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Influence of culture conditions on the production of extra-cellular 5-aminolevulinic acid (ALA) by recombinant *E. coli*

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Abstract

Extra-cellular production of 5-aminolevulinic acid (ALA) by recombinant *E. coli* harboring a fusion gene *hemA:egfp* was investigated in shake flasks and a bioreactor with chemically defined media by varying physiological factors such as the concentrations of precursors (glycine and succinic acid), and levulinic acid (LA). Optimum concentrations of glycine and succinic acid were found to be 15 and 30 mM, respectively. When 30 mM LA was added to the culture medium at the end of the exponential cell growth phase, intra-cellular ALA dehydratase activity was suppressed. Some imbalances might be caused by the addition of titrants to the cultivation. Therefore, the recombinant *E. coli* used in this study were grown with no control over the culture pH in the beginning phase. The recombinant *E.coli* produced significant ALA in the initial pH ranges of 6.1 and 6.5, i.e. without the addition of titrants. IPTG induction for the expression of the fusion gene did not enhance the production of extra-cellular ALA. Repeated addition of glycine, succinic acid, and LA increased the production of extra-cellular ALA and the inhibition of intra-cellular ALA activity, with up to 1.3 g/l ALA having been produced in the cultivation.

Keywords: Recombinant; *E. coli*; Fusion gene *hemA:egfp*; 5-Aminolevulinic acid (ALA); Fermentation

1. Introduction

5-Aminolevulinic acid (ALA) is an intermediate in the biosynthesis of tetrapyrroles such as prophyrin, vitamin B12 and chlorophyll in living cells. It has recently received attention as selective and biodegradable herbicide and insecticide [1] and growth promoting factor [2]. ALA also has medical applications for photodynamic cancer therapy and tumour diagnosis [3]. Due to the broad application range for ALA, a number of ALA production methods have been developed [4]. Some micro-organisms such as *Clostridium* [3], *Chlorella sp*. [5], photosynthetic bacteria [6] have been employed in the biological production of ALA. Extensive reviews of the biotechnological production of ALA and its agricultural and medical applications have been well reported by Sasikala et al. [1], Nishikawa and Murooka [3] and Sasaki et al. [7].

Recently, a few recombinant strains have been developed for the mass production of ALA [8–11]. Van der Mariet and Zeikus [8] cloned the *hemA* gene of *R. sphaeroides* into a plasmid and transformed the recombinant plasmid to *E. coli*. Choi et al. [9] transferred the *hemA* gene of *Bradyrhozobium japonicum* to *E. coli* and produced extra-cellular ALA. Kiatpapan et al. [10] recombined the *hemA* gene for *R. sphaeroides* and transformed it to *Propionibacterium freudenreichii*.

However, production of ALA on an industrial scale can be improved by monitoring the process as well as by regulating the *hemA* gene in ALA synthesis. Previous research [12] coupled the *hemA* gene with an enhanced green fluorescent protein gene (*egfp*) in order to monitor the biological process on-line, based on the fluorescent intensity of the fusion protein. For the purpose of this research cell growth of the recombinant *E. coli* harbouring the plasmid with the fusion gene, *hemA:egfp*, and the extra-cellular production of ALA are characterized and investigated under various conditions.

2. Material and methods

2.1. Bacterial strain and plasmid

Escherichia coli BL21(DE3)pLysS (Invitrogen, USA) was used with the plasmid pFLS 45 in the production of

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extra-cellular ALA. The plasmid pFLS 45 was made from the plasmid pET-28b/ALAS harbouring *hemA* gene for aminolevulinate synthetase from *Bradyrhizobium japanicum* (provided by Professor Back, KyungHwan in CNU) and the plasmid pEGFP (Invitrogen, USA) harbouring the *egfp* gene for green fluorescent protein. For the construction of pFLS45, a forward primer containing *Not*I sites (5′-AAGGAAAAAAGCGGCCGCATGGATTACAG-CCAGTTCTT-3′) and a reverse primer containing *Eco*RI site (5′-GGAATTCCTACTCCGCCGCCAGCGA-3′) were employed. The plasmid pET-28b/ALAS was used as a template for PCR. The PCR product was digested with *Not*I and *Eco*RI, gel-purified, and ligated into pEGFP. The plasmid pFLS45 coded for the fusion protein of enhanced green fluorescent protein (egfp) and the aminolevulinate synthetase (ALAS) under the transcription control of the isopropylthiogalactoside (IPTG)-inducible Lac promoter. The plasmid carried the resistance gene against ampicillin and its restriction map was shown in Fig. 1. The $CaCl_2$ method was used for the transformation of the plasmid pFLS 45 into the host cell. Recombinant cells were maintained at −80 °C as a 30% (v/v) glycerol stock solution.

2.2. Cultivation conditions

For the preparation of the first inoculum, 100–200 μl recombinant *E. coli* glycerol stock solution was plated out onto an LB (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl) agar plate containing 75 mg/l ampicillin. After incubation at 37 °C overnight a colony of green coloured cells was washed from the surface of the agar plate and transferred to 20 ml of LB medium with 75 mg/l ampicillin. The cells were cultivated in 100 ml by shaking the flasks with two baffles at 37 °C and 180 rpm for 6–8 h. For the preparation of the second inoculum, 99 ml of the fermentation medium, MS8 medium with the supplement of some precursors and inhibitors were incubated with 1 ml of the first inoculum for 12–14 h in 250 ml by shaking the flasks as previously described.

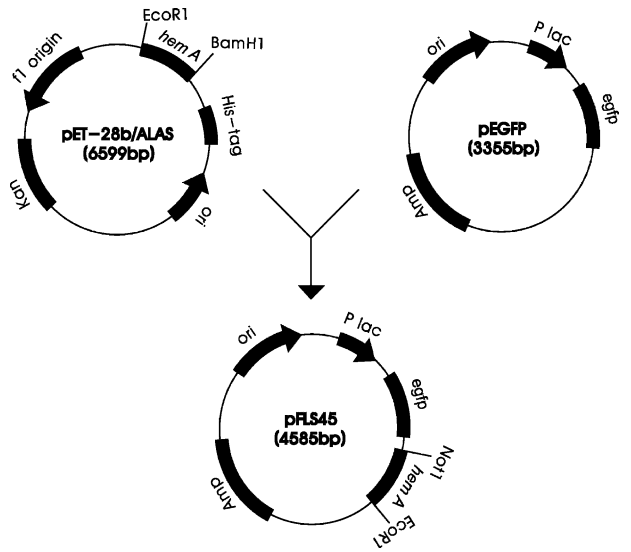

Fig. 1. Restriction map of the plasmid pFLS 45.

The fermentation medium (MS8 medium) were prepared as follows: 6 g/l glucose, 0.1 g/l $MgSO_4{\cdot}7H_2O$, 0.02 g/l NH_4Cl, 0.2 g/l $(NH_4)_2SO_4$, 1.3 g/l K_2HPO_4, 1.0 g/l KH_2PO_4, 0.6 g/l NaH_2PO_4, 10 ml/l silicone antifoam (non-ionic emulsifier, Sigma, USA), 4 ml/l of trace element stock solution, 4 ml/l of vitamin stock solution and 75 mg/l ampicillin (trisodium salt). The composition of the trace element stock solution was 10 g/l $CaCl_2{\cdot}2H_2O$, 0.5 g/l $ZnSO_4{\cdot}2H_2O$, 0.25 g/l $CuSO_4{\cdot}5H_2O$, 2.5 g/l $MnSO_4{\cdot}5H_2O$, 1.75 g/l $CoCl_2{\cdot}6H_2O$, 0.125 g/l H_3BO_4, 2.5 g/l $AlCl_3{\cdot}6H_2O$, 0.5 g/l $Na_2MoO_4{\cdot}2H_2O$, and 10 g/l $FeCl_3{\cdot}6H_2O$. The vitamin stock solution was prepared with 2 mg/l folic acid, 100 mg/l riboflavin, 1500 mg/l thiamine–HCl, 500 mg/l nicotinic acid, 500 mg/l pyridoxine–HCl, 500 mg/l Ca-panthothenate, 1 mg/l biotin and 10 mg/l vitamin B12. The solution of glucose, nutrient salts and trace elements were autoclaved separately and mixed in shake flasks or in a bioreactor. The vitamin stock solution and ampicillin were sterile filtered and added to the bioreactor after autoclaving the fermentation medium. Solutions of precursors (glycine, succinic acid) and inhibitors (levulinic acid) were also separately autoclaved and introduced into shake flasks or a bioreactor.

2.3. Bioreactor and on-line monitoring

A 2.5 l (or 7.0 l) stirred tank reactor (KoBiotech, South Korea) with 1.0 l (or 4.5 l) working volume was provided with pH- and DO-meters (Mettler-Toledo, USA), air flow meter as well as with temperature-, stirrer speed-, antifoam- and pH-controllers. An O_2/CO_2 analyser (Lokas, South Korea) was employed to monitor on-line the concentrations of O_2 and CO_2 in the exhaust gas. The on-line data logging was performed using LabView-software (version 6.1, National Instruments, USA). The Oxygen uptake (OUR) and Carbon dioxide production rates (CPR) were evaluated using the concentrations of O_2 and CO_2 in the inlet and outlet gas. A 2-D fluorescence spectroscopy (Hitachi, Japan) was connected by a 2 m bifurcated liquid light conductor (Lumatec, Germany) to a quarz window of the bioreactor made of stainless steel. The configuration and controls of the spectrofluorometer, data acquisition and saving were made using a computer with home-made software, so that the results monitored on-line were displayed simultaneously on a PC monitor.

2.4. Off-line analysis

Cell mass concentration was determined off-line by weighing the dried cell mass (DCW) or by measuring the optical density at 600 nm (OD_{600}). A linear correlation between DCW and OD_{600} was made, $DCW = 0.3457^* OD_{600} + 0.1757$ ($R^2 = 0.9983$) [13]. The number of