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Production of natural indirubin from indican using non-recombinant *Escherichia coli*

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ABSTRACT

Indirubin is an important natural substance and has positive effects on various diseases. However, the current process of producing indirubin is inefficient, making it difficult to produce indirubin of high purity; thus, it is commercially unavailable. In this study, a method of indirubin using non-recombinant *Escherichia coli* as a whole cell enzyme with indican as a substrate was developed. After confirming that indirubin was produced from indican by non-recombinant *E. coli* under general conditions, attempts to compare the yield and purity of indirubin were conducted under various pH, temperature and culturing media conditions. Under the optimum conditions, the yield was reliably determined to be about 25–35%, and it was further increased (1.8–2.1 fold) by replenishing the catalyst with freshly prepared whole cells. Since the established method was simple and reproducible, high purity indirubin would expected to be produced efficiently through improvement of whole cell enzymes and development of scale-up processes.

1. Introduction

Indirubin, a red-colored indigoid compound, is found in the traditional Chinese curative medicine, danggui longhui wan. This compound is currently known to be pharmacologically active against various diseases, including leukemia, inflammation, psoriasis and skin rashes (Hoessel et al., 1999). Studies of cell physiology have reported that indirubin or its derivatives could intervene in the CDK and Stat3 signaling pathway, thereby suppressing the differentiation of cancerous cells and then inducing apoptosis (Leclerc et al., 2001; Nam et al., 2005). Accordingly, studies are under way to produce a variety of derivatives as anticancer agents (Marko et al., 2001). Owing to its high therapeutic potential, various attempts have been made to produce indirubin from a typical substrate, indole, using recombinant cells transformed with tryptophanase and/or oxygenase (Han et al., 2008; Lim et al., 2005; Singh et al., 2010; Rui et al., 2005; McClay et al., 2005; Guengerich et al., 2004; Hart et al., 1992). This is a straightforward method that occurs via the activity of two enzymes linked to the synthetic pathway of tryptophan in a host. However, it was well known that only a small amount of indirubin is unevenly produced, and that this property was mainly linked with the substrate specificity, position of indole oxidation and dimerizing dependence on oxygen. Accordingly, a production route of indirubin with reproducibility and high purity has not been established to date. Under such circumstances, it is difficult to apply this process to a practical scale because of the low yield, use of antibiotics as a selection marker, high cost of fermentation (inducer, tryptophan or indole) and difficulty associated with indirubin separation (Lim et al., 2005).

Unlike processes supplemented with indole or tryptophan as a substrate, a natural fermentation process has used indican as a substrate, which is abundant in the plant *Polygonum tinctorium* (Gilbert et al., 2004). In this process, indican is converted into indoxyl by the activity of β-glucosidase, after which indigo is generated by spontaneous oxidation of indoxyl (Fig. 1). A small amount of indirubin could also be produced as the end product of the oxidation between indoxyl and isatin (a derivative generated by an oxygenase) in this process (Maugard et al., 2001). Although these enzymes are known to originate from the plant *P. tinctorium*, it is still possible that these products could be partly produced by resident microbes found frequently in the solution produced during natural fermentation. It was recently reported that a variety of microbes were found and involved in the reduction of indigo during natural fermentation (Aino et al., 2010). Furthermore, it has also been reported that various microbes used indican as a sole carbon source (Kim et al., 2009). Accordingly, microbes capable of producing indirubin are expected to be discovered by screening the solution of natural fermentation or the strain pool used indican as a sole carbon source. This possibility has partially been supported by fact that the relative ratio of indirubin to indigo was found to differ significantly depending on the fermentation conditions.

In this study, we attempted to screen microorganism capable of producing indirubin from the strain pools of natural fermentation and with indican-hydrolyzing activity. During the screening

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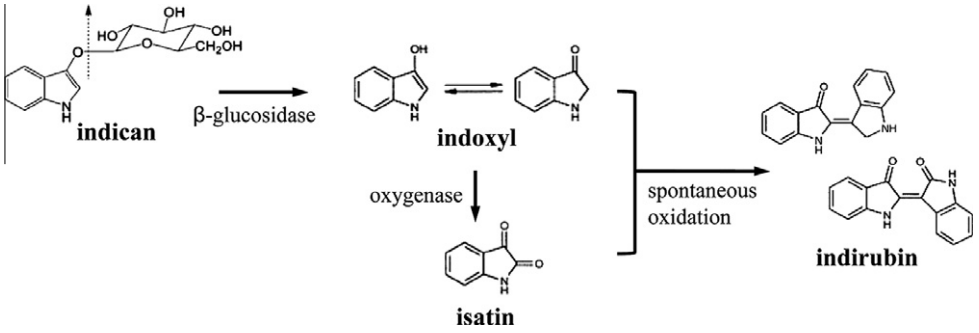

Fig. 1. The reaction pathway of bioconversion from indican to indirubin during the natural fermentation of indigo.

procedure, we found that non-recombinant *Escherichia coli* was capable of producing indirubin from indican without any additives under normal conditions. Accordingly, the results presented here strongly suggested that various species of non-recombinant *E. coli* could be used as whole cell enzymes to produce indirubin reliably.

2. Methods

2.1. Chemicals and reagents

Indican was purchased from TCI (Japan). Synthetic indirubin and indigo as standards were purchased from Alexis Biochemicals (USA) and Sigma (USA), respectively. Thin layer chromatography, potassium phosphate, sodium bicarbonate, Trizma-base and Triton X-100 were purchased from Sigma (USA). All other chemicals and solvents used were of analytical grade.

2.2. Bacterial strains and culture conditions

Unless otherwise stated, all reactions were conducted using *E. coli* XL1-Blue as a whole cell enzyme. All *E. coli* strains used in this study are listed in Table 1. *Salmonella typhimurium* 14028S was also used for comparative analysis of indirubin production. All strains were grown in typical LB medium containing 1% tryptone, 0.5% yeast extract and 1% NaCl. Each bacterial strain was primarily streaked onto LB agar plates. Well isolated single colonies were seeded in LB liquid medium and then incubated at 37 °C for 6–8 h with constant shaking (200 rpm). Subsequently, 2–4% pre-culture was reseeded and further grown in the same medium under identical conditions. To compare the effects of culture medium on the yield of indirubin production, typical media (SOB, SOC, 2xYT, TB, M9) for *E. coli* were also used (Sambrook and Russell, 2001).

2.3. Optimization of reaction conditions for whole cell enzyme

Bacterial cells were harvested by centrifugation (4000 rpm for 10 min) when the optical density at 600 nm reached 2.5–2.75. The harvested cells were then washed with DDW and used as whole cell enzymes. The buffers (50 mM) used for whole cell reaction were prepared as follows: potassium phosphate monobasic/dibasic for a range of pH 5.5–6.5, Tris–HCl for pH 7.0–9.0 and sodium bicarbonate for pH 9.5–10.0. Each buffer was mixed with 1 mM indican and 1% Triton X-100 and then used as a reaction solution. To determine the optimum pH, harvested cells (1 mg, DCW) were resuspended in each reaction solution (500 μl) and then incubated at 37 °C for 3 days with constant shaking (200 rpm). Experiments to determine the optimum temperature were conducted in a reaction buffer (50 mM Tris–HCl, pH 8.0) at varying temperatures (22–47 °C) under the same conditions as described above.

The effects of culture media on indirubin production were analyzed by using each strain cultivated in a typical medium (LB, SOB, SOC, 2xYT, TB and M9) under the same conditions as described for the reaction used to determine the optimum temperature.

2.4. Recovery and identification of indirubin

After reaction with whole cell enzymes, indigo and indirubin were extracted with 500 μl of ethyl acetate and then dried at 100 °C for 1 h 30 min (Lim et al., 2005). The resulting powder was subsequently dissolved in DMSO (150 μl) and then subjected to analysis by thin layer chromatography (TLC) using synthetic indigo and indirubin as standards. The developing solvents used were toluene:acetone:chloroform (2:1:1). The absorbance of the extract at 620 and 552 nm was also measured using a spectrophotometer (UV-1700, Shimadzu) to determine the quantity of indigo and indirubin, respectively. High performance liquid

Table 1
The strains of *E. coli* used in this work.

Strain	Name	Genotype
K12	K12[a]	*Wild type*
	XL1-Blue	*recA1 endA1 gyrA46 thi-1 hsdR17 supE44 relA1 lac F'[proAB laclqZM15 Tn10(Tetr)c]*
	DH5αF'	*F'/endA1 hsdR17(rK-mK+) glnV44 thi-1 recA gyrA(Nalr) relA Δ(lacIZYA-argF)U169 deoR (φ80dlac Δ(lacZ)M15)*
	HB101	*F- Δ(gpt-proA)62 leuB6 glnV44 ara-14 galK lacY1 Δ(mcrC-mrr) rpsL20 (Strr) xyl-5 mtl-1 recA13*
	JM109	*(F'traD36proA + B + laclqΔ(lacZ)M15/Δ(lac-proAB) glnV44 e14- gyrA96(Nalr) recA1 relA1 endA1 thi hsdR17(rK-mK+)*
	SURE	*F' proA + B + laclq Δ(lacZ)M15 Tn10(Tetr)/e14-(McrA-) Δ(mcrCB-hsdSMR-mrr)171 endA1 glnV44(supE44) thi-1 gyrA96(Nalr) relA1 lac recB recJ sbcC umuC::Tn5(Kanr) uvrC*
	TOP10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-*
	CY15000[b]	*F- λ- IN(rrnD-rrnE)1 rph-1 tnaA5*
B	BL21	*F- ompT gal [dcm] [lon] hsdSB (rB-mB-; an E. coli B strain)*
C	C[c]	*Wild type*

[a] CGSC 6300.
[b] CGSC 7682.
[c] KCTC 2571.