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Enhanced indirubin production in recombinant *Escherichia coli* harboring a flavin-containing monooxygenase gene by cysteine supplementation

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ABSTRACT

In our previous study, a batch fermentation of recombinant *Escherichia coli* DH5α cells harboring the *fmo* gene from *Methylophaga aminisulfidivorans* MP^T produced indirubin (5.0 mg/L) and indigo (920 mg/L) in a 5 L fermenter containing tryptophan medium (2 g/L tryptophan, 5 g/L yeast extract, 10 g/L NaCl). In this study, it was found that indirubin production greatly increased when 0.36 g/L cysteine was added to the tryptophan medium, although cysteine inhibited the growth of the recombinant *E. coli* harboring the *fmo* gene. However, the addition of cysteine did not inhibit the expression level and activity of FMO in the cell. Indigo was synthesized by the dimerization of two 3-hydroxyindole molecules under the non-enzymatic reaction. Cysteine influenced the regioselectivity of FMO and enhanced the synthesis of 2-hydroxyindole instead of 3-hydroxyindole, which might function to increase indirubin production. The optimal culture conditions for indirubin production in tryptophan medium were determined from the response surface methodology analysis: 2 g/L tryptophan, 5 g/L yeast extract, 10 g/L NaCl, 0.36 g/L (3 mM) cysteine, pH 8.0 at 35 °C. Under these conditions, the recombinant *E. coli* cells were capable of producing 223.6 mg/L of indirubin from 2 g/L of tryptophan. The intracellular accumulation of the indirubin crystals might stress the cell, which may be a main reason for the poor growth of the recombinant *E. coli* pBlue 1.7.

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1. Introduction

Indigoid compounds such as indirubin and indigo have been employed as natural drugs and dyes since ancient times (Kohda et al., 1990; Xia and Zenk, 1992). Traditionally, Danggui Longhui wan, which contains indirubin, has been used for the treatment of numerous chronic diseases including chronic granulocytic leukemia (Han, 1994; Nam et al., 2005; Tang and Eisenbrand, 1992). Recently, indirubin was found to be a potent inhibitor of cyclin dependent kinases (CDKs) and glycogen synthase kinase-3β (GSK-3β), which suggests that indirubin may play an important role in the treatments of leukemia and Alzheimer's disease (Bradford, 1976; Hoessel et al., 1999; Leclerc et al., 2001).

In general, indirubin and indigo compounds are extracted from the plant cell cultures of *Indigofera tinctoria*, *Isatis tinctoria*, *Polygonum tinctorium*, and *Lonchocarpus cyanescens* (Ensley et al., 1983; Fitzhugh et al., 1997; Seldes et al., 1999). Recently, several investigations have reported that indirubin and indigo can be produced from recombinant micro organisms expressing oxygenase (Doukyu et al., 2002; Ensley et al., 1983; Madsen and Bollag, 1988; McClay et al., 2005; Murdock et al., 1993; Rui et al., 2005). Rui et al. reported that they convert 0.5 mM indole to 0.2 mM indirubin and 0.1 mM indigo in Tris–HCl buffer using *Escherichia coli* TG1cells harboring a toluene *ortho*-monooxygenase gene of *Burkholderia cepacia* G4. Our previous studies showed that recombinant *E. coli* DH5α cells harboring a flavin-containing monooxygenase (FMO) gene from *Methylophaga aminisulfidivorans* MP^T (Choi et al., 2003) produced indirubin (≤5 mg/L) and indigo (920 mg/L) in a 5 L fermenter containing tryptophan (2 g/L) medium (Han et al., 2008, 2011).

FMOs belong to a family of FAD, NADPH, and molecular oxygen-dependent enzymes. FMOs are involved in a wide range of oxidative biological processes, including drug detoxification and the biodegradation of aromatic compounds by the catalyzation of the oxygenation of many nitrogen-, sulfur-, phosphorous-, selenium-, and other nucleophilic heteroatom containing chemicals and drugs (Krueger and Williams, 2005). In addition to cytochrome P450s (CYPs), FMOs are considered to be important monooxygenase enzymes for metabolism in both prokaryotes and eukaryotes (Zhou and Shephard, 2006).

It is known that recombinant *E. coli* harboring oxygenases can produce indigoid compounds from glucose or indole. Glucose is readily available, but its metabolic pathway is so complicated that

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the efficiency of the indigoid compound production is low (Berry et al., 2002). Indole (>1–6 mM) inhibits the growth of *E. coli*, thus the indole concentration in the culture medium must be maintained at a low level to avoid toxicity (Chant and Summers, 2007). Therefore, tryptophan was used in this study as an alternative substrate for indirubin synthesis to overcome the aforementioned problems.

A modified synthetic pathway for several indigoid compounds in the recombinant *E. coli* containing FMO (AF494423) is represented in Fig. 1. In a tryptophan rich condition, extracellular tryptophan is transported into the cell by tryptophan permease and thereafter converted into indole, pyruvate, and ammonia by tryptophanase (TnaA; EC 4.1.99.1) (Newton and Snell, 1965). FMO catalyzes the hydroxylation of indole to 2-hydroxyindole and 3-hydroxyindole by using the reducing power of NADPH in the presence of oxygen (Choi et al., 2003; Eaton and Chapman, 1995; Maugard et al., 2002). In the non-enzymatic reaction, indigo is produced from the combination of two 3-hydroxyindole molecules, whereas indirubin is made from the dimerization of 2-hydroxyindole and 3-hydroxyindole (Berry et al., 2002; Cho et al., 2011; Choi et al., 2003; Eaton and Chapman, 1995; Maugard et al., 2002; McClay et al., 2005). Currently, it is thought that the disproportional production of indigo and indirubin is due to random combinations of the various hydroxyindole molecules under different conditions, but any enzyme activity in the combination reaction has not been discovered yet.

Herein we reported that the enhanced production of indirubin through the supplementation of cysteine in tryptophan medium for the growth of recombinant *E. coli* DH5α cells harboring the *fmo* gene. In addition, the effects of cysteine on the growth of *E. coli* and the FMO activity were investigated. Finally, the optimal conditions for maximum production of indirubin was analyzed using response surface methodology (RSM).

2. Materials and methods

2.1. Bacterial strains and chemicals

A restricted facultatively methylotrophic marine bacterium, *M. aminisulfidivorans* MP^T (KCTC 12909^T = JCM 14647^T), was isolated in our lab and was cultivated in a standard mineral base (SMB) medium containing 3% (w/v) sodium chloride and 1% (w/v) methanol, at 30 °C (Choi et al., 2003; Kim et al., 2007). Flavin-containing monooxygenase gene (*fmo*) was cloned from *M. aminisulfidivorans* MP^T and sequenced (GenBank No. AF494423). *E. coli* DH5α was used for the cloning and expression studies. Recombinant *E. coli* pBlue 1.7 harboring pBluescriptSKII(+) (Fermentas, Glen Burnie, MD, USA) that contains a *fmo* gene was cultivated in LB medium containing 50 μg/mL ampicillin at 30 °C. Tryptophan medium (0.2% tryptophan, 0.5% yeast extract, 1.0% sodium chloride (w/v), and 50 μg/mL ampicillin) was used for the production of indigoid derivatives. Dimethyl sulfoxide (DMSO), isatin, indole, 2-oxindole, 3-hydroxylacetate, and indigo were purchased from Sigma–Aldrich (St. Louis, MO, USA), and indirubin standard was from Biomol (Biomol Research Laboratories, Plymouth Meeting, PA, USA). All other chemicals were of analytical grade.

2.2. Cloning, expression, and purification of FMO

The *fmo* from *M. aminisulfidivorans* MP^T was subcloned into the *Nde*I and *Xho*I restriction sites of the expression vector pET30a(+). The expression of *fmo* was induced by the addition of 0.2 mM isopropyl-D-1-thiogalactopyranoside (IPTG) to the bacterial culture medium and incubation at 18 °C for 20 h. The cells were harvested (10,000 × *g*), disrupted by French Press (30,000 psi, French Pressure Cells 40k, Thermo Electron Corporation, Needham, MA, USA) and centrifuged at 12,000 × g. Then, the recombinant FMO protein in the supernatant fraction was purified through Ni-NTA affinity column chromatography (Qiagen GmbH, Hilden, Germany) and Superdex G-200 gel filtration column chromatography (GE Healthcare, Piscataway, NJ, USA). The purity of the FMO was determined by SDS-PAGE analysis, and the purified protein was concentrated to 45 mg/mL in 40 mM Tris–HCl (pH 8.0) by centrifugal ultrafiltration (100,000 Da, Vivaspin, Satourius Stedim, Germany) and stored at −20 °C for further study.

2.3. Enzyme assay and Western blot analysis of FMO

FMO activity was determined spectrophotometrically by the use of indole as substrates. The assay mixture (1 mL) contained 0.1 mM EDTA and 0.1 mM NADPH dissolved in 0.1 M Tricine/KOH buffer (pH 8.5), and an aliquot of the purified FMO solution was added to the mixture. The reaction rates of the enzyme were monitored by following NADPH oxidation at 340 nm. Protein concentrations were determined by the Bradford method (Bradford, 1976).

For Western blot analysis, harvested cells (1×10^9) were resuspended in 300 μL SDS gel-loading buffer (50 mM Tris–HCl [pH 6.8], 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, and 8 mM $MgCl_2$) and placed in boiling water for 3 min. The resulting crude cell lysate (10 μL) was loaded into 8% polyacrylamide gels and electrophoresed, and Western blotting was performed as described in the manual (Sambrook and Russell, 2001). The antibody against FMO was prepared and provided by Abfrontier Co. (South Korea).

2.4. Determination of tryptophan and cysteine concentrations

For the quantitative analyses of tryptophan and cysteine were performed using the Waters Associates PICO-TAG methods (Bidlingmeyer et al., 1984), high performance liquid chromatography (HPLC) with a Pico-tag column (4 μm, 3.9 mm × 300 mm, Waters) was performed. The HPLC pump (Waters 510, Milford, MA, USA) was used for the time-dependent mixing of the mobile phases of solvent A (140 mM sodium acetate and 6% acetonitrile) and solvent B (60% acetonitrile). The gradient program consisted of two solvent mixtures: 0–9 min, 100% solvent A and 0% solvent B; 9–9.2 min, 86% A and 14% B; 9.2–17.5 min, 80% A and 20% B; 17.5–17.7 min, 54% A and 46% B; 17.7–21.0 min, 100% B; 21.0–30.0 min, 100% A. The flow rate was 1.0 mL/min. All solvents used for the mobile phase in HPLC were filtered through a 0.45 μm cellulose membrane filter (Life Sciences, Inc., Newtown, PA, USA) and degassed in the ultrasonic bath. The peak responses from each chemical were monitored at 254 nm by a variable wavelength photodiode UV detector (Waters 2487 UV, Milford, MA, USA).

2.5. Effect of reducing agents and amino acids on indirubin synthesis

To examine the effect of the reducing agents and amino acids on indirubin biosynthesis, variable concentrations (0–5 mM) of cysteine, methionine, serine, tyrosine, phenyalanine, arginine, aspartic acid, glutathione, dithiothreitol, ascorbic acid, thioglycolic acid, and isatin were added to the tryptophan medium.

2.6. Indigoid compound analysis

The 3-hydroxyindole formation was detected by a luminescence spectrometer (excitation at 365 nm and emission at 470 nm, Perkin Elmer LS 45, Perkin Elmer, Santa Clara, CA, USA) (Woo et al., 2000). The *in vitro* assay was performed in a 100 mM Tricine–KOH buffer (pH 8.5) containing various concentrations of cysteine,

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