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Short communication

# Bio-indigo production in two different fermentation systems using recombinant *Escherichia coli* cells harboring a flavin-containing monooxygenase gene (*fmo*)

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ABSTRACT

Bio-indigo was produced in 3000-L batch and 5-L continuous fermentation of a recombinant *Escherichia coli* DH5α harboring a novel gene encoding flavin-containing monooxygenase (FMO). Batch fermentation in a 3000-L fermenter produced $911 \pm 22$ mg $L^{-1}$ of indigo from 2 g $L^{-1}$ tryptophan as a substrate (yield 46.9%) under the following culture conditions as follows: culture temperature 30 °C, pH 7.0, agitation speed 200 rpm, and aeration 3 vvm. Sufficient oxygen (aeration rate and agitation speed) was critical for bio-indigo production. For continuous fermentation in a 5-L fermenter, the volumetric productivity was found to be 11.3 mg $L^{-1}$ $h^{-1}$ up to 110 h (final accumulated bio-indigo was 23 g) with a constant dilution rate ($D$) of 0.084 $h^{-1}$ (constant feeding rate of 0.167 L $h^{-1}$ with medium containing 3 g $L^{-1}$ tryptophan). Recombinant *E. coli* cells have the ability to withstand the toxicity of high concentration of accumulated indigo in batch fermentation. In continuous fermentation, the recombinant cells exhibited high plasmid stability up to 110 h, after which they lost the plasmid.



## 1. Introduction

Indigo is one of the oldest blue dyes used by man. Microbially produced indigo (bio-indigo) can be exploited as an alternative to chemically synthesized indigo. Although several studies regarding the biological production of indigo from indole using recombinant microorganisms expressing mono- or di-oxygenase have been reported [1–6], no attempt has yet been made for the large-scale and continuous production of indigo utilizing recombinant microorganisms. In our previous studies, tryptophan was used as a substrate for bio-indigo production using recombinant *Escherichia coli* DH5α cells harboring a novel gene encoding flavin-containing monooxygenase (FMO) [7,8]. In these cells, tryptophan was converted to indole by *E. coli* tryptophanase, and indole was then oxidized to 2-hydroxyindole, 3-hydroxyindole, and isatin by FMO. The indole derivatives were then dimerized into indigo or indirubin, depending on the oxygen concentration [7,9,10].

In the present study, two systems for industrial bio-indigo production, 3000-L batch fermentation and 5-L continuous fermentation, were demonstrated, and different culture conditions for process optimization were investigated. This is the first report describing a large-scale or continuous production system for bio-indigo using recombinant *E. coli* harboring a novel *fmo* gene. Important factors, such as product toxicity, plasmid stability, medium oxygen concentration, and the volumetric productivity of bio-indigo, are also described.

## 2. Materials and methods

### 2.1. Bacterial strains, chemicals and seed culture

Recombinant *E. coli* strain DH5α, which harbors a pBlue 1.7 construct with a novel bacterial flavin-containing monooxygenase gene (*fmo*) derived from *Methylophaga aminisulfidivorans* MP$^T$ (KCTC 12909$^T$ = JCM14647$^T$), was used in this study [7,8]. All chemicals were of analytical grade and commercially available. All fermentations were performed in tryptophan medium (2 g $L^{-1}$ tryptophan, 5 g $L^{-1}$ yeast extract, and 10 g $L^{-1}$ sodium chloride), unless stated otherwise [11]. The recombinant *E. coli* cells were grown on LB agar plates containing 100 μg $mL^{-1}$ ampicillin at 37 °C overnight. A single colony was inoculated in 100 mL of tryptophan medium (seed culture) and incubated at 30 °C for 16 h. The seed culture was inoculated in a 10-L fermenter (Bioflow 3000 model, New Brunswick, NJ, USA) with a 2-L working volume, cultured and transferred to a 100-L fermenter (tank height/tank diameter = 900/500 mm, impeller diameter = 300 mm, speed range 20–300 rpm, Rushton-turbine impeller, KSB 6231 model, KoBioTech, Seoul, Korea) for the 3000-L commercial batch culture seed (tank height/tank diameter = 3780/1800 mm, speed range 20–300 rpm, top-driven type, 6-bladed disc turbine impeller) using a made-to-order fermenter (BestKorea, Seoul, Korea).

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### 2.2. Batch and repeated-batch fermentation

Large-scale batch fermentation was carried out in a 3000-L commercial reactor. The culture prepared in the 100-L bioreactor was transferred to the 3000-L bioreactor containing 2000-L of fresh tryptophan medium. The basic operation conditions were derived from the results of batch fermentation experiments, which were carried out at a 100-L scale by varying the culture conditions of temperature, pH, agitation speed (100–250 rpm), and aeration (1–3 vvm). Based on the initial results, the final batch fermentation was performed for 24 h in a 3000-L fermenter. After 24-h of fermentation, 95% of the culture fluid was drained from the reactor, and freshly sterilized tryptophan medium was supplied to continue the repeated batch fermentation.

### 2.3. Continuous fermentation

Continuous fermentation was performed in a 5-L fermenter (KF-5L, KoBioTech) with an initial 2-L volume of sterile tryptophan medium. Bio-indigo production under different fermentation conditions, such as temperature (30–37 °C), agitation speed (100–300 rpm), dilution rate ($D$), and tryptophan concentration (2–4 g $L^{-1}$), were determined during operation. Continuous culture was started at the time when the indigo concentration in the medium reached 800 mg $L^{-1}$. Fresh tryptophan medium was added to the fermenter at different dilution rates and tryptophan concentrations, and the product was drained into a separate vessel connected to the main instrument.

### 2.4. Analytical methods

#### 2.4.1. Indigo concentration

Indigo was precipitated by centrifuging the culture broth at 10,000 × $g$ for 1 min, washed at least five times with distilled water, and dried under a vacuum for 6 h. The amount and purity of bio-indigo was estimated as described previously by Han et al. [11].

#### 2.4.2. Tryptophan consumption

Fermentation broth (10 mL) was centrifuged for 10 min at 10,000 × $g$, and the supernatant was filtered through a 0.45-μm membrane filter (Millipore, France). The filtered sample was subjected to high performance liquid chromatography (HPLC) (Agilent 1200 HPLC, CA, USA) to measure its tryptophan concentration. The HPLC operation conditions were as follows: symmetry ODS $C_{18}$ column (Agilent, CA, USA); solvent mixture containing 0.1% aqueous trifluoroacetic acid and acetonitrile, 90:10 (v/v); flow rate, 1.0 mL $min^{-1}$; column temperature, 25 °C; and UV detection, 340 nm [12]. The bio-indigo production yield $Y$(%) was calculated using the following equation:

$$Y(\%) = \frac{I}{Ti - Tr} \times 100,$$

where $I$ represents the total indigo amount produced (g $L^{-1}$), and $Ti$ and $Tr$ are the initial and residual tryptophan concentrations (g $L^{-1}$) in the medium, respectively.

#### 2.4.3. Indigo toxicity

Exponentially growing *E. coli* cells in 50 mL of tryptophan medium were treated with different concentrations of purified indigo (0.5–2.0 g $L^{-1}$) and incubated at 37 °C. The survival rate of the recombinant *E. coli* strain was immediately evaluated by plating the samples onto LB agar plates and counting the colony-forming units (CFUs).

#### 2.4.4. Plasmid stability

The stability of the pBlue 1.7 construct in the recombinant *E. coli* DH5α cells during fermentation was determined by the method of Friehs et al. [13,14]. After dilution, the cells were spread on nonselective (ampicillin-negative) and selective (ampicillin-positive) LB agar plates, and incubated at 37 °C for 14–16 h. The percentage of plasmid containing cells, P(+), was estimated by calculating the number of colonies formed on the selective plate divided by the number of colonies formed on the nonselective plate and multiplying by 100.

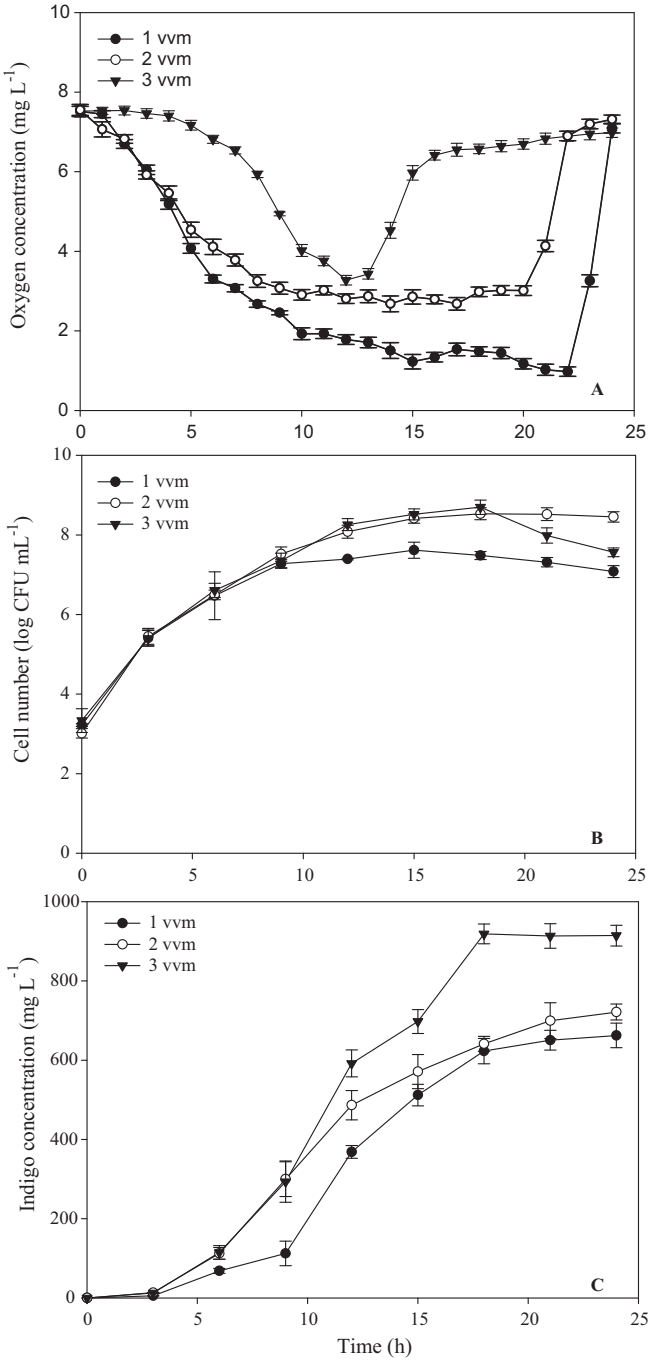


**Fig. 1.** The effect of aeration rate on the profiles of dissolved oxygen, cell growth and bio-indigo production. Profiles of oxygen concentration (A), cell number (B) and indigo concentration (C) under different air supply conditions. Symbols: (●) 1 vvm, (○) 2 vvm, and (▼) 3 vvm.

## 3. Results and discussion

### 3.1. Effect of aeration and agitation rate on the profiles of dissolved oxygen, cell growth and indigo production

As described in Section 1, oxygen concentration in the medium is an important factor for bio-indigo production [7,9,10]. Fig. 1A and B show the growth-dependent dissolved oxygen concentrations in the batch condition. To study the effect of aeration rate on cell growth and indigo production, three different amounts of air were supplied to the 100-L fermenter. When 1 vvm of air was supplied to the culture medium, the oxygen concentration decreased significantly and was kept at a low level, based on cell growth. When 2 vvm of air was supplied, the dissolved oxygen also decreased significantly but stayed at a higher level than that of 1 vvm. Thus a higher cell number and more indigo could be obtained. However, when 3 vvm of air was fed into the medium, the oxygen concentration decreased sharply until 12 h of cultivation and then recovered to a saturated level thereafter. The fact that the total viable cell number was decreased after 18 h of cultivation indicates that the energy source was depleted in the medium. However, a maximum of 910 mg $L^{-1}$ of bio-indigo was obtained under this condition (Fig. 1B and C).

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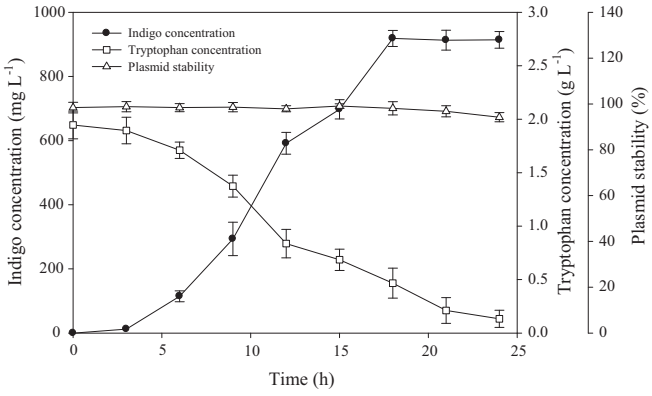


**Fig. 2.** The bio-indigo production profile during batch fermentation in a 100-L fermenter. Symbols: (●) indigo concentration, (□) tryptophan concentration, and (□) plasmid stability.

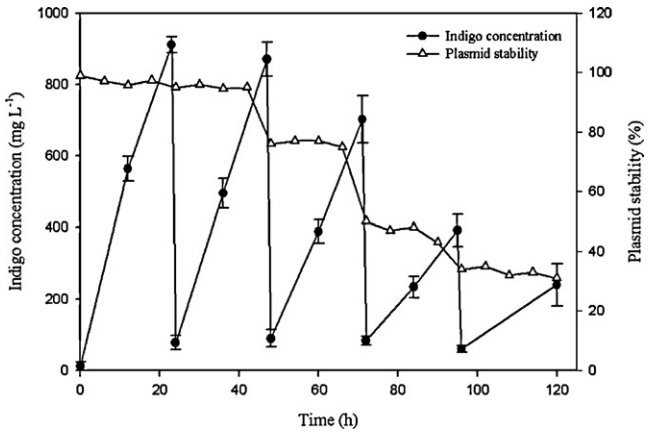


**Fig. 3.** Repeated batch fermentation for bio-indigo production in a 3000-L reactor. Symbols: (●) indigo concentration, (□) plasmid stability, and (↓) the time that fresh medium was fed into the reactor for the next batch fermentation.

The effects of both aeration and agitation rates on indigo production were also investigated. As the rates increased, indigo production increased as follows: $640 \pm 40\ mg\ L^{-1}$ at 100 rpm and 1 vvm, $870 \pm 50\ mg\ L^{-1}$ at 150 rpm and 2 vvm, and $910 \pm 50\ mg\ L^{-1}$ at 200 rpm and 3 vvm (data not shown). These results imply that oxygen was used to not only grow cells but also supply enough oxygen for the conversion of indole to indigo [9,10]. Therefore, 200 rpm and 3 vvm of air were supplied to the culture medium in all subsequent fermentation procedures.

A 100-L fermentation was performed with a 70-L working volume of tryptophan medium at 30 °C, pH 7.0, an agitation speed of 200 rpm, and an aeration rate of 3 vvm. As shown in Fig. 2, bio-indigo production increased as the tryptophan concentration in the medium decreased. Although a slight decrease in the tryptophan consumption rate was observed at around 12 h of cultivation, and matched the oxygen concentration in medium, maximum bio-indigo production ($912 \pm 50\ mg\ L^{-1}$) and cell number were obtained after 18 h incubation. Afterwards, no further increases in bio-indigo were observed and the number of viable cell significantly decreased, probably due to the high concentration of bio-indigo in the medium.

### 3.2. 3000-L batch and repeated-batch fermentation

A pilot-scale batch fermentation was carried out in a 3000-L commercial fermenter with a working volume of 2000-L. At this volume, under the conditions described above, $911 \pm 22\ mg\ L^{-1}$ of bio-indigo was produced during the initial 24 h of fermentation (Fig. 3). Repeated batch fermentations (5 fermentations, a total of 120 h) were also carried out in a 3000-L fermenter to increase the volumetric productivity per fermenter (Fig. 3). After each 24-h cultivation, 95% of the culture fluid was drained from the fermenter, and the same amount of fresh tryptophan medium was added to the reactor to repeat cultivation. Interestingly, the outputs of second and third batches decreased to $870 \pm 48$ and $702 \pm 66\ mg\ L^{-1}$, respectively, and the fifth fermentation only produced $240 \pm 60\ mg\ L^{-1}$. Given the operational stability of bio-indigo production, the repeated batch fermentations should be carried out in fewer than three runs. Determination of the bio-indigo production yield $Y(\%)$ was carried out in a 3000-L batch culture. 97% of the initial tryptophan (4 kg) was consumed within 24 h, and 1.82 kg of bio-indigo was produced (Fig. 3). Therefore, the production yield was estimated to be 46.9%. Berry et al. produced $23.0\ g\ L^{-1}$ of bio-indigo from $200\ g\ L^{-1}$ of glucose using the naphthalene dioxygenase system [15]. In this case, the volumetric productivity could not be calculated because the experiment was done in fed-batch mode.

### 3.3. Continuous fermentation

The dilution rate ($D$) and bio-indigo concentration were important factors for bio-indigo accumulation, where accumulation of indigo at more than $900\ mg\ L^{-1}$ inhibited cell growth. When the concentration of bio-indigo reached $800\ mg\ L^{-1}$ in batch mode, 4-L of fresh medium containing different concentrations of tryptophan (2, 3 or $4\ g\ L^{-1}$) was added to the fermenter for 24 h at a constant feeding rate of $0.167\ L\ h^{-1}$. The initial operation conditions were as follows: temperature 30 °C, pH 7.0, aeration rate 3 vvm, agitation speed 200 rpm, and dilution rate $0.084\ h^{-1}$. However, when the indigo concentration in the culture medium reached $800\ mg\ L^{-1}$, the operating temperature was changed to 35 °C to increase productivity. Under these conditions, a maximum of $937.4 \pm 24\ mg\ L^{-1}$ bio-indigo was produced in 24 h of fermentation, and the final accumulated bio-indigo and volumetric productivity were 23 g and $11.3\ mg\ L^{-1}\ h^{-1}$, respectively, after 110 h of fermentation (Fig. 4). To the best of our knowledge, these are among the highest values ever reported (Table 1).

No significant mortality was observed after 5–60 min of incubation in the medium containing 0.5–$1.0\ g\ L^{-1}$ of previously purified indigo. In the presence of $1.5\ g\ L^{-1}$ bio-indigo in the medium, cell growth was inhibited as a function of incubation time (e.g., approximately 80% growth inhibition was observed after 60 min of incubation). When the cells were incubated with $2.0\ g\ L^{-1}$ of bio-indigo, only 15% of the initial cells survived after 40 min, and 100% mortality was observed after 60 min incubation (data not shown).

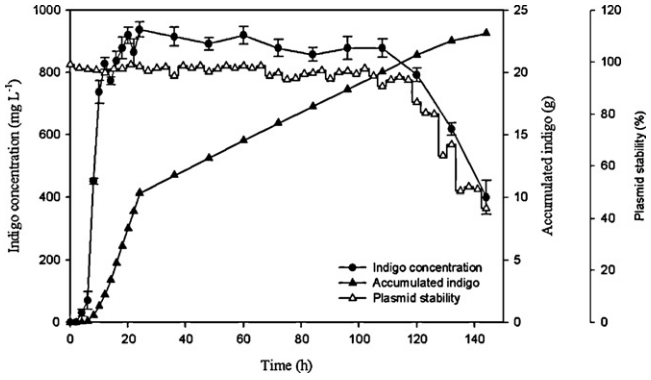


**Fig. 4.** Bio-indigo production by continuous fermentation in a 5-L fermenter. When the bio-indigo concentration reached $800\ mg\ L^{-1}$, $2.8\ mL\ min^{-1}$ of fresh medium containing $3\ g\ L^{-1}$ tryptophan was added. Symbols: (●) indigo concentration, (▲) total indigo accumulation over 140 h, and (□) plasmid stability of recombinant *E. coli* DH5α.

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**Table 1**
Summary of bio-indigo production from different production systems.

| No. | Enzyme/host strain | Substrate | Indigo concentration (scale/type) | Reference |
|---|---|---|---|---|
| 1 | Naphthalene dioxygenase/*E. coli* | Tryptophan | 25 mg $L^{-1}$ (500 mL/batch) | [3] |
| 2 | Naphthalene dioxygenase/*E. coli* | Glucose | 135 mg $L^{-1}$ (500 mL/batch) | [2] |
| 3 | Styrene monooxygenase/*P. putida* S12 | Styrene/indole | 17 mg $L^{-1}$ (500 mL/batch) | [16] |
| 4 | Unknown/*Acinetobacter* sp. ST-550 | Indole | 292 mg $L^{-1}$ (500 mL/batch) | [4] |
| 5 | Naphthalene dioxygenase/*E. coli* | Glucose/tryptophan | 23.0 g $L^{-1}$ (6 l/fed-batch) | [15] |
| 6 | Toluene ortho-monooxygenase/*E. coli* | Indole | 69.9 mg $L^{-1}$ (500 mL/batch) | [5] |
| 7 | Cytochrome P450 BM3 and glucose dehydrogenase/*E. coli* | Indole | 759.8 mg $L^{-1}$ (500 mL/batch) | [17] |
| 8 | Flavin-containing monooxygenase/*E. coli* | Tryptophan | 911 ± 22 mg $L^{-1}$ (3000 L/batch) 23 g (5 L/continuous, productivity was 11.3 mg $L^{-1}$ $h^{-1}$) | This study |

### 3.4. Plasmid stability

In the batch culture, the percentage of plasmid-containing P(+) cells was counted every 3 h. As shown in Fig. 2, a slight reduction in the P(+) cell percentage was observed after 24 h in a 100-L fermenter. However, a dramatic decrease in the P(+) cell percentage was observed after repeated batch fermentation in a 3000-L fermenter (Fig. 3). Although plasmid stability was kept constant at 95% until 42 h of cultivation, plasmid stability decreased significantly each time the culture medium was replaced with fresh medium. A sudden reduction in the P(+) cell percentage was observed between 18 and 24 h after each medium replacement and start of cultivation, which is the time when the maximum amount of indigo accumulated in the medium. In the continuous culture, plasmid stability was maintained up to 110 h, and bio-indigo was continuously produced (Fig. 4). As the number of P(+) cells decreased after 110 h of incubation, bio-indigo production also decreased significantly. The reason why plasmid stability decreased seems to be that the cells without the plasmid have a growth advantage, while the cells with the plasmid grow poorly due to the indigo inside the cell. The plasmid stability data gave us several insights regarding how to best choose the appropriate fermentation mode for bio-indigo production. For bio-indigo productivity, we recommend a continuous culture system, because this system exhibited a high level of indigo productivity and long-term plasmid stability.

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