration on growth of *E. coli* Rosetta(DE3)/pET28a(+)-*hemA*. Glycine: –▾– 1 g/l; –▴– 2 g/l; –●– 5 g/l; –▪– 10 g/l

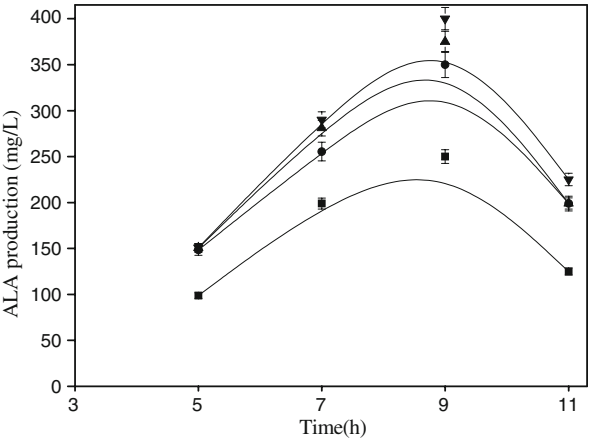

Fig. 6 Effect of initial succinic acid concentration on ALA production in *E. coli* Rosetta(DE3)/pET28a(+)-*hemA*. Succinic acid: –▾– 9 g/l; –▴– 6 g/l; –●– 3 g/l; –▪– 1 g/l

increased from 1 g/l and 3 g/l (A in Fig. 7) to 2 g/l and 9 g/l (F in Fig. 7), the ALA yield increased about 22%. However, when succinic acid concentration was increased from 3 g/l (A in Fig. 7) to 9 g/l (C in Fig. 7) and glycine was kept at 1 g/l, the ALA yield only increased about 8.8%. In conclusion, the suitable concentration of glycine and succinic acid in the initial medium was 2 g/l and 3 g/l, respectively.

ALA Production with *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* in a Fermenter using Fed-Batch Culture System

It was desirable to perform the fermentation in a fermenter. In this work, a 15.0-l fermenter was applied and fed-batch fermentation was carried out using *E. coli* Rosetta(DE3)/pET28a (+)-*hemA* The initial conditions for the ALA production were 3.0 g/l succinic acid, 2.0 g/l glycine, and 2.0 g/l glucose added initially. And 2.0 h after incubating, IPTG was added and culture temperature was adjusted to 28 °C. The pH was controlled at 5.9 with 10% H_2SO_4 initially and at 6.2 with the mixture of glycine and succinic acid (4.0 g/l glycine and 7.0 g/l succinic acid) after

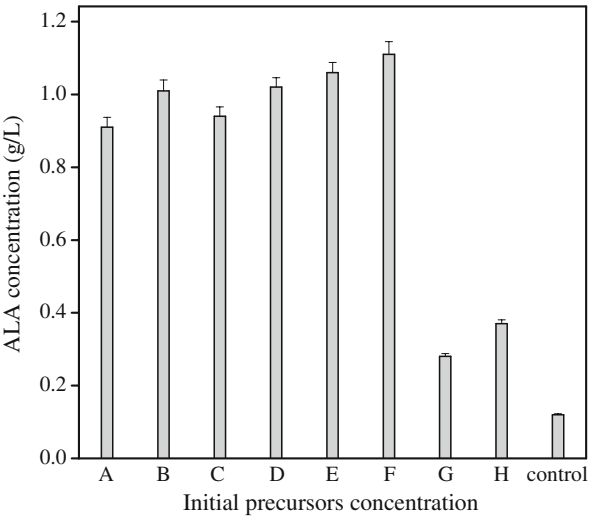

Fig. 7 The effect of initial precursor concentration on ALA production in Rosetta(DE3)/pET28a(+)-*hemA*. *control* = without glycine and succinic acid. *A* represents 1 g/l glycine and 3 g/l succinic acid. *B* represents 1 g/l glycine and 6 g/l succinic acid. *C* represents 1 g/l glycine and 9 g/l succinic acid. *D* represents 2 g/l glycine and 3 g/l succinic acid. *E* represents 2 g/l glycine and 6 g/l succinic acid. *F* represents 2 g/l glycine and 9 g/l succinic acid. *G* represents 1 g/l glycine and no succinic acid. *H* represents 3 g/l succinic acid and no glycine

6.0 h of incubating. Additional 4.00 g/l glucose was added at 9.0, 12.0, and 15.0 h, respectively. The final concentration of ALA was 6.5 g/l, equivalent to 50 mM (Fig. 8).

Discussion

The *hemA* gene has been cloned mainly from photosynthetic bacterium *R. sphaeroides* [7, 12] and *B. japonicum* [8, 10] to construct recombinant *E. coli* for mass ALA production. *A. radiobacter* is a good producer of vitamin B_{12} [13]. And the *hemA* gene from *A. radiobacter* was expressed in *E. coli* BL21 (DE3) firstly in our previous work [9] for ALA production. But the ALA synthase activity was low and ALA production was only 3.01 g/l under optimal fed-batch fermentation conditions [14]. In this work, a rare codon optimizer, *E. coli* Rosetta(DE3), was applied to express the *hemA* gene. The results showed that the ALA synthase activity with *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* was 35.3 U/mg protein, which was about 20% higher than that with ordinary *E. coli* BL21(DE3)/pET28a(+)-*hemA*, which explained that the codon favorability of the host strain was an important factor for enzyme activity expressed by a foreign gene. And the higher ALA synthase activity would improve the ALA synthesis. The ALA synthase activity from *A. radiobacter* 4718 was only about 2 U/mg protein [13] under the optimal conditions which revealed that this new recombinant ALA synthase from *A. radiobacter* zju-0121 might be potential for higher ALA production. Moreover, suitable IPTG concentration was only 0.05 mM in this work. It was probably because *E. coli* Rosetta(DE3) was a derivate of BL21(DE3), contained a mutation of *lacY1*, and IPTG could infiltrate through all the cells of *E. coli* Rosetta(DE3) equably. Low concentration of IPTG could induce the expression of *hemA* gene carried with plasmid pET-28a(+) completely.

Glucose was thought to be an inhibitor for ALA dehydratase [20]. The results in this study indicated that the inhibition of ALA dehydratase with initial glucose was favorable for ALA production, which was the result of preventing the degradation of synthesized ALA. Then the addition of glucose in the late stage of fermentation acted as the inhibitor of ALA dehydratase.

The expression of ALA synthase was improved in rare codon optimizer strain *E. coli* Rosetta(DE3); however, it is not enough for higher ALA production because there were not enough precursors (both glycine and succinic acid) in the medium for high ALA

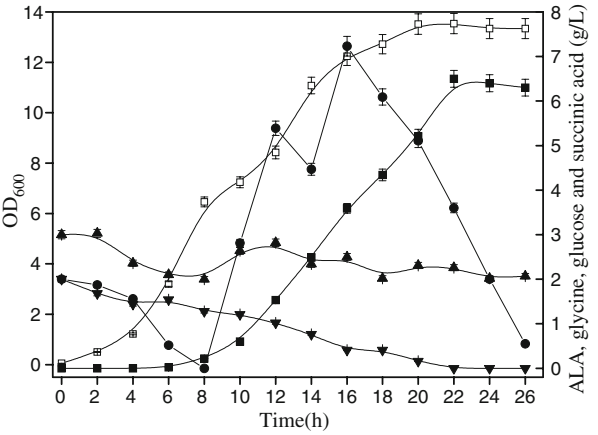

Fig. 8 5-aminolevulinic acid (ALA) production in *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* using fed-batch culture method. Mixture of glycine and succinic acid was added continuously from 6.0 h. Additional 4.00 g/l glucose was added at 9.0, 12.0, and 15.0 h, respectively. ▲ Succinic acid, ▼ glycine, ■ ALA, □ OD, ● glucose. The pH was controlled at 5.9 with 10% H_2SO_4 initially and at 6.2 with the mixture of glycine and succinic acid after 6.0 h of incubating

productivity. Thus, it is necessary to supply these precursors in the medium. Considering that high initial concentration of glycine was harmful to *E. coli* growth, initial glycine was optimized with lower concentration.

In the fed-batch fermentation using the recombinant *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* in a 15-l fermenter, the final ALA yield reached 6.5 g/l, which was about 116% higher than that of our previous work [14]. It is expected to get higher ALA production by further study of fed-batch fermentation with recombinant *E. coli* Rosetta(DE3)/pET28a(+)-*hemA*.

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