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**| RESEARCH ARTICLE**

# Effect of Temperature on Coenzymes Associated with Cytochrome P450

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**| ABSTRACT**
Cytochrome P450 are heme-containing enzymes that have been deemed to be the most active group of monooxygenases reaction. It is a self-sufficient protein whose activities rely on the various associated coenzymes. Expression of the catalytic module of cytochrome P450 gene (CYP102A2) containing pET-28a in *Escherichia coli* BL21 (DE3) across temperatures ranging from 19 ºC to 37 ºC. The solubility of the expressed recombinant proteins on SDS-PAGE showed that the recombinant protein is both secreted and membrane-bound protein across all the temperature treatments. The highest expressed recombinant protein of interest (P.O.I.) band observed in lane 15 was membrane-bound and was optimum at 28 ºC, while the most prominent expressed P.O.I. bands in lane 16 from the secreted proteins was optimum at 30 ºC. The estimated flavin adenine dinucleotide (F.A.D.) and flavin mononucleotide (FMN) showed optimum concentrations of 15 mM and 14 mM respectively at the optimum temperature of 25 ºC, while the estimated heme optimum concentration (2.4 mM) was recorded at the optimum temperature of 35 ºC. These observations showed that it is little or no connection between the quantity of the expressed recombinant cytochrome P450 and the estimated coenzymes and cofactors concentrations in response to the temperature parameter. The enzymatic activity of the recombinant P.O.I. at a selected temperature of 25 ºC and 35 ºC showed exponential slope within the period of 60 seconds at 550 nm wavelength of reaction; however, a wide range of absorbance ranging from 0.1 A to 0.7 A and 0. 689 A - 0.998 A was recorded, respectively. The increase in activity may also be attributed to the fact that higher temperature tends to support an increase in the rate of reactions, hence the rate of monoxygenation.

**| KEYWORDS**

Cytochrome P450, flavin mononucleotide (FMN), a flavin adenine dinucleotide (F.A.D.)

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## 1. Introduction

Cytochrome P450 are heme-containing enzymes that catalyze the oxidation of many endogenous and exogenous compounds such as drugs, carcinogens and xenobiotic chemicals and have been deemed to be the most active group of monooxygenases recognized (White-house *et al*., 2008; White-house *et al*., 2012). They have been studied to be widely distributed in all forms of life, including prokaryotes and eukaryotes (Pflug *et al*., 2007) hence regarded as generally recognized as safe status (GRAS). It has been studied to have two major domains; the catalytic domain, where substrate oxidation processes take place and the diflavin reductase domain, which is involved in the electron transport chain. This has given it a wide range of ability of catalysis that enabled it to alter and manipulate the region and stereo structure of both natural products and xenobiotics substrate. Some of these processes range from hydroxylation, dealkylation, epoxidation, oxidation, dehalogenation, dehydrogenation and reduction (Nguyen *et al*., 2020). Hence cytochrome P450 monooxygenases have shown promiscuity for a wide range of substrates, thanks to the auxiliary redox partners such as flavoenzymes or iron-sulfur proteins that are often co-expressed to obtain electrons from N.A.D. (P)H (Hannemann *et al.,* 2007; Lamb and Waterman, 2013) This makes it a self-sufficient protein which has two major classes N-terminal P450 heme domain is connected to a C-terminal NADPH cytochrome P450 reductase (C.P.R.) that enclosed a flavin mononucleotide (FMN)- binding flavodoxin and a flavin adenine dinucleotide FAD/NADPH binding domain, hence the electron transport chain in these P450s is NADPH→FAD→FMN→heme (Zhang *et al*., 2020).

The movement of the electrons from the cytochrome P450 reductase NADPH through F.A.D. and FMN as the cofactors which receives and subsequently transfer the electrons in a cascade and stepwise manner are eventually received by the heme center where the activation of the molecular oxygen takes place, leading to the insertion into the organic substrates to form water (Paine *et al*., 2005; Pflug *et al*., 2007). Therefore, the coenzymes play a vital role in the activities of cytochrome P450. Liu and Kong (2017) studied the response of cytochrome P450 monooxygenase from *Bacillus megaterium* BM3 mutant 139-3 hydroxylation activity to thermal variation and studied the activity over a broad temperature range from $4^0$C to $50^0$C where optimum hydroxylation was observed at 37 $^0$C with a drastic decline in the activity beyond this temperature. This present research focuses on the optimization of a fed-batch process for increased production cytochrome P450 monooxygenase CYP102A2 from the strain *Bacillus megaterium* expressed in *E. coli.* Little information is available on the expression and quantification of this protein and its associated coenzymes. The temperature was used as a parameter for optimizing the output and quality of the CYP102A2. Hence this research is aimed to exploit the response of coenzymes associated with cytochrome P450 to temperature.

## 2. Materials and methods

### *2.1 Transformation of E. coli BL21 (DE3) with the catalytic module of cytochrome P450 gene (CYP102A2) containing pET-28a*

Transformation of recombinant *Escherichia coli* BL21 (DE3) to pick up the recombinant pET28a carrying CYP102A2 was carried out following the descriptions of (Das and Dash, 2015; Ge *et al*. 2018). The recombinant organism was cultured on auto-induction medium Luria Bertani broth (L.B.) for 18 hours at 37 $^0$C with the addition of 1M IPTG to induce protein expression. The recombinant organism was lysed and the expressed proteins extracted via centrifuge at 24,000 x g for 30 minutes at 4ºC in a Sorvall centrifuge with a rotor SS-34 (15,000 rpm) to separate the lysate pellets proteins.

### *2.2 Molecular weight estimation of CYP450 protein from isolates and recombinant point of interest (P.O.I.)*

The secretory and membrane-bound protein molecular weight of the crude extracts from the recombinant cytochrome P450 proteins was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis SDS-PAGE as described by Ge *et al*. (2018**)**. To the electrophoresis wells, equal volumes 20 µl of 1 x S.D.S. and test sample preheated at 100 ºC in a test tube for 30 minutes and marker (17.671 – 103.142 kDa) respectively were loaded in the gel. The gel was then run in 100 V for 5 h, after which it was stained with silver stain for secretory and membrane-bound proteins, respectively, for easy distinction. The apparent molecular weights of the samples were determined by comparison with the mobility of the standard weight protein markers.

### *2.3 Cytochrome P450 and corresponding cofactor estimation*

The cytochrome P450 estimation was carried out following the description of Guengerich *et al*. (2009). A cytochrome reduction assay used in this evaluation was the 200 mM imidazole fraction from the 30 °C samples of L.B. media. A 2.0 ml of 100 mM of potassium phosphate buffer (pH 7.4–7.7) containing 1.0 mM of EDTA, 20% glycerol (vol/vol), 0.5% sodium cholate (wt/vol) and 0.4% (wt/vol) non-ionic Triton N-101) was used to dilute the partially purified expressed recombinant CYP450 in a small test tube. This was mixed homogeneously in an airtight capped test tube with paraffin by inverting and reinverting 10 times. The sample was divided into two 1 ml glass cuvettes. The two cuvettes were placed in the spectrophotometer (Jenway 6300 VRS), and baselines were recorded between 400 and 500 nm. The sample cuvette was removed from the spectrophotometer, and carbon monoxide bubbled into the samples cuvette containing the recombinant protein CYP450 using Pasteur pipette. One milligram of solid $Na_2S_2O_4$ and dithionite was added to both cuvettes each and was mixed by inverting and reinverting the capped test tube to dissolve $Na_2S_2O_4$ without vigorous shaking. The mean absorbance was read and recorded across the spectrum from 400 to 500 nm. The absorbance at 450 and 490 nm was used to estimate and evaluate the cytochrome P450 concentration using the following formula to calculate (ΔA450 − ΔA490)/0.091 = nmol of P450 per ml). The heme, flavin adenodinucleotides (FAD) and flavinmononucleotides (FMN) concentration (mM) were estimated using Beer-lambert's principles, A = εCl (Where, A= absorbance; ε=molar extinction coefficient; C=concentration; l = path length) (Extinction coefficients: Heme, ε at 425 = 154 $mM^{-1}cm^{-1}$; FAD, ε at 450 = 11.3 $mM^{-1}cm^{-1}$; FMN, ε at 450 = 12.2 $mM^{-1}cm^{-1}$, ε for CYP450 = 0.091 $mM^{-1}cm^{-1}$). A biochemical assay was used to determine the spectrophotometric rate of F.A.D. and FMN due to the high absorbance of oxidized heme near 450 nm, masking the absorbance of F.A.D. and FMN.

### *2.4 Enzymatic activity of the recombinant CYP450 protein and the crude protein extract from the selected isolates from soil polluted with crude oil*

The nicotinamide adenine diphosphate hydrogen (NADPH) –cytochrome c reduction activity was estimated as described by Guengerich *et al*. (2009). An aliquout of 80 µl of a 0.5 mM solution of the horse heart cytochrome c (in 10 mM of potassium phosphate buffer, pH 7.7) was pipetted into a 1-ml of glass cuvette with the path length of 10 mm. A 1 µg and 10 µl of the partially purified recombinant CYP450 and 10-mM NADPH solution were added respectively into the glass cuvette containing the cytochrome c and properly capped and homogeneously mixed by inverting and reinverting. The absorbance was recorded at an absorbance of 550 nm in a spectrophotometer (Jenway 6300 VRS) for a period of 3 minutes 30 seconds (210 seconds). The NADPH–cytochrome *c* reduction activity was estimated via the formula

$$\frac{\Delta A_{550}/\text{min}}{0.021}$$ = nmol of cytochrome c reduced per min

**3. Results and Discussion**

The cytochrome P450 expressed in the recombinant *E. coli* showed that the proteins are soluble and can be secreted outside the system in a similar way to the quantity expressed within the membrane of the organisms. Temperature seems to have an effect on the expression of the recombinant proteins (Plate 1). The importance of this is that this protein can easily be harvested without the need to lyse and kill the recombinant organisms during and after production, which can act as a starter culture for future production. This is achievable since it has been secreted by the culture media. At a temperature of 28 ºC, the expressed protein of interest was observed in lanes 14 and 15. The biggest protein band was observed at the membrane-bound extract lane 15 loaded with the lysed recombinant protein extracted from the recombinant *E. coli*, which showed the biggest recombinant protein bands among the evaluated temperatures. Below this temperature, the protein bands tend to be obvious but not as pronounced as the 28 ºC and above this temperature, the protein bands diminished. This observation technically opposed the report of Liu and Kong (2017), where the percentage relative activity of recombinant cytochrome P450 contained in pET 28a expressed in *E. coli* DE3 BL21 was observe to show the highest hydroxylation activity at $37^0$C. One would expect that the relative activity of the expressed protein should be directly proportional to the quantity of the protein. However, the expressed voluminous bands may not be all active proteins as inclusion bodies have been reported in microbial proteins, which tend to serve as storage vessels for insoluble aggregated of the protein lacking biological activity (Wang *et al.*, 2015). Likewise, the active coenzyme may not peak at the $28^0$C observed for the cytochrome P450.

Figure 1 showed the response of the estimated quantity of the coenzymes (heme, flavin adenodinucleotides and flavin mononucleotides) associated with cytochrome P450 to temperature. The F.A.D. and FMN estimated quantity was optimum at 25 $^0$C to be 15 mM and 13.5 mM; however, the optimum heme quantity was observed at 35 $^0$C. This heme concentration optimum temperature was close to the percentage relative activity of hydroxylation reported by Liu and Kong (2017). Since it is the heme that produces the oxygen atom, which oxidizes the electron transfer through the cofactors F.A.D. and FMN, the quantity of the heme may have a great influence on the relative activities of the cytochrome P450 protein.

Figures 2 and 3 showed the enzymatic activity of the expressed purified cytochrome p450 in the presence of horse cytochrome c and cytochrome p450 reductase at optimum temperature for the estimated cofactor (F.A.D. and FMN) and heme associated with the expressed recombinant cytochrome P450. A gradual increase in activity per second was observed from 0.689 A to 0.988 A within the interval of one minute at 550 nm wavelength at 35 $^0$C, where the heme concentration recorded optimum temperature that supported the highest concentration recorded. This absorbance showed increased activity due to its high value; however, a wide range of absorbance ranging from 0.1 A to 0.7 A was recorded at 25 $^0$C. This wide range is nevertheless low in absorbance compared with the optimum temperature for the heme concentration ($35^0$C). This increase in activity may also be attributed to the fact that higher temperature tends to support an increase in the rate of reaction.

**4. Conclusion**

There is no connection between the quantity of the expressed recombinant cytochrome P450 and the estimated coenzymes and cofactors concentrations in response to the temperature parameter. Plate 1 showed that at approximately 28 ºC and 30 ºC (lane 15 and 16 respectively), the expressed recombinant proteins recorded the most prominent bands; however, these protein band sizes do not correspond to the optimum coenzymes temperature at 25 ºC. The optimum temperature recorded for recombinant cytochrome P450, associated cofactors (FAD/FMN) and heme is 25 $^0$C, 25 $^0$C and 35 $^0$C, respectively. Nevertheless, this temperature range may favour the natural process since it is within the atmospheric temperature.

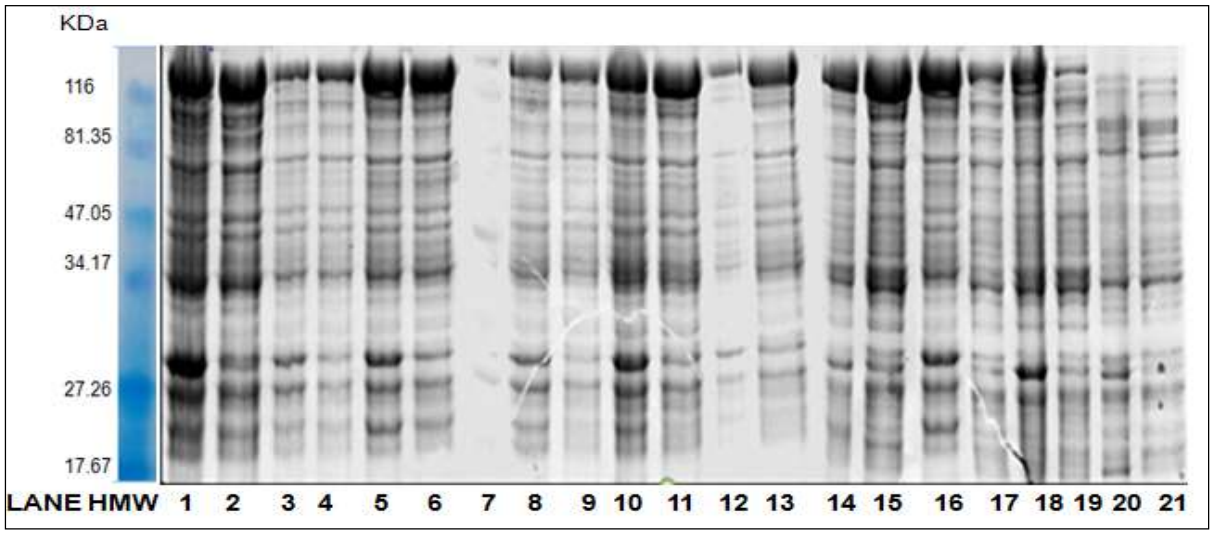


Plate 1: SDS-PAGE of the crude extract of the recombinant *E. coli* DE3 BL21 containing pET28a containing CYP102A2 at varied temperature

**Key:** 1 &2 at 19$^0$C, 3 & 4 at 20$^0$C, 5 & 6 at 22$^0$C, 7 at Ladder Marker, 8 & 9 at 24$^0$C, 10 & 11 at 26$^0$C, 12 & 13 at 27$^0$C, 14 & 15 at 28$^0$C, 16 & 17 at 30$^0$C and 18 & 19 at 37$^0$C 20&21 wild type *E. coli* in secretary and membrane bound order

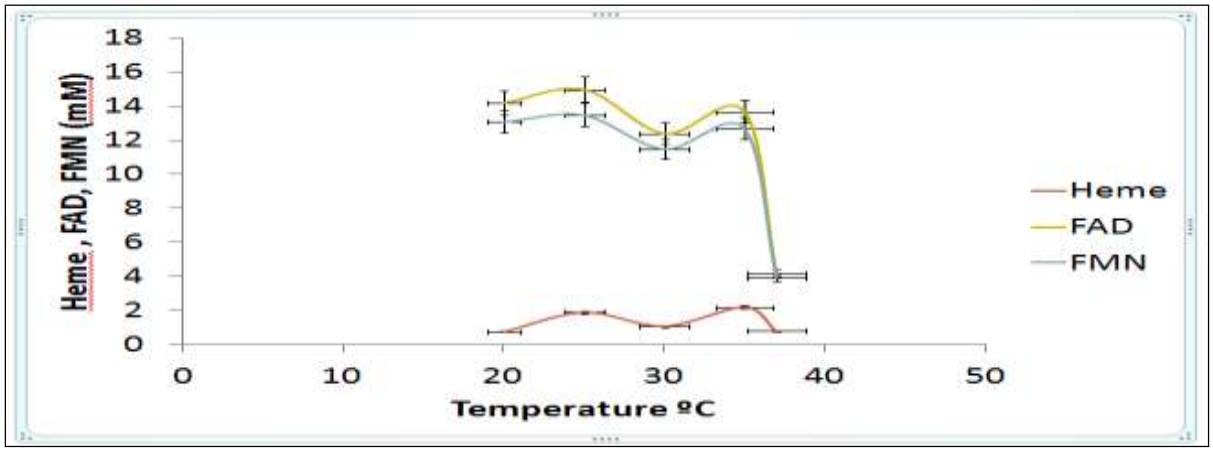


Figure 1: The Heme, FMN and F.A.D. content (mM) of various expressed recombinant protein at different fermentation temperatures in L.B. medium

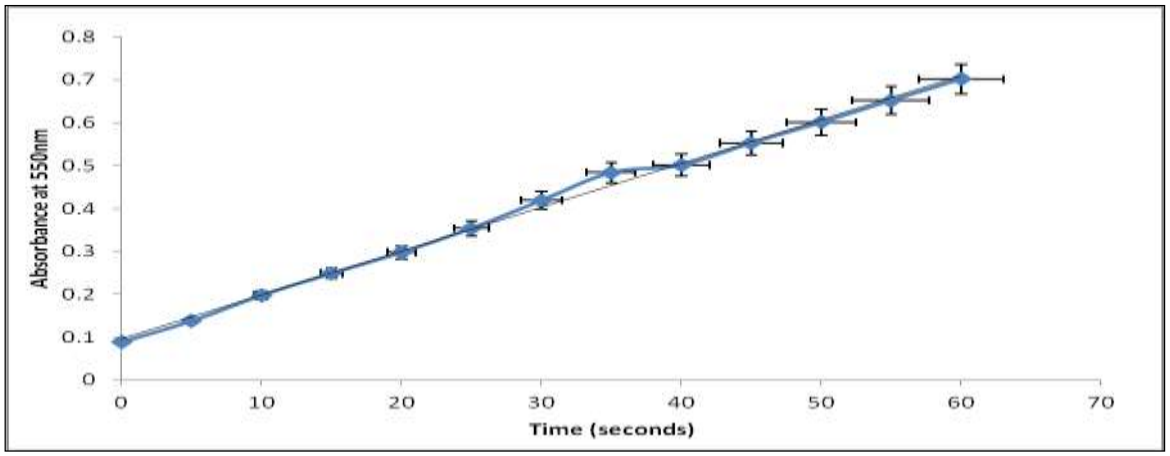


**Figure 2:** Enzymatic activity of recombinant CYP450 at 25$^0$C

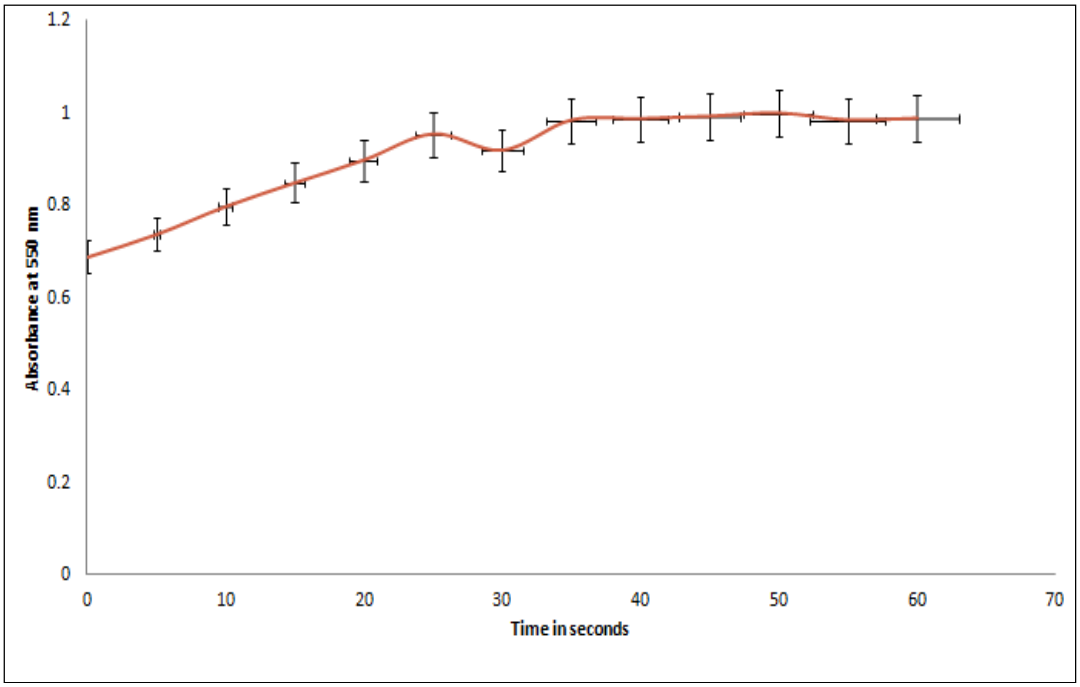


**Figure 3:** Enzymatic activity of recombinant CYP450 at 35 $^{0}$C

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