**Abstract**

Indigo is a natural dye that is widely used in the textile industry worldwide. However, the synthesis of indigo using toxic compounds such as aniline, formaldehyde, and hydrogen cyanide leads to environmental pollution and poses a threat to workers' health. To overcome these challenges, bio-indigo or indigo biosynthesis has been proposed to replace indigo synthesized from aniline. Among the various biosynthesis methods, Flavin-containing Monooxygenases (FMOs) have been found to achieve the highest yield (1700 mg/L) of bio-indigo. However, the commercialization of indigo biosynthesis still faces several challenges. This review explores the history of indigo biosynthesis by FMOs and identifies the expensive substrate L-tryptophan, unsuitable chassis Escherichia coli, and relatively low yield and high cost compared with the chemical method as the main reasons. Additionally, this paper summarizes the strategies to improve the yield and applications of indigo synthesized by FMOs.

**Keywords:** Flavin-containing Monooxygenase, Indigo

**Introduction**

Indigo, a natural dye commonly extracted from plants, has a history of over 6,000 years of traditional extraction techniques. Indigo is widely used in the textile industry for dyeing jeans and other fabrics. However, the production of around 80,000 tonnes of indigo each year involves the use of non-renewable petrochemicals and results in the generation of toxic compounds (Linke, J.A. et al., 2023). To address these issues, bio-indigo, which involves the use of synthetic biology to synthesize indigo from tryptophan or indole, has been proposed as a sustainable and eco-friendly alternative. Although several countries, such as China, America, and France, have companies that are making efforts to commercialize bio-indigo, the process has been hindered by low yields. FMOs, which have been shown to possess the ability to synthesize bio-indigo, are regarded as promising enzymes to overcome this limitation.

Flavin-containing monooxygenase (FMO, EC 1.14.13.8), a type of class B flavoprotein monooxygenases (FPMOs), is encoded by a single gene and contains a tightly bound FAD cofactor. FMO retains the coenzyme NADP+ bound during catalysis and is composed of two dinucleotide binding domains (Rossmann fold) that bind FAD and NADPH, respectively. Ongoing studies have described the complete catalytic model of FMOs (Ziegler, 1993) (Fig. 4E). The procedure involves five steps, including (1) FAD reduction to FADH2 by NADPH and an additional H+, (2) interaction of FADH2 with O2 to form a stable C4a-(hydro)peroxide intermediate, (3) single oxidation of the closing substrates without particular binding, (4) discharge of the residuary oxygen atom as H2O, and (5) the whole cycle returns to the initial state when NADP+ is separated from FMO. Since substrates do not need to provide reducing potential and specifically bind to FMO, they are extensive. Theoretically, any nucleophilic compound that could get close to FAD-OOH can become a substrate of FMO. However, molecular size and charges affect the selectivity of FMO. Substrates with one positive charge have the highest affinity to FMO (Ziegler, 2002).

Other plant dyes like indirubin, an indole alkaloid used as a drug in chronic myeloid leukemia, has shown anticancer effects (Yang et al., 2022), and traditional production methods rely on plant cell culture extraction (Han et al., 2012). Biosynthetic processes are needed to improve production efficiency and conditions (Berry et al., 2002; Choi et al., 2003; Cho et al., 2011).

**1. The Research History of Indigo Biosynthesis by FMOs**

Bacterial FMO, also known as trimethylamine monooxygenase (TMM), besides oxidizing many compounds containing nitrogen and sulfur, is also able to oxidize indole, (TMA), dimethylsulfate (DMS), and dimethylsulfoxide (DMSO) (Chen et al., 2011; Choi et al., 2003).

The first bacterial FMO, Methylophaga sp. strain SK1 (mFMO) (Choi et al. 2003), was used in engineered Escherichia coli to produce indigo. Further research suggested that it also catalyzes indole derivatives into indigoid dyes (Rioz-Martinez et al., 2011). It is important to note the mechanism of FMOs in the synthesis of indigo. In bacteria, tryptophan is converted to indole by tryptophanase (TRP), and FMOs subsequently oxidize indole to 2-hydroxyindole or 3-hydroxyindole, which are oxidized to isatin by oxygen. Isatins then dimerize into indigo or indirubin (Fig. 5C).

The earliest record of indigo biosynthesis arises from the study of naphthalene dioxygenases (Ensley et al., 1983). In 1989, the pBS959 plasmid was constructed based on the naphthalene dioxygenase gene and pUC19 plasmid, which realized the synthesis of indigo in recombinant E. coli (Boronin et al., 1989). In 1993, a recombinant E. coli capable of producing indigo from glucose was also developed (Murdock et al., 1993). However, the yield was only 135 mg/L. The indigo synthesis pathways of other aromatic hydrocarbon-degrading bacteria were also identified. In 1997, two indigo-synthesizing strains, Pseudomonas putida S12 and CA-3, were identified (O'Connor et al., 1997). The toluene dioxygenases from P. putida NCIB11767 (Stephens et al., 1989) and P. putida F1 (Woo et al., 2000) both demonstrated the ability to synthesize indigo. However, the ability to synthesize indigo of naphthalene dioxygenases and toluene dioxygenase is limited, and their production capacity fails to exceed 300 mg/L. Other classes of oxidases, such as toluene-4-monooxygenase from P. mendocina and functional enzymes similar to Multicomponent phenol hydroxylases in Acinetobacter sp. ST-550 (Doukyu et al., 2002; Doukyu et al., 2003) can also synthesize indigo, but their yield are inferior to mFMO, which has been widely studied for indigo biosynthesis. Choi et al. were the first to report synthesizing indigo by mFMO effectively in 2003 (Choi et al., 2003). Subsequent research on mFMO-catalyzed indigo biosynthesis was based on this study. With further optimization of the sequence and fermentation process (Han et al., 2008), Han et al. reported an indigo yield of 911 mg/L in recombinant E. coli (Han et al., 2011). By adjusting cysteine concentration in the tryptophan medium, the production of indigo was increased. In later studies, it was found that cysteine also had a significant impact on the catalytic selectivity of mFMO. During the oxidation catalytic process, the concentration of cysteine increased, and the reaction tended towards the indirubin synthesis (Kim et al., 2019), which is of great significance to the synthesis of indirubin by mFMO. Numerous indigo-producing bacterial FMOs were also reported (Ameria et al., 2015; Lončar et al., 2019a). However, their yields were not comparable to mFMO. To overcome the limitations of indigo and indirubin biosynthesis by FMOs, various methods were employed, including enzyme engineering and fermentation engineering (Chen et al., 2021; Hsu et al., 2018; Lončar et al., 2019b; Sun et al., 2022).

**2. The Substrates of Indigo Biosynthesis by FMOs**

**3. The Difference between Synthetic Technologies**