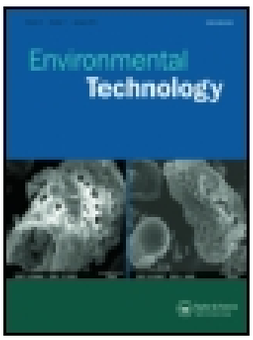

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# Multisubstrate specific Flavin containing monooxygenase from *Chlorella pyrenoidosa* with potential application for phenolic wastewater remediation and biosensor application

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## Abstract

Microbial degradation of phenolic pollutants in industrial wastewater is dependent on enzymatic pathway comprising a cascade of phenol metabolizing enzymes. Phenol hydroxylase is the first enzyme of the pathway catalyzing the initial attack on phenol in green algae *Chlorella pyrenoidosa*. The present work reports cost effective production of partially purified microalgal phenol hydroylase by single step purification and characterization of its kinetic properties with the view of application for enzyme based remediation of phenolic wastewater or in phenolic biosensor. The enzyme with molecular weight of 25 kDa shows all characteristics of phenol hydroxylases i.e. hydroxylation of phenol to catechol (confirmed by HPLC), substrate dependent NADPH oxidation, absorption spectrum typical of flavoproteins and peptide mass fingerprint corresponding to flavoprotein hydroxylase. The enzyme utilizes phenol with apparent Michealis constant ($K_m$) of 1.71 µM, maximal velocity ($V_{max}$) of 0.4 µM/min with optimal activity at pH 7 and 35$^o$C. $Fe^{2+}$chelators (Phenanthroline and sodium arsenate), heavy metals, denaturants and oxidizing agents showed inhibitory effect on phenol hydroxylation activity of the enzyme. The enzyme has broad substrate specificity against isomeric diphenols, isomeric methylphenols, halogen substituted phenols, amino substituted phenols, nitrophenols, hydroxybenzaldehyde and hydroxylbenzoic acid. The enzyme shows remarkable storage stability at room temperature and at 4 $^o$C. The multisubstrate specificity coupled to remarkable storage stability of the microalgal phenol hydroxylase opens up avenues

for its application in remediation of a wide range of phenolics released in industrial wastewater or phenolic biosensor application.

**Keywords:** Algae, Phenol hydroxylase, Flavoprotein, Phenol, Biodegradation pathway, Broad substrate specificity

## 1. Introduction

Phenols constitute the second largest group of natural compounds following carbohydrates. Phenols are widely found in nature in peptides, proteins, steroids, terpenes, glucosides, ethers, esters, lignin, melanin and tannins (Enroth et al. 1998) [1]. However, anthropogenic sources of phenol in environment such as chemical, petroleum and pharmaceutical industries substantially increase the natural pool of phenols which is matter of grave environmental concern owing to carcinogenic or mutagenic nature of these compounds. The situation is further worsened by resistance of phenols to natural degradation leading to its accumulation in environment and their biomagnification through the food chain [2]. This calls for efficient technologies for remediation of phenols at its anthropogenic source before its enters into the environmental ecosystem. Microbial degradation has gained attention for treatment of phenols owing to its ability of complete mineralization and cost effectiveness. The microbes capability of phenol degradation is conferred by metabolic pathway comprising of a cascade of enzymes which had been well reported in bacteria [3-6], fungi [7-11] as compared to that in algae [12-14]. Phenol hydroxylase is the first enzyme of the phenol degradation pathway catalyzing the most crucial step of opening the stable phenol ring by hydroxylation of phenols to corresponding diols. This position specific hydroxylation of phenol ring is a tremendous challenge to perform in vitro [1]. The use of enzymes in phenolic treatment is seen as a potential alternative owing to its advantages over conventional methods as solvent extraction, microbial degradation, chemical oxidation and activated carbon adsorption as its applicability over a wide range of phenol concentrations, pH and temperatures; broad substrate specificity; simple process control than microbial systems; operation in catalytic manner and low sludge formation [15-17]. Klibanov et al. [18] first proposed utilization of horse-radish peroxidase (HRP) to treat phenol contaminated wastewater. HRP oxidse phenols with hydrogen peroxide as an oxidant generating phenoxy radicals which react forming higher less soluble oligomers and polymers which can be easily removed from wastewater. However, inactivation of HRP is a main drawback of the phenol treatment process. Wu et al. [19] reported addition of polyethylene glycol (PEG) for significant protective effect on the activity of HRP for phenol removal. Laccase and tyrosinase which utilize molecular oxygen instead of hydrogen peroxide was proposed as another alternative for treatment of phenol. Tyrosinase (also known as polyphenol oxidase) converts phenols to adsorbable o-quinones which are removed by a second step of chemisorption [20]. However,

contamination due to non-precipitated products in the aqueous solution after an enzymatic treatment is a matter of concern which is much less discussed [17]. In order to solve this issue, membrane immobilization of multiple enzymes of the microbial phenol degradation pathway to convert phenol via catechol resulting in formation of linear chain metabolites and end products with low energy level was proposed [17,21]. The development of enzymatic processes from an engineering perspective is hindered by high enzyme production costs owing to expenses associated with enzyme isolation and purification [16, 22]. Each purification step has its own specific requirements and will contribute to purification cost of the enzyme. Purification schemes with more than three steps have significant impact on overall cost [23]. Thus, the application of crude enzyme extracts for phenol remediation has been proposed recently since application of purified enzymes for phenol remediation is not economical. Kameda et al. [24] employed crude extract from *Agaricus bisporus* for remediation of 90% phenol of 100 mg/L using 200 U/mL tyrosinase activity suggesting it as a potential and economic biocatalyst for phenol remediation. However, application of crude enzyme suffers from the drawback of less active catalysis since the key element of protein purification is to achieve increased specific activity of the enzyme [23]. Hence, cost effective strategies with minimal protein purification steps is required to produce enzyme with increased specific activity.

Keeping in view the potential of phenol hydroxylase to achieve specific hydroxylation reactions of aromatic ring which is difficult to achieve in vitro, the knowledge of catalytic properties of novel phenol hydroxylases is indispensible. To the best of our knowledge, there are no reports that endorses characterization of any microalgal phenol hydroxylase and the present research efforts to explore the phenol ring hydroxylation kinetics by microalgal phenol hydroxylase. Cost effective strategies for production of phenol hydroxylases combining properties of increased specific activity and high broad substrate specificity is need of the hour to overcome the bottlenecks associated with enzyme based wastewater treatment. The present research efforts to produce partially purified microalgal phenol hydroylase with increased specific activity by cost effective single step purification and explore its kinetic properties which is quintessential for exploiting it for potential applications as enzyme based phenolic remediation technology or phenolic biosensor.

## 2. Materials and Methods

### *2.1 Chemicals*

$MgSO_4$, $CaCl_2$, $K_2HPO_4$,$FeSO_4$,$Na_2EDTA$,$H_3BO_3$,$MnCl_2.4H_2O$,$ZnSO_4.7H_20$,$Na_2MoO_4.2H_2O$,$CuSO_4.5H_2O$, $C_6H_50H$,$(NH_4)_2SO_4$,SDS,$(NH_4)_2S_2O_8$,$H_2O_2$ were of analytical grade and obtained from Merck, India.

Catechol (HPLC grade), phenanthroline, sodium arsenate, Tiron, sodium diethyldithiocarbamate were obtained from Sigma-Aldrich, India. NADPH (HPLC grade), NADH (HPLC grade), dialysis membrane, acrylamide, bis-acrylamide, tris base, bromophenol blue, mercaptoethanol, glycine, TEMED, silver nitrate, resorcinol, quinol, o-cresol, m-cresol, p-cresol, 2,4-dichlorophenol, 4-nitrophenol, 2-aminophenol, 3-aminophenol, p-bromophenol, 4-chlorophenol, 2-chlorophenol were obtained from HiMedia Laboratories, India. $AgCl_2$ and Urea were obtained from CDH, India respectively. p-hydroxylbenzoic acid, p-hydroxylbenzaldehyde, m-hydroxylbenzaldehyde was obtained from CDH, India.

### *2.2. Culture of microorganisms*

Axenic culture of *C. pyrenoidosa* (NCIM 2738) obtained from NCIM, Pune was cultured in Fog's medium (pH 7.5) composed of 0.2 g/L $MgSO_4$, 0.2 g/L $K_2HPO_4$, 0.1 g/L $CaCl_2.H_20$, 1 mL/L micronutrient solution and 5 mL/L Fe-EDTA solution. The micronutrient solution is composed of 2.86 mg $H_3BO_3$, 181.0 mg $MnCl_2.4\ H_20$, 22.0 mg $ZnSO_4.7H_2O$, 39 mg $Na_2MoO_4.2H_2O$ and 8 mg $CuSO_4.5\ H_2O$ dissolved in 100 mL of distilled water. *C.pyrenoidosa* was cultured in 125 mg/L phenol containing Fog's medium with continuous orbital shaking of 140 rpm under illumination of 3500 lux for a photoperiod of 14 hours light/10 hours dark. The residual phenol concentration was determined by HPLC (Prostar,Varian, USA) with the UV-Visible detector operating at 270 nm using a C-18 column with mobile phase of acetonitrile:water (30:70) at a flow rate of 0.5 mL/min.

### *2.3. Analysis of phenol hydroxylase activity in crude enzyme extract*

The *C.pyrenoidosa* cells were cultured in 125 mg/L phenol as at this particular concentration the specific growth and phenol degradation rate was reported to be highest [12]. *C.pyrenoidosa* cells were harvested for preparation of crude enzyme extract after 70 % phenol consumption. The cells were harvested by centrifugation at 3000 rpm for 10 min ($4^oC$) and washed thrice with potassium phosphate buffer (50 mM). The washed cells were ground in 50 mM potassium phosphate buffer (1 gm biomass/mL) using mortar-pestle in ice bath, extract was centrifuged (10,000 r.p.m. at $4^oC$) and the supernatant obtained was for enzyme activity analysis. The total protein in the crude enzyme extract was analyzed as per Lowry et al. [25]. The efficiency of crude enzyme extract to show phenol hydroxylase (PH) activity was analyzed by determination of two characteristic properties of PHs: a) Hydroxylation of phenol to catechol in the assay mixture. b) Decrease in absorbance at 340 nm of NADPH in assay mixture owing to substrate dependent oxidation. HPLC analysis of the samples were carried out to determine phenol utilization and concomitant production of the reaction product catechol [26,27].

### *2.4. Single step partial purification of phenol hydroxylase*

The crude enzyme extract was subjected to ammonium sulphate fractionation in the salt concentration range of 10-70%. For salt fractionation, various salt concentrations were dissolved in the crude enzyme extract by continuous stirring for 30 minutes with incubation in water-ice slurry. After all salts got dissolved, enzyme extract was centrifuged at 12000 rpm for 20 minutes at 4$^{o}$C resulting in a pellet containing the fractionated protein. The pellet was resuspended in 1-2 pellet volumes of buffer and then dialyzed overnight against 50 mM potassium phosphate buffer at 4$^{o}$C. Total protein and phenol hydroxylase activity of different ammonium sulphate fractions was analyzed as mentioned in Section 2.3 respectively.

### *2.5. Enzyme homogeneity, molecular weight determination and peptide mass finger printing*

The homogeneity of the purified enzyme as well as its molecular mass was determined by SDS-PAGE using 12.5 % gel.The different molecular weight markers used as reference were MBP-paramayosin (80 kDa), MBP-CBD (58 kD), CBD-Mxe Intein-2CBD (46 kD), CBD-MxeIntein (30 kDa), CBD-BmFKBP13 (25 kD) and lysozyme (17 kD). The molecular weight of the purified enzyme was determined by a plot of log of molecular weight vs mobility. Protein spot corresponding to the purified enzyme in the SDS-PAGE gel was excised followed by in gel digestion by trypsin and peptide mass fingerprinting by MALDI-TOF (UltrafleXtreme, Bruker, Daltonics, USA) [28]. The resulting tryptic maps were identified by MASCOT (www.matrixscience.com) using NCBInr and JGI Chlorella genome portal [29].

### *2.6. Absorption characteristics of flavoproteins*

Phenol hydroxylase is known to show characteristic spectral properties of flavoproteins with maxima around 271 nm, 345 nm (Fe-S clusters), 450 nm (flavins) and shoulder between 465-470 nm (Fe-S clusters). The absorption spectrum of the purified protein was analyzed for characteristic of flavoproteins by UV-Visible spectrophotometry (Cary 100, Agilent Technologies, USA) [10,26,30].

### *2.7.Cofactor requirement of the enzyme*

Phenol hydroxylase requires nicotinamide adenine dinucleotide as co-substrate for phenol hydroxylation activity. However phenol hydroxylase shows specific ability to use NADH or NADPH or both as cofactors [11-13]. NADPH being very expensive it is desirable to look for phenol hydroxylase with ability to use NADH as cheaper alternative [31]. The effectiveness of two co factors NADPH and NADH on phenol hydroxylase activity was analyzed as per the phenol hydroxylase assay mentioned in section 2.3 except for replacement of NADPH with NADH for determining the applicability of NADH as cofactor.

### *2.8. Substrate affinity of the enzyme towards phenol*

Measurement of velocity of enzyme catalyzed reaction at different substrate concentration helps understand the affinity of the enzyme for the substrate. The substrate affinity of phenol hydroxylase towards various phenol concentration and the values of maximal velocity ($V_{max}$) and apparent Michealis constant ($K_m$) for phenol oxidation was calculated from Lineweaver Burk plots [10,26].

### *2.9.Effect of chelators, heavy metals, denaturant and oxidizing agent on enzyme activity*

The presence of iron or copper in the enzyme was verified by inhibition of enzyme activity by iron or copper chelators. The effect of phenanthroline and sodium arsenate ($Fe^{2+}$ chealator), sodium diethyldithiocarbamate (Cu chelator) on phenol hydroxylation activity was determined. Heavy metals, denaturants and oxidizing agent are commonly found in industrial wastewater where we intend to utilize the patially purified microalgal phenol hydroylase for remediation of phenolics. Thus, the analysis of the effect of heavy metals as $CuSO_4$, $AgCl_2$, $HgCl_2$, denaturants as SDS and urea; oxidizing agent as $H_2O_2$ on enzyme activity was quite indispensible to view its potential application for remediation of phenolic industrial wastewater.

### *2.10. Multisubstrate specificity of Phenol hydroxylase*

The broad substrate specificity of phenol hydroxylase was determined in addition to phenol against isomeric diphenols (catechol, resorcinol, quinol), isomeric methylphenols (o- cresol, m-cresol, p-cresol), halogen substituted phenols (2-chlorophenol, 4-chlorophenol, 2,4-chlorophenol, 2-aminophenol, 3-aminophenol, p-bromophenol, 4-nitrophenol), p and m-hydroxybenzaldehyde, p-hydroxylbenzoic acid and 4-nitrophenol. Phenol hydroxylase activity was analyzed in an assay mixture containing 50 mM potassium phosphate buffer solution (pH 7.2), 500 µg protein, 1.5 µM substrate, 1.5 µM NADPH incubated in a water bath at 35 $^{o}$C. The substrate specificity of phenol hydroxylase was measured by disappearance of its cosubstrate NADPH (decrease of absorbance at 340 nm) as per Neujhar and Varga [32].

### *2.11. Stoichiometry of reaction with phenol*

Stoichiometry helps determine the optimal ratio of reactants for a chemical reaction so that all reactants are fully used. An understanding of appropriate stoichiometry of phenol hydroxylase reaction will help determine the optimal ratio of phenol and co-substrate NADPH at which complete phenol concentration will be hydroxylated to its reaction product catechol. The appropriate stochiometry of phenol hydroxylase reaction was determined using various combination of initial concentration of phenol and NADPH [10].

### *2.12. Effect of pH and temperature on enzyme activity*

Several factors as binding of the substrate to enzyme, ionization states of amino acid residues in enzyme active site, substrate ionization and enzyme structure variation are effected by pH. Temperature has effects on enzyme reaction rate constant and denaturation of enzyme at high temeperature. Thus, the dependence of phenol hydroxylating activity on pH and temperature was studied. To determine effect of pH, phenol hydroxylase activity was analyzed in an assay mixture containing 50 mM potassium phosphate buffer solution with pH varying in range of 5.5-8.0, 500 µg protein, 1.5 µM phenol and1.5 µM NADPH incubated in a water bath at 35 $^{o}$C. Similarly, the effect of temperature on phenol hydroxylation activity was studied by incubating the phenol hydroxylase assay mixture in temperature range of 25-85 $^{o}$C.

### *2.13. Storage stability of phenol hydroxylase*

Phenol hydroxylase with good storage stability is required so that it can be used for prospective practical applications as phenol remediation or biosensors. Thus, the effect of storage at room temperature as well as 4$^{o}$C on phenol hydroxylase activity was determined over a period of seven days.

## 3. Results and discussion

### *3.1. Induction of phenol hydroxylation activity*

The crude enzyme extracted from *C.pyrenoidosa* biomass cultured on phenol showed characteristic properties of phenol hydroxylase activity as shown in Figure 1a. Figure 1a shows steep hydroxylation of 1.2 µM phenol by 4 minutes followed by decreased phenol hydroxylation of 0.1 µM phenol till 6 minutes finally leading to concomitant accumulation of 0.77 µM reaction product catechol by six minutes. The reaction product catechol was identified by HPLC on basis of its identical retention time of 10.9 min with that of standard catechol. The decreased catechol accumulation is due to activity of catechol cleaving enzymes catechol-1,2-dioxygenase (orthocleavage) and catechol-2,3-dioxygenase (meta cleavage) present in the crude enzyme extract. The activity of both the catechol cleaving enzymes in the phenol degradation pathway of *C. pyrenoidosa* have been reported in our previous publication [12]. Vilmikova et al. [33] reported similar decreased catechol accumulation following hydroxylation of phenol by crude enzyme extract owing to activity of catechol-1,2-dioxygenase. Figure 1a also shows decreased absorbance at 340 nm due to NADPH oxidation concomitantly with phenol hydroxylation suggesting substrate dependent oxidation of NADPH. Except for crude extract a minimal endogenous oxidation of NADPH of 0.67% was detected as shown in Figure 1b. Similar results of NADPH dependent hydroxylation of phenol to catechol by phenol hydroxylases have been reported in a variety of microbes as *Pseudomonas* sp., *Acinetobacter radioresistens*, *Ralstonia eutropha*, *T. cutaneum*, *Candida tropicalis* [10,26,34-38]. Control incubation

using heat killed enzyme extract showed no characteristic phenol hydroxylation activity. Crude enzyme extract obtained from *C. pyrenoidosa* grown on phenol free media showed no phenol hydroxylation activity indicating inducible nature of phenol hydroxylase in response to phenol. Stiborova et al. [39] and Paca et al. [26] also reported phenol dependent inducibility of phenol hydroxylase in *Candida tropicalis* which supports our present findings. On basis of the detection of substrate consumption and reaction product formation, the crude extract of phenol grown cells indicates presence of phenol hydroxylase catalyzing the ortho-hydroxylation of phenol.

### *3.2 Purification and peptide mass fingerprinting of phenol hydroxylase*

Phenol hydroxylase in crude extract was partially purified by ammonium sulphate fractionation. Table 1 showed phenol hydroxylation activity of 15-40% the ammonium sulphate fractions. The highest specific activity of 0.39 µmol/min/mg obtained for 25% ammonium sulphate fraction was comparatively higher to that of 0.13µmol/min/mg in crude enzyme extract. Figure 2 a indicates that the 25% ammonium sulphate fraction showed characteristic properties of phenol hydroxylase as hydroxylation of phenol to catechol with concomitant oxidation of NADPH. Figure 2b shows the homogeneity of 15-40% ammonium sulphate fractions which showed positive phenol hydroxylation activity. The 15-40% ammonium sulphate fractions shows a prominent single protein band of 25 kD. The molecular mass of the protein band is evident from the log molecular weight versus relative mobility plot as shown in Figure 2c. Peptide mass fingerprinting of the 25 kDa protein band in SDS- PAGE gel corresponding to the purified enzyme was carried out to identify the enzyme on basis of their constituent peptide masses. MALDI-TOF analysis of the 25 kDa band (Figure 3) revealed a protein hit ChlNC64A_1 49348 with protein sequence coverage of 39 % in the *Chlorella* sp. NC64A genome. Table 2 shows that the protein hit ChlNC64A_1 49348 is a flavoprotein hydroxylase involved in hydroxylation of aromatic rings. The results correspond well with the phenol hydroxylation ability and flavoprotein nature of the purified protein.

### *3.3 Spectral properties of the enzyme*

Flavoproteins are known to have a quite distinct UV-Visisble spectra [40]. The purified enzyme shows characteristic spectral properties of flavoproteins with maxima around 271 nm, 345 nm (Fe-S clusters), 450 nm (flavins) and shoulder between 465-470 nm (Fe-S clusters) (Figure 4). This confirms the flavoprotein nature as predicted by peptide fragment spectra of the purified protein (Table 2). The presence of Fe-S clusters as denoted by the spectra is known for their role in oxidation reduction reactions. The presence of flavin and Fe-S cluster of the type [2Fe-2S] is important for functioning of aromatic hydroxylases. The Fe-S cluster may be present as a separate ferrodoxin or as part of an iron sulfur flavoprotein that interacts with NAD (P) directly [41]. The spectral characteristics of the purified enzyme

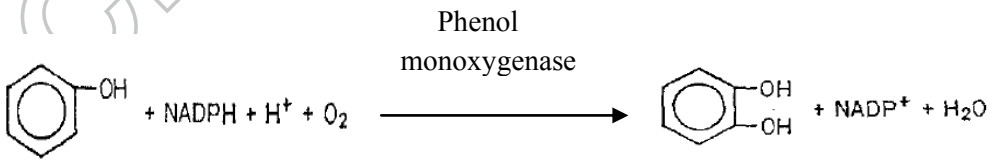
is supported by similar spectral properties of phenol hydroxylase from fungal species as *Candida tropicalis*, *Trichosporon cutaneum* and bacterial species as *Acinetobacter radioresistens*, *Pseudomonas pickettii* [10,26,30,34].

### *3.4 Cofactor requirement for phenol hydroxylation activity*

Phenol hydroxylases use electrons from cofactor nicotine adenine dinucleotide to activate and cleave a molecule of oxygen through the formation of an intermediate flavin hydroperoxide and enable the incorporation of an oxygen atom into the substrate [42]. Phenol hydroxylase shows specific ability to use NADH or NADPH or both as cofactors [10,26,43]. Figure 5 shows phenol is being hydroxylated to catechol with concomitant oxidation of NADH. The specific activity of phenol hydroxylase reaction with NADPH as cofactor (0.39 µmoles/min/mg) was higher as compared to specific activity of 0.14 µmoles/min/mg with NADH as cofactor suggesting NADPH as better cofactor. The specificity for NADPH or NADH as cofactor has been found to be species specific. Nakagawa and Takeda [44] reported reduced activity of phenol hydroxylase obtained from *Brevibacterium fuscum* phenol hydroxylation activity when using NADH as cofactor compared to that with NADPH which accords with our findings. On the other hand, phenol hydroxylase from *Candida tropicalis* [26] and *Trichosporon cutaneum* [10] was found to be strictly dependent on NADPH for activity. The ability of the enzyme to use NADH as a cheap cofactor could add to its application for phenol remediation or biosensor application.

### *3.5 Stoichiometry of the reaction with phenol*

The stoichiometry of reaction of phenol hydroxylase is being shown in Table 3. A fairly close equivalence can be seen between the concentration of phenol utilized and its hydroxylation product catechol. The oxidation of NADPH increased with the increasing ratio of NADPH: phenol in the assay mixture. From Table 3, it can be concluded that with appropriate concentrations of NADPH (1.5 µM), phenol (1.5 µM) and the enzyme (500 µg) the phenol hydroxylase reaction proceeds with the following stoichiometry:




Thus the stoichiometric study helped us determine ratio of the substrate (phenol) and co-substrate (NADPH) at which there will be optimal conversion of all the substrate (phenol) to its reaction product

catechol. The enzyme shows monooxygenase behavior since it transfers one oxygen atom to the substrate phenol resulting in production of catechol while the other oxygen atom reduced to water.

### *3.6 Substrate affinity of the enzyme towards phenol*

Figure 6 shows that the phenol utilization by the partilally purified enzyme follows Michealis- Menten kinetics. The apparent Michealis constant ($K_m$) for oxidation of phenol was estimated to be 1.71 µM and the maximal velocity ($V_{max}$) to be 0.4 µM/min. The $K_m$ value of 1.7 µM obtained for bacterial phenol monoxygenase from *Pseudomonas mendocina* was equal to that obtained for the purified enzyme indicating similar phenol affinity. The $K_m$ value of the partially purified enzyme was lower to that obtained for phenol monoxygenase from bacterial species as *Pseudomonas mendocina* ($K_m$= 1.7 µM), *Pseudomonas flourescens* ($K_m$= 1.9 µM), *Pseudomonas putida* ($K_m$= 2.4 µM), *Pseudomonas flourescens* strain F PC20 ($K_m$= 13.1 µM), *Pseudomonas flourescens* strain C PC24 ($K_m$= 21.4 µM), *Pseudomonas flourescens* strain F P69 ($K_m$= 27.1 µM) [43] and fungal species as *Candida tropicalis* ($K_m$= 450 µM) [26], *Trichosporon cutaneum* ($K_m$= 18 µM) [10], *Candida tropicalis* HP 15 ($K_m$= 5 µM) [9] and *Candida albicans* TL3 ($K_m$= 1700 µM) [7] suggesting higher affinity of the purified enzyme for phenol. High phenol affinity of the enzyme adds to its catalytic efficiency which is indispensible for its phenol remediation applications.

### *3.7 Effect of pH and temperature on phenol hydroxylase activity*

Figure 7 and Figure 8 shows the dependence of phenol hydroxylation on pH and temperature respectively. The optimal pH and temperature for enzyme activity was found to be pH 7 and 35$^{o}$C respectively. The optimal pH for enzyme activity found in neutral range is comparable to optimal pH range obtained in previous studies as *Brevibacterium fuscum* (pH 7.5), *Comamonas testosteroni* (pH 7.6), *Trichosporon cutaneum* (pH 7.6), *Acinetobacter radioresistens* (pH 7.5), *Brevibacterium fuscum* (pH 7.5), *Candida tropicalis* (pH 7.6-8) [9,10,26,44,45] Similarly, the optimal temperature for activity of the enzyme falls within the optimal temperature range reported by previous studies as *Acinetobacter calcoaceticus* (33 $^{o}$C), *Candida tropicalis* HP 15 (40 $^{o}$C), *Candida albicans* (25 $^{o}$C), *Acinetobacter radioresistens* (24 $^{o}$C) (Krug et al. 1986; Paller et al. 1995; Tsai et al. 2005; Divari et al. 2003) [7, 45-47].

### *3.8 Effect of chelators on phenol hydroxylation activity*

To verify the presence of iron or copper in the partially purified enzyme, the inhibition of enzyme activity was studied using iron and copper chelators. Figure 9 a and b shows that phenanthroline and sodium arsenate (chelator $Fe^{2+}$) shows an increased inhibition of phenol hydroxylation with increase in concentration of both chelators. It was observed that 30 µM phenanthroline and sodium arsenate depresses the phenol hydroxylation reaction by 42.31% and 84.62% respectively. Figure 9 c denotes that sodium

diethyldithiocarbamte (Cu chelator) has no inhibitory effect on phenol hydroxylation activity. From these results, it becomes evident that the purified enzyme contains iron and lacks copper. The presence of iron is an important characteristic of phenol hydroxylase. Pessione et al. [30] reported that iron is present in Fe-S clusters of phenol hydroxylase from *Acinetobacter radioresistens*. Griva et al. [48] studied the multicomponent phenol hydroxylase in *Acinetobacter radioresistens* and showed that Fe-S cluster containing reductase component is essential for electron transfer from NADH to the dinuclear iron centre of the oxygenases which binds oxygen and mono-hydroxylates phenol.

### *3.9. Effect of heavy metals on phenol hydroxylation activity*

Heavy metals can cause harmful effects on tertiary structure of enzymes or sulfur-sulfur cross bridge interference. This makes it essential to study the effect of heavy metals on enzyme activity. The effect of heavy metals as $FeSO_4$, $CuSO_4$, $AgCl_2$ and $HgCl_2$ on phenol hydroxylase activity was analyzed. Heavy metals as $FeSO_4$, $CuSO_4$, $AgCl_2$ and $HgCl_2$ at a concentration of 30 µM inhibits the enzyme activity by 84.61%, 82.05 %, 74.36% and 82.05 % respectively (Figure 10 a, b, c and d). Neujhar and Gall [3] reported severe inhibition of phenol hydroxylase activity by 10 µM of heavy metals as $CuSO_4$, $AgNO_3$ and $HgCl_2$ which supports our present finding. Similar results of inhibition of phenol hydroxylation activity by heavy metal as $CuSO_4$ (93% inhibition), $HgCl_2$ (100% inhibition), $AgNO_3$ (30 % inhibition) and $FeSO_4$ (50 % inhibition by 0.1 mM $FeSO_4$) were also reported by Liu and Chapman [49].

### *3.10 Effect of denaturant on phenol hydroxylation activity*

Denaturants change the enzyme structure and an enzyme with changed conformation will have reduced optimal fit of substrate in enzymes active site. Thus, the analysis of effect of denaturants on enzyme activity is important.1M SDS while 3M urea inhibit phenol hydroxylation activity by 82% and 80.77% respectively (Figure 11 a and b). The present results are in accord with the findings of inhibitory effect of denaturants on 3-hydroxybenzoate-6-hydroxylase [50]. Neujhar and Gaal [10] reported irreversible inhibition of phenol hydroxylase activity in *Trichosporon cutaneum* by 70-80% on treatment with 2 M Urea which accords with the present findings. Sumathi and Dasgupta [50] reported similar loss 3-hydroxybenzoate-6-hydroxylase activity by 90% and 98% on treatment with 2M Urea and and 0.01% SDS respectively. They proposed that denaturants led to conformational changes in 3-hydroxybenzoate-6-hydroxylase perturbing the microenvironment of aromatic amino acid residues. Such changes results in reduction or loss of catalytic activity of the enzyme.

### *3.11 Effect of oxidizing agent on phenol hydroxylation activity*

Oxidising agents cause oxidation of –SH group in the enzymes resulting in inhibition of enzyme activity by forming (S-S) disulphide linkages [51]. 20 μM of oxidizing agent $H_2O_2$ depresses the activity of the purified enzyme by 83.33% (Figure 12). Neujhar and Gall [10] have reported 71% inhibition of activity of phenol hydroxylase from *Trichosporon cutaneum* by 0.1 M $H_2O_2$ which supports our findings.

### *3.12. Storage stability of the enzyme*

Storage stability of enzyme is indispensible in order to exploit it for any potential applications. The partially purified enzyme shows remarkable storage stability at room temperature and at 4ºC. A minimal loss of phenol hydroxylation activity of 5.13% and 1.28% have been obtained after storage of 7 days at room temperature and 4$^{o}$C respectively (Figure 13). Neujhar and Gaal [10] studied the stability of cell free preparations of phenol hydroxylase from *Trichosporon cutaneum*. They reported that phenol hydroxylase retained original activity for atleast 7 days when stored at 5 $^{o}$C in 0.1 M potassium phosphate buffer supplemented with protective agents mercaptoethanol, EDTA and FAD. Contary to this, the present study stored the enzyme in potassium phosphate buffer with no addition of protective agents. The significant retention of enzyme activity for 7 days on storage even at room temperature apart from that at 4 $^{o}$C highlights its potential to be used for enzyme based phenol remediation or phenol biosensor applications.

### *3.13. Multisubstrate specifity of the enzyme and application thereafter*

The activity of the enzyme towards a broad range of phenolics (unsubstituted and substituted phenols) would be beneficial for its application in remediation technologies of phenol. Apart from this broad substrate specificity for a variety of phenolics would further highlight its potential application for developing phenolic biosensors. This makes the study of multisubstrate specificity of phenol hydroxylase important. In addition to phenol, the purified enzyme has broad substrate specificity acting on 4-nitrophenol; isomeric diphenols as catechol and resorcinol, isomeric methylphenols as o-cresol, m-cresol and p-cresol; halogen substituted phenol as 2-chlorophenol, 4-chlorophenol, 2,4-chlorophenol and p-bromophenol; amino substituted phenols as 2-aminophenol and 3-aminophenol; m-hydroxybenzaldehyde and p-hydroxybenzaldehyde and p-hydroxylbenzoic acid (Figure 14). Neujhar and Gall [10] reported similar substrate specificity of phenol hydroxylase from *Trichosporon cutaneum* against phenol, isomeric diphenols, halogen substituted phenol, amino substituted phenol and to lesser extent on cresols and hydroxybenzoates. Figure 13 shows that the purified enzyme shows higher activity (38.34%) with 4-chlorophenol as substrate as compared to 2-chlorophenol. This is in accordance with the findings of Neujhar and Gall [10]. Purified phenol hydroxylase from *Trichosporon cutaneum* showed higher activity for 4-chlorophenol (29%) over 2-chlorophenol (10 %). The purified enzyme had comparatively lower activity against 2,4-dichlorophenol since higher chlorinated phenols are degraded less rapidly than lower

chlorinated phenols [52]. As evident from Figure 13, the activity of the purified enzyme against three isomers of cresol was in the order: o-cresol > p-cresol> m- cresol. Ahmad and Kunhi [53] reported similar difference in cresol isomer degradation rates in *Pseudomonas* sp. CP4. Similarly, the activity of the purified enzyme against 2-aminophenol was higher by 12.12% than that of 3-aminophenol as seen in Figure 13. The activity of purified enzyme against isomeric diphenols is of the order: quinol > resorcinol > catechol which is in accordance to catalytic activity of phenol hydroxylase from *Trichosporon cutaneum* against isomeric diphenols [10].

The multisubstrate nature of an enzyme makes it a desirable candidate for various applications. Kjellen and Neujhar [54] exploited the multi-substrate specific nature of phenol hydroxylase from *Trichosporon cutaneum* for developing an enzyme electrode for detection of phenolics. The phenol hydroxylase showed reaction towards many monosubstituted phenols carrying halogen, hydroxyl or methyl group while lacking activity against carboxy groups. The purified microalgal phenol hydroxylase described in the present study showed activity against carboxy-group substituted phenol as well as against isomeric diphenols, isomeric methylphenols, halogen substituted phenols, amino substituted phenols, nitrophenols and hydroxybenzaldehyde. This multi substrate nature brightens the prospect of exploiting the phenol hydroxylase from *Chlorella pyrenoidosa* for many different applications. One suggested application is development of phenolic biosensors which has been taken up by the group as an independent study. The phenol hydroxylase was attached to top of an oxygen electrode and oxygen consumption monitored since the enzyme utilize molecular oxygen for oxidation of substrate. Another prominent application is phenolic waste water remediation. The group has reported earlier the complete degradation of 50-200 mg/L phenol by using the same strain of *Chlorella pyrenoidosa* [12]. The strain also had the ability to metabolize both alkanes and aromatics (along with phenol) present in petroleum refinery wastewater. Petroleum refinery waste phenol was used as mixotrophic growth substrate with high neutral lipid accumulation potential for biodiesel feedstock. The findings reported is covered by a patented process which combined dual benefits of environmental protection by phenol remediation and generation of algal biodiesel feedstock as byproduct [12]. The multisubstrate nature of the phenol hydroylase from *C.pyrenoidosa* implies that microalgal strains rich in such enzyme holds promise for application in bioremediation of both unsubstituted and substituted phenols in industrial wastewaters and the spent biomass with high neutral lipid content could be exploited as biodiesel feedstock. As an alternative to bioremediation by pure or mixed microbial cultures, the multisubstrate specific microalgal phenol hydroxylase could be utilized for developing enzyme based technology for remediation of broad range of phenolics found in refinery wastewaters.

## 4. Conclusion

The present work endorses for the first time the phenol ring hydroxylation kinetics by microalgal phenol hydroxylase. The knowledge of catalytic properties of this novel phenol hydroxylases is indispensible owing to the potential of phenol hydroxylase to achieve specific hydroxylation reactions of aromatic ring which is difficult to achieve in vitro. The multisubstrate specificity coupled to remarkable storage stability of the microalgal phenol hydroxylase opens up avenues for its application in remediation technologies of a wide range of phenolics released in industrial wastewater and for phenolic biosensor applications. To realize these potential applications of the enzyme, the production of partially purified microalgal phenol hydroylase with increased specific activity by cost effective single step purification will be indispensible. Further, broad substrate specific nature of the microalgal phenol hydroxylase implies that microalgal strain rich in such enzyme holds promise for application in bioremediation of phenolics in industrial wastewaters. Along with this, there is possibility of generation of high neutral lipid containing spent microalgal biomass after phenolic wastewater remediation that could serve as biodiesel feedstock [12].

## Disclosure Statement

The authors report no potential conflict of interest.

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## Figures

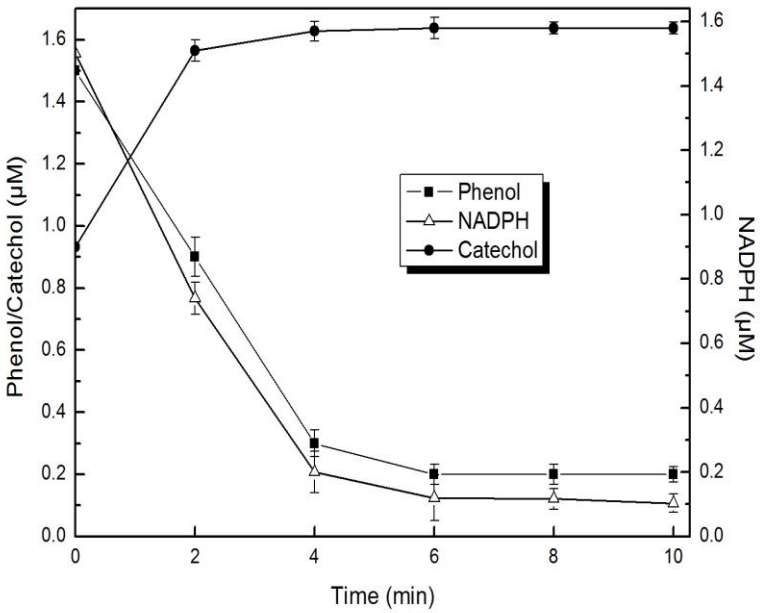



**Figure 1 a.** Phenol hydroxylase activity of crude extract

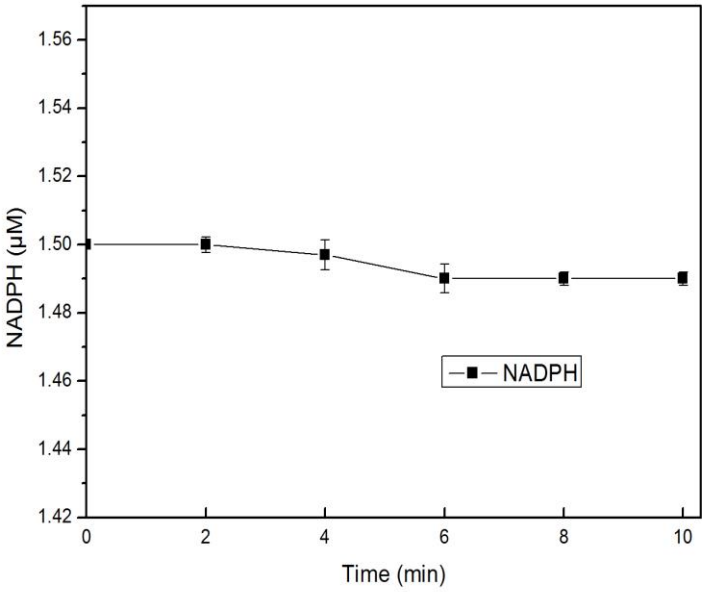



**Figure 1 b.** Autooxidation of NADPH

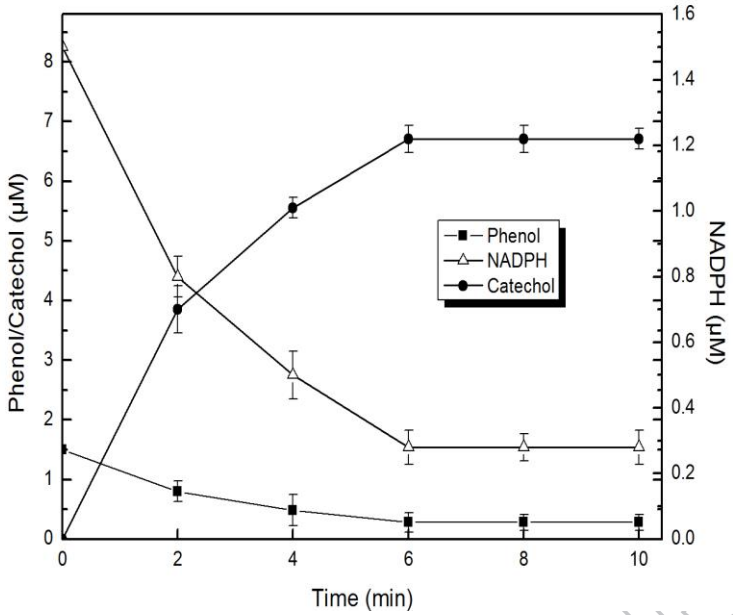



**Figure 2 a.** Phenol hydroxylase activity of 25% Ammonium Sulphate fraction.

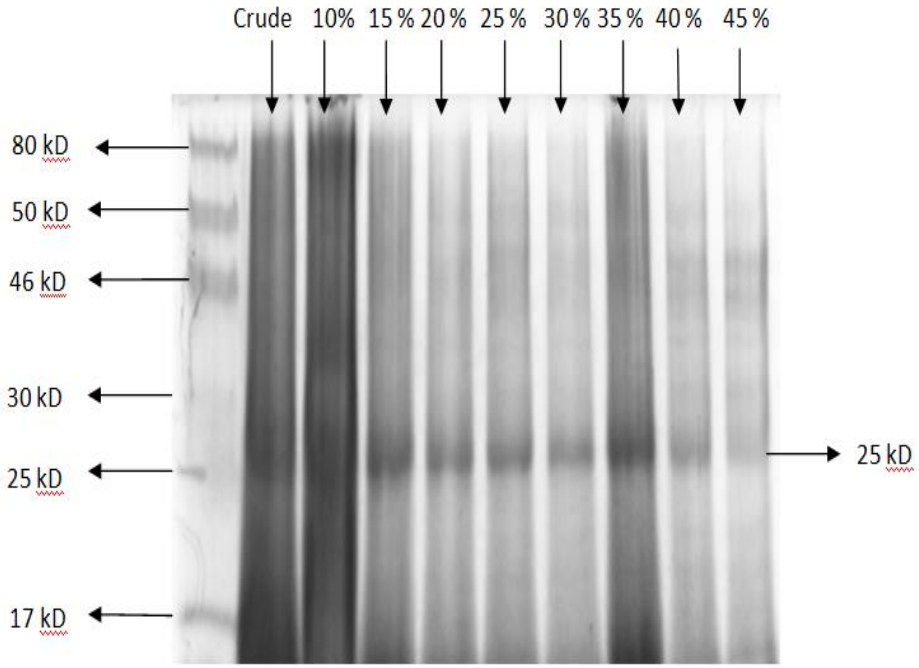



**Figure 2 b.** SDS PAGE image of ammonium sulphate fractions (10-45%).

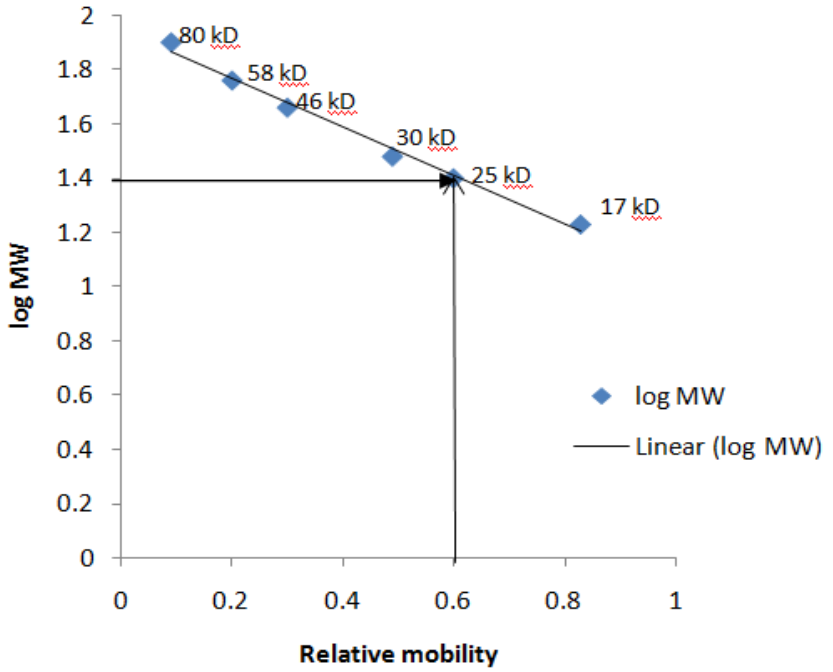



**Figure 2 c.** Molecular weight determination of purified enzyme by log molecular weight (log MW) versus relative mobility plot.

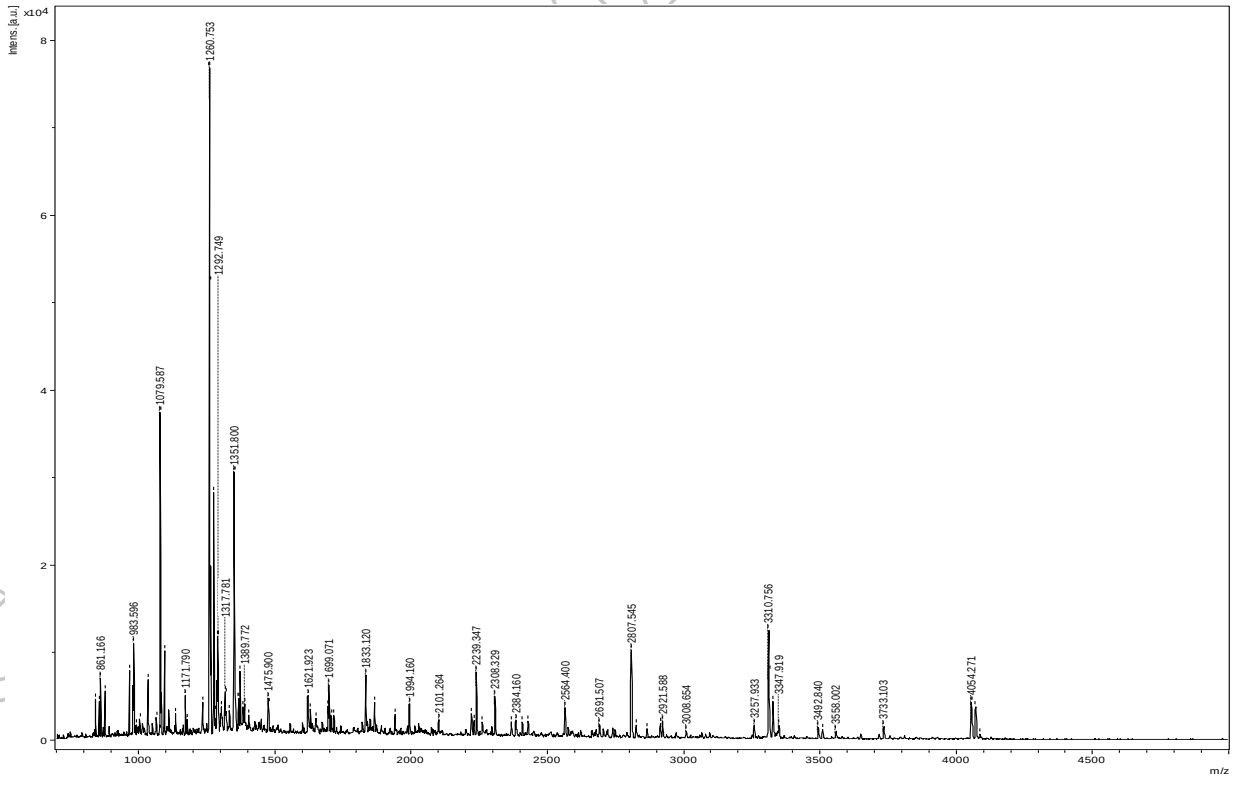



**Figure 3.** Peptide fragment spectra of Phenol hydroxylase obtained by MALDI TOF.

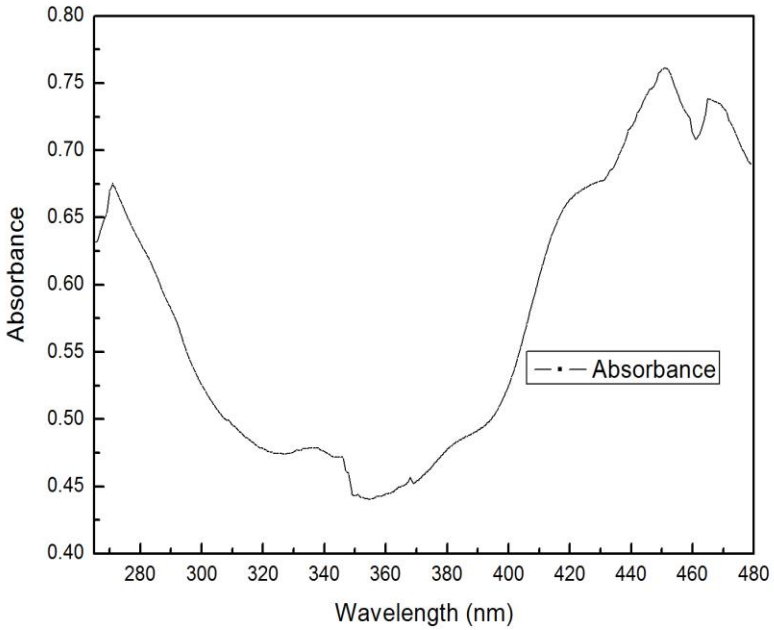



**Figure 4.** UV-Visible spectrum of purified phenol hydroxylase

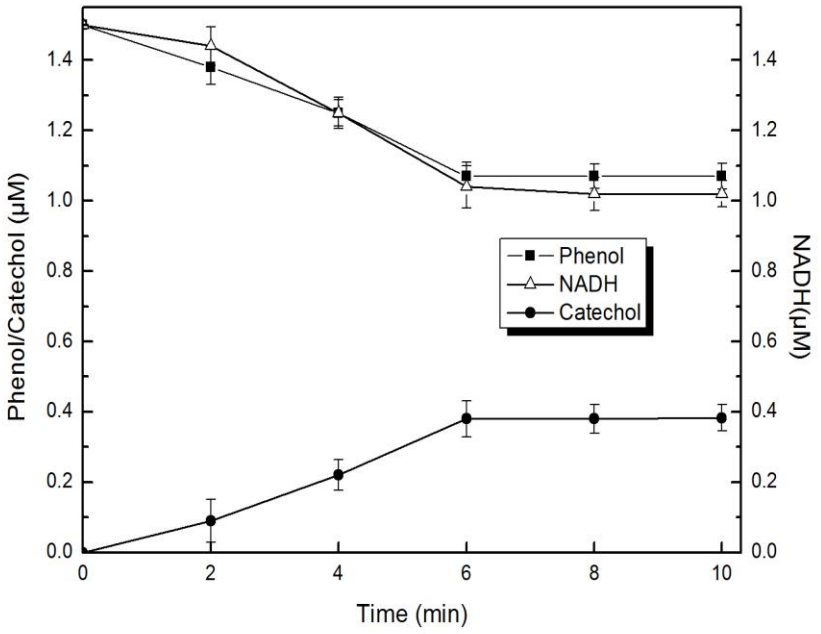



**Figure 5.** Phenol hydroxylase assay using NADH as cofactor.

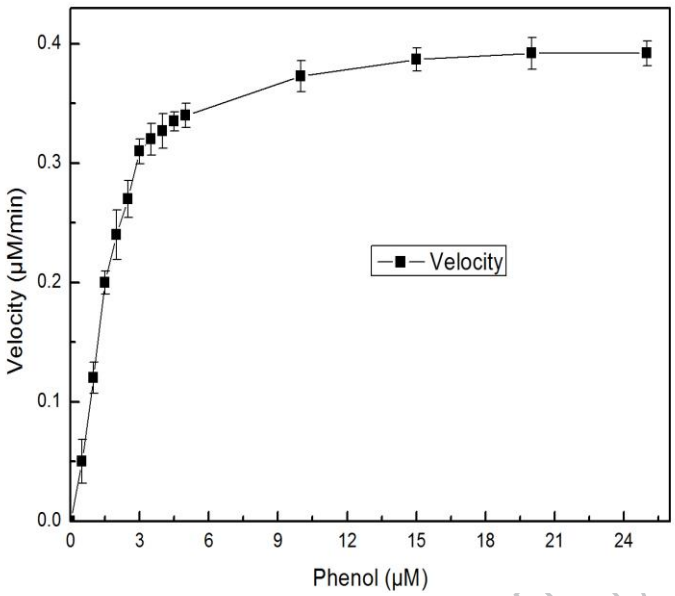



**Figure 6.** Substrate affinity of purified phenol hydroxylase towards phenol

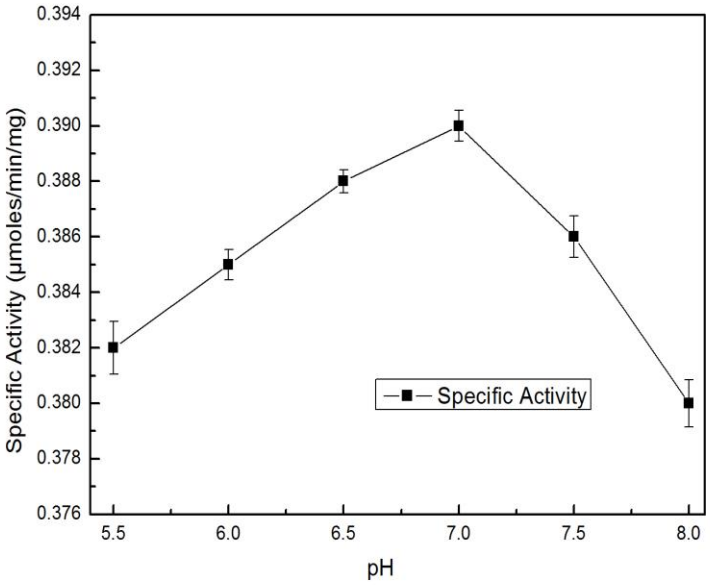



**Figure 7.** Effect of pH on phenol hydroxylase activity

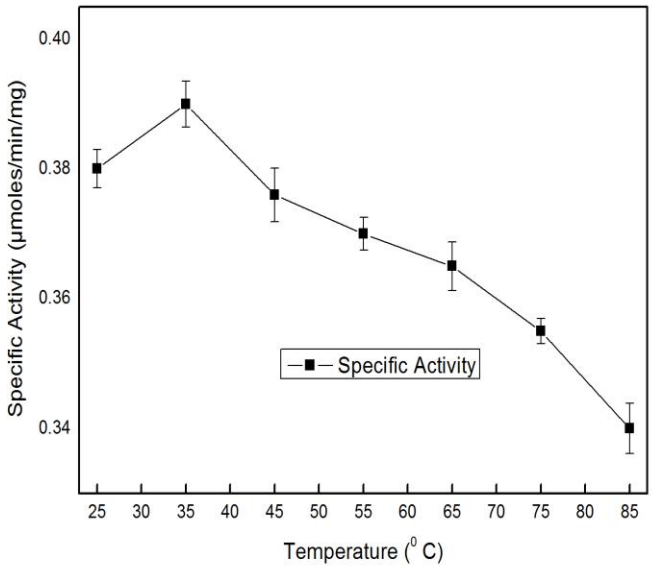



**Figure 8.** Effect of temperature on phenol hydroxylase activity.

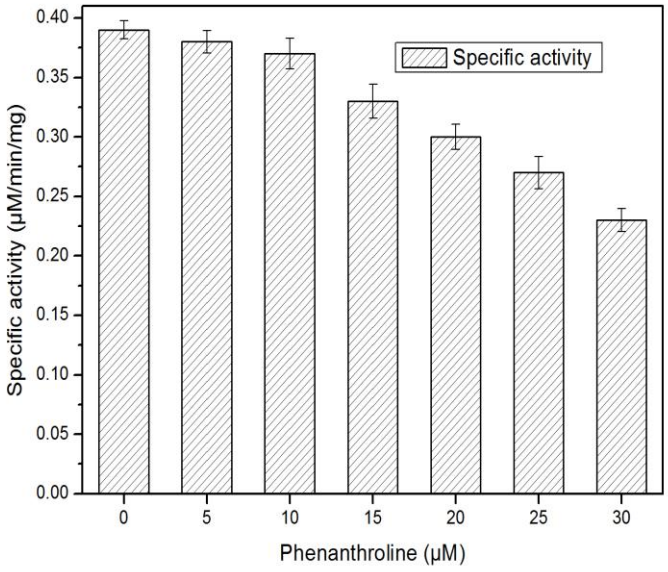



**Figure 9 a.**

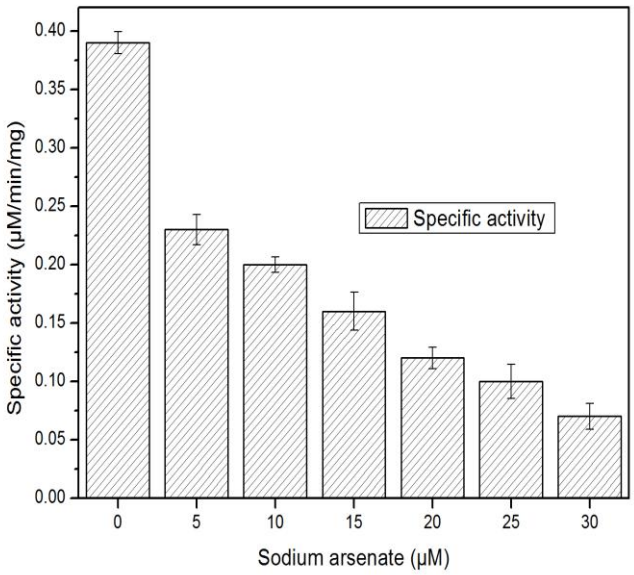



**Figure 9 b.**

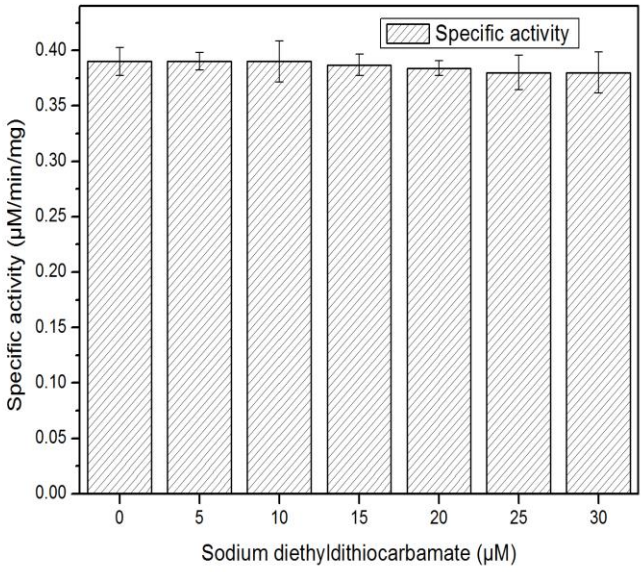
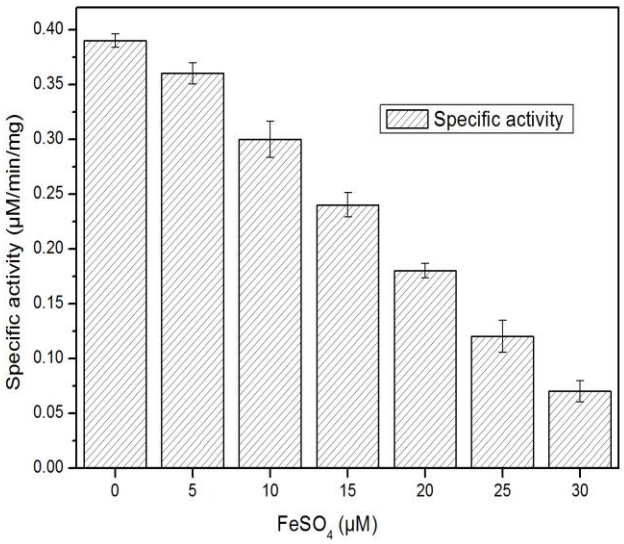
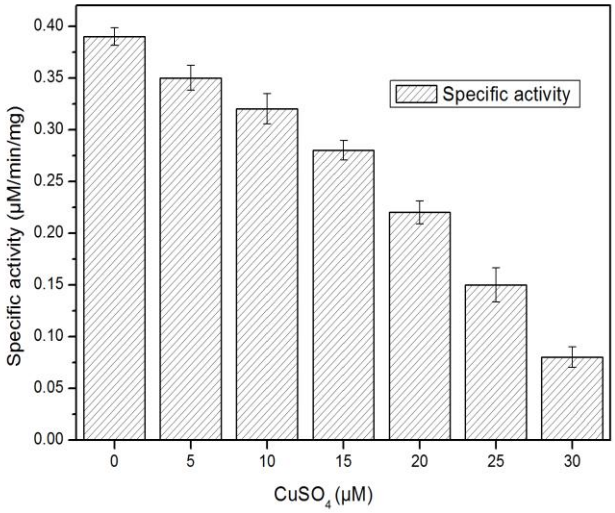



**Figure 9 c.**

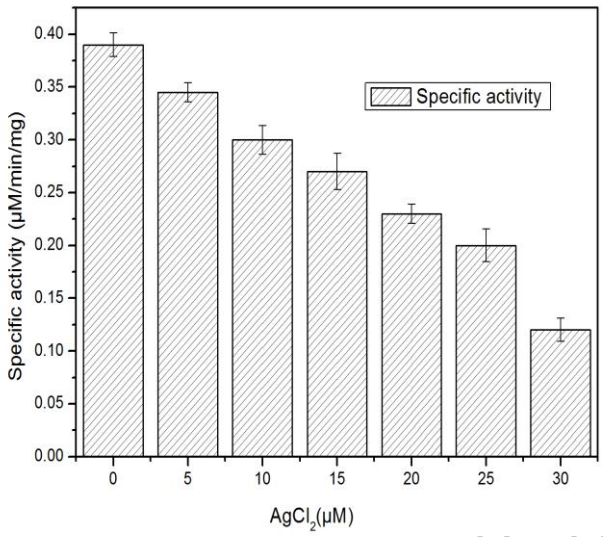
**Figure 9.** Effect of chelators on phenol hydroxylase activity: **a)** phenanthroline (chelator of $Fe^{2+}$) **b)** Sodium arsenate (chelator of $Fe^{2+}$) **c)** Sodium diethyldithiocarbamate (Cu chealator)



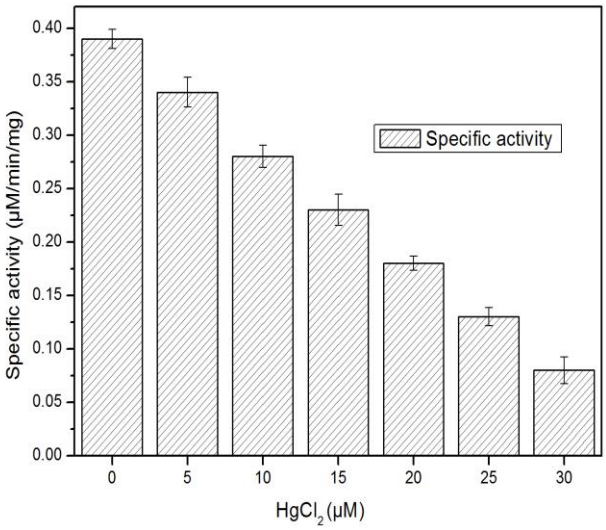

**(a)**




**(b)**

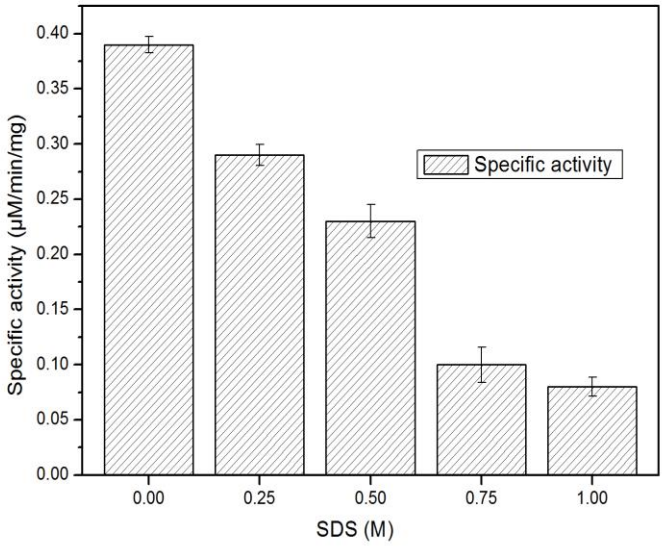



**(c)**

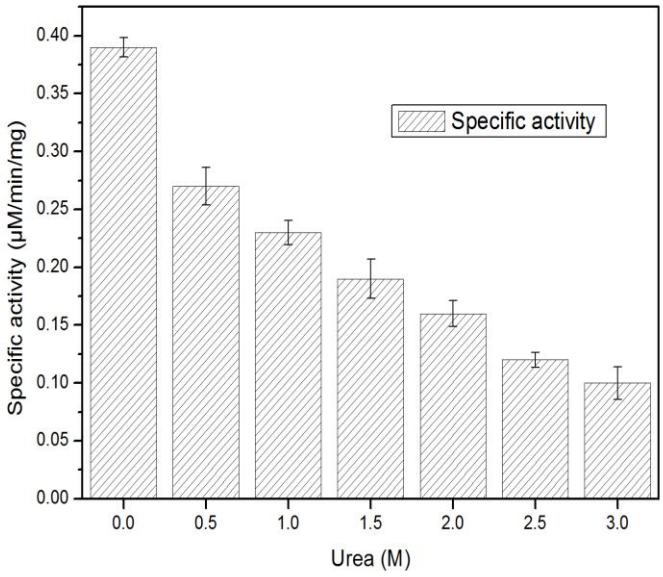



**(d)**

**Figure 10.** Effect of heavy metals on phenol hydroxylase activity: a) $FeSO_4$ b) $CuSO_4$ c) $AgCl_2$ d) $HgCl_2$

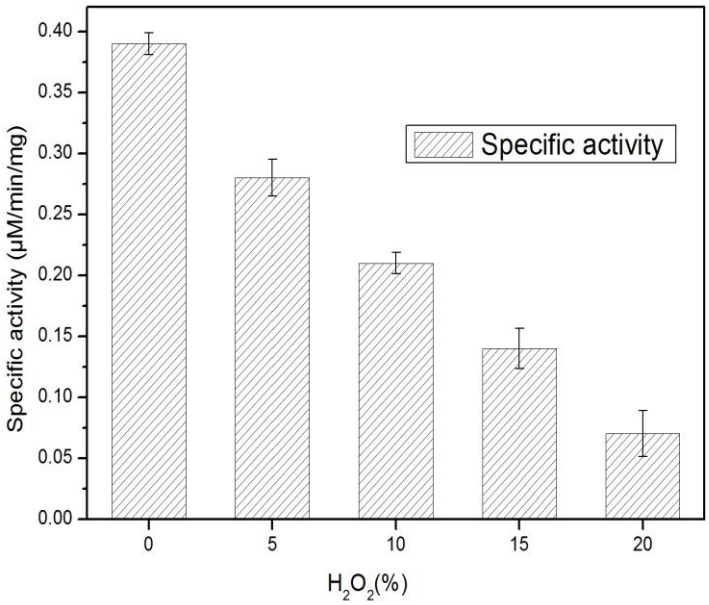



**(a)**

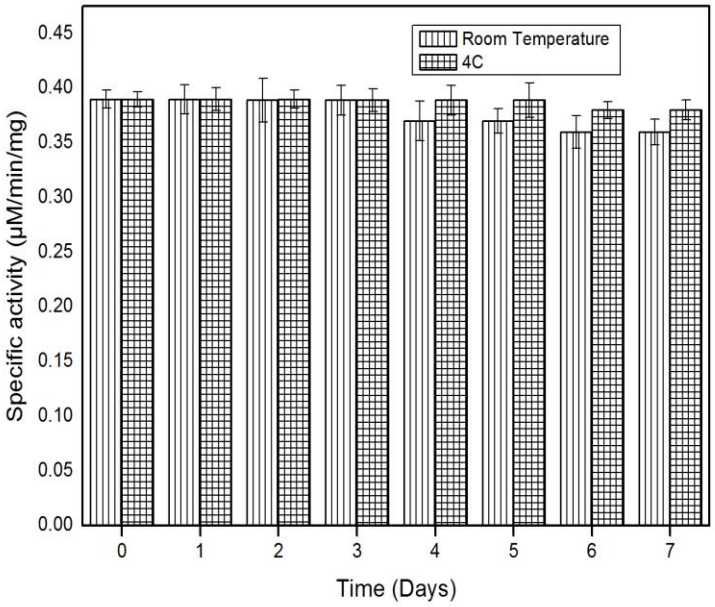



**(b)**

**Figure 11 a)** Effect of denaturant SDS on Phenol hydroxylase activity **b)** Effect of denaturant Urea on phenol hydroxylase activity




activity.




ylase

**Figure 13.** Storage stability of phenol hydroxylase

## Tables

**Table 1.** Specific activity of active ammonium sulphate fractions

| Purification Step | Specific activity ( µmol/min/mg) |
|---|---|
| Crude enzyme extract | 0.13 |
| 15% Ammonium sulphate precipitate | 0.24 |
| 20% Ammonium sulphate precipitate | 0.34 |
| 25% Ammonium sulphate precipitate | 0.39 |
| 30% Ammonium sulphate precipitate | 0.36 |
| 35% Ammonium sulphate precipitate | 0.26 |
| 40% Ammonium sulphate precipitate | 0.14 |

**Table 2.** MASCOT search based identification of purified 25 kD protein band using peptide fragment spectra obtained by MALDI TOF

| Protein Sequence ID hit | Score | E Value | % Identity | % Coverage | Organism | Description |
|---|---|---|---|---|---|---|
| ChlNC64A_1 49348 | 460 | 1.41E-032 | 46.5 | 39.0 | *Chlorella* sp. NC64A | Flavoprotein hydroxylase involved in aromatic ring hydroxylation, carries out oxidation reduction reaction |

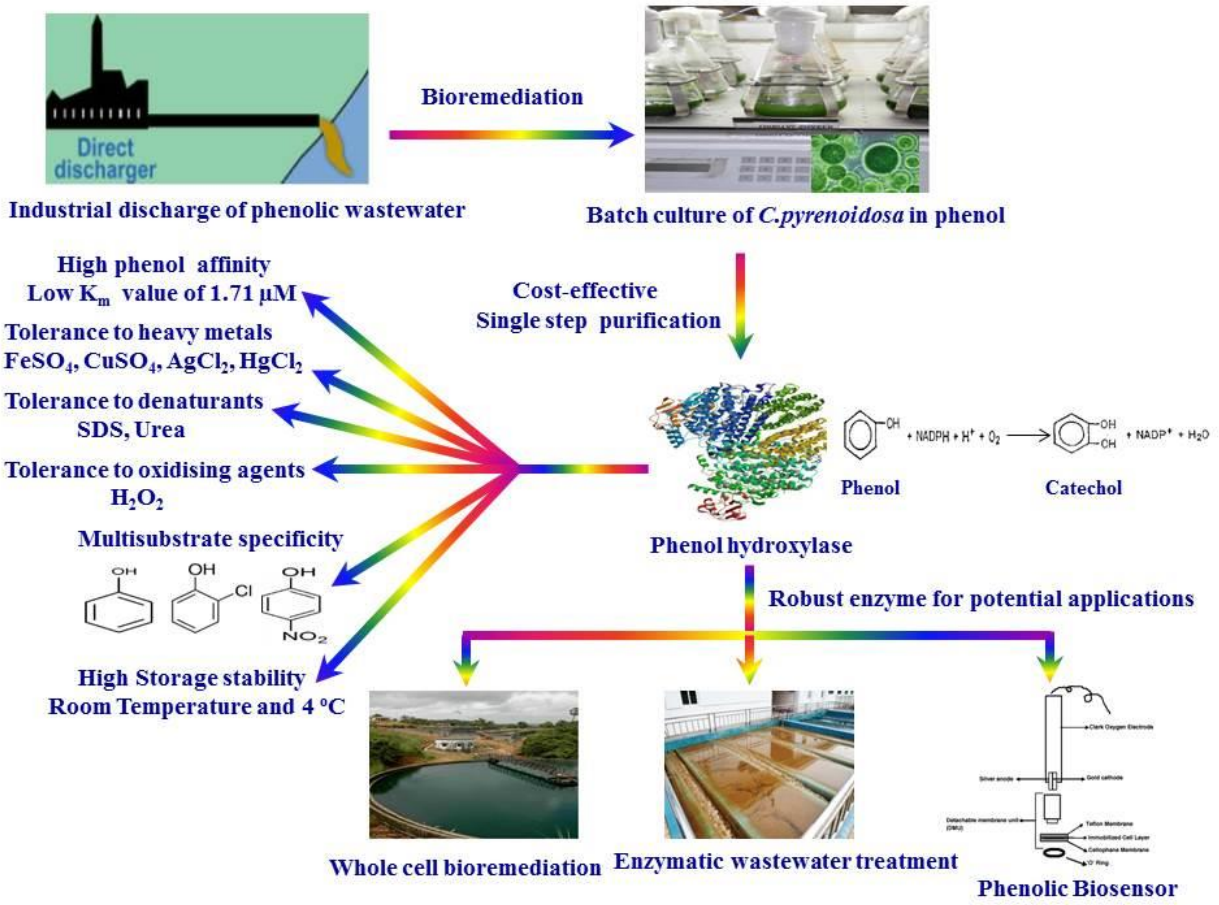
**Table 3.** Stoichiometry of phenol hydroxylase reaction

| **Intial concentration phenol/NADPH** | **Ratio (NADPH/Phenol)** | **NADPH (µM)** | **Phenol Utilized (µM)** | **Ratio utilized (NADPH/Phenol)** | **Catechol produced (µM)** |
|---|---|---|---|---|---|
| 0.5 µM Phenol/0.5 µM NADPH | 1.0 | 0.25 | 0.3 | 0.83 | 0.30 |
| 0.5 µM Phenol/ 0.25 µM NADPH | 0.5 | 0.19 | 0.27 | 0.70 | 0.27 |
| 0.25 µM Phenol/0.5 µM NADPH | 2.0 | 0.29 | 0.17 | 1.71 | 0.16 |
| 1 µM Phenol/1 µM NADPH | 1.0 | 0.80 | 0.70 | 1.14 | 0.70 |
| 1 µM Phenol/0.5 NADPH | 0.5 | 0.45 | 0.69 | 0.65 | 0.67 |
| 0.5 µM Phenol/1 µM NADPH | 2.0 | 0. 71 | 0.43 | 1.65 | 0.42 |
| **1.5 µM Phenol/1.5 µM NADPH** | **1.0** | **1.20** | **1.20** | **1.0** | **1.20** |
| 1.5 µM Phenol/0.75 µM NADPH | 0.5 | 0.65 | 1.10 | 0.59 | 0.09 |
| 0.75 µM Phenol/1.5 µM NADPH | 2.0 | 1.11 | 0.64 | 1.77 | 0.64 |

Continued

Continued

| Intial concentration phenol/NADPH | Ratio (NADPH/Phenol) | NADPH (µM) | Phenol Utilized (µM) | Ratio utilized (NADPH/Phenol) | Catechol produced (µM) |
|---|---|---|---|---|---|
| 2 µM Phenol/2µM NADPH | 1.0 | 1.5 | 1.45 | 1.03 | 1.14 |
| 2 µM Phenol/1 µM NADPH | 0.5 | 0.89 | 1.18 | 0.75 | 1.40 |
| 1 µM Phenol/2 µM NADPH | 2.0 | 1.23 | 0.86 | 1.43 | 0.83 |
| 2.5 µM Phenol/2.5 µM NADPH | 1.0 | 1.80 | 1.63 | 1.10 | 1.42 |
| 2.5 µM Phenol/1.25 µM NADPH | 0.5 | 0.90 | 1.53 | 0.60 | 1.50 |
| 1.25 µM Phenol/2.5 µM NADPH | 2.0 | 1.50 | 0.94 | 1.60 | 0.91 |
| 3 µM Phenol/ 3 µM NADPH | 1.0 | 2.30 | 1.90 | 1.21 | 1.83 |
| 3 µM Phenol/1.5 µM NADPH | 0.50 | 1.18 | 1.86 | 0.63 | 1.82 |
| 1.5 µM Phenol/3 µM NADPH | 2.0 | 1.93 | 1.13 | 1.70 | 1.09 |


Direct discharger
Bioremediation
Industrial discharge of phenolic wastewater
Batch culture of C.pyrenoidosa in phenol
High phenol affinity
Low $K_m$ value of 1.71 µM
Tolerance to heavy metals
$FeSO_4$, $CuSO_4$, $AgCl_2$, $HgCl_2$
Tolerance to denaturants
SDS, Urea
Tolerance to oxidising agents
$H_2O_2$
Multisubstrate specificity
High Storage stability
Room Temperature and 4 °C
Cost-effective
Single step purification
Phenol hydroxylase
+ NADPH + H+ + O2
+ NADP+ + H2O
Phenol
Catechol
Robust enzyme for potential applications
Whole cell bioremediation
Enzymatic wastewater treatment
Phenolic Biosensor