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**Biotransformation of indole to indigo and indirubin by
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Methylophaga aminisulfidivorans SK1**

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Abstract

In this study, the methylotrophic yeast *Komagataella phaffii* GS115 was engineered to express the wild-type flavin-containing monooxygenase from *Methylophaga aminisulfidivorans* strain SK1 (MaFMO), enabling the biocatalytic conversion of externally supplied indole into the bisindole pigments indigo and indirubin. The effects of indole concentration, methanol concentration, induction time, agitation speed, and supplementation with L-cysteine or Tween 80 were individually evaluated. In the substrate concentration experiment, 3 mM indole resulted in the highest indigo titer (19.97 ± 0.68 mg/L) and an indirubin titer of 2.29 ± 0.19 mg/L. Increasing the indole concentration to 5 mM markedly decreased pigment formation, suggesting substrate-associated inhibition and/or indole-induced cellular stress. Methanol concentrations ranging from 0.5 to 2.0% (v/v) had relatively limited effects on pigment titers. In a separate time-course experiment, extending the induction period to 72 h increased the indigo and indirubin titers to 38.93 ± 3.77 and 2.70 ± 0.96 mg/L, respectively. Supplementation with L-cysteine altered the apparent product distribution, whereas supplementation with 7% (v/v) Tween 80 strongly inhibited pigment production. These results demonstrate the functional activity of MaFMO in *K. phaffii* and establish a proof-of-concept yeast platform for the biotransformation of indole into indigo and indirubin. Further optimization of substrate feeding, oxygen transfer, redox balance, and host metabolism will be necessary to improve pigment titer, productivity, and product selectivity.

Keywords

Indigo; Indirubin; *Komagataella phaffii* GS115; MaFMO flavin-containing monooxygenase; indole biotransformation; yeast host

1. Introduction

Indigo is one of the most widely used textile dyes, particularly in denim manufacturing, owing to its characteristic blue color and distinctive fading properties [1-3]. In contrast, indirubin, a structural isomer of indigo, is best known as a bioactive constituent of *indigo naturalis*. Indirubin and several of its derivatives have been reported to modulate protein kinases and other signaling pathways involved in cell proliferation, inflammation, and neuronal function [4-7].

Industrial indigo production is dominated by chemical synthesis, whereas indirubin and related derivatives are produced through chemical synthesis, extraction from natural materials, or specialized biocatalytic processes [1-3]. Conventional synthetic processes may involve hazardous reagents, organic solvents, and waste-generating reaction steps, which has motivated the development of alternative biocatalytic production methods [1-3,8].

In microbial systems, oxygenase-mediated oxidation of indole generates reactive hydroxylated intermediates, including indoxyl and other oxidized species [1,9,10]. Oxidative coupling of indoxyl molecules predominantly yields indigo, whereas coupling between indoxyl and isatin-related intermediates can lead to indirubin [1,10-12]. Because these downstream reactions are largely non-enzymatic, product distribution can be strongly influenced by oxygen availability, redox conditions, intermediate stability, and the extracellular or intracellular reaction environment [11-13]. Several oxygenases have been used to oxidize indole in recombinant microorganisms, including naphthalene dioxygenases, toluene monooxygenases, styrene monooxygenases, cytochrome P450 enzymes, and flavin-containing monooxygenases [1,9,10,14-17].

Recent studies have demonstrated the feasibility of monooxygenase-based indigo biosynthesis in yeast and established analytical approaches for characterizing pigments in yeast-derived extracts [18]. Building on these advances, FMO-based platforms are particularly

attractive because FMOs are single-component flavoprotein monooxygenases that use FAD, molecular oxygen, and NADPH-derived reducing equivalents without requiring a separate reductase component [2,19–21]. In highly optimized recombinant *Escherichia coli* systems, FMO-mediated indole oxidation has achieved substantially higher indigo titers, including gram-per-liter production under specialized cultivation or feeding conditions [13,22,23]. However, the applicability of MaFMO-based indigoid production in *Komagataella phaffii* remains insufficiently characterized.

Among FMO-based systems, the flavin-containing monooxygenase from *Methylophaga* sp. strain SK1, hereinafter referred to as MaFMO, has been investigated as an efficient biocatalyst for indole oxidation [19]. In bacterial hosts, MaFMO-based systems have been extensively optimized through genetic construct engineering, medium optimization, and process development [13,19,22,23]. Furthermore, strategies such as L-cysteine supplementation, redox engineering, structure-guided enzyme engineering, and cofactor regeneration have been shown to enhance indirubin formation and improve overall pigment yields [11,12,24,25].

To date, however, most studies on MaFMO-mediated indigoid biosynthesis have been conducted in prokaryotic hosts, particularly *E. coli* [13,19,22–24]. Although yeast-based indigo biosynthesis has recently been demonstrated in *Saccharomyces cerevisiae* [18], the applicability of MaFMO-mediated indole oxidation in *K. phaffii* remains largely unexplored. *K. phaffii*, formerly known as *Pichia pastoris*, is widely used for recombinant protein production because it supports strong inducible expression, high-cell-density cultivation, robust eukaryotic protein folding, and scalable fermentation [26–30]. These features make it an attractive host for developing whole-cell biocatalytic processes for the production of value-added compounds [28–30]. However, previous studies have not systematically examined the feasibility of implementing MaFMO-mediated indole oxidation in *K. phaffii* or determined

99 whether this yeast host can support the formation of both indigo and indirubin. Thus, *K. phaffii*
100 represents a promising platform for the sustainable production of value-added pigments and
101 bioactive compounds.

102 Therefore, this study sought to determine whether MaFMO-mediated indole oxidation
103 could be functionally implemented in *K. phaffii* GS115 and whether the resulting recombinant
104 strain could convert externally supplied indole into both indigo and indirubin. We further
105 evaluated the individual effects of indole concentration, methanol concentration, induction
106 time, agitation speed, and supplementation with L-cysteine or Tween 80 on pigment titers and
107 product distribution. This work was designed to establish a proof-of-concept *K. phaffii* platform
108 for MaFMO-mediated indigoid pigment production and to identify process variables that could
109 guide subsequent strain and fermentation development.

2. Materials and Methods

2.1 Strains, plasmid, and media

E. coli DH5 α was used for plasmid propagation and maintenance. *K. phaffii* strain GS115 served as the expression host for MaFMO. The pPIC9 expression vector was used to construct the methanol-inducible MaFMO expression cassette. Luria–Bertani (LB) medium (Difco™, BD, Sparks, MD, USA; Cat. No. 244620), supplemented with ampicillin to a final concentration of 100 μ g/mL, was used for *E. coli* cultivation and for selecting transformants carrying pPIC9::MaFMO. YPD medium (MBcell, Kisan, Korea; Cat. no. MB-Y0617) was used for routine cultivation of *K. phaffii* and preparation of pre-cultures. For biomass accumulation and induction of *K. phaffii*, buffered glycerol-complex medium (BMGY) and methanol-complex medium (BMMY) were prepared according to standard *Pichia* expression protocols.

2.2 Gene construction and *E. coli* transformation

The wild-type MaFMO coding sequence from *Methylophaga aminisulfidivorans* SK1 (GenBank accession no. AF494423.2; 1,371 bp) was amplified using the primers MaFMO-F (5'-AAA GGA TCC ATG GCA ACT CGT ATT GCG ATA CTT GGT-3') and MaFMO-R (5'-AAA ACT CGA GTA AGC TTC TTT AGC CAC AGG AAT TTC GCT C-3') and inserted into pPIC9 between the (BamHI) and (XhoI) restriction sites to generate pPIC9::MaFMO. Competent *E. coli* DH5 α cells were transformed with pPIC9::MaFMO by heat shock. Transformants were selected on LB agar plates containing ampicillin and cultivated in liquid LB medium supplemented with ampicillin for plasmid amplification. After overnight cultivation, plasmid DNA was isolated using a standard miniprep kit according to the manufacturer's instructions. The MaFMO insert and flanking regions were further confirmed by sequencing to ensure sequence integrity before yeast transformation.

2.3 Transformation of *K. phaffii* and clone selection

For genomic integration, pPIC9::MaFMO was linearized at the AOX1 locus using the (PmeI) restriction site. To enable intracellular expression, the α -factor secretion signal sequence in pPIC9 was removed, and the MaFMO coding sequence containing its native start codon was inserted downstream of the AOX1 promoter. Electrocompetent *K. phaffii* GS115 cells were prepared according to an established protocol for *Pichia* electroporation. Briefly, cells were grown in YPD, harvested during the mid-exponential phase, washed with ice-cold sterile water followed by 1 M sorbitol, and resuspended in 1 M sorbitol. Approximately 5–10 μ g of linearized pPIC9::MaFMO DNA was mixed with 50 μ L of electrocompetent *K. phaffii* GS115 cells. Electroporation was performed in a pre-chilled 0.2-cm cuvette at 1.5 kV, 25 μ F, and 200 Ω . Immediately after pulsing, 1 mL of ice-cold 1 M sorbitol was added, and the cells were recovered at 30 °C for 1–2 h. Transformants were selected on minimal dextrose (MD) agar plates lacking histidine and incubated at 30 °C until colonies appeared.

Integration of the MaFMO expression cassette into the *K. phaffii* genome was confirmed by colony PCR using MaFMO-specific primers together with vector- or genome-flanking primers. Positive transformants were further streak-purified and stored as glycerol stocks at –80 °C.

2.4 Cultivation and induction of recombinant *K. phaffii*

Cultivation of MaFMO-expressing *K. phaffii* GS115 was performed in three successive stages: inoculation from a glycerol stock, subculture in BMGY, and induction in BMMY, following the general workflow of standard *Pichia* expression protocols with modifications for this system. Glycerol stocks of biotransformed *K. phaffii* GS115 (final glycerol concentration, 25%) were thawed on ice. For each cultivation, YPD medium was

prepared in a culture tube, and an inoculum corresponding to 1% (v/v) of the glycerol stock was added to the medium. Cultures were incubated at 30 °C and 250 rpm for approximately 18–20 h to allow sufficient biomass accumulation. This pre-culture served both to revive the cells from the frozen state and to provide an actively growing inoculum for subsequent subculture in BMGY. BMGY was prepared with the following final composition: 1% yeast extract, 2% peptone, 0.1 M potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base (YNB), 0.0004% biotin, and 1% glycerol. The YPD pre-culture was used to inoculate BMGY at 4% (v/v) of the final BMGY volume. Subcultures were grown at 30 °C and 250 rpm until the OD₆₀₀ reached 2–6. For induction, BMGY cultures grown the previous day were harvested by centrifugation at 4 °C and 6,000 × g (rcf) for 10 min. The supernatant was discarded, and the cell pellet was washed twice with fresh BMMY medium to remove residual glycerol because glycerol represses AOX1 promoter activity and can interfere with methanol induction. Buffered BMMY was identical in composition to BMGY except that glycerol (1%) was replaced with methanol. In this study, BMMY contained 0.5% (v/v) methanol as both the carbon source and inducer, which was replenished daily at 0.5% (v/v) to compensate for consumption. After two washes, the pellet was resuspended in BMMY, and the suspension was adjusted to an OD₆₀₀ of 1.0. Substrate (indole) and other additives, including L-cysteine and Tween 80, were then added to the cultures to achieve the desired final concentrations. Unless otherwise indicated, all experiments were performed in baffled shake flasks with a working volume equivalent to 10% of the total flask volume to ensure sufficient aeration during cultivation.

2.5 Process variables and additives

Indole was supplied at final concentrations ranging from 1 to 5 mM to assess the effect of substrate loading on pigment formation. Methanol was initially added at final concentrations

of 0.5, 1.0, 1.5, or 2.0% (v/v) to evaluate the effect of inducer concentration on pigment production and was replenished every 24 h at the corresponding concentration throughout the induction period. Induction periods ranging from 24 to 72 h were examined to evaluate time-dependent pigment production. The agitation speed was varied from 0 to 250 rpm to modulate oxygen transfer, an important variable in monooxygenase-catalyzed reactions. L-cysteine was supplemented at final concentrations ranging from 1 to 5 mM to investigate the effects of thiol-containing compounds and redox modulation on the indirubin-to-indigo ratio, based on previous reports of cysteine-enhanced indirubin formation in *E. coli* FMO systems. Tween 80 was added at concentrations of up to 7% (v/v) to investigate the effects of a nonionic surfactant on cell physiology, substrate solubilization, and pigment production. Under baseline conditions, experiments were conducted using 3 mM indole, 1.0% (v/v) methanol, 30 °C, 250 rpm, and a 24-h induction period as the standard condition. For each variable experiment, only the indicated parameter was changed, while all other conditions were kept constant.

2.6 Pigment extraction

At designated time points, culture samples were collected for pigment analysis. For pigment extraction, 1 mL of culture broth was centrifuged to collect the cell pellet. The pellet was resuspended in 1 mL of dimethyl sulfoxide (DMSO), vortexed vigorously for 1 min, and centrifuged at $13,000 \times g$ for 10 min. The resulting supernatant was collected, and a 0.1-mL aliquot was diluted with 0.9 mL of acetonitrile. The diluted sample was filtered through a 0.22- μ m PTFE syringe filter prior to chromatographic and mass spectrometric analysis. Pigment titers were expressed as mg/L of culture, and yields were calculated relative to the supplied indole when appropriate.

2.7 LC/MS verification of indigo and indirubin

LC/MS analysis was performed to verify the identities of the pigments produced by the MaFMO-expressing *K. phaffii* GS115 strain. Chromatographic separation was carried out using a Thermo DIONEX UltiMate 3000 HPLC system equipped with a Waters CORTECS C18 column (2.1 × 50 mm, 2.7 μm) maintained at 40 °C. The injection volume was 5 μL. Mobile phase A consisted of 0.1% formic acid in deionized water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The flow rate was maintained at 0.300 mL/min throughout the analysis. The gradient program was as follows: 0–1 min, 5% B; 1–40 min, linear increase from 5% to 95% B; 40–45 min, 95% B; 45–45.5 min, decrease from 95% to 5% B; and 45.5–50 min, 5% B for column re-equilibration.

Mass spectrometric detection was performed using a Thermo LTQ XL mass spectrometer equipped with an electrospray ionization source operated in positive ion mode. The ion spray voltage was set to 3.5 kV. The source and capillary temperatures were maintained at 300 °C and 320 °C, respectively. The sheath gas, auxiliary gas, and sweep gas were set to 42, 15, and 0 arbitrary

3. Results and discussion

3.1 Visual confirmation of pigment production

Recombinant *K. phaffii* GS115 expressing MaFMO developed a pronounced blue coloration, primarily in the cell pellet, and the pigmentation was retained in DMSO extracts. In contrast, the wild-type control showed no visible color change under identical conditions. This visual phenotype provided an initial qualitative indication that the engineered yeast was capable of producing indole-derived pigments.

The observed blue pigmentation is consistent with MaFMO-mediated oxidation of indole to reactive intermediates that subsequently undergo nonenzymatic coupling to form indigoid pigments. Because indigo formation depends on both enzymatic indole oxidation and subsequent chemical coupling reactions, the observed pigmentation suggests that *K. phaffii* can support the key steps required for MaFMO-mediated indigoid biosynthesis. These results demonstrate the feasibility of using *K. phaffii* as a eukaryotic host for the production of indole-derived pigments.

3.2 Identification and quantification of extracts

DMSO extracts from induced recombinant cultures showed peaks corresponding to indigo and indirubin. The detected peaks exhibited retention times and mass spectral profiles consistent with those of authentic standards analyzed under identical conditions, confirming the production of both pigments by the MaFMO-expressing *K. phaffii* GS115 strain. Because indigo and indirubin are constitutional isomers with the same molecular mass, chromatographic separation and comparison with authentic standards were used for reliable compound assignment [11,12,25].

Thus, the visual pigmentation observed in the recombinant strain was attributable to the formation of indigoid compounds rather than nonspecific colored by-products. The

simultaneous detection of indigo and indirubin indicates that MaFMO-mediated indole oxidation generated indoxyl-type intermediates capable of undergoing multiple nonenzymatic coupling reactions. Indigo was the dominant product, whereas indirubin was detected at lower levels, suggesting that the reaction environment in *K. phaffii* favored indigo formation under the tested conditions. This product distribution is consistent with the general chemistry of indole oxidation, in which indigo formation is often favored unless the redox environment, intermediate availability, or coupling chemistry is specifically tuned to promote indirubin formation [11,12]. Together, the visual and LC/MS data support the functional activity of MaFMO in *K. phaffii* and confirm the formation of indigo and indirubin.

3.3 Effect of indole concentration

Indole concentration had a strong influence on pigment production. As the indole concentration increased from 1 to 3 mM, indigo titer increased, reaching a maximum of 19.97 mg/L at 3 mM indole (Table 1). Under these conditions, indirubin was also detectable, although at substantially lower levels than indigo, indicating that both branches of indigoid pigment formation were active in the recombinant *K. phaffii* strain.

However, when the indole concentration was further increased to 5 mM, both indigo and indirubin titers decreased sharply. This decrease may reflect substrate-associated stress or inhibition, as elevated indole concentrations have been reported to affect microbial biotransformation systems and have often required controlled feeding or two-phase strategies to improve conversion [17,31,32]. However, direct measurements of cell growth, viability, and residual indole would be required to confirm this interpretation.

These results indicate that 3 mM indole was the optimal substrate concentration within the tested range under the present batch conditions. The non-linear relationship between substrate concentration and pigment titer suggests that simply increasing the initial indole

concentration is unlikely to improve production. Instead, fed-batch addition, staged substrate feeding, or controlled-release strategies may be required to maintain indole at concentrations sufficient for enzymatic conversion while avoiding inhibitory levels [31,32]. The decrease observed at 5 mM indole may therefore reflect substrate-associated stress or inhibition, although direct measurements of cell growth, viability, and residual indole would be needed to confirm this interpretation.

3.4 Effect of methanol concentration

Variation in methanol concentration between 0.5 and 2% (v/v) resulted in only minor changes in indigo and indirubin titers. Pigment production remained relatively stable across this range, suggesting that increasing the methanol concentration did not substantially enhance MaFMO-dependent pigment formation under the tested conditions. Therefore, within the tested range, methanol concentration did not appear to be a primary determinant of pigment productivity.

This result may indicate that AOX1-driven expression was sufficient across the tested methanol range, although direct protein-level analysis would be required to determine whether MaFMO abundance limited overall productivity. In *K. phaffii*, AOX1-driven expression is closely associated with methanol utilization, and methanol metabolism can impose oxygen-transfer and metabolic burdens during recombinant protein production [26,34,35]. Thus, higher methanol concentrations may not necessarily improve recombinant pathway performance and could instead adversely affect cellular fitness.

The limited effect of methanol concentration suggests that other factors, such as indole toxicity, NADPH availability, oxygen transfer, and the stability or fate of reactive intermediates, may play more dominant roles in determining overall pigment yields. Future improvement of this system may therefore benefit more from optimizing substrate-feeding strategies, redox

metabolism, and oxygen supply than from further increasing the methanol induction level [24,34,35].

3.5 Effect of induction time

Induction time had a pronounced effect on pigment accumulation. Extending the induction period from 24 to 72 h resulted in continuous increases in both indigo and indirubin titers, with the highest pigment levels observed at 72 h. This trend suggests that sustained MaFMO expression and prolonged catalytic activity promoted continued conversion of indole into pigment-forming intermediates.

The increase in pigment levels over time indicates that recombinant *K. phaffii* maintained sufficient metabolic activity during prolonged induction. Under the tested conditions, the cells appeared to tolerate methanol induction and indole exposure for at least 72 h while supporting MaFMO activity, cofactor regeneration, and pigment accumulation. However, prolonged methanol induction can impose metabolic and oxygen-transfer burdens in *K. phaffii* processes, particularly in high-cell-density or fed-batch systems [34,35]. This consideration is important for biotransformation-based pigment production because product formation may require extended reaction times due to the combined effects of enzyme expression, substrate oxidation, and subsequent non-enzymatic pigment formation.

Nevertheless, prolonged induction can impose a metabolic burden on the host. Recombinant protein expression, methanol metabolism, NADPH consumption, oxygen demand, and the accumulation of reactive intermediates may collectively reduce cellular fitness over time. Although pigment titers increased up to 72 h in this study, further extension of the induction period could eventually lead to nutrient depletion, oxidative stress, accumulation of toxic by-products, or reduced enzyme activity. Therefore, 72 h represented the best-performing induction condition within the tested time range. Further time-course analysis combined with

measurements of cell viability, residual indole, and MaFMO activity would be useful for defining the optimal balance among final titer, productivity, and cellular stress [34,35]. Although the highest titers were obtained after 72 h, volumetric productivity may not increase proportionally with induction time. Therefore, both final titer and time-dependent productivity should be considered in future process optimization.

3.6 Effect of agitation and oxygen availability

Agitation was required for detectable pigment production, as no pigment was detected under static conditions. This observation is consistent with the oxygen-dependent nature of FMO-catalyzed monooxygenation and with previous bio-indigo fermentation studies showing that oxygen supply, aeration, and agitation can strongly influence indigo production [13,19,21]. However, increasing agitation from 100 to 250 rpm produced only a modest change in indigo titer and did not improve indirubin formation. These results suggest that oxygen transfer contributed to MaFMO-mediated indole oxidation, although it was not the sole determinant of pigment accumulation within the tested range.

The modest difference between 100 and 250 rpm further suggests that oxygen availability was not severely limiting under the baffled shake-flask conditions, whereas the absence of agitation was unfavorable for pigment formation. However, the effect of agitation was less pronounced than the effects of indole concentration and induction time. This suggests that oxygen transfer was not severely limited in baffled shake flasks within the tested range, although it still contributed to pigment formation. The moderate response to agitation also indicates that oxygen availability interacts with other potentially limiting factors, including substrate toxicity, NADPH supply, and intermediate coupling chemistry.

Oxygen supply is expected to become more important during scale-up, where oxygen transfer can differ substantially from that under shake-flask conditions [13,35]. In larger

bioreactors, agitation, aeration rate, dissolved oxygen control, and potentially oxygen-enriched gas feeding could be used to improve MaFMO activity. At the same time, excessive oxygen availability may promote undesired oxidative stress or alter the balance of reactive intermediates. Therefore, precise control of oxygen transfer will be important not only for increasing total pigment production but also for tuning the distribution between indigo and indirubin.

3.7 Effect of L-cysteine and Tween 80 supplementation

L-cysteine supplementation altered the apparent product distribution without increasing total pigment production under the tested conditions. In particular, the indirubin-to-indigo ratio shifted at specific L-cysteine concentrations, suggesting that L-cysteine influenced the fate of indole-derived intermediates. Previous FMO-based *E. coli* studies showed that cysteine can enhance indirubin formation by altering the chemistry of indoxyl- and isatin-derived intermediates, although cysteine may also affect cell growth and overall pigment production [11,12]. This suggests that L-cysteine influenced the fate of indole-derived intermediates and altered the balance between indigo and indirubin formation.

The effect of L-cysteine is likely associated with thiol-mediated modulation of the redox environment and interactions with reactive intermediates. Cysteine may affect intracellular reducing power, scavenge reactive oxygen species, or participate in intermediate stabilization, thereby changing the probability of coupling reactions that lead to indirubin rather than indigo [11,12]. The response of indirubin formation to L-cysteine supplementation suggests that redox conditions and intermediate chemistry may influence product distribution in addition to MaFMO-mediated indole oxidation.

From an application perspective, this result is potentially important because indirubin has greater pharmaceutical relevance than indigo. The consistently lower indirubin titers

observed under standard conditions indicate that the MaFMO-mediated reaction network in *K. phaffii* is biased toward indigo formation. Therefore, process variables that alter the redox state, such as L-cysteine concentration, oxygen availability, pH, and cofactor regeneration capacity, may be useful for redirecting product formation toward indirubin [11,12,24].

In contrast, Tween 80 supplementation at 7% severely reduced overall pigment production. This inhibitory effect may be attributable to adverse effects on membrane integrity, altered substrate and product partitioning, changes in indole availability, or detrimental effects on enzyme stability and cell physiology. Similar process-level challenges associated with hydrophobic substrates and indigoid products have been reported in two-phase or solvent-assisted indigo production systems [17,31]. Under some supplementation conditions, indirubin was not detectable, further indicating that indirubin formation is sensitive to perturbations in the reaction environment. Because 7% Tween 80 represents a relatively high surfactant concentration, the observed inhibition may reflect surfactant-induced physiological stress rather than improved substrate solubilization. Future studies should examine lower Tween 80 concentrations or alternative solubilization strategies to improve indole availability without compromising cell viability.

Taken together, these results suggest that L-cysteine can be used as a process variable to modulate pigment composition, whereas high concentrations of Tween 80 are unfavorable for MaFMO-mediated pigment production in *K. phaffii*. Fine-tuning thiol supplementation and redox-related parameters may provide a practical strategy for improving indirubin selectivity in future studies.

3.8 Implications for yeast-based indigoid production

The production of both indigo and indirubin in MaFMO-expressing *K. phaffii* supports the feasibility of implementing MaFMO-mediated indigoid biotransformation in a eukaryotic yeast host. Most previously reported MaFMO-based indigoid production systems have relied on prokaryotic hosts, particularly *E. coli*. Although yeast-based indigo biosynthesis has recently been demonstrated in *S. cerevisiae* through tryptophan flux redirection and heterologous expression of an indigo-forming module [18], the applicability of MaFMO-mediated indole oxidation in *K. phaffii* has remained largely unexplored.

Although yeast-based indigo biosynthesis has recently been demonstrated in *S. cerevisiae* through tryptophan flux redirection and heterologous expression of an indigo-forming module [18], the present study extends yeast-based indigoid biotransformation to *K. phaffii* and focuses on the MaFMO-mediated conversion of externally supplied indole. Importantly, both indigo and indirubin were detected in the recombinant *K. phaffii* system, indicating that this host can support not only indigo formation but also the competing coupling route leading to indirubin under the tested cultivation conditions. Although the pigment titers obtained in this initial *K. phaffii* system were lower than those reported for highly optimized bacterial platforms and the recently reported *S. cerevisiae*-based indigo biosynthesis system, the primary objective of this study was to determine whether MaFMO-mediated indole oxidation could be implemented in *K. phaffii* [18,21–24].

The results also identify several process variables that should be prioritized in future optimization, including controlled indole feeding, induction duration, oxygen availability, and redox-modulating additives. Further improvements may require optimization of oxygen transfer, redox and cofactor engineering, and high-cell-density fermentation strategies, in addition to controlled indole feeding, as suggested by optimized bacterial FMO systems and established *K. phaffii* fermentation studies [11–13,24,34,35].

4. Conclusion

This study demonstrates that *K. phaffii* GS115 can support the MaFMO-mediated conversion of externally supplied indole into indigo and indirubin, establishing a proof-of-concept *K. phaffii* platform for indigoid pigment production. Visual pigmentation and LC/MS analysis confirmed the formation of both pigments in the recombinant strain, providing evidence of MaFMO-dependent indole oxidation in vivo.

Among the process variables examined, indole concentration and induction time had the most pronounced effects on pigment accumulation. Within the tested range, 3 mM indole resulted in the highest pigment titer in the substrate concentration experiment, whereas extending the induction period to 72 h further increased the titers of both indigo and indirubin. Methanol concentration had only a limited effect on pigment formation, whereas agitation and L-cysteine supplementation influenced pigment production and apparent product distribution. In contrast, supplementation with 7% Tween 80 strongly inhibited pigment production.

The present study should be considered an initial proof-of-concept demonstration rather than a fully optimized production process. Further improvements will require controlled indole feeding, direct evaluation of MaFMO expression and activity, optimization of oxygen transfer and redox balance, and metabolic engineering strategies aimed at increasing pigment titer and productivity while improving indirubin selectivity.

Author contributions

Kwon D.H. and Yeo C.S. performed the engineering of the pigment-producing host and the experiments for indigo and indirubin production. They also contributed to drafting the manuscript. Choi K.Y. conceived the study, contributed to drafting the manuscript, and revised the paper.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Availability of data and materials

Data supporting the findings of this study are included in the article.

Supporting information

5. References

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589 **Table Legends**

590 Table 1. Production of indigo and indirubin

Variable	Levels	Indigo			Indirubin		
		mean ± SD (mM)	Yield ± SD (%)	mg/L ± SD	mean ± SD (mM)	Yield ± SD (%)	mg/L ± SD
Indole (mM)	1	0.013 ± 0.0022	2.6 ± 0.44	3.4 ± 0.58	-	-	-
	2	0.038 ± 0.004	3.8 ± 0.4	9.96 ± 1.04	-	-	-
	3	0.0761 ± 0.0026	5.08 ± 0.17	19.97 ± 0.68	0.0087 ± 0.0007	0.58 ± 0.05	2.29 ± 0.19
	5	0.0221 ± 0.0052	0.89 ± 0.21	5.8 ± 1.37	-	-	-
MeOH (%)	0.5	0.0536 ± 0.002	3.58 ± 0.13	14.07 ± 0.51	0.0031 ± 0.0006	0.21 ± 0.04	0.82 ± 0.16
	1	0.0528 ± 0.0065	3.52 ± 0.43	13.86 ± 1.7	0.0033 ± 0.0008	0.22 ± 0.05	0.88 ± 0.2
	1.5	0.0517 ± 0.0073	3.45 ± 0.49	13.55 ± 1.91	0.004 ± 0.0013	0.27 ± 0.08	1.05 ± 0.33
	2	0.0467 ± 0.0058	3.11 ± 0.38	12.25 ± 1.51	0.0035 ± 0.002	0.23 ± 0.13	0.91 ± 0.52
Supplementation	1 mM L-cysteine	0.0385 ± 0.006	2.57 ± 0.4	10.1 ± 1.57	0.0019 ± 0.0005	0.13 ± 0.03	0.51 ± 0.13
	3 mM L-cysteine	0.0332 ± 0.0035	2.22 ± 0.23	8.72 ± 0.91	0.0022 ± 0.0005	0.15 ± 0.04	0.58 ± 0.14
	5mM L-cysteine	0.0366 ± 0.0093	2.44 ± 0.62	9.6 ± 2.44	0.0037 ± 0.0029	0.25 ± 0.19	0.98 ± 0.75
	7% Tween 80	0.004 ± 0.0063	0.27 ± 0.42	1.05 ± 1.64	-	-	-
	3 mM L-cys+7% Tween 80	-	-	-	-	-	-
Induction time (h)	24	0.0807 ± 0.0091	5.38 ± 0.61	21.16 ± 2.39	0.003 ± 0.001	0.2 ± 0.06	0.78 ± 0.25
	48	0.0875 ± 0.0024	5.83 ± 0.16	22.95 ± 0.63	0.0075 ± 0.0063	0.5 ± 0.42	1.98 ± 1.65

Agitation speed (rpm)	72	0.1485 ± 0.0144	9.9 ± 0.96	38.93 ± 3.77	0.0103 ± 0.0036	0.69 ± 0.24	2.7 ± 0.96
	0	-	-	-	-	-	-
	100	0.0637 ± 0.016	4.25 ± 1.07	16.71 ± 4.2	0.0044 ± 0.0044	0.29 ± 0.29	1.15 ± 1.15
	250	0.0667 ± 0.0083	4.45 ± 0.56	17.5 ± 2.19	0.0025 ± 0.0007	0.17 ± 0.04	0.66 ± 0.17

Figure Legends

Figure 1 Mechanism of methanol-induced AOX1 promoter activation and recombinant protein production in *Komagataella phaffii* (formerly *Pichia pastoris*).

Figure 2 (A) Transformation of *Komagataella phaffii* strain GS115 with linearized pPIC9::MaFMO via electroporation and homologous recombination at the AOX1 locus. (B) Biocatalytic conversion of indole to indigo and indirubin in *Komagataella phaffii* strain GS115 expressing MaFMO.

Figure 3 Visual comparison of wild-type *Komagataella phaffii* GS115 and *K. phaffii* GS115 pPIC9::MaFMO cultures showing pigment formation in cell pellets and DMSO extracts.

Figure 4 LC–MS analysis of authentic indigo, authentic indirubin, and the biotransformation reaction extract, showing matching retention times and diagnostic m/z values that confirm the production of indigo and indirubin.

Figure 5 Effects of process variables on indigo (IND) and indirubin (INR) production in *Komagataella phaffii* GS115 pPIC9::MaFMO cultures.

Figure 6 Indigo (IND) and indirubin (INR) production by *Komagataella phaffii* GS115 pPIC9::MaFMO under different process conditions.

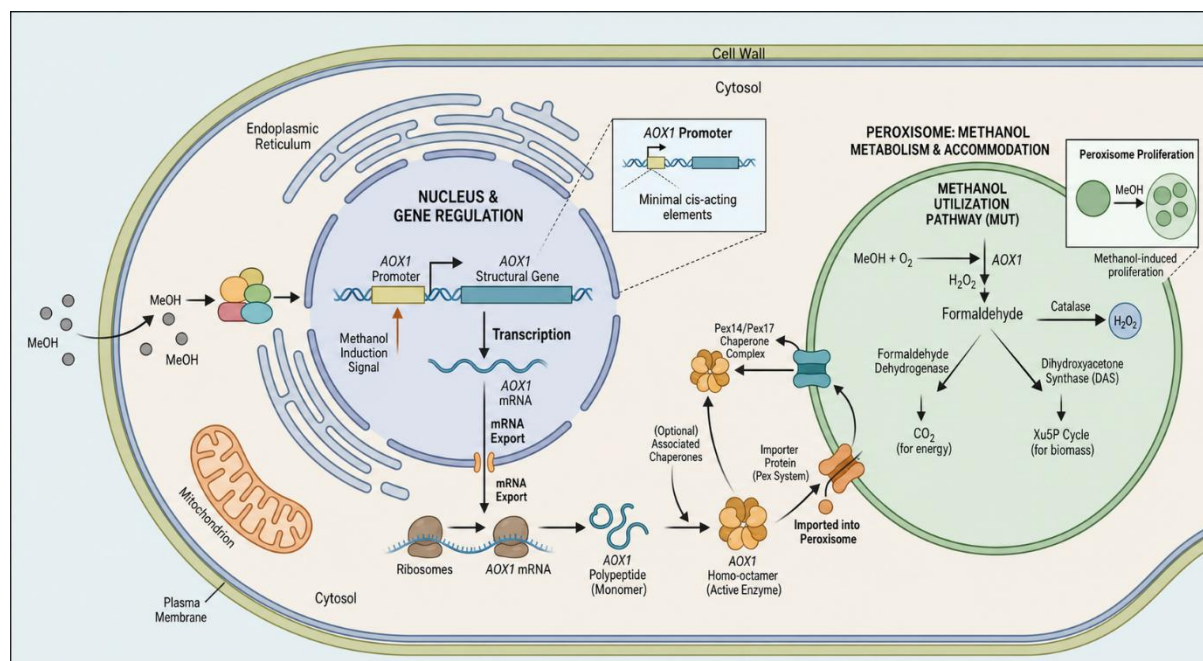
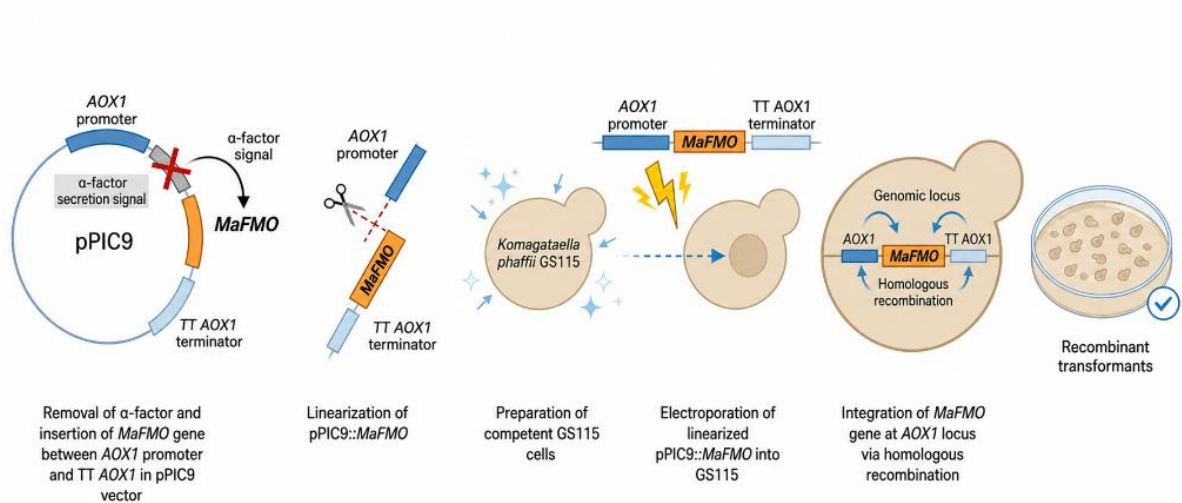


Figure 1

(A)



(B) Biocatalytic conversion of indole to indigo and indirubin in *Komagataella phaffii* strain GS115 expressing MaFMO.

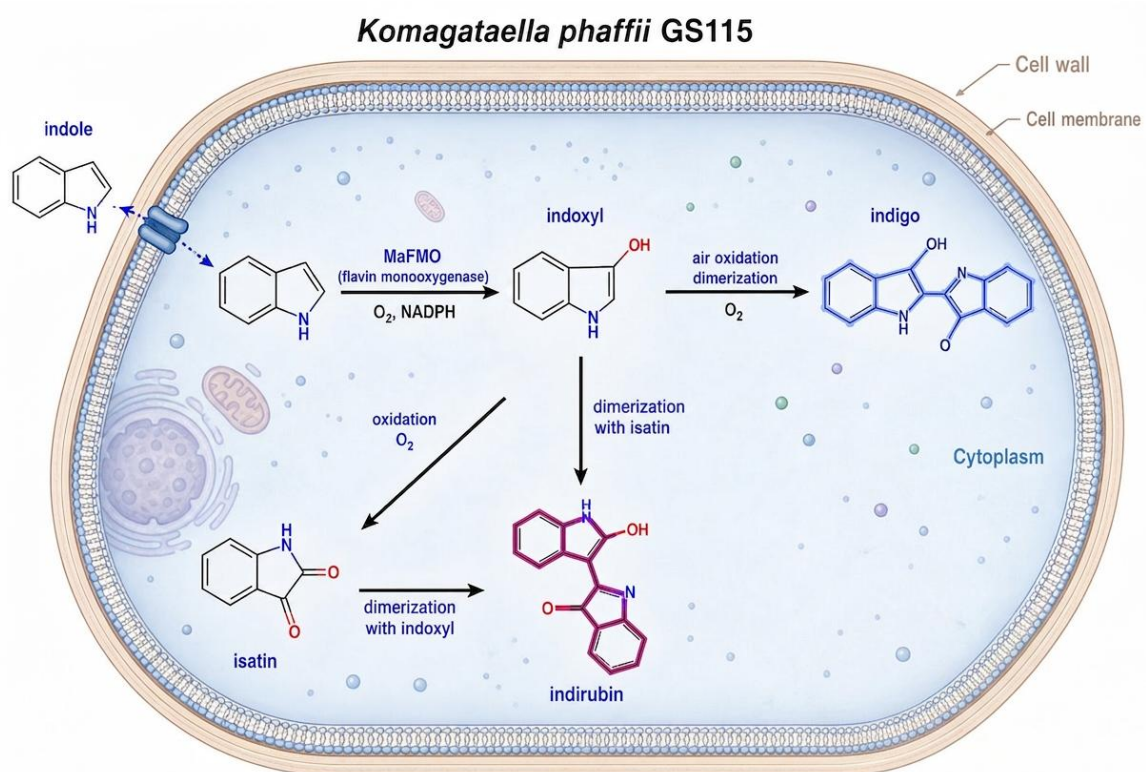
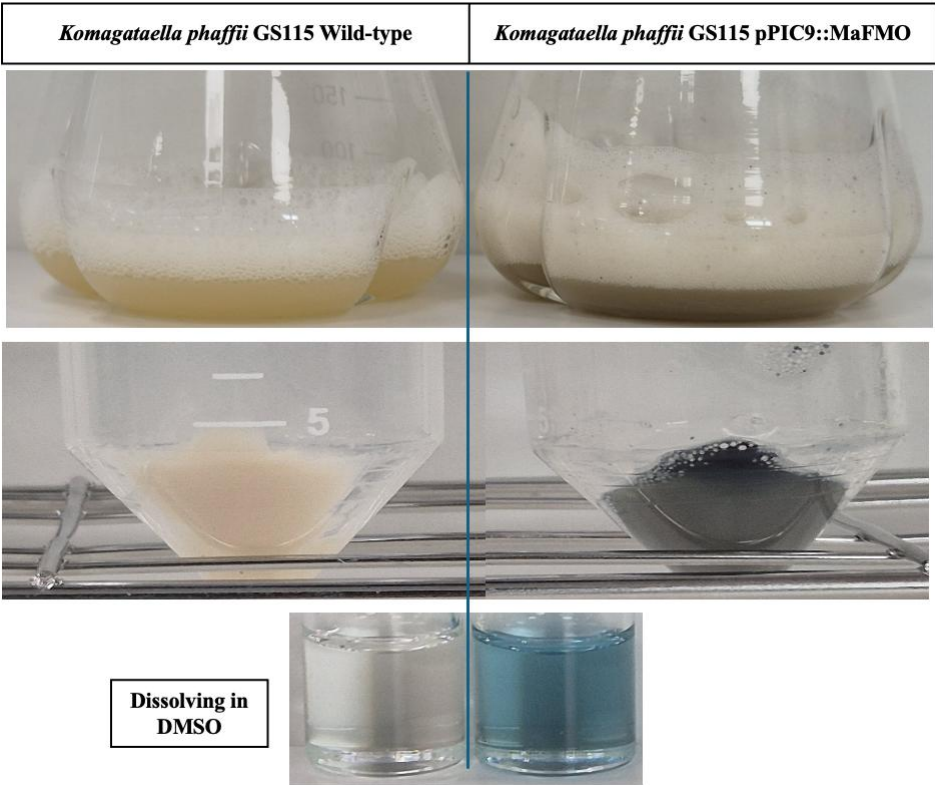


Figure 2

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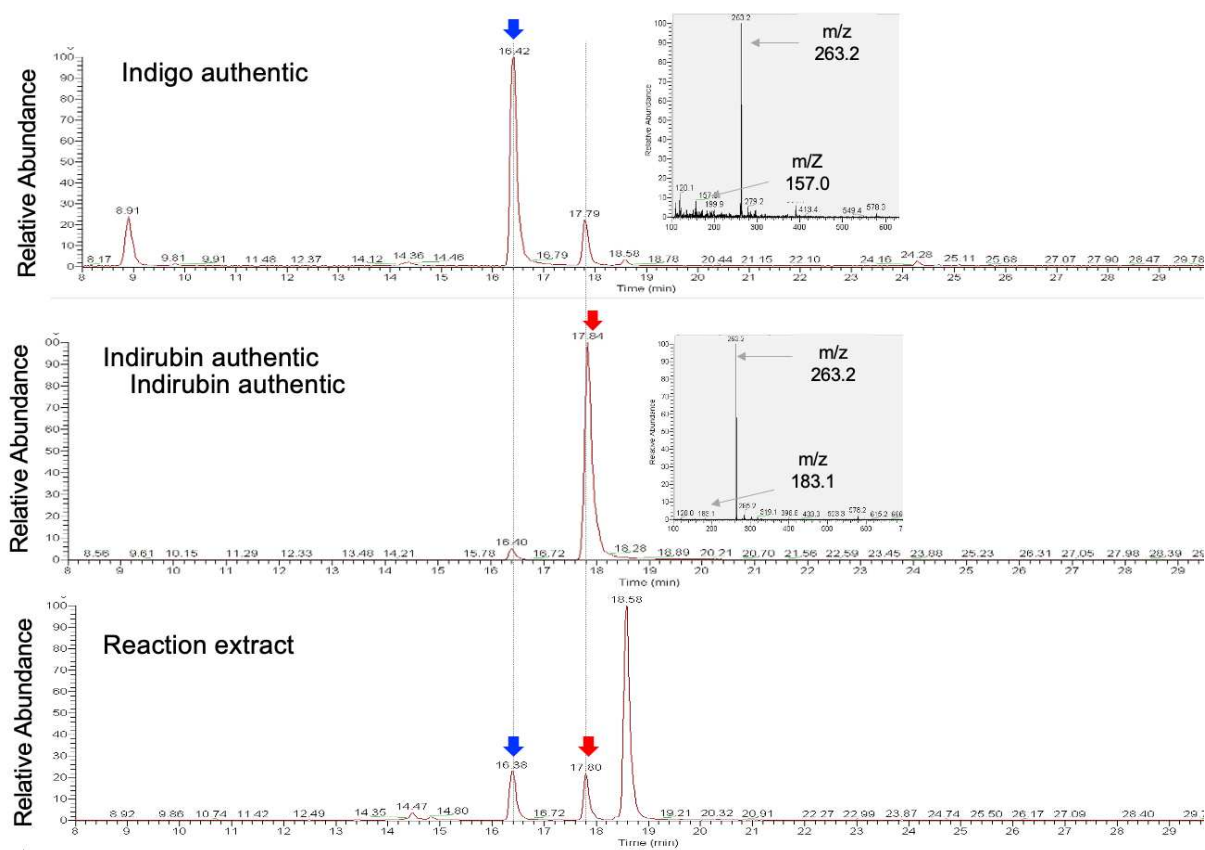
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Figure 3

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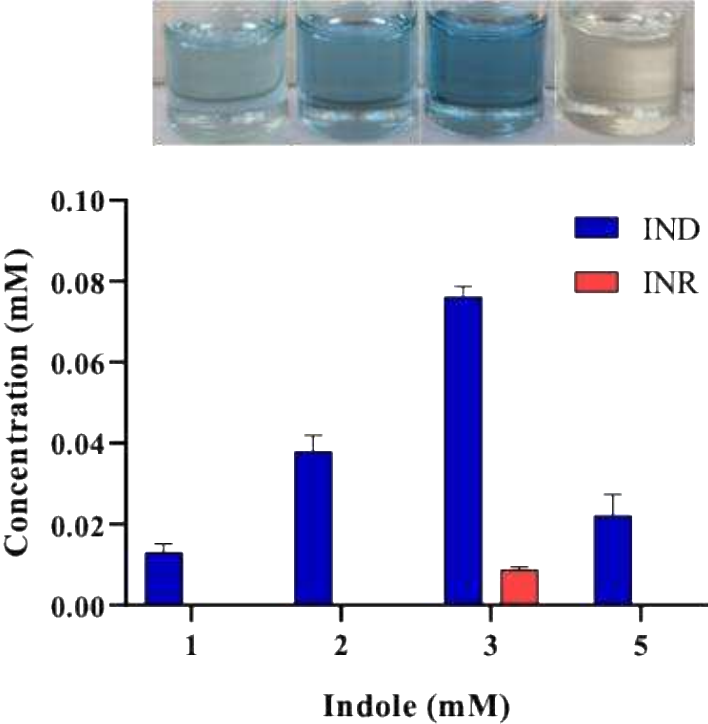
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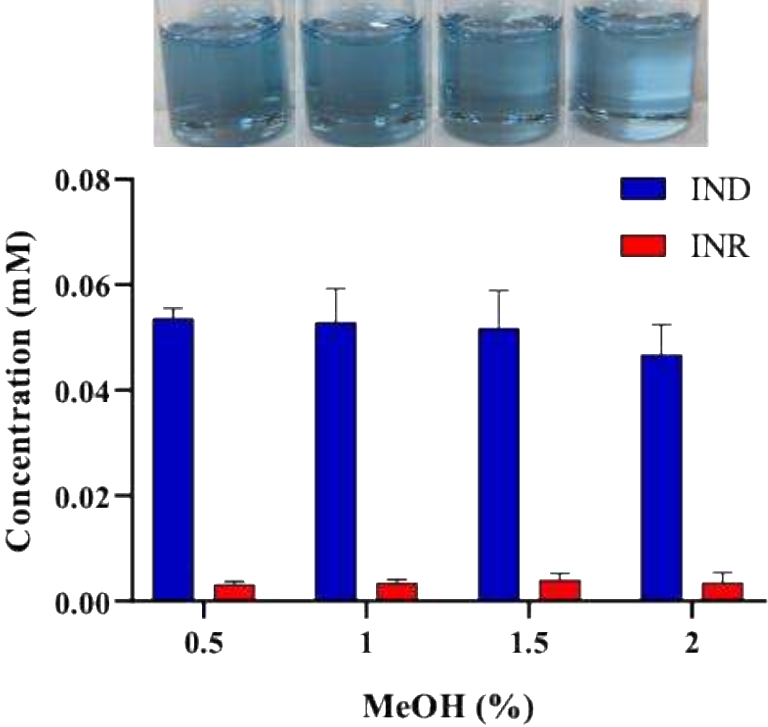
639 **Figure 4**

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641 (A) Effect of indole concentration on IND and INR production.

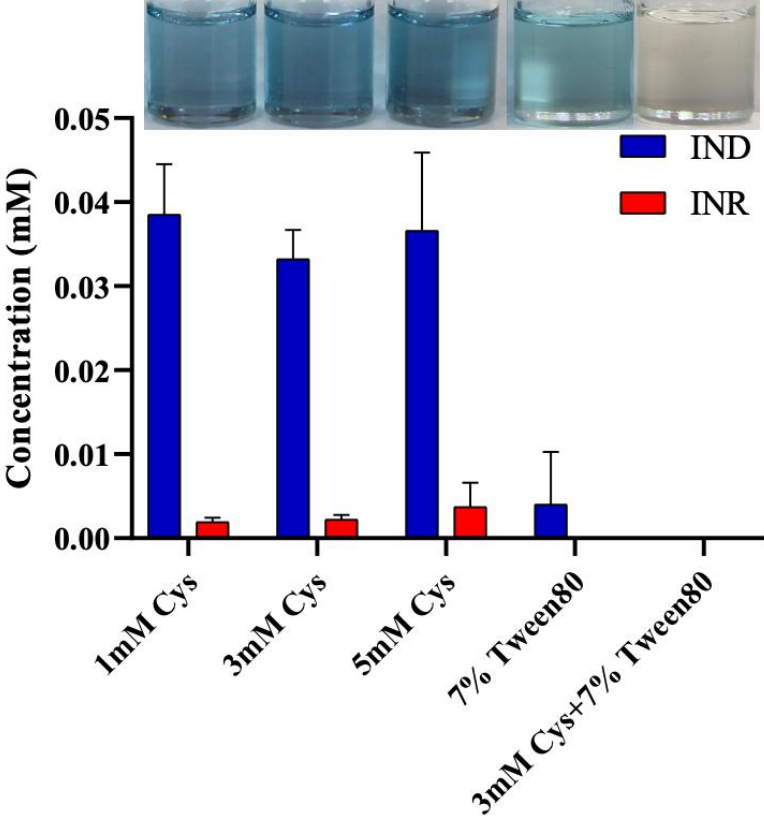


642 (B) Effect of methanol (MeOH) concentration on IND and INR production.
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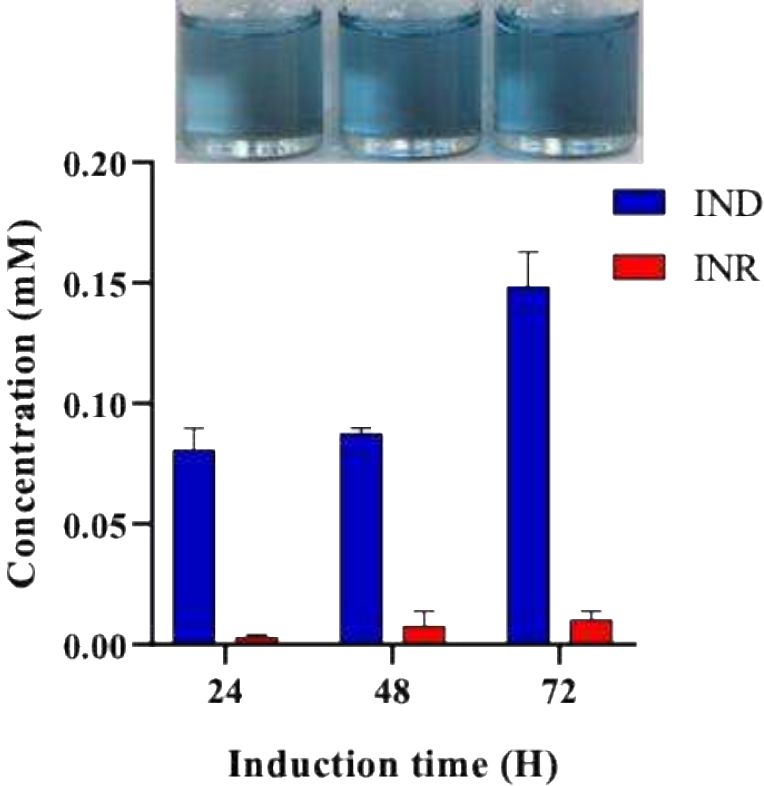


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647 (C) Effects of L-cysteine and Tween 80 concentrations on IND and INR production.



648 (D) Effect of induction time on IND and INR production.



650 (E) Effect of agitation speed on IND and INR production.

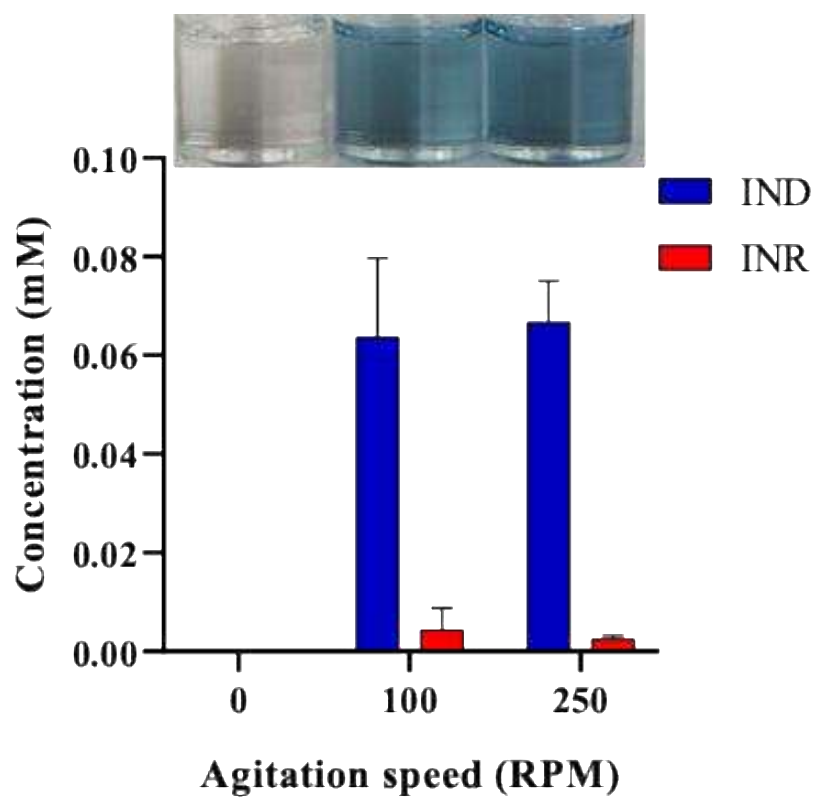
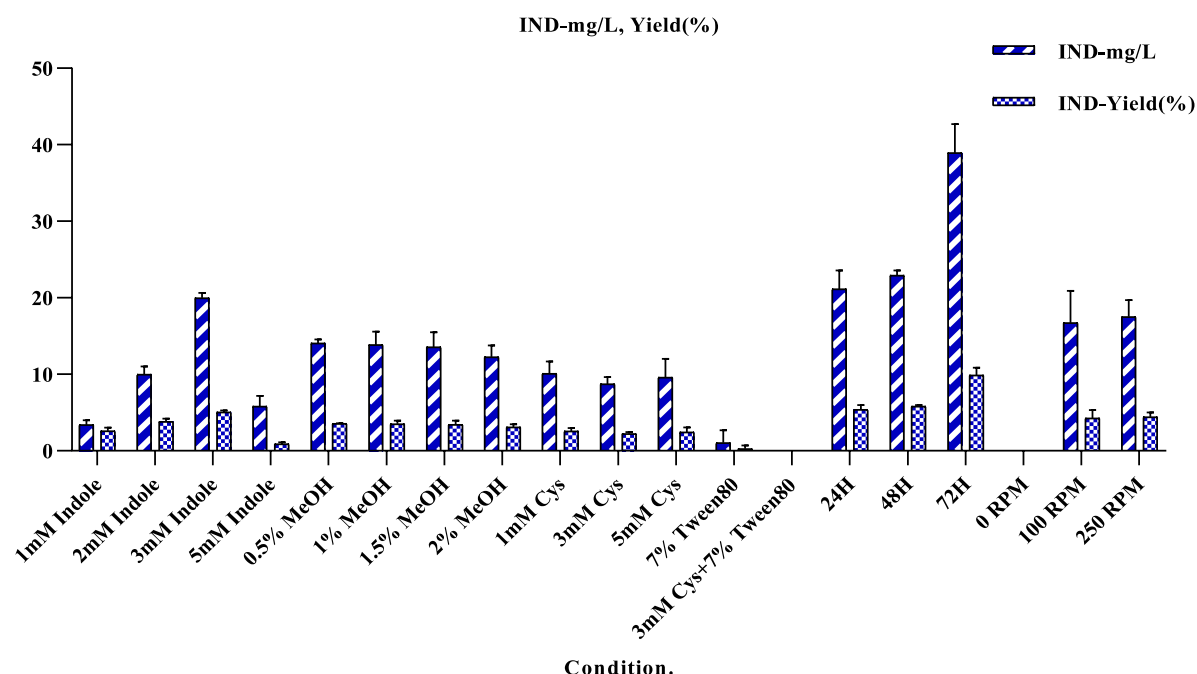


Figure 5

(A) IND titer (mg/L) and yield (%) in *K. phaffii* GS115 pPIC9::MaFMO cultures under various process conditions, including indole concentration, methanol concentration, L-cysteine and Tween 80 supplementation, induction time, and agitation speed.



(B) INR titer (mg/L) and yield (%) in *K. phaffii* GS115 pPIC9::MaFMO cultures under the same process conditions.

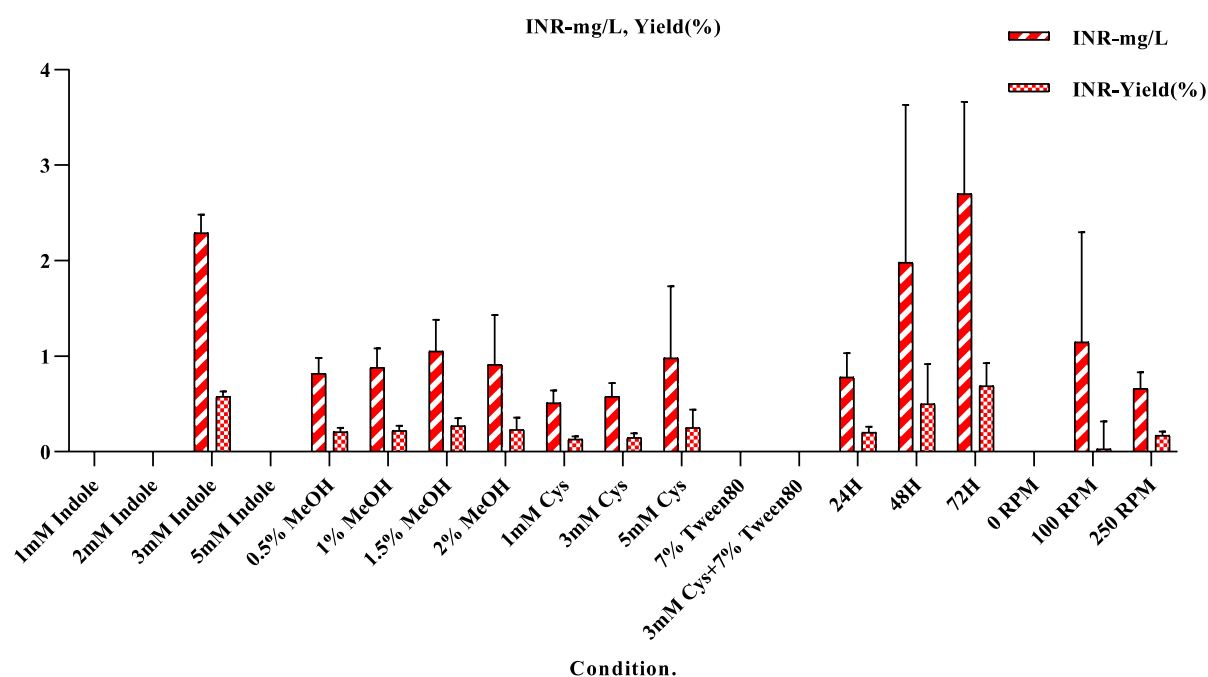


Figure 6