**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 naturally occurring dye derived from botanical sources, boasts a historical legacy of more than 6,000 years, with extraction methods deeply rooted in tradition. Its prominent application in the textile industry, notably in the dyeing of jeans and various fabrics, underscores its industrial significance. However, the prevailing manufacturing paradigm, yielding approximately 80,000 tonnes of indigo annually, relies extensively on non-renewable petrochemicals. This conventional approach concurrently precipitates the formation of deleterious compounds (Stasiak et al. (2014) and Linke, J.A. et al. (2023)). In response to the environmental ramifications intrinsic to such processes, the conceptualization of bio-indigo has emerged as a prospective avenue. This innovative paradigm employs synthetic biology methodologies to effectuate the synthesis of indigo from tryptophan or indole, presenting a promising trajectory towards a sustainable and environmentally benign indigo production methodology.

While numerous countries, including China, the United States, and France, host companies actively engaged in the commercialization of bio-indigo, progress has been impeded by suboptimal yields. Addressing this challenge, flavin-containing monooxygenases (FMOs) have emerged as focal points of investigation, given their demonstrated capability to synthesize bio-indigo. Notably, FMOs have become the subject of extensive research over the past decades, positioning them as one of the pivotal enzymatic entities in endeavors to overcome the limitations associated with bio-indigo production.

Flavin-containing monooxygenase (FMO, EC 1.14.13.8), classified as a member of the class B flavoprotein monooxygenases (FPMOs), is encoded by a singular gene and features a tightly bound flavin adenine dinucleotide (FAD) cofactor. Recent investigations have brought to light a comprehensive indigo-catalytic model of FMOs, as detailed by Ziegler in 1993 (Fig. 4E). The catalytic process unfolds in five discrete stages: (1) the NADPH-driven reduction of FAD to FADH2, accompanied by an additional H+; (2) the interaction of FADH2 with O2, yielding a stable C4a-(hydro)peroxide intermediate; (3) the substrate undergoes unassisted oxidation without specific binding; (4) release of the residual oxygen atom as H2O; and (5) the entire cycle resets to the initial state upon the dissociation of NADP+ from FMO.

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

In the quest for indigo biosynthesis, exploration into microbial synthesis pathways has been proposed. Engineered *Escherichia coli* (*E. coli*) has notably demonstrated its efficacy as a viable platform for indigo biosynthesis, as evidenced by seminal studies (Ensley et al., 1983; Murdock et al., 1993; Berry et al., 2002; Doukyu et al., 2003). Throughout the research discussed in this chapter, *E. coli* predominantly serves as the chosen chassis for investigating indigo synthesis pathways.

Following the oxidation of indole to indoxyl, the subsequent spontaneous dimerization of two indoxyl molecules, accompanied by a reaction with oxygen, results in the formation of indigo. Consequently, a pivotal aspect in the biosynthesis of indigo revolves around the judicious selection of an appropriate oxidase to catalyze the conversion of indole to indoxyl. Therefore, this review will explore the history of indigo biosynthesis in the order of the utilization of different oxidases in this transformative process.

**1.1 Early Research on Indigo Biosynthesis**

The earliest record of indigo biosynthesis arises from the study of naphthalene dioxygenases (NDO) form *Pseudomonas putida* PpG7. In 1983, Ensley pioneered the construction of the initial synthetic indigo-producing Escherichia coli (E. coli) strain by introducing NDO, yielding an indigo production of 25 mg/L. Notably, native E. coli tryptophanase (TRP/TnaA) was utilized to convert L-tryptophan to indole, subsequently catalyzed by NDO to form indigo (Ensley et al., 1983). Building upon this foundation, Ensley and colleagues further explored NDO expression by employing various plasmids and inducers in 1988, providing crucial insights for subsequent investigations. The construction of the pBS959 plasmid in 1989, based on the nahA gene (NDO expressing gene) and pUC19 vector, marked a milestone in realizing indigo synthesis within recombinant E. coli (Boronin et al., 1989). However, the initial expression levels of indigo in recombinant *E. coli* was much lower compared to *Pseudomonas putida*.

In 1993, Murdock et al. enhanced indigo production by incorporating a lac promoter and operator into the plasmid, approaching expression levels comparable to *Pseudomonas putida*, resulting in a yield of 135 mg/L—a notable 5.4-fold increase. The engineered E. coli strain also demonstrated the capacity to produce indigo from glucose by inactivating the trpB gene. Nevertheless, a feedback effect was identified during the formation of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP), indicating the downstream product indigo could inhibit DAHP formation. To overcome this challenge, Berry et al. in 2002 achieved a 60% increase in indigo production by engineering a feedback-resistant DAHP synthase (Berry et al., 2002).

Research extended to explore NDO from different species. In 2010, NDO from *Pseudomonas sp.* HOB1 demonstrated the capability to transform 0.3 mM indole in 8 hours, yielding an impressive indigo production of 246 mg/L (Pathak and Madamwar, 2010). Subsequently, Qu et al. conducted a study in 2012 utilizing an indigo-producing strain named *Comamonas sp.* MQ, which, with the consumption of 0.05 g/L indole and 0.2 g/L naphthalene, produced 32.2 mg/L of indigo (Qu et al., 2012b).

Another extensively explored oxidase for indigo synthesis is toluene dioxygenases. In 1989, Stephens et al. integrated a toluene dioxygenase from *Pseudomonas putida* NCIB 11767 into E. coli HB101 using pIG, achieving the synthesis of approximately 60 mg/L of indigo through the supplementation of 5 mM tryptophan (Stephens et al., 1989).

Some recombinant *E. coli* systems for indigo synthesis have been constructed based on multicomponent phenol hydroxylases. From *Acinetobacter sp.* ST-550, Doukyu et al. identified a functional enzyme akin to multicomponent phenol hydroxylases, demonstrating the capacity for indigo synthesis. In 2002, they assessed the performance of *Acinetobacter sp.* ST-550 cultured with 10% diphenylmethane and 0.9 mg/ml indole, resulting in an indigo production of 292 mg/L (Doukyu et al., 2002). Subsequently, in 2003, they engineered the recombinant *E. coli* strain OST3410 with this functional enzyme, yielding a production of 52.1±1.6 mg/L of indigo under the conditions of 10% diphenylmethane and 0.9 mg/ml indole (Doukyu et al., 2003). In 2012, Qu et al. identified a multicomponent phenol hydroxylase from *Arthrobacter sp.* W1 (PHw1), including the measurement of its kinetic parameters to indole (Qu et al., 2012a).

However, it is crucial to note that the practical applications of multicomponent enzymes are constrained by their fermentation conditions. The yield of indigo synthesized by these enzymes has not surpassed 300 mg/L due to the requisite stability of each component under specific conditions, rendering flavin-containing monooxygenases (FMOs) with simpler components more operationally practical.

Moreover, evidence suggests that class D flavoprotein monooxygenases, closely related to FMOs, are capable of synthesizing indigo. Notably, an indole oxygenase (indAB) identified in *Cupriavidus sp.* SHE has demonstrated the ability to synthesize indigo, achieving a production level of 307 mg/L with the provision of 1.0 g/L tryptophan (Dai et al., 2019). However, further research is needed to elucidate the underlying biosynthetic mechanisms at play in this process.

**1.2 Indigo Biosynthesis by Bacterial FMO**

Researchers aspire to enhance bio-yield and simplify production conditions, with current emphasis on Flavin-containing monooxygenase (FMO) standing out prominently in these endeavors. Bacterial FMO, also referred to as trimethylamine monooxygenase (TMM), exhibits a versatile substrate profile, encompassing the oxidation of various nitrogen and sulfur-containing compounds. Notably, it can catalyze the oxidation of indole, trimethylamine (TMA), dimethylsulfate (DMS), and dimethylsulfoxide (DMSO) (Chen et al., 2011; Choi et al., 2003).

Of great significance is understanding the mechanism by which FMOs contribute to indigo synthesis. In bacterial systems, tryptophan is initially converted to indole by tryptophanase (TRP), and subsequently, FMOs oxidize indole to form either 2-hydroxyindole or 3-hydroxyindole. These intermediates are further oxidized to indoxyl by oxygen. The indoxyls subsequently undergo dimerization, yielding either indigo or indirubin (Fig. 5C). This intricate process underscores the pivotal role of FMOs in the biosynthesis of indigo.

**1.2.1 Bio-indigo Synthesis with mFMO**

The first bacterial FMO employed in engineered *Escherichia coli* for indigo production was *Methylophaga aminisulfidivorans* MPT (mFMO/bFMO). This enzyme was introduced into *E. coli* DH5α via the pBlue 2.0 vector. Following a 12-hour fermentation period supplemented with 2.00 g/L tryptophan, the recombinant E. coli successfully generated 160 mg/L of indigo (Choi et al., 2003). The kinetic parameters of mFMO to indole were measured by Alfieri et al. in 2008 (Alfieri et al., 2008)

Nevertheless, the productivity of the original plasmid harboring mFMO exhibited limitations, prompting the implementation of optimization strategies. Through the removal of redundant sequences, the enhanced plasmid demonstrated a significant improvement in indigo yield, reaching 662 mg/L—a remarkable increase of 413% compared to the original production level of 160 mg/L (Han et al., 2008). Subsequently, through culture optimization with the method of response surface methodology (RSM), a notable milestone was achieved with a yield of 920 mg/L in a 5L fermenter. Further advancements led to the development of a larger-scale fermentation system, culminating in an impressive yield of 911 ± 22 mg/L of indigo in a 3,000 L/batch setup, with an input of 2 g/L of tryptophan (Han et al., 2011). Han et al. also reported a continuous fermentation conducted in a 5L system supplied with 3 g/L tryptophan, in which 23 g of indigo was produced for 110 hours, at a rate of 11.3 mg/L/h (Han et al., 2011).

The redox reaction of the tightly linked flavin adenine dinucleotide (FAD) cofactor, intrinsic to flavin-containing monooxygenases (FMO), is contingent upon the assistance of nicotinamide adenine dinucleotide phosphate (NADPH) (Alfieri et al., 2008; Eswaramoorthy et al., 2006; Krueger and Williams, 2005). However, in extracellular enzyme reaction systems or recombinant cells, the rate of NADPH regeneration may constrain the overall reaction rate. To address this, an optimized strategy involved the fusion of mFMO and phosphate dehydrogenase (PTDH), exploiting phosphate as a cost-effective and sacrificial substrate (Rioz-Martinez et al., 2011).

In

Rioz-Martinez et al. illustrated the efficacy of mFMO-PTDH by catalyzing the oxidation of indole and indole thioanisole derivatives, resulting in the synthesis of a diverse array of indigo compounds, each manifesting distinctive colors (Rioz-Martinez et al., 2011). Notably, mFMO-PTDH exhibited the capability to synthesize modified indigo, including halogenated indigo variants (Schnepel et al., 2021). This bifunctional enzyme, mFMO-PTDH, not only showcases proficiency in oxidizing indole and its analogs but also demonstrates potential in chirality selection, particularly in the biocatalytic sulfoxide oxidation of prochiral sulfides (Pereira et al., 2022; Wojaczyńska and Wojaczyński, 2020).

Indirubin, an indole alkaloid with applications as a drug in chronic myeloid leukemia, has exhibited anticancer effects (Yang et al., 2022). Traditional production methods have relied on plant cell culture extraction. However, with the utilization of the improved mFMO (Han et al., 2008), the biosynthetic pathway of indirubin has been further explored. Cysteine was identified to enhance the selectivity of FMO for 2-hydroxyindole (Han et al., 2013), resulting in a yield of 223.6 mg/L under optimized conditions. In a fed-batch fermentation conducted by Du et al., mFMO and *Escherichia coli* tryptophanase (TRP) were employed to biologically synthesize indirubin using glucose as the primary substrate. This system yielded 56 mg/L of indirubin and 640 mg/L of indigo with a fed glucose concentration of 5.4 g/L (Du et al., 2018). Subsequent studies revealed that cysteine significantly influenced the catalytic selectivity of mFMO. As the concentration of cysteine increased during the oxidation catalytic process, the reaction tended towards indirubin synthesis (Kim et al., 2019), holding considerable significance for the synthesis of indirubin by mFMO. In 2022, a semi-rational design, combined with modifications in two regions (K223R/D317S) of FMO, was applied. Through metabolic engineering technology, E. coli strains were screened to achieve high indirubin production, reaching the current maximum yield of 860.7 mg/L (Sun et al., 2022).

Additionally, the enzyme tryptophanase (TRP) can convert tryptophan into indole and pyruvate (Shimada et al., 2004). As pyruvate can be used by the cell to regenerate NADPH, a bifunctional fusion enzyme of mFMO-TRP was developed to produce indigo from L-tryptophan. This approach resulted in the complete conversion of 2.0 g tryptophan into 1.7 g indigo per liter of culture (Fabara and Fraaije, 2020).

Moreover, directed evolution was applied for mFMO modification. A computational library design protocol named FRESCO is a structure-based enzyme engineering tool for stabilization (Wijma et al., 2018). FRESCO was utilized to improve mFMO’s thermostability. After screening a relatively small number of enzyme mutants, the kcat for indole was improved by 1.5-fold, and some mutants exhibited higher Kcat and lower Km, showing higher indole affinity and superior catalytic efficiency at low substrate concentrations (Lončar et al., 2019b). Furthermore, in 2022, the catalytic efficiency of mFMO was enhanced by 6.6-fold using FRESCO (Sun et al., 2022).

**1.4 Optimization of Biosynthetic Conditions of mFMO**

The toxicity of indole has been shown to decrease indigo production of the producer microorganisms (Murdock et al., 1993; O'Connor et al., 1997). To address this issue, a two-phase culture system was constructed comprising organic solvent-tolerant microorganisms and non-aqueous media, which enriched indole in the hydrophobic organic solvent and reduced its concentration in the medium (Fig. 9C) (Doukyu et al., 2003).

For instance, a consecutive two-cell reaction system was constructed using mFMO, 6-halogenase SttH, and tryptophanase TnaA to synthesize 6,6'-dibromoindigo (6BrIG) with a yield of 315.0 mg/L from tryptophan (Lee et al., 2021).

To reduce the substrate cost of indigo fermentation, a system based on the co-cultivation of microorganisms was developed to convert renewable carbon substrates to indigo (Chen et al., 2021). The system can be divided into upstream and downstream parts: the upstream engineered E. coli is responsible for the synthesis of tryptophan from glucose or glycerol; the downstream system is responsible for the conversion of tryptophan to indole and then to indigo. Besides, the metabolism of the two strains was specially optimized, and the final indigo yield reached 104.3 mg/L, which was more than eleven folds higher than the original strain.

Recently, a strategy was implemented to increase the cyclopropane fatty acid (CFA) composition of phospholipid fatty acids in the cell membrane to counteract the cytotoxic effects of indole. This approach resulted in a 1.5-fold increase in indigo production compared to the control strain (Ham et al., 2023).

**1.5 Other Bacterial FMOs**

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).

For instance, FMO motifs encoded by *Mesorhizobia* and *Sphingomonas* isolated from wastewater sludge by metagenomic methods exhibit biocatalytic activity for indigo synthesis (Singh et al., 2010).

PtFMO was cloned from *Polygonum tinctorium* and heterologously expressed in *E. coli* BL21 (DE3), achieving a 30 mg/L indigo yield (Inoue et al., 2021).

The cFMO, cloned from *Corynebacterium glutamicum*, reaches the biosynthetic indigo yield of 685 mg/L (Ameria et al., 2015) in engineered *E. coli*. Mutants F170Y, A210G, A210S, and T326S cFMOs were fused with maltose-binding protein (MBP), and the fusion protein produced 1,040 mg/L indigo and 112 mg/L indirubin with 2.5 g/L tryptophan (Jung et al., 2018).

While NiFMO from *Nitrincola lacisaponensis* has a comparably lower yield of indigo, it exhibits unique thermal stability, and the optimum catalytic temperature reaches 51 °C, which is about 8 °C higher than mFMO (Lončar et al., 2019a).

**1.6 Other plant pigment synthesized by FMOs**

**1.7 After Synthesis Optimization**

Indigo's intrinsic insolubility in water necessitates the addition of reductants, such as sodium sulfate, to transform it into a water-soluble dye during practical production. However, this conventional approach brings about economic and environmental burdens. To overcome the insolubility challenge of indigo in water, a novel strategy involving chemical group protection has been proposed. This innovative method entails the co-expression of a glycosyltransferase from Polygonum tinctorium with mFMO in Escherichia coli. This co-expression introduces a glucose group to protect the active hydroxyl group of hydroxyindole, resulting in the creation of a water-soluble and stable pigment named indican (Hsu et al., 2018). Through fed-batch fermentation, the recombinant E. coli strain TMH011 successfully produced 2.9 g/L of indican. Given that two indoxyl molecules are required to produce one indigo molecule, the fermentation result can be converted to 1.4 g/L of indigo production. This approach offers a promising solution to enhance the environmental sustainability and economic viability of indigo synthesis.

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

**Table 1.** The Kcat and Km of different FMO subtypes on various substrates

In the biosynthetic strategy, indole is used as a substrate or intermediate in the pathway. With the help of heterologous FMO, indole is oxidized to 3-hydroxyindole (indophenol`/hydroxyindole), which further forms indigo by spontaneous dimerization under aerobic conditions (Choi, 2020; Han et al., 2008).

**3. The Difference between Synthetic Technologies**

**3.1 Technologies performed to mFMO**

Numerous studies have been conducted to improve the properties of the enzyme and optimize biosynthetic conditions (Table 3).