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Short Communication

Characterization of 5-aminolevulinate synthase from *Agrobacterium radiobacter*, screening new inhibitors for 5-aminolevulinate dehydratase from *Escherichia coli* and their potential use for high 5-aminolevulinate production

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ABSTRACT

The *hemA* gene encoding 5-aminolevulinate synthase (ALAS) from *Agrobacterium radiobacter* zju-0121 showed 92.6% homology with that from *A. radiobacter* ATCC4718 and contained several rare codons. To enhance the expression of this gene, *Escherichia coli* Rosetta(DE3), which is a rare codon optimizer strain, was used as the host to construct an efficient recombinant strain. And the encoded protein was over-expressed as fusion protein and was purified by affinity purification on Ni-NTA agarose and by gel filtration chromatography on Sephadex G-25 Medium resin. The recombinant protein was partly characterized, and D-glucose, D-fructose, D-xylose, D-mannose, L-arabinose, D-galactose, lactose, sucrose and maltose were detected to have no distinct inhibition on this recombinant ALAS. Meanwhile, 20 mM D-glucose or D-xylose inhibited about 20% activity of ALA dehydratase (ALAD) from *Escherichia coli* Rosetta(DE3). Combining D-xylose as a new inhibitor for ALAD with D-glucose in fed-batch culture and based on the optimal culture system using Rosetta(DE3)/pET28a-*hemA*, the yield of ALA achieved was 7.3 g/l (56 mM) under the appropriate conditions in the fermenter.

1. Introduction

5-Aminolevulinate (ALA) is generally known as an essential intermediate in the biosynthesis of tetrapyrroles such as heme, porphyrin, chlorophyll, and vitamin B_{12} (Sasaki et al., 2002). As a kind of photodynamic chemical, ALA had been approved by the Food and Drug Administration (FDA, USA) in 1999 as a photodynamic medicine for the treatment of skin cancer. And ALA has recently received great attention as a new selective and biodegradable herbicide and insecticide (Sasaki et al., 2002). In non-plant eukaryotes and some bacteria, 5-aminolevulinate synthase (ALAS) (EC 2.3.1.37) catalyzes the condensation of glycine and succinyl-CoA to form ALA, CoA, and carbon dioxide in the presence of the cofactor, pyridoxal 5′-phosphate (PLP). The genes encoding ALASs have been named as *hemA* and characterized in several bacteria: *Rhodobacter sphaeroides* (Bolt et al., 1999), *Paracoccus denitrificans* (Page and Ferguson, 1994), *Rhodopseudomonas palustris* KUGB306 (Choi et al., 2004), etc. And the production of ALA using recombinant *Escherichia coli* containing the *hemA* gene has been studied by several groups (Lee et al., 2005; Chung et al., 2005; Fu et al., 2007, 2008).

Agrobacterium radiobacter is a good producer of vitamin B_{12}. Researchers (Drolet and Sassarman, 1991) previously cloned the *hemA* gene from *A. radiobacter* ATCC4718 and expressed it in *E. coli* K12. But the property of the enzyme ALAS was not studied in detail and the ALA yield of the recombinant *E. coli* K12 was not published. In our previous work (Liu et al., 2005), a *hemA* gene (Bankit766399, DQ352146) from *A. radiobacter* zju-0121 was cloned and expressed in *E. coli* BL21 (DE3). And it showed only 92.6% homology with that of *A. radiobacter* ATCC4718. The low homology suggested that the properties of these two ALASs might not be the same and required further investigation. Moreover, the activity of recombinant ALAS from *A. radiobacter* zju-0121 was relatively low and the ALA yield was 3.01 g/l (Qin et al., 2006) with recombinant *E. coli* BL21 (DE3) under optimal fed-batch fermentation conditions.

It is reported that the addition of D-glucose (Lee et al., 2003) in the fermentation medium as a competitive inhibitor for ALA dehydratase (ALAD) can greatly improve the accumulation of ALA, and that D-glucose may also act as an inhibitor (Xie et al., 2003) for ALA synthase (ALAS). In our previous study (Fu et al., 2007), it was indicated that a low initial glucose concentration (2 g/l) was favorable for ALA production with recombinant *E. coli*, whereas the addition of glucose (Fu et al., 2008) in the later exponential phase of fermentation was also beneficial for high ALA production.

It is found that the *hemA* gene from *A. radiobacter* zju-0121 contains several rare codons, which should be unfavorable for ALAS

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expression, therefore, an 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, was used as the host to construct an efficient recombinant strain. Purified recombinant fusion ALAS was obtained after Ni-NTA agarose affinity purification and Sephadex G-25 medium resin gel filtration chromatography. And the molecular weight and purity of the enzyme were determined by SDS-PAGE. Then the properties of the recombinant ALAS expressed in *E. coli* Rosetta(DE3) were characterized.

In recombinant *E. coli* Rosetta(DE3)/pET28a-*hemA*, the expression of ALAS is improved in genetic engineering cells, however, the ALAD may catalyze the degradation of ALA. It is necessary to inhibit the degradation of ALA by adding some suitable inhibitors for ALAD in the fermentation medium. And some new inhibitor such as D-xylose for ALAD from *E. coli* Rosetta(DE3) was selected from all the carbohydrates evaluated in this study. Then ALA production using recombinant *E. coli* Rosetta(DE3)/pET28a-*hemA* was conducted with the addition of the new inhibitor for ALAD from *E. coli* Rosetta(DE3) in a fermenter.

2. Methods

2.1. Strain and plasmid

The host strains applied in this work are *E. coli* Rosetta(DE3) (Novagen, Germany) [F^-ompT $hsdS_B(r_B^-m_B^-)$ gal dcm lacY1(DE3) pRARE(Cm^R)] and *E. coli* BL21(DE3) (Novagen, Germany) [F^-ompT $hsdS_B(r_B^-m_B^-)$ gal dcm]. Recombinant pET28a(+)-*hemA* (*hemA* from *A. radiobacter*) was constructed in our previous work (Liu et al., 2005). And the expression plasmid pET28a(+)-*hemA* was transformed into *E. coli* Rosetta(DE3) to obtain *E. coli* Rosetta(DE3)/pET28a(+)-*hemA*.

The *hemB* gene was amplified using polymerase chain reaction (PCR), where the genome of *E. coli* Rosetta(DE3) was used as the DNA template. Then the recombinant pET28a(+)-*hemB* was constructed and the recombinant *E. coli* BL21(DE3)/ pET28a(+)-*hemB* was obtained in this work.

2.2. Expression of the recombinant hemA gene and purification of ALAS

For recombinant *E. coli* cultivation, Luria-Bertani (LB) medium containing (per liter): 5.00 g yeast extract, 10.00 g tryptone, and 10.00 g NaCl was used. Also, 30 mg/l kanamycin and 34 mg/l chloramphenicol were added in the medium.for *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* After incubation for 2 h at 37 °C, isopropyl-b-D-thiogalactopyranoside (IPTG) was added to induce the expression of ALAS at the final concentration of 0.05 mM, and the mixture was incubated for 6 h at 28 °C. Cells were harvested by centrifugation (10,000*g* for 10 min at 4 °C). The cells were resuspended in 100 mM Tris-buffer (pH 7.0), and then were disrupted with an Ultrasonic Instrument (Ningbo Scientz Biotechnology, China) for 6 min. After re-centrifugation (10,000*g* for 10 min at 4 °C), the 6 his-tagged ALAS in the supernatant was recovered by affinity purification on Ni-NTA according to the manufacture's instruction (Qiagen, Germany), and was eluted with imidazole and NaCl. Sephadex G-25 Medium resin (GE healthcare, USA) was used for gel filtration chromatography to remove imidazole and NaCl from the ALAS solution. The purified ALAS was freeze-dried by a freeze dry system (Labconco, USA).

2.3. Gel electrophoresis and the determination of protein concentration

The proteins of the cell extracts were analyzed by Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A 12% (w/v) separation slab gel was prepared (Laemmli 1970). Proteins were stained with Coomassie brilliant blue G. The images of gels were scanned with GEL-DOC 2000 gel documentation system (Bio-Rad, USA) and were analyzed with Quantity One software, Version 4.4.0 (Bio-Rad, USA).

Protein concentrations were measured using a Pierce BCA Protein Assay Kit (Sigma, St. Louis, Mo.) with bovine serum albumin as the standard.

2.4. Assay for enzyme activity of ALAS

Crude enzyme assay was carried out according to our previous work (Fu et al., 2007). Purified enzyme assay was also performed at the same conditions with about 0.1 mg/ml ALAS. The assays were terminated by the addition of trichloroacetic acid. After reaction with acetylacetone for 15 min at 100 °C, the mixtures were cooled down to room temperature and mixed with modified Ehrlich's reagent for 30 min. Then the absorbance at 554 nm was measured. One unit of ALA synthase activity was defined as the amount of enzyme needed to produce 1 nmol of ALA in 1 min.

2.5. Enzyme properties of ALAS

The enzymatic reaction was assayed at various pH values and temperatures. The effects of various metal ions and chemicals on the enzyme activity were also evaluated. The effects of different carbohydrates on the activity of recombinant ALAS were investigated. All the measurements were performed with 0.1 mg/ml ALAS in 50 mM Tris-HCl buffer. Different concentrations of glycine or succinyl-CoA were used to examine the relationship between the initial velocity and the substrate concentration. Glycine concentration and succinyl-CoA concentration were set at 100 mM and 4 mM, respectively, when the other substrate was changed with different concentrations. The parameters in Michaelis-Menten equation, K_m and V_{max}, of the purified ALAS were determined by double-reciprocal Lineweaver-Burk plot.

2.6. Assay for enzyme activity of ALAD

Cell-free extracts of induced *E. coli* BL21(DE3)/pET28a(+)-*hemB* were prepared, and the activity of ALAD was determined by a calorimetric assay based on the reaction between porphobilinogen (PBG) and modified Ehrlich's reagent according to the process of assay for enzyme activity of ALAS. The purified ALAD was also prepared by the same method as the purification of recombinant ALAS by affinity purification on Ni-NTA agarose and by gel filtration chromatography on Sephadex G-25 Medium resin.

The assay for recombinant *E. coli* ALAD involved a total volume of 500 μl, consisting of 50 mM of potassium phosphate buffer (pH = 7.5), 50 μM of $ZnCl_2$, 1 mM of $MgCl_2$, and 5 mM of ALA. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of an equal volume of stop reagent (20% trichloroacetic acid). One unit of ALA dehydratase activity was defined as the amount of enzyme needed to produce 1 nmol of PBG in 1 h.

2.7. Fermentation conditions

The Luria-Bertani (LB) medium was used for seed culture. Seed cultures were incubated for 8 h at 200 rpm and at 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). And the basic conditions for the fermentation were as same as that of our previous work (Fu et al., 2008).

2.8. Assay for ALA and PBG

ALA was analyzed according to our previous work (Fu et al., 2008). And PBG was analyzed using modified Ehrlich's reagent

(Burnham, 1970). Specifically, 2 ml of sample or standard was mixed with 2 ml of freshly prepared modified Ehrlich's reagent (Burnham, 1970). After 30 min, the absorbance at 555 nm was measured.

3. Results and discussion

3.1. Effect of host strain on the expression of ALAS

It is well known that rare codons are harmful for the expression of protein in host cell. The *hemA* gene from *A. radiobacter* contains several rare codons when expressed in *E. coli* BL21(DE3), therefore, in our previous work (Liu et al., 2005), *E. coli* BL21 (DE3) with recombinant plasmid of pET28a(+)-*hemA* (*hemA* from *A. radiobacter*) showed relatively low ALAS activity and low ALA yield (about 3.01 g/l in optimized fed-batch fermentation) (Qin et al., 2006). Total synthesis of gene and point mutation with favorable codons are currently applied in genetic engineering manipulation (Galazka et al., 2006; Jo et al., 2007; Carpenter et al., 2008). But it was difficult to carry out point mutation for this *hemA* gene since there are 5 rare codons (CCC) and 4 rare codons (GGA) separately in the *hemA* gene (406 codons) (Liu et al., 2005). In this work, the expression plasmid pET28a(+)-*hemA* was transformed into *E. coli* Rosetta(DE3) to obtain *E. coli* Rosetta(DE3)/ pET28a(+)-*hemA*. And the optimal conditions determined for ALA synthase expression were as follows: the fermentation temperature was initially maintained at 37 °C, and after 2.0 h of batch fermentation, 0.05 mM IPTG was added to induce the expression of ALAS, in the mean time, the culture temperature was lowered to 28 °C. And ALAS activity was 35.3 U/mg protein in the *E. coli* Rosetta(DE3)/pET28a(+)-*hemA*, which was about 20% higher than that in the *E. coli* BL21(DE3)/ pET28a(+)-*hemA* (29.1 U/mg protein) under the appropriate conditions.

3.2. Purification of recombinant ALAS in E. coli

To study the function of the enzyme, it is necessary to obtain adequate purified ALAS protein. Thus, after inducing with IPTG for 6.0 h, about 47 kDa fusion protein (45 kDa ALAS with 6 his-tagged and T7-tagged) band from the cell-free extracts of *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* was observed during SDS-PAGE (Fig. 1, lane 3). The results indicated that the *hemA* gene was expressed in large quantity as fusion protein in *E. coli*. The 6-his-tagged ALAS fusion protein was purified by Ni-NTA affinity chromatography, and then was eluted with imidazole and NaCl solution (Fig. 1, lane 4). Gel filtration chromatography with Sephadex G-25 Medium resin was further applied to remove the imidazole and NaCl from the ALAS solution. Finally, the purified ALAS was freeze-dried (Fig. 1, lane 2). The ALAS was purified 9.3-fold from the cell-free extracts with an enzyme specific activity of about 117.6 U/mg, and the purity of the protein was found to be about 95% (w/w) by SDS-PAGE (Fig. 1, lane 2).

3.3. Basic properties of recombinant ALAS

The effects of temperature on the activity of recombinant ALAS were determined in 50 mM Tris-HCl buffer (pH 7.5). According to our study, the optimum temperature of the recombinant ALAS was 37 °C. The stability of the ALAS is shown in Fig. 2. After storage for 1 h at 57 °C or 67 °C, ALAS lost all its activity. And the enzyme retained only about 30% of its activity after storage for 1 h at 47 °C, whereas, about 66% of its activity remained after storage for 3 h at 27 °C. It is obvious that the ALAS should be stored at low temperature. The optimum pH value of the recombinant ALAS was 7.5, which was similar to that of native ALAS purified from *R. sphaeroides* (Burnham 1970). The recombinant ALAS was more stable in the alkaline pH range; approximately 90% of its activity was retained at pH 9.5 after storage for 3 h.

Because PLP is the cofactor for the ALAS-catalyzed reaction, the existence of PLP in the reaction system was found to be essential for the activity of recombinant ALAS. ALAS showed no activity without the addition of PLP (data not shown). A similar result was observed for *R. sphaeroides* (Burnham 1970).

The Michaelis-Menten constant, K_m, for ALA synthesis reaction catalyzed by recombinant ALAS from glycine and succinyl-CoA was obtained. The K_m value for glycine and succinyl-CoA was 9.71 mM and 257.3 μM, respectively. Previously, the K_m value was reported as 1.88 mM for glycine and 17 μM for succinyl-CoA for the recombinant *R. sphaeroides* ALAS (Bolt et al., 1999) and as 2.01 mM and 49.55 μM for the recombinant *Rhodopseudomonas palustris* KUGB306 ALAS (Choi et al., 2004).

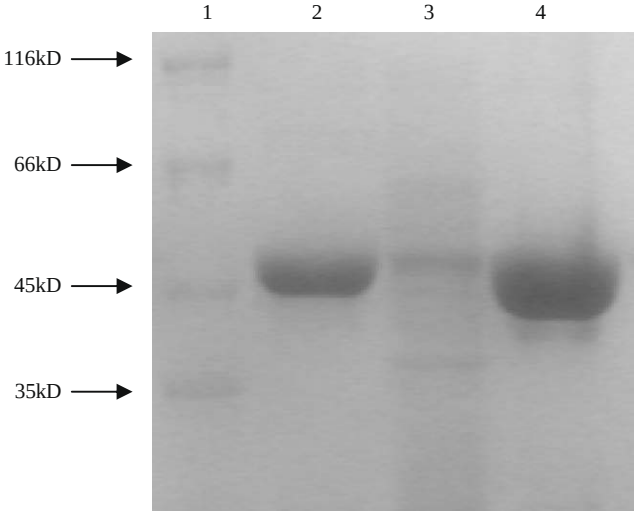

Fig. 1. Crude and purified ALA synthase of the recombinant *E. coli* Rosetta(DE3)/ pET28a(+)-*hemA*, Lane 1; Marker, Lane 2; Purified ALAS, freeze-dried powder, Lane 3; Cell-free extracts of induced *E. coli* Rosetta(DE3)/pET28a(+)-*hemA*, Lane 4; Purified ALAS from the imidazole and NaCl elution after affinity purification.

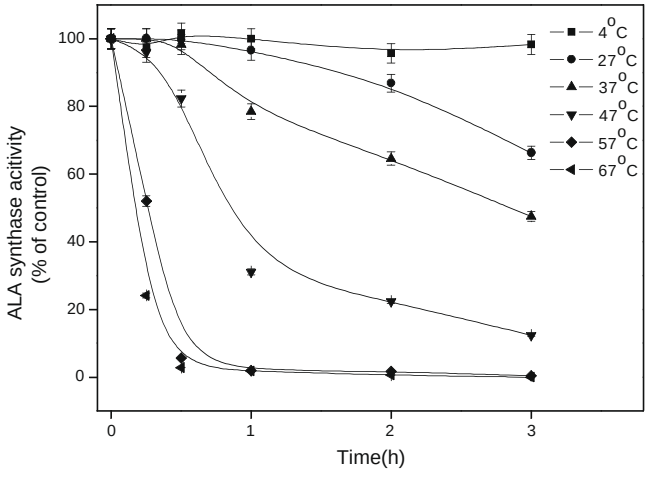

Fig. 2. Effects of storage at various temperatures for 3.0 h on the activity of recombinant ALAS. The activity of the non-treated reference sample assayed at 37 °C was arbitrarily set as 100%.

3.4. Effects of different chemicals and ion intensity on the recombinant ALAS

The effects of various metal ions on the activity of recombinant ALAS were examined when the concentration of metal ions was set as 20 mM. As shown in Table 1, the inhibitory effects of different metal ions on the reaction rate are in the following order: Ba^{2+}<Co^{2+} <Zn^{2+} <Cu^{2+}. Particularly, Cu^{2+} inhibited about 90% of the activity. The results are similar to those of the ALAS purified from *R. sphaeroides* (Burnham 1970). And ALAS activity was effectively promoted by Mn^{2+}, but was slightly affected by K^{+}, Li^{+} and Ca^{2+} (less than 10%). And our study indicated that the ALAS activity changed a little when the addition of NaCl was increased from 20 mM to 320 mM.

Also surfactants such as SDS and EDTA showed strong inhibitory effect on the ALAS activity, almost 80% of the activity was lost.

3.5. Effects of different carbohydrates on the recombinant ALAS and recombinant ALAD

Glucose was regarded as an inhibitor for ALA dehydratase, which is beneficial to improve the accumulation of ALA (Lee et al., 2003), whereas D-glucose (Xie et al., 2003) was also regarded as an inhibitor for ALA synthase. Our previous study (Fu et al., 2007) indicated that a low initial glucose concentration of 2 g/l was favorable for ALA production with recombinant *E. coli*. In this work, the effect of glucose on the activity of recombinant ALAS was evaluated. The results showed that glucose concentration had little effect on the activity of recombinant ALAS when different concentrations of D-glucose were added to the enzyme activity assay system. When the concentration of D-glucose was changed from 10 mM to 80 mM, the enzyme activity changed less than 4% with respect to the control under the same conditions, but in the absence of D-glucose. The effects of other carbohydrates such as D-glucose, D-fructose, D-xylose, D-mannose, L-arabinose, D-galactose, lactose, sucrose and maltose were also tested under the same conditions. The results showed that these sugars only brought about slight effects on the activity of recombinant ALAS.

5-Aminolevulinate dehydratase (ALAD) can catalyze the degradation of ALA, therefore, the inhibition of the activity of ALAD is desirable for higher ALA yield. One research group (Caballero et al., 1998) previously indicated that the activity of ALAD from red blood cells was 80% inactivated when preincubated with 500 mM D-glucose or D-xylose at 37 °C for 20 hr. Our preliminary study (Table 1) showed that the activity of ALAD from *E. coli* Rosetta(DE3) (cell-free extracts of induced recombinant *E. coli* BL21(DE3)/pET28a(+)-*hemB*) was also inhibited by D-glucose and D-xylose. About 20% of ALAD activity was lost by adding 20 mM D-glucose or D-xylose to the reaction system compared with the blank control under the same conditions. Then our further study indicated that D-glucose and D-xylose possessed distinct inhibitory effects on purified recombinant ALAD from *E. coli* Rosetta(DE3). When the concentration was set as 20 mM, D-glucose and D-xylose showed 14.1% and 21.1% inhibition on ALAD, respectively. The inhibition D-glucose and D-xylose on ALAD activity increased when the concentration of the two carbohydrates was changed from 10 mM to 80 mM. And the results also showed that D-xylose was a better inhibitor for ALAD than D-glucose in ALA production.

Table 1
Effects of various metal ions on the activity of recombinant ALAS and some different carbohydrates (their concentrations were all 20 mM) on the activity of crude recombinant ALAD. The values are the mean of triplicate experiments. The activity of the non-treated reference sample in the assay was arbitrarily set as 100%.

Chemicals	Relative activity (%)
Blank control for ALAS	100
EDTA	18.6
SDS	19.2
$ZnCl_2$	17.2
$CaCl_2$	109.1
$CuCl_2$	10.1
$MnCl_2$	122.3
$CoCl_2$	22.0
$BaCl_2$	75.6
KCl	107.6
LiCl	102.0
Blank control for ALAD	100
D-Glucose	83.7
D-Fructose	94.8
D-Xylose	85.4
L-Arabinose	99.3
D-Mannose	101.9

3.6. ALA production with addition of new ALAD inhibitor in fed-batch system

Based on the above results, D-xylose may be a better ALAD inhibitor in ALA production. Also, xylose is present in abundance in certain lignocellulosic biomass including waste crop residues such as corn stover (Schell et al., 2004). So it was desirable to

Table 2
Different addition strategies with some inhibitors for ALAD using recombinant *E. coli* Rosetta(DE3) /pET28a(+)-*hemA* in ALA production.

	Addition strategy		
	Additional 4.00 g/l glucose was added at 9.0 h, 12.0 h and 15.0 h	Additional 3.00 g/l xylose was added at 9.0 h, 12.0 h and 15.0 h	Additional 3.00 g/l xylose was added at 6.0 h and additional 4.00 g/l glucose was added at 9.0 h, 12.0 h and 15.0 h
Fermentation time (h)	28.0	26.0	30.0
OD_{600}	17.5	8.25	17.7
Maximum yield of ALA (g/l)	6.5	3.0	7.3

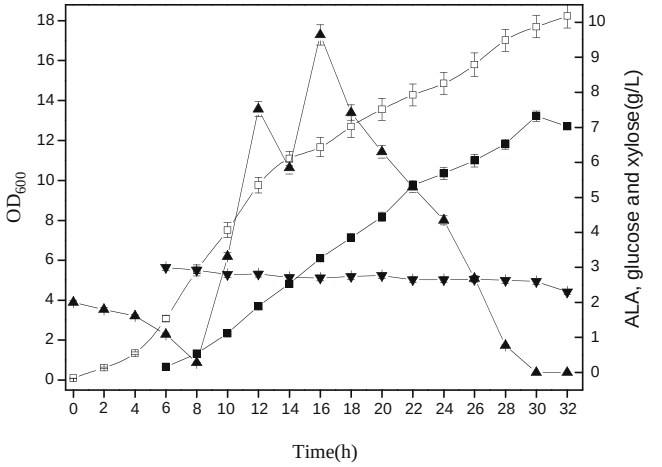

Fig. 3. 5-aminolevulinic acid (ALA) production in *E. coli* Rosetta(DE3)/pET28a(+)-*hemA* using fed-batch culture method. Mixture of glycine and succinic acid was added continuously for 6.0 h. Additional 3.00 g/L xylose was added at 6 h and 4.00 g/L glucose was added at 9.0 h, 12.0 h and 15.0 h, respectively. ■ ALA, □ OD, ▲ glucose, ▼ xylose. The pH was controlled at 5.9 with10% H_2SO_4 initially and at 6.2 with a mixture of glycine and succinic acid after 6.0 h of incubation.

carry out the fermentation with the addition of D-xylose. In this work, a 15.0 l fermenter was used, 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, which were added initially in LB medium (Fu et al., 2008). And after incubating for 2.0 h, 0.05 mM IPTG was added and the 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 a mixture of glycine and succinic acid (4.0 g/l glycine and 7.0 g/l succinic acid) after 6.0 h of incubation. Table 2 shows that without the addition of glucose in the fermentation medium, the recombinant cells grew much worse and that the ALA yield was only 3.0 g/l. With the addition strategy that 3.0 g/l (20 mM) xylose be added at 6.0 h and 4.0 g/l glucose be added at 9.0 h, 12.0 h and 15.0 h, respectively (Fig. 3), 7.3 g/l concentration of ALA was reached at 30.0 h when glucose was exhausted and much xylose, which was not a preferable carbon source (Govindaswamy and Vane, 2007) for *E. coli*, still remained. Addition of D-xylose as a new inhibitor combined with D-glucose for ALAD in ALA production increased the ALA yield over 10% than fed-batch system with the addition of only D-glucose as ALAD inhibitor.

4. Conclusions

The adoption of the host strain *E. coli* Rosetta(DE3) (Novagen, Germany) [F^-ompT $hsdS_B(r_B^-m_B^-)$ gal dcm lacY1(DE3) pRARE(Cm^R)], which is capable of expressing genes with rare codons, was favorable for ALAS production. The expressed ALAS was purified, and its basic properties were characterized. The results showed that the optimal temperature and pH values for ALAS were 37 °C and 7.5, respectively. The cofactor PLP was necessary for enzyme activity. The Michaelis-Menten constant, K_m, for glycine and succinyl-CoA was 9.71 mM and 257.3 μM, respectively. Among the examined metal ions, only Mn^{2+} was able to promote the ALAS activity, while Cu^{2+} was the most strong inhibitor.

All the carbohydrates evaluated showed no distinct effects on the ALAS activity, and D-xylose was picked out as a new inhibitor for ALAD from all the carbohydrates evaluated in this study. And combining D-xylose with D-glucose in fed-batch culture and based on the optimal culture system using Rosetta(DE3)/pET28a-*hemA*, the yield of ALA achieved was 7.3 g/l (56 mM) under the appropriate conditions in fermenter, which suggests that addition of some new suitable inhibitors for ALAD may lead to high ALA production in the future.

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