Effect of pH and Temperature on Recombinant CYP450

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Abstract: **Cytochromes P450 (CYPs) has been studied to be of great importance to humans, plants and environment as a whole. Hence it has been recognised as GRAS i.e., generally regarded as safe status. The optimum pH and temperature for the production and stability of recombinant CYP450 proteins was evaluated after expression using *Escherichia coli* BL21 (DE3) with the catalytic module of cytochrome P450 gene (CYP102A2) containing pET-28a and elution of the recombinant protein of interest (recombinant CYP450 proteins) from the expressed total recombinant proteins. Highest biomass yield of the recombinant organism expressing the POI was observed at 30 ºC followed by declination with further increase in temperature. Total protein yield of 1.5 M was observed at 25 ºC while after elution for the POI, highest estimated concentration was observed to be 2.4 mM concentration at optimum temperature of 30 ºC. The highest recombinant POI concentration 2.4 mM after elution was observed at optimum purification elution buffer concentration of 150 mM 30 ºC however the temperature that showed the highest purification of the eluted protein was 37 ºC at elution buffer concentration of 150 mM. The pH also showed broad influence on the enzymatic activity of the recombinant POI ranging from acidic to alkaline. The optimum pH of recombinant CYP450 activity was near neutrality where maximum activity was recorded. However, when pH was at both extremes, the biological activity was still visible. The biological activity increased significantly from pH 2 till neutral pH but decline at pH 9. Crystallization result reveals the presence of rod cluster crystals shape, in the well with 0.3 M, Na acetate salt 25% PEG and 2 K MME.**

***Keywords*: Cytochromes P450, optimum, pH and temperature**

I.INTRODUCTION

Cytochromes P450 (CYPs) has been studied to belong to a very bulky group of enzyme superfamilies and are commonly found in all living organisms like bacteria, fungi, plants and animals (Werck-Reichhart, and Feyereisen, 2000; Nelson, 2018) hence are regarded as generally safe (GRAS). This group of enzymes work in synergistic cascade process leading to catalyzing more than 20 known types of reactions in regio- and stereoselective manners such as hydroxylation, dealkylation, epoxidation, oxidation, dehalogenation, dehydrogenation and reduction (Nguyen *et al.,* 2020). It has also been studied to effectively catalyse the breaking down of complex substrates, toxic substances and xenobiotic (Bernhardt, 2006) hence has been a potential bioremediation prospect.

Bacterial CYP450s has shown capacity in the oxidative degradation of the natural substances and artificial chemicals through hydroxylation which is the addition of the hydroxyl group to modify the configuration of the substrate or epoxidation by converting the carbon-carbon double bonds into oxirane (Urlacher and Schmid, 2002). In spite of the importance of many enzymes in the catalytic process especially bioremediation, one of the major constrain in the application of this technology is the concern of thermostability and environmental optimal pH that will favour the stability of the expressed microbial protein. This factor has low durability concern in the application on industrial scale (Niehaus *et al*., 1999). Hence, the need to source for thermostable CYP450 has become paramount. The CYP450 from thermophiles source archaea *Sulfolobus solfataricus* has been found to be stable at thermophilic temperature, melting at approximately 90^0C (Wright *et al*., 1996). Adesina, (2016) also reported crude microbial proteins extracts to retain their 100% thermo-stability activity at 68 ºC for the period of 10 minutes. *In situ* microbial proteins production may also be influenced greatly on many physicochemical factors not only limited to pH and temperature, but also water activity (aw), carbon di oxide, (CO_2), oxygen concentration (O_2), redox potential, time of incubation, as well as other substrate intrinsic factors (such as the structure — fluidity, particulate matter, emulsions and buffering capacity). Parada *et al*., (2007) reported that pH can also have a significant role in the suitability, functionality and production of particular microbial proteins. Aymerich *et al*., (2000) also reported that both temperature and pH influenced enterocin production with optima between 25 and 35°C, and from 6.0 to 7.5 of initial pH while crude bacteriocins of the Lactic acid bacteria (LAB) isolates were reported active in a pH range of 2 - 9, but the maximum activity was observed at acidic pH (pH 2.0 and 5.0). This disparity in various microbial protein responses to temperature and pH showed that various microbial proteins have specific environment for optimal production and activities (Lye *et al.*, 2003; Islam *et al*., 2007). Expression of bacterial cytochrome CYP102A2 from *Bacillus megaterium* and the human CYP2B subfamily and CYP3 family were logically premeditated for thermostability (Harris *et al.,* 2018; Tavanti *et al*., 2018; Gumulya *et al*., 2018). Therefore, there is a need to study more on the stability to temperature and unstable environmental pH especially of indigenous sources if a resounding success will be achieved in the scaling up process in the utilisation of this protein for bioremediation process.

II. METHODOLOGY

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

Chemically competent BL21 (DE3) cell membrane was perforated following standard procedure order to pick up the recombinant pET28a containing the gene of interest CYP102A2 (Das and Dash, 2015; Ge *et al*., 2018). Fifty millilitre cultures of CYP102A2 pET28-containing pET-plasmid DNA was pipetted into the centrifuge tube containing the competent cells (*E. coli* BL21 DE3) and mixed by swirling with the pipette tip. It was incubated on ice for 20 minutes and alternated with hot temperature incubation at 42 ºC for 90 seconds. It was further cooled on ice for 1 minute. A 200 µl of luria bertani LB medium was added to the tube and incubate in a water bath set at 37 ºC for 45 minutes after 48 hours. A portion of 50 g/ml kanamycin was added to prevent contaminants. The culture was inoculated on LB agar plate using spread plate techniques and incubated at 37 ºC overnight for 48 hours. The biomass yield (g/ 725 ml) was calculated by multiplying the net cellular mass (g/ 725 ml) of the 725 ml culture, with the 1000/750 rapport. Cellular yield g/ 725ml = Net mass X 1000/725 (Zhu and Levins, 2011)

Inoculation of auto-induction medium with BL21 (DE3) cells carrying the catalytic module of the cytochrome P450 gene sequence (CYP102A2)-containing pET-28a

To express the CYP102A2 protein, the procedures described by Ge *et al.* (2018) was adopted. The recombinant cells were stimulated in auto-induction medium luria bertani broth (LB) medium (10 ml) with the addition of 50 mg/ml kanamycin, incubated at 37 ºC for 16 hours with shaking at 200 rpm. The culture was further inoculated into 500 ml of LB medium containing kanamycin (50 mg/ml) and grown at 25 ºC, 200 rpm. The optical density was monitored at 600 nm (OD_{600}) until the cells reached 0.6 A. 7.5 ml of isopropyl b-D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 0.5 mmol/L to induce protein expression. After incubation at 16 ºC for 16–20 hours, the cells were harvested by centrifugation at 3000 x g for 10 minutes at 4 ºC. The cell pellets were washed with 10 mL of 50 mmol/L Tris-HCl buffer (pH 7.0) and re-suspended in the same buffer. The cell pellets and crude cell lysates were used directly to determine protein expression. Each prepared conical flask containing 740 ml of the substrate was subjected to sterilization for 60 minutes. A 10 ml of starter cultures were injected into the fermenter. After 2-3 hours, the optical density (OD) of the fermenting substrate was measured, at 600 nm using the spectrophotometer to be 0.6. The protein expression was induced with 7.5 ml of isopropyl-thio-β galacto pyranoside (IPTG) and the temperatures were set to range from 20 ºC, 30 ºC and 37 ºC for each media. After the overnight fermentation, the fermented medium was removed and subjected to harvesting.

Cell harvesting and production of clarified lysate

A standard procedure described by Ge *et al*. (2018) with some modification was employed in the harvesting and production of clarified lysates of experimental microbial cells. The cell harvesting was done in two sets. Firstly, 25 ml was centrifuged at 5000 x g, for 10 minutes, the supernatant was discarded and the pellet stored in ice. The remaining media was centrifuged at 8000 x g for 10 minutes and the obtained pellets were re-suspended by adding 40 ml of the spent medium and again centrifuged at 8000 x g for 10 minutes. Finally, the pellet from this medium was stored on ice after weighing the net mass of the pellet. The pelleted cells were re-suspended in 10 ml/g lysis buffer (50 mM Tris.HCl, pH 7.9; 0.5 M NaCl); disrupted by mechanical cell disruption via thermal shock by successive rotation at 43 ºC for 3 minutes and cooled to the ambient temperature as described in the provided standard operating procedure. Afterwards, 50 µl of benzamidine and phenylmethylsulfonyl fluoride (PMSF) were added to the final concentration and the samples were evaluated for total protein profile detection. Centrifugation was further carried out at 24,000 x g for 30 minutes at 4 ºC in a Sorvall centrifuge with a rotor SS-34 (15,000 rpm) to obtained pellets, and stored on ice for further use.

Molecular weight estimation of CYP450 protein from isolates and recombinant point of interest (POI)

The expressed recombinant cytochrome P450 proteins molecular weight of the crude extracts from recombinant *E.coli* was determined using SDS-PAGE as described by Ge *et al*. (2018**)**. To the electrophoretic wells, equal volumes 20 µl of 1 x SDS 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 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.

Protein solubility and quantification

The theoretical molecular weight of cytochrome P450 is approximately within 118 kDa (Glieder *et al.,* 2002). The heterologous expression of pET28a containing CYP102A2 was induced by IPTG in *E. coli* strain Transetta (DE3) at temperature range from 20 ^{0}C to 37 ^{0}C for 16 hours. The solubility properties of the recombinant protein were explored using the supernatant obtained after centrifugation. Using SDS-PAGE analysis, total soluble secretory protein and total membrane bound protein from the total cellular lysate were compared. The SDS-PAGE containing 10% of acrylamide resolving gel and a 4% in stacking gel were loaded with 20 µg of protein per well along with High Range Sigma Aldrich Marker. Protein concentration was enumerated using Bradford technique (Bradford, 1976). Protein assay kit from BioRad Laboratories (California, USA) and bovine serum albumin (BSA) as standard was used in this analysis. The expression of

the recombinant protein in the optimised fermentation environment was purified using nickel affinity chromatography and was eluted using different concentrations of imidazole and the size was determined by SDS-PAGE. The recombinant protein purification procedure was carried out following standard procedure (Bornhorst and Falke, 2000).

Optimization of pH for the biological activity of recombinant cytochrome P450 expressed in E. coli BL21 (DE3) pET 28a

The biological activity of the expressed recombinant protein was observed following the addition of NADPH-cytochrome p450 reductase, horse cytochrome c and the recombinant CYP450. This activity was monitored within the space of three minutes thirty seconds, within the pH range of 2-9 (Joshi *et al*., 2006).

Recombinant CYP450 protein crystallization

Protein crystallization was carried out as described by Bijelic and Rompel (2018) using a hanging drop setup. A 24 well microtitre plates was set up to contain the 5 µL of both purified recombinant cytochrome P450 and varied concentration of crystalisation cocktail solution comprising of polyethylene glycol (PEG), monomethyl ether (MME), 1 K, 1.5 K, 2 K, 4 K, 5 K, 6 K, 8 K and 20 K correspond to the molecular weight, in thousands of Daltons, of PEG. Six different selected precipitating salt 0.3 M Na acetate, 0.2 M Li_2SO_4, 0.2 M $MgCl_2$, 0.2 M KBr, 0.2 M KSCN, 0.8 M Na formate, placed on a siliconized cover slide which then covers a well containing only the crystallization cocktail (5 µL) and sealed using grease as the sealant to prevent evaporation into the air and enhanced precipitation of the protein crystals. The crystalisation tray was analyses for 7 days consecutively using binocular microscope and observed crystals were photographed

III. RESULTS AND DISCUSSION

To investigate whether the recombinant CYP450 could be industrially used, an optimization processes of production conditions was performed. The effect of temperature and pH on enzyme activity was thus tested. The biomass weight of the recombinant *E. coli* DE3 BL21 pET 28a containing the gene of interest showed varied growth response at various experimental temperature with 30^0C showing the highest biomass yield on incubation for 24 hours across mesophilic temperature range. There was also a correlation between the biomass and the recombinant protein yield as both expressed optimal yield at 30 ^{0}C (Figure 1). The recombinant CYP450 expressed in *E. coli* DE3 BL21 pET 28a displayed activity over a broad mesophilic temperature range from 25 ^{0}C to 37 ^{0}C. At 25 ^{o}C, 1.5 M concentration of the total expressed protein was recorded after which a decline concentration was observed following the increase temperature (Figure 2). This high quantity in concentration is associated with other expressed proteins associated with wild type *E. coli* and recombinant protein of interest (POI) CYP450 expressed in *E. coli* DE3 BL21 pET 28a under the T8 promoter embedded in the DE3 coding. T8 promoter has been documented to be highly efficient in expression of recombinant proteins (Jiang *et al*., 2016). After elution of the POI (CYP450 proteins) form the total expressed proteins, a gradual increase in quantity of recombinant protein POI (CYP450) with temperature from 25 ^{0}C – 28 ^{0}C was recorded (Figure 3). The recombinant CYP450 cultured in LB medium showing highest protein yield of 2.35 mM with optimum temperature range spanned across 28 ^{0}C to 30.5 ^{0}C. The peak concentration observed at this temperature range could be due to the fact that the recombinant organism expressing the protein is known to range in the mesophilic temperature. The temperature range is stable at temperature higher than normal atmospheric temperature (20 ^{0}C) however, this is low compare with the CYP450 from *Sulfolobus solfataricus* which was found to be stable at thermophilic temperature, melting at approximately 90 ^{0}C (Nguyen *et al*., 2020).

Plate 1 showed that an overnight fermentation at 30 °C, in LB media of the recombinant *E. coli* produced the highest quantity of purified and functional recombinant CYP102A2 protein. The specific protein with approximately 118 kDa (Figure 3) was observed by SDS-PAGE detection, validating the soluble expression of the recombinant expressed protein in bacteria. It was observed that the higher the temperature the lower the concentration of purified recombinant CYP450 protein. This could be due to the fact that the proteins are being denatured as the temperature increases. Also, at imidazole concentration of 150 mM to 200 mM the recombinant proteins of interest were more purified compared to the lower concentrations used at incubation temperature of 37 ºC. Liu and Kong (2017) reported maximum hydroxylation activity of cytochrome p450 at 37 ºC. This maximum activity may be related to the optimum biomass and correesponding recombinant protein production observed at the similar temperature range.

In addition, the elution of non-specific protein were observed to be high at lower imidazole concentrations compared to higher imidazole concentrations, however the most purified crude recombinant protein observed was at 37 ^{0}C with 150 mM concentration of elution imidazole treatment.

The effect of pH on the expressed recombinant protein of interest was showed in Figure 4. It clearly showed that recombinant CYP450 had a broad pH tolerance, ranging from acidic to alkaline. The optimum pH of recombinant CYP450 activity was near neutrality where maximum activity was recorded. However, when pH was at both extremes, the biological activity was still visible. The biological activity increased significantly from pH 2 till neutral pH but decline at pH 9. Similar trend was observed by Liu and Kong (2017) who evaluated the hydroxylation activity of enzyme catalyzed by the monooxygenase BM mutant 139-3. The presence of crystals after crystalisation showed high level of purification. The rod cluster and single 3-D crystals shape indicated good level of purity however, the quantity was small. The two shapes observed crystals could imply expression of more than one protein. This could be attributed to human factor during

purification process especially the elution stage. Hence automated purification process is recommended to achieve a high quality and quantity of the protein. Crystallization result reveals the presence of rod cluster crystals shape, in the well with 0.3 M, Na acetate salt 25% PEG and 2 K MME, although it is not certain that crystals are from CYP450 protein due to incomplete purification. As a matter of fact, the quantity of the recombinant protein was obviously higher (1.5 M) at 25 ºC than other eluted protein (2.4 mM) at approximately 30 ºC, hence it is most likely to be corresponding to the crystals. Plate 2 further reveals the presence of crystalised protein which are most likely to be the protein of interest since it is the most expressed in the recombinant organisms.

Figure 1: Total biomass of E. coli DE3 BL21pET28a cultured containing recombinant CYP450 in LB broth at different fermentation temperatures

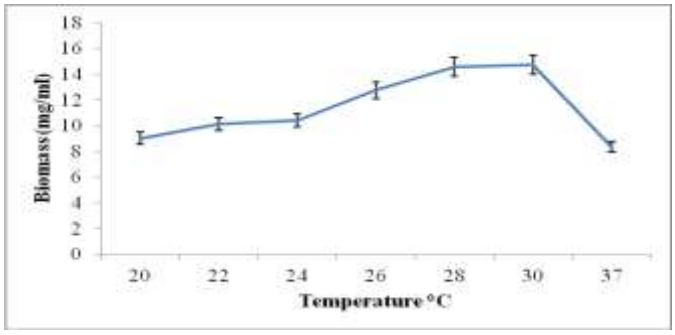
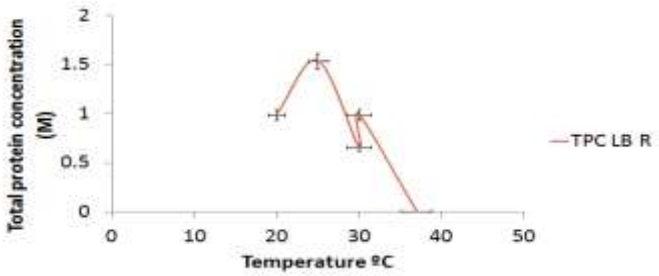


Figure 2: Total recombinant protein concentrations at different fermentation temperatures expressed in E. coli DE3 BL28 PET system in LB



Key: TPC LB R= Total Protein Content in Recombinant E. coli

Figure 3: Total estimated recombinant CYP450 at different fermentation temperatures expressed in E. coli DE3 BL28 cultured in LB

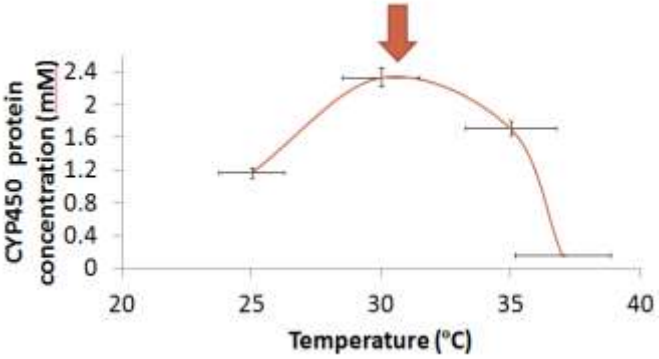
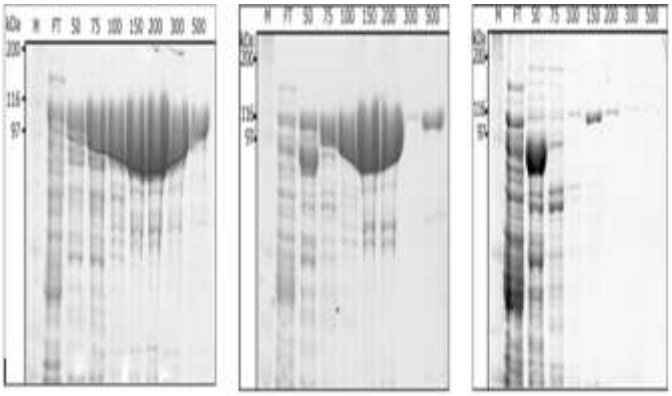


Plate 1: Purification gels (SDS-PAGE) derived from LB pellet, eluted using different imidazole concentrations in mM for 20 ºC, 30 ºC and 37 ºC fermentation temperatures respectively.



Key: M= Marker, FT= Filtrate without imidazole

Figure 4: Effect of pH on recombinant CYP450 enzymatic activity

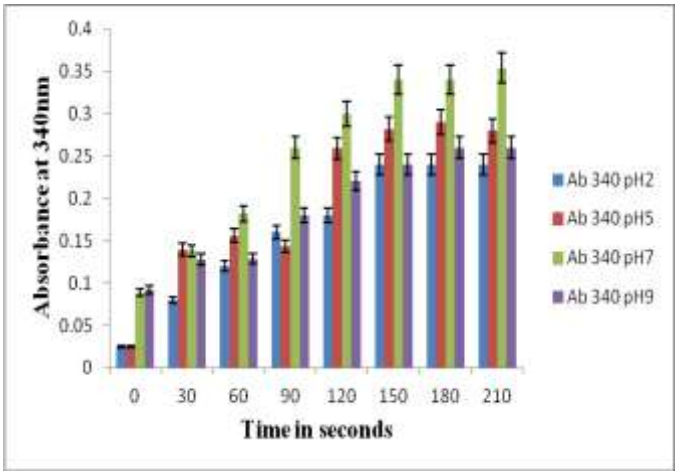
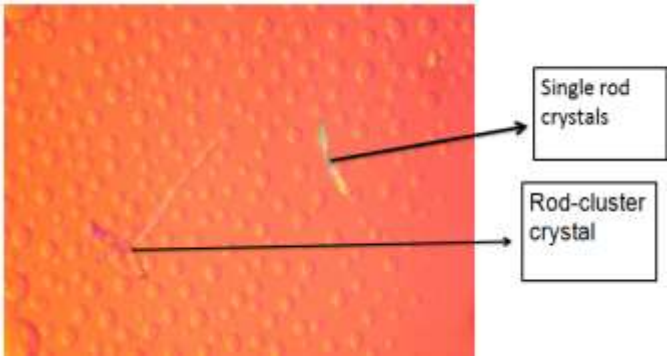


Plate 2: Cytochrome p450 protein crystals



IV. CONCLUSION

Findings from this study revealed that the optimum pH and temperature for the production and stability of recombinant CYP450 proteins are pH7 and 30 ºC respectively where the highest activity of protein production was recorded. Based on the findings from this study, it is further recommended that production of recombinant CYP450 proteins at the optimum pH and temperature should be augmented by automated

purification processes in other to obtain high quantity of the protein in its purest form.

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