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Optimizing Heterologous Expression of CYP124 in *Escherichia coli* using Box-Behnken Design

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Abstract

Introduction: CYP124 is a cytochrome P450 enzyme from *Mycobacterium tuberculosis* capable of catalyzing ω-hydroxylation of methyl-branched lipids, isoprenoid alcohols, sterols, cholesterol analogs, and vitamin D3. It is also suspected to be involved in the bacterium's resistance to macrophages. Due to its capabilities, CYP124 has potential as a drug target and biocatalyst. Therefore, there is a need to produce sufficient quantities of CYP124 for biochemical assays, protein engineering, and drug development. The study optimized CYP124 production in *Escherichia coli* DH5α using Response Surface Methodology (RSM) with a Box-Behnken Design (BBD).

Materials and Methods: $FeCl_3$, δ-aminolevulinic acid (5-ALA), and IPTG concentrations were optimized using BBD. A total of 15 experimental runs with various factor combinations were performed, and a three-dimensional response surface was generated to analyze the effects and identify the optimal concentrations of each factor, with R/Z value and CYP124 yield as responses.

Results: The optimal supplement combination for CYP124 production included 0.10 mM $FeCl_3$, 1 mM 5-ALA, and 0.2427 mM IPTG. The model predicted the highest CYP124 yield to be 0.041 mg/L. Purified CYP124 bound to farnesol, its substrate, with a dissociation constant of 1.00 ± 0.14 μM.

Conclusions: The findings underscore the significance of ALA supplementation in regulating CYP450 expression and emphasize the need to optimize $FeCl_3$ and IPTG concentrations as cofactors and inducers, respectively.

Keywords: Cytochrome P450, Medium Supplements, Response Surface Methodology, *Mycobacterium tuberculosis*

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Introduction

Cytochrome P450s (CYP450) are heme-containing enzymes involved in various metabolisms of xenobiotics and biosynthesis of metabolites through reactions that essentially convert lipophilic to hydrophilic compounds.[1-5] CYP124 of *Mycobacterium tuberculosis* is known for its ability to catalyze the ω-hydroxylation reaction of methyl-branched lipids, including isoprenoid alcohols, sterols, cholesterol analogs, and vitamin D3.[1,6-9] There is another source of CYP124 from *Mycobacterium marinum*, but the variant is less efficient at binding and oxidizing cholesterol.[6]

Though the essential role is still unknown, CYP124 is presumed to implicate the resistance of *M. tuberculosis* defense against the human immune system, particularly during its residence in macrophages.[7,10] In addition to its role in macrophage survival, this CYP is supposed to have a role against xenobiotics exposed to the bacterium.[10] As one example, SQ109, an analog of ethambutol, is subject to hydroxylation catalyzed by the enzyme into its monohydroxylated derivative, which has better binding to mycobacterial protein Large 3 (MmpL3) compared to its parent compound.[11,12] CYP124 can also metabolize 20S-hydroxycholesterol, which plays a role in the Hedgehog signaling pathway-activated response to the pathogen.[8] Given its potential as a biocatalyst and drug target, large quantities of CYP124 are required for biochemical assays and drug development.

The availability of cofactors such as iron and 5-aminolevulinic acid (5-ALA), as well as the level of inducer (such as IPTG) play essential roles in the expression of CYP450.[13-15] 5-ALA and $FeCl_3$ provide the precursor for heme formation.[14] Hence, the addition of those supplements would be necessary to ensure the proper CYP conformation and functionality.[14,16-20]

Optimization of CYP124 protein expression was designed using Box-Behnken Design (BBD) with a variation in $FeCl_3$, δ-aminolevulinic acid (5-ALA), and IPTG concentration in addition to terrific broth (TB) medium. This approach helps

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optimize CYP124 expression based on several factors simultaneously. To ensure the functionality and characteristics of CYP124, a binding assay with farnesol as the substrate was conducted on the purified protein.

Materials and Methods

Microorganism and Plasmid

The synthetic gene of *cyp124* (GenScript, USA), adapted from NCBI with protein sequence NP_216782.1, was inserted into the pCWORI expression vector using the circular polymerase extension cloning (CPEC) method.[21] Primers were designed to add flanking regions to the *cyp124* gene and linearize the pCWORI vector to remove A13AMO-aaCPRct,[22] as shown in Table 1. The pCWORI construct containing the *cyp124* gene (referred to as pCWORI-cyp124) was transformed into *E. coli* DH5α and confirmed by sequencing using a set of primers listed in Table 1. The results of the circular polymerase extension cloning are provided in the Supplementary Data.

Small Scale Expression

A single colony of *E. coli* containing pCWORI-cyp124 was inoculated into Luria-Bertani (LB) broth supplemented with ampicillin (Sigma-Aldrich, USA) (100 µg/ml) and incubated at 37 °C overnight. Then, 2.5 ml of this culture was used to inoculate 50 ml of Terrific Broth (TB) with ampicillin (100 µg/ml) in a 250 ml flask. The culture was incubated at 37 °C and 210 rpm until OD600 reached 0.8 for induction. Then, $FeCl_3$ and 5-ALA (Sigma-Aldrich, USA), and IPTG (Promega, USA), were added during the induction period. The culture was further incubated for 48 hours at 25 °C and 130 rpm.

Table 1. List of Primers

Primer	Nucleotide Sequence	Function
F-Lin-pCWORI	5′–GTC GAC CTG CAG CCC AAG CTT AT–3′	Forward primer to linearize pCWORI vector
R-Lin-pCWORI	5′–ATG ACC TCC TAA GCA TCG ATG GAT CC–3′	Reverse primer to linearize pCWORI vector
F-cyp124-pCWORI-CPEC	5′–CCA TCG ATG CTT AGG AGG TCA TAT GGG CCA TCA TCA CCA TCA CCA C–3′	Forward primer to added flanking on *cyp124* gene
R-cyp124-pCWORI-CPEC	5′–GAT AAG CTT GGG CTG CAG GTC GAC TTA TGA CCA TGT AAC CGG GAG TGT TTT AAT TC–3′	Reverse primer to added flanking on *cyp124* gene
F-seqpCWORI	5′–CGA TCC ATC GAT GAG CTT AC– 3′	Forward primer for pCWORI-cyp124 sequencing
R-seqpCWORI	5′–GGC CCT TTC GTC TTC AAG C– 3′	Reverse primer for pCWORI-cyp124 sequencing

Table 2. Independent Factor Used in Box-Behnken Design

Factor	Level		
	-1	0	+1
$FeCl_3$	0.1 mM	0.55 mM	1 mM
IPTG	0.1 mM	0.55 mM	1 mM
5-ALA	0.1 mM	0.55 mM	1 mM

Experimental Design and Statistical Analysis

To determine the optimal culture media for heterologous CYP124 expression, the Box-Behnken design was employed, using CYP124 mass and R/Z value as the response variables. The mass of CYP124 was determined by comparing the intensity of its band to that of BSA on the SDS-PAGE gel. Meanwhile, the R/Z value was calculated from the ratio of absorbance at 420 nm to 280 nm, which indicates the purity of CYP.[23-25] A design matrix with 15 experimental runs was generated using the software Minitab® 21 (Minitab, Inc.). The polynomial equation was generated from the Box–Behnken experimental design. Each experiment was performed under different $FeCl_3$, 5-ALA, and IPTG concentrations, as described in Table 2.

Purification and Protein Quantification

The expression culture was centrifuged at 3750 rpm for 20 minutes to obtain a cell pellet. The cell pellet was resuspended in 6 ml of lysis buffer containing LEW buffer, cOmplete® protease inhibitor (Roche, Germany), and 25 mM imidazole (Sigma-Aldrich, Germany), followed by cell lysis using an ultrasonicator with a pulse of 15:15 and 60% amplitude for 20 minutes. The supernatant was collected by centrifugation at 10,000 g for 15 minutes. During the purification step, 200 µl of Ni-NTA resin (GenScript, USA) slurry was used and incubated with 4 mL of supernatant for 4 hours, then washed with 1 ml of LEW buffer 5 times, followed by five washes, each with 500 µl of 25 mM imidazole. Elution was performed four times, each with 500 µl of 75 mM imidazole. Elution samples were checked using a UV-Vis spectrophotometer at 280 nm and 420 nm. The samples were also run on SDS-PAGE under reducing conditions. The intensity of the CYP124 band was compared to the bovine serum albumin (BSA) (Sigma-Aldrich, USA) band, whose concentration is known, and calculated using ImageJ software.

CYP124 Characterization and Binding Assay

CYP450 characterization can be done using UV-Vis spectrophotometry. Purified CYP450 is in a low-spin ferric state, which presents an absorbance peak at 418-421 nm.[7,26] Under reduced conditions, when sodium dithionite (Sigma-Aldrich, USA) is added, CYP450 should produce a Soret band at 450 nm after binding to carbon monoxide (CO). Purified CYP124 was titrated with farnesol as the substrate for the binding assay, with concentrations ranging from 0.5 mM to 80 mM. Absorbance at 390 nm and 420 nm was recorded, and a curve was plotted between the absorbance difference of 390 and 420 nm against the substrate concentration. The dissociation constant (Kd) was determined by non-linear regression and fitted to the hyperbolic function:

$$\Delta Absorbance\ (390\ nm - 420\ nm) = Absorbance\ max\ \times \frac{Farnesol\ concentration}{(K_d + Farnesol\ concentration)}.$$

Absorbance max refers to the maximum absorbance difference at 390 nm and 420 nm (peak to trough difference).

Statistical Analysis

Results from the binding study are expressed as mean ± SEM. Data of CYP124 mass from the SDS PAGE and R/Z value were further analyzed by one-way ANOVA. The value of p less than 5% ($p<0.05$) was considered statistically significant.

Results

Experimental Design and Modelling for CYP124 Production

CYP124 expression was initially carried out in TB medium supplemented with 1 mM ALA, 0.1 mM $FeCl_3$, and 0.55 mM IPTG.[26] The expression yielded 0.017 mg/L of CYP124 in the medium. In order to determine the optimal levels of these three different supplements simultaneously, we utilized experimental design methodology. The Box-Behnken method has been successfully employed to optimize protein overproduction in *E. coli*.[27,28] The experimental design followed the Box-Behnken method with three factors at three different levels. Each factor had concentrations of 0.1 mM, 0.55 mM, and 1 mM. This experiment aimed to establish a mathematical model for the optimum conditions of CYP124 expression in response to various ALA, $FeCl_3$, and IPTG concentrations. The experimental design used in this study included a total of 15 runs, incorporating triplicate center points (Table 3). The typical absorption peak at 450 nm, following CO binding under reducing conditions, was recorded from all lysates derived from expression cultures with different supplement compositions.

CYP Mass and R/Z value were set as two response variables. Results from 15 sets combinations of 5-ALA, IPTG and $FeCl_3$ provided models with the following two quadratic equations:

CYP Mass (ng) = 216.7 – 73 $FeCl_3$ + 115 IPTG + 160 5-ALA –52$FeCl_3$.$FeCl_3$ –101IPTG.IPTG +225-ALA.5-ALA +242$FeCl_3$.IPTG –75$FeCl_3$.5-ALA -316IPTG.5-ALA

R/Z = 0.4590 + 0.245$FeCl_3$ + 0.001IPTG – 0.0175-ALA – 0.077 $FeCl_3$.$FeCl_3$ –0.031 IPTG.IPTG +0.119 5-ALA.5-ALA –0.028 $FeCl_3$.IPTG –0.190$FeCl_3$.5-ALA +0.182 IPTG.5-ALA

Table 3. Experimental Result for CYP124 Expression

Experiment	$FeCl_3$ (mM)	IPTG (mM)	5-ALA (mM)	R/Z	CYP Mass (ng)*	A450 (nm)
1	0	+1	-1	0.53	295.09	0.133
2	+1	0	-1	0.55	270.51	0.132
3	0	0	0	0.60	271.03	0.134
4	+1	0	+1	0.60	176.98	0.115
5	-1	+1	0	0.55	142.33	0.094
6	0	-1	+1	0.59	287.05	0.143
7	-1	0	-1	0.50	266.71	0.145
8	-1	-1	0	0.47	324.24	0.133
9	0	+1	+1	0.68	175.78	0.096
10	0	0	0	0.56	205.45	0.157
11	-1	0	+1	0.70	234.22	0.200
12	+1	-1	0	0.57	183.64	0.183
13	0	0	0	0.57	253.09	0.110
14	+1	+1	0	0.64	198.08	0.093
15	0	-1	-1	0.58	150.60	0.122

*CYP mass was calculated based on intensity of the band compared to intensity of BSA band which concentration is approximated using ImageJ.

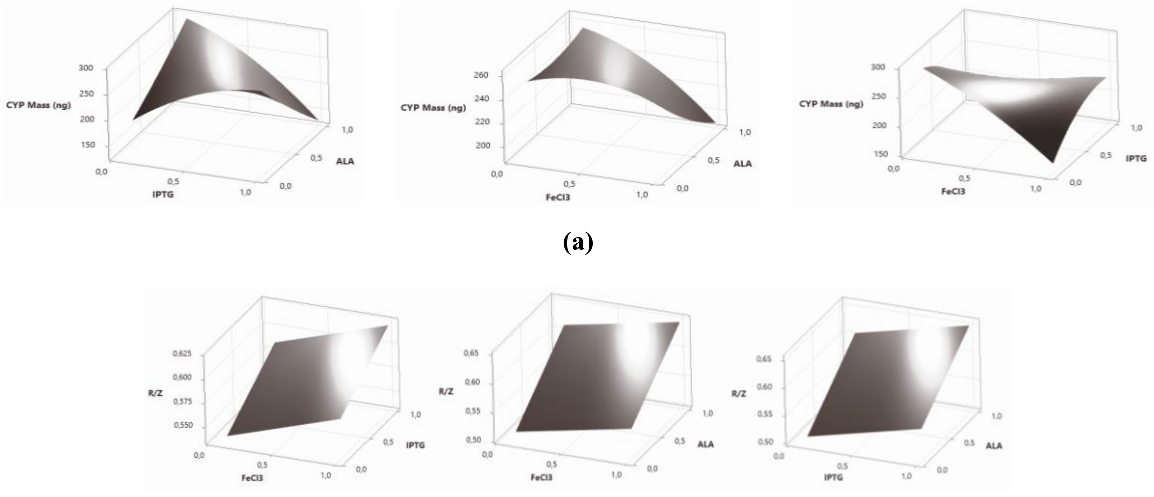
Table 4. p-value for CYP124 Mass and R/Z

Factor	CYP124 Mass *p-value*	R/Z *p-value*
$FeCl_3$	0.324	0.301
IPTG	0.337	0.204
5-ALA	0.429	0.027*
$FeCl_3$.$FeCl_3$	0.669	0.539
IPTG.IPTG	0.418	0.804
5-ALA.5-ALA	0.855	0.355
$FeCl_3$.IPTG	0.080	0.812
$FeCl_3$.5-ALA	0.526	0.151
IPTG.5-ALA	0.036*	0.164
Lack-of-Fit	0.323	0.138

*significant at $p<0.05$

Data of p-values and lack of fit for CYP124 mass and R/Z are shown in Table 4. The interaction between IPTG and 5-ALA had a substantial effect on CYP124 mass, while for the R/Z value, 5-ALA had a significant impact. Figure 1 illustrates the interaction plot depicting the influence of three factors on CYP124 mass and R/Z. While the increase in 5-ALA proportionately improved CYP124 mass, the increase in $FeCl_3$ and IPTG caused negative effects. On the other hand, the R/Z value enhanced as the concentration of the three factors increased.

The response optimizer was used to optimize the CYP124 mass and R/Z value. A high R/Z value indicates high purity of cytochrome P450, as it is calculated from the ratio of absorbance at 420 nm compared to 280 nm, with a value around 1 being preferable.[29,30] However, based on the response surface plot, increasing the R/Z value might lead to a decrease in CYP124 mass. Therefore, the targets for CYP124 mass and R/Z value were set at 335 ng and 0.61, respectively. The results of the response optimizer are shown in Figure 2.



(a)

(b)

Figure 1. Surface Plot of Interaction between 2 Combination Factors: $FeCl_3$ and IPTG, $FeCl_3$ and 5-ALA, IPTG and 5-ALA for (a) CYP124 mass and (b) R/Z.

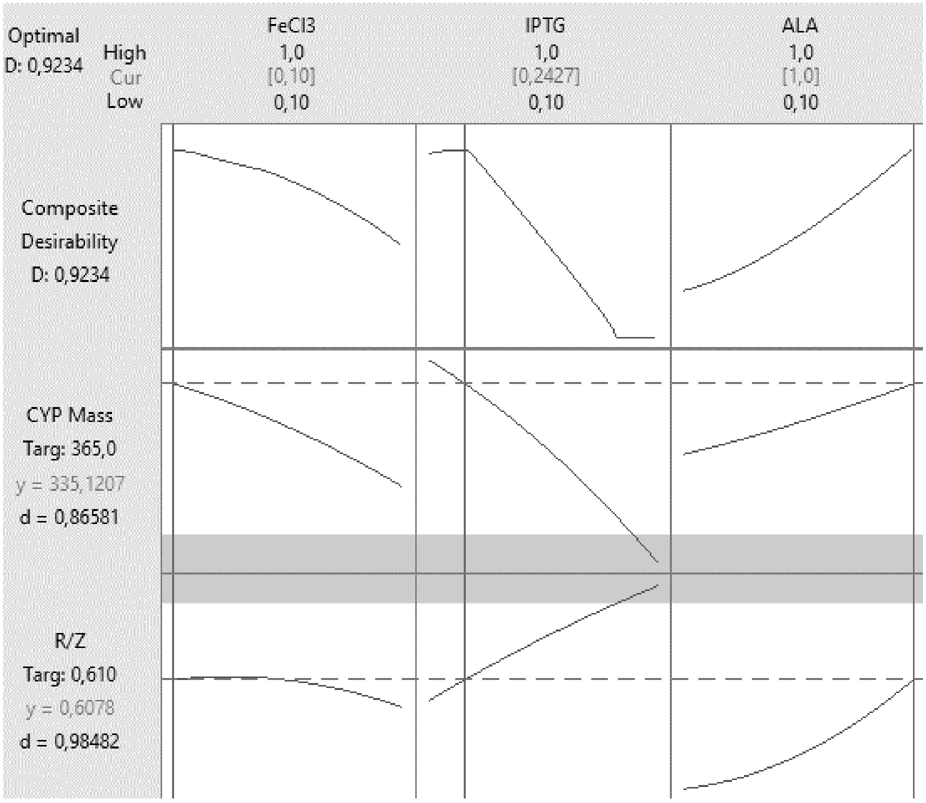


Figure 2. Response Optimizer Result.

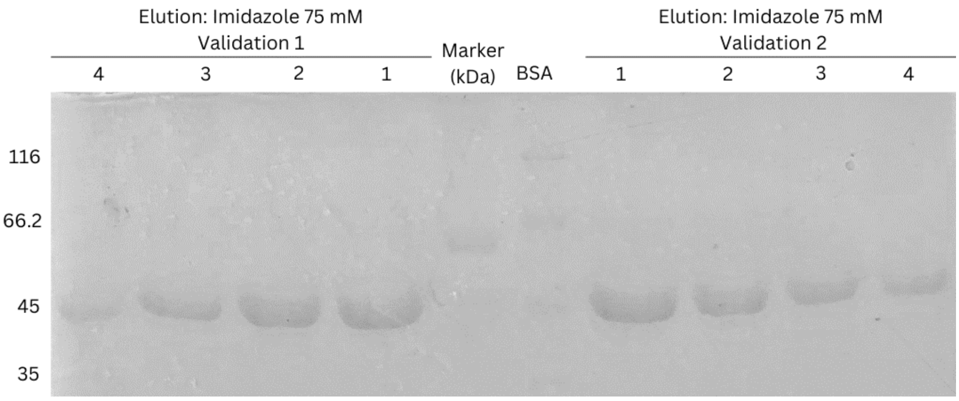


Figure 3. SDS-PAGE Purified CYP124 from Validation Experiment.

Table 5. Validation Result of Box-Behnken Design

Repetition	Prediction		Actual		Error	
	R/Z	CYP Mass (ng)	R/Z	CYP Mass (ng)	R/Z	CYP Mass (ng)
1	0.61	335.12	0.61	339.95	0.03%	1.44%
2			0.61	333.56	0.30%	0.47%

A set expression with the optimized medium composition based on the CYP mass and R/Z equations was conducted to validate the mathematical model. The supernatant from the validation sample contained CYP124, which was confirmed by the presence of absorbance at 450 nm. The purified CYP124, obtained through four elutions using a 75 mM imidazole concentration, was run on SDS-PAGE and showed a single band at around 47.8 kDa (Figure 3). The results from the validation of the mathematical model are shown in Table 5. The CYP124 mass obtained from two validation experiments was 339.95 ng and 333.56 ng. Thus, the mathematical model could forecast the optimum CYP124 yield with 0.033%-1.441% error. The CYP124 yield obtained from this validation experiment was 0.041 mg/L medium, approximately 1.41 folds higher compared to the initial condition.

A binding assay was conducted to confirm the functionality and specificity of CYP124. Farnesol was chosen due to its high affinity for the enzyme. The assay showed that purified CYP124 could bind with farnesol with a dissociation constant of 1.00 ± 0.14 µM (Figure 4).

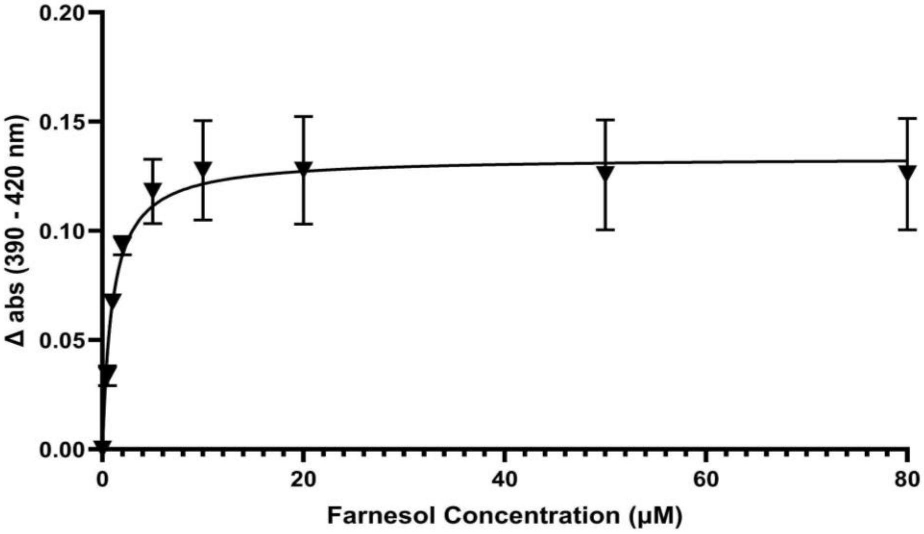


Figure 4. Binding Profile of Purified CYP124 Toward Farnesol. The result is expressed as the mean ± standard error of the mean (SEM) (n = 3).

Discussion

CYP124, a member of the CYP450 family found in *Mycobacterium tuberculosis*, is believed to contribute not only to host-pathogen interactions but also to the metabolism of methyl-branched lipids.[7] Notably, CYP124 is hypothesized to activate SQ109 into a potent antimicrobial agent against *M. tuberculosis*.[12] Beyond its physiological role, CYP124 exhibits intriguing biocatalytic potential, particularly toward methyl-branched fatty acids and isoprenoids such as farnesol, farnesyl diphosphate (FDP), and steroids.[7] To fully harness its potential, efficient heterologous expression of CYP124 in *E. coli* is crucial. While bacterial CYP450s generally pose fewer expression challenges compared to their eukaryotic counterparts,[31,32] optimization is necessary to achieve high yields and functional protein.

The design of experiments provides an efficient and effective way to optimize protein expression.[33,34] Currently, BBD is widely employed to forecast and enhance optimization power that includes several variables simultaneously in various sectors of research.[34,35] Here, we employed BBD to find the optimum medium supplement composition for CYP124 expression in *E. coli*. The calculated *p*-value result was less than 0.05,[36] indicating that the assigned factors significantly influenced the expression of CYP124. Based on the p-value, it can be concluded that the interaction between IPTG and 5-ALA concentration significantly affected CYP124 mass. Meanwhile, 5-ALA concentration significantly affected R/Z ratio reflecting the abundance of CYP124 compared to the total protein. The lack of fit value indicated a non-significant result, which means that the equation is appropriate.[37]

While the 5-ALA concentration correlated positively with the increase in CYP124 mass and R/Z value, the $FeCl_3$ concentration was only proportional to the rise in CYP124 mass and its R/Z value at a minimum level. This indicates that the iron supply was sufficient and that a higher concentration of $FeCl_3$ might lead to negative feedback, reducing the protein concentration.[16] This finding is consistent with a study showing that the addition of 5-ALA up to 1 mM can enhance the expression of CYP450.[38]

Expression of heterologous protein is usually hampered by improper folding and cofactor integration.[39-41] This issue can be mitigated by expressing the protein at lower temperatures with an optimal inducer concentration.[14,42] IPTG plays an important role in increasing protein expression, with its transcription process controlled by the lac operator, as seen in pCWORI.[13,14,31] In our experience, instead of increasing CYP124 expression, a higher concentration of IPTG reduced the protein's mass. However, excessive addition of IPTG can also cause toxicity in *E. coli*.[15,43,44] According to a study by Uchida et al.,[38] adding IPTG concentrations above 0.5 mM led to a decrease in CYP450 expression.

A binding assay with farnesol was performed to characterize the purified CYP124. The dissociation constant of purified CYP124 with farnesol in this study is comparable to that in another study.[7] Therefore, it can be concluded that purified CYP124 retains its biochemical affinity for one of its substrates.

Conclusion

In the present work, Response Surface Methodology (RSM) was applied to evaluate the effect of three fermentation variables. The optimal condition for CYP124 expression was successfully determined by RSM using a Box-Behnken design. From the validation result, 0.1 mM $FeCl_3$, 1 mM 5-ALA, and 0.2427 mM IPTG were the optimum medium composition for CYP124 expression, resulting in 0.041 mg/L of the protein. The purified protein was able to bind to farnesol as a substrate at a Kd of 1.00 ± 0.14 μM, confirming the characteristic of CYP124.

Authors' Contributions

HP: Conceptualization, Methodology, Original draft preparation, Manuscript review and editing, Funding acquisition, Supervision; RAU: Methodology, Supervision, Manuscript review, and Editing; CMG & MHN: Perform research, Data analysis, Write and revise the manuscript. All the authors read and approved the final manuscript for submission.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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