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Production of 5-aminolevulinic acid by cell free multi-enzyme catalysis

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ABSTRACT

5-Aminolevulinic acid (ALA) is the precursor for the biosynthesis of tetrapyrroles and has broad agricultural and medical applications. Currently ALA is mainly produced by chemical synthesis and microbial fermentation. Cell free multi-enzyme catalysis is a promising method for producing high value chemicals. Here we reported our work on developing a cell free process for ALA production using thermostable enzymes. Cheap substrates (succinate and glycine) were used for ALA synthesis by two enzymes: 5-aminolevulinic acid synthase (ALAS) from *Laceyella sacchari* (LS-ALAS) and succinyl-CoA synthase (Suc) from *Escherichia coli*. ATP was regenerated by polyphosphate kinase (Ppk) using polyphosphate as the substrate. Succinate was added into the reaction system in a fed-batch mode to avoid its inhibition effect on Suc. After reaction for 160 min, ALA concentration was increased to 5.4 mM. This is the first reported work on developing the cell free process for ALA production. Through further process and enzyme optimization the cell free process could be an effective and economic way for ALA production.

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1. Introduction

5-Aminolevulinic acid (ALA), a derivative of five-carbon amino acid, is the universal precursor for synthesis of tetrapyrroles, including heme, chlorophyll, cytochrome and vitamin B12. As a high value chemical (around $600 per kg), ALA has broad applications in the fields of medicine (photodynamic drugs which have a great effect on diagnosis and treatment of cancer) and agriculture (biodegradable herbicide, plant growth regulators, and pesticide) (J. Zhang et al., 2015).

Currently there are mainly two methods for ALA production. Chemical synthesis of ALA is complicated and low-yielding, biological synthesis (fermentation) appears to be a good choice (Liu et al., 2014). For instance, the *Rhodobacter sphaeroides hemA* representing the gene name of 5-aminolevulinic acid synthase (ALAS) in the pUC18/19 vector system was transformed into *Escherichia coli*, and in recombinant *E. coli* extracts the ALA production was up to 22 mM (Vanderwerf and Zeikus, 1996). The *hemA* gene from *Bradirhyzobium japonicum* was transformed into *E. coli* and ALA accumulation was nearly 20 mM (Choi et al., 1999). *E. coli* expressing *hemA* gene from *Rhodopseudomonas palustris* produced 44 mM ALA (Zhang et al., 2013). The ALA production by recombinant *E. coli* Rosetta (DE3) (contained *hemA* from *Agrobacterium radiobacter*) reached 56 mM (Lin et al., 2009). Notably, the production of ALA is significantly increased by applying a full factorial design model. However, application of complex medium and cultivation process would be unbeneficial to industrial production.

Cell free multi-enzyme catalysis is a novel method for the production of high value chemicals by the assembly of a number of enzymes and coenzymes catalyzing a series of biochemical reactions (Hodgman and Jewett, 2012). Cell free catalysis has received much attention because it has several distinctive advantages com-

Abbreviations: ALA, 5-aminolevulinic acid; ALAS, 5-aminolevulinic acid synthase; LS-ALAS, 5-aminolevulinic acid synthase from *Laceyella sacchari*; Suc, succinyl-CoA synthase; Ppk, polyphosphate kinase.

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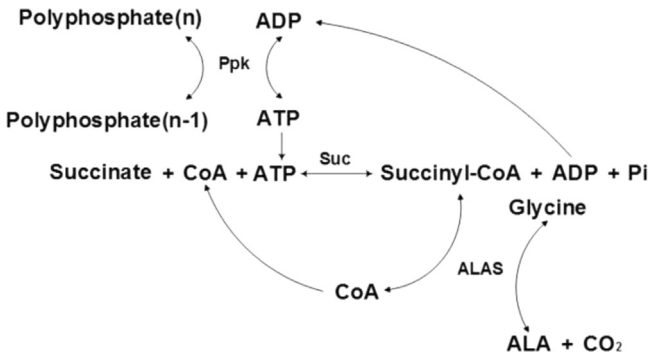

Fig. 1. The multi-enzyme reactions for cell free 5-aminolevulinic acid (ALA) synthesis. The enzymes used were 5-aminolevulinic acid synthase (ALAS), succinyl-CoA synthase (Suc) and polyphosphate kinase (Ppk).

paring to microbial fermentation, such as fast reaction rate, great engineering flexibility, high product titer and high product yield. From the economical aspect, although extra costs for enzyme purification and stabilization are spent, the stabilized enzymes have more than 3 orders of magnitude higher weight-based total turnover numbers than microbial biocatalysts (Zhang, 2010). Therefore, screening thermostable enzymes is of great importance for the development of cell free enzymatic process for the production of high value chemicals like ALA.

Two ALA biosynthesis pathways have been reported: The C4 pathway and C5 pathway (Woodard and Dailey, 1995). The C5 pathway is difficult to be realized *in vitro* as t-RNA is an intermediate of the pathway. Therefore, the C4 pathway is a better choice for cell free ALA synthesis. ALAS is the key enzyme in the C4 pathway, which catalyzes the succinyl-CoA and glycine with pyridoxal phosphate (PLP) as a cofactor (succinyl-CoA + glycine ⇔ ALA + CoA + CO_2). We have previously screened three thermostable ALASs and found that LS-ALAS (ALAS from *Laceyella sacchari*) was the most thermostable one and also with the highest specific activity (Meng et al., 2015).

In this study, we developed cell free process for ALA production from succinate and glycine by three enzymes including LS-ALAS, succinyl-CoA synthase (Suc) and polyphosphate kinase (Ppk, for ATP regeneration). Through fed-batch addition of succinate the ALA concentration was increased and could be further improved by optimization of the enzymes used in the cell free system.

2. Materials and methods

2.1. Materials

Succinyl-CoA, glycine, ALA, succinate and CoA were all purchased from Sigma (USA). The kits and markers used for construction of clones were from Transgen (Beijing, China). The SDS-PAGE gels were purchased from life technologies (USA) and the protein markers were purchased from New England Biolabs (UK). Other chemicals used in this article otherwise demonstrated were purchased from Solarbio (Beijing, China).

2.2. Plasmids and strains

E. coli BL21 (DE3) was used as the host strain for his-tagged protein expression. The encoding sequences of LS-ALAS were codon optimized for *E. coli* and synthesized by Generay Company (Meng et al., 2015). Suc contains two subunits encoded by *sucC* and *sucD* respectively in *E. coli*, and the gene of Suc (named as *suc*, including *sucC* and *sucD* and the location of *sucC* is next to *sucD* in the genome of *E. coli*) from *E. coli* MG1655 was amplified with primers *sucC*-F1 and *sucD*-R1. The *ppk* gene (code for Ppk) from *R. sphaeroides* was amplified with primers *ppk*-F1 and *ppk*-R1. The above primers were shown in Supplementary Table 1 and all these genes were attached with 6 his tags (6 his tags in *suc* was located on *sucC*). These three sequences were inserted into pET28a to construct enzyme expression plasmids named as pET28a-*hemA*, pET28a-*suc* and pET28a-*ppk*, respectively. Then these plasmids were transformed into *E. coli* BL21 (DE3) for enzyme expression. The plasmids and strains used were shown in Supplementary Table 2.

2.3. Enzyme expression and purification

To purify the enzymes, single colony was grown in LB medium (contained 50 μg/ml kanamycin) at 37 °C until OD_{600} of the culture reached 0.6–0.8. Then cultures were cooled to 16 °C and IPTG was added to a final concentration of 1 mM. After further growth at 16 °C for 14–16 h, the cells were harvested by centrifugation at 6000*g* for 30 min at 4 °C and the pellet was washed three times with binding buffer A (Tris-HCl 50 mM, NaCl 150 mM and imidazole 20 mM, pH 7.5). The pellet was suspended with buffer A and the cells were disrupted by high pressure homogenizer. Then the cell lysate was centrifuged at 12,000*g* for 30 min at 4 °C, the supernatant was loaded onto Ni-NTA His-Bind column and some supernatant

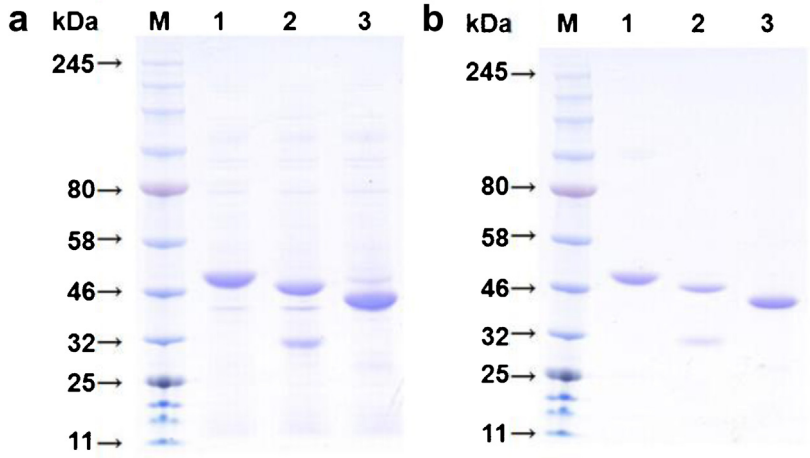

Fig. 2. SDS-PAGE analysis of enzymes. (a) SDS-PAGE analysis of soluble total protein of *E. coli* BL21 (DE3) expressing LS-ALAS, Suc and Ppk. (b) SDS-PAGE analysis of purified LS-ALAS, Suc and Ppk purified by Ni-NTA affinity chromatography. *Lane M* protein marker, *Lane 1* LS-ALAS (44.7 kDa), *Lane 2* Suc (Suc contains two subunits SucC and SucD, SucC is 41.4 kDa and SucD is 29.8 kDa), *Lane 3* Ppk (38.2 kDa).

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