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Exploring substrate promiscuity of chlorophenol hydroxylase against biphenyl derivatives

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Abstract

A 2,4-dichlorophenol hydroxylase, whose gene was derived from the metagenomic library of polychlorinated biphenyls (PCBs)-contaminated soil had been found to exhibit broad range of activity for single ring aromatic contaminants including chlorophenols (CPs) and their homologues. In this study, we intended to explore its activity to aromatic bicyclic compounds such as biphenyl and its derivatives which are also important persistent environmental contaminants. Results demonstrated that the enzyme exhibited broad substrate specificity to selected biphenyl derivatives including hydroxylated biphenyls, halogenated biphenyls, PCBs and hydroxylated PCBs, which extended its substrate promiscuity apart from CPs and their homologues. The enzymatic activities against these aromatic bicyclic compounds were congener dependent and the position and type of the substituent on biphenyl derivatives greatly affected the substrate priority of this enzyme. The hypothesis of the catalysis preference of the enzyme on the aromatic ring was preliminarily proposed on the basis of the analyses of the enzymatic activities against biphenyl derivatives. The high activity and removal ability of this enzyme against selected aromatic contaminants would make it a very promising catalyst for biphenyl derivatives bioremediation.

Keywords: Biphenyl derivatives; Chlorophenol hydroxylase; Hydroxylation; Substrate promiscuity; Bioremediation

1. Introduction

Enzyme promiscuity is the engine of evolutionary innovation and it has attracted significant attention from chemists and biochemists.[1-4] It is increasingly being perceived as immensely useful phenomenon which can dramatically enhance utility of biocatalysis in biotechnology.[2] Broad specificity of an enzyme in terms of catalysis of the same reaction with range of substrates is called substrate promiscuity (also known as substrate ambiguity or broad substrate specificity).[3] Apart from catalytic promiscuity and condition promiscuity, substrate promiscuity is one of the most important parts of enzyme promiscuity and it might lead to improvements in existing catalysts and results in far larger ranges of organic compounds which can be obtained by biocatalysis.[3,5-7]

Substrate promiscuity have been reported for numerous enzyme classes including cytochrome P450s,[8,9] kinase,[10,11] phosphatases,[12] acylaminoacyl peptidase,[13] DNA methyltransferase,[14] cyclic dipeptide prenyltransferase,[15] glutathione S-transferases,[16] laccases[17] and lipases.[18] Among these enzymes, oxidoreductase such as cytochrome P450 superfamily (EC 1.14) and laccases (EC 1.10.3.2) have been increasingly used in the enzymatic-catalyzed degradation of polycyclic aromatic hydrocarbons (PAHs) contaminants due to their high degree of substrate promiscuity.[19] PAHs contaminants such as biphenyl and its derivatives including hydroxylated biphenyls, halogenated biphenyls, PCBs and hydroxylated PCBs (OH-PCBs) are found to be persistent pollutants with high toxicity, bioaccumulation and widespread distribution in the environment.[20] Enzymatic degradation of these compounds formally could only be

conducted by biphenyl dioxygenases.[21] Recent literatures reported that successfully biotransformation of these compounds could also be achieved by monooxygenase cytochrome P-450.[22]

2,4-dichlorophenol (2,4-DCP) hydroxylase (EC 1.14.13.20) is another monooxygenase which have been reported to display high degree of substrate promiscuity against chlorophenols (CPs) and their homologues.[23] This enzyme and multifunction biocatalysis cytochrome P-450, are classified in the same category (EC 1.14) in enzyme commission number. It catalyzes the FAD-dependent oxidative hydroxylation of 2,4-DCP and its homologues, in the presence of O_2 and NADPH/NADH as an electron donor, into the corresponding 3,5-dichlorocatechol/CPs, $NADP^+/NAD^+$, and H_2O.[23] Since the hydroxylation activities of this enzyme against chlorophenol congeners were in general much higher than whose of the reported cytochrome P-450s and laccases, there has been substantial interest in expanding the substrate scope of 2,4-DCP hydroxylase apart from CPs and their homologues.

Our previous research found that 2,4-DCP hydroxylase exhibited broad substrate spectrum against chlorophenols (CPs) and excellent CPs removal ability at both mild and low temperatures, which might make this catalyst more attractive for bioremediation and industrial use.[23] However, the use of this enzyme in the biotransformation was only observed in the biodegradation of single ring aromatic contaminants including above mentioned CPs and their homologues. Limited research has been carried out on its biodegradation of PAHs so far. To explore further the substrate promiscuity of 2,4-DCP hydroxylase, we sought to investigate its ability to

degrade biphenyl and its derivatives in this study. Since enzymatic degradation of compounds with higher substituent group was usually reported to be less effective, and many biphenyl derivatives were not commercially available, only lower chlorinated and hydroxyl (each bearing at most two substitutes at different position on the aromatic ring) substitutional bicyclic aromatic compounds were used in this study (structures and names shown in Fig. 1). Cofactors, such as FAD, required for the hydroxylase activities of biphenyl and its derivatives were also investigated because this enzyme exhibits a high sequence and structural similarity to FAD-dependent hydroxylase.[24]

2. Material and methods

2.1. Material

Eight biphenyl and its derivatives: Biphenyl, 4-Chlorobiphenol, 4,4'-Dichlorobiphenyl, 4-Hydroxybiphenyl, 4,4'-Dihydroxybiphenyl, 4-Hydroxy-3-chlorobiphenyl, 4-Hydroxy-2-chlorobiphenyl, 4-Hydroxy-4'-chlorobiphenyl and 2,4-DCP of analytical grade were purchased from J&K Scientific Ltd. (Shanghai, China). Other chemicals of analytical grade were obtained from Sigma. Recombinant *Escherichia coli* DH5α containing the TfdB-JLU gene for 2,4-DCP hydroxylase expression was from our lab. BugBuster protein extraction reagent was from Novagen (Nottingham, UK).

2.2. Methods

2.2.1. Protein expression and purification

The recombinant *E. coli* was cultivated in LB medium containing 30 μg kanamycin/ml and 34 μg chloramphenicol/ml at 37°C. Protein expression was induced at 18°C by the addition of 0.2 mM isopropyl-β-D-1-thiogalactoside (IPTG) (Fisher Scientific, Fairlawn, NJ) at an OD_{600} of 0.4. After 15 h incubation, the cell pellets were harvested by centrifugation at 12000 rpm and washed with 50 mM sodium phosphate buffer, pH 8.0. For the preparation of crude extract, cells (1.11 g of *E. coli* cell paste from 400 ml fermentation culture) were suspended in 4 ml pH 8.0 Bugbuster Protein Extraction Reagent with 1 mM dithiothreitol (DTT) and 0.6 mM PMSF (phenylmethylsulfonyl fluoride) to yield a higher specific activity compared to ultrasonication. The protein extraction was performed for 10 min at 20°C at 150 rpm. Then, the lysate was centrifuged at 12,000 rpm for 10 min using a Thermo Sorvall WX Ultracentrifuge (Fisher Scientific, Fairlawn, NJ, USA) at 4°C. The supernatant was transferred onto a Hislink™ column (Promega, Madison, WI, USA), rinsed with wash buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mM imidazole, pH 8.0), and eluted with elution buffer (10 mM HEPES, 1 M imidazole, pH 8.0). The protein supernatant was loaded onto a nickel-nitrilotriacetic agarose resin (Qiagen, Germany) equilibrated with the same buffer. After washing with 5 column volumes of the wash buffer (40 mM imidazole), the bound enzyme was eluted with the elution buffer (200 mM imidazole). The fractions containing 2,4-DCP hydroxylase activity were pooled and concentrated by ultrafiltration by using Amicon Ultra-15 centrifugal filter units (Millipore, USA) and then were diafiltered with 50 mM sodium phosphate buffer, pH 7.5, containing 10% (v/v) glycerol. A total of 4 ml protein solution

(300 μg ml^{-1}) was obtained after 4000 rpm centrifugation. Samples were stored at -80°C for further analysis.

2.2.2. Enzymatic removal of biphenyl and its derivatives

The experiments on conversion of biphenyl and its derivatives were performed in a 500 μl eppendorf tube. The reaction mixture was placed into the air-bath constant temperature oscillation incubator (HZQ-F160, Beijing donglian har lnstrument manufacture co.,ltd). Unless otherwise indicated, standard reactions were performed by incubating purified enzyme (final concentration 12 μg ml^{-1}) with 0.1 mM biphenyl and its derivatives (dissolved in acetone), 0.2 mM NADPH (nicotinamide adenine dinucleotide phosphate) in 50 mM sodium phosphate buffer (pH 7.5) and 5 μM FAD (Flavin adenine dinucleotide) at 25 and 0°C (immersed in ice water) with mild shaking for 1 h. After the reaction, samples were quickly moved to 100°C hot water to deactivate the enzyme. All the removals were performed for three times and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test.

2.2.3. Cofactor requirement for hydroxylase activity and removal of biphenyl and its derivatives

2,4-Dichlorophenol hydroxylase is bright yellow and its visible absorption spectrum is typical of a flavoprotein. The prosthetic group is FAD since FAD alone reconstituted active enzyme from apoenzyme. The FAD requirement experiment at 25

and 0°C were conducted the same as that of the enzymatic biphenyl and its derivatives removal method described above. Experiments without addition of FAD were used as control.

2.2.4. Enzyme assay and characterization

The enzyme assay for biphenyl and its derivatives during the reaction was determined by monitoring the decrease in absorbance at 340 nm (ε340 = 6,220 M^{-1} cm^{-1}) following the substrate-dependent oxidation of NADPH. Unless otherwise indicated, standard enzyme activity assays were performed by incubating the purified enzyme with 0.1 mM biphenyl or its derivatives, 5 μM FAD and 0.2 mM NADPH in 50 mM sodium phosphate buffer (pH 7.5) at 25 or 0°C in 500 μl eppendorf tube. The total volume of the reaction mixture is 200 μl. One unit of activity was defined as the amount of enzyme required to consume 1 μmol NADPH per min at 25°C. The kinetic parameters of the purified enzyme for biphenyl and its derivatives at 25°C were obtained using NADPH at 0.2 mM, 5 μM FAD and varying biphenyl or its derivatives from 0.5 to 200 μM. The kinetic constants were calculated from Lineweaver–Burk plots via non-linear regression using GraphPad Prism 5 (GraphPad, San Diego, CA).[24] Protein concentrations were determined by the BCA method (Novagen® BCA Protein Assay Kit) using bovine serum albumin as the standard. Biphenyl and its derivatives removal were measured after 1 h reaction using UV spectrometry. The removal of biphenyl and its derivatives was calculated by dividing the concentration of the amount of reduction of NADPH by the amount of the initial NADPH.

3. Results and discussion

3.1. Substrate specificity of 2,4-DCP hydroxylase against biphenyl and its derivatives

Although 2,4-DCP hydroxylase exhibited broad substrate to certain chlorophenol congeners and derivatives, its substrate specificity to biphenyl and its derivatives at low and moderate temperature has yet to be investigated.[23] In our study, 2,4-DCP hydroxylase activities to biphenyl and its derivatives were investigated at 25 and 0^{o}C. The reported possibility of product inhibitory effect was not evaluated in this study. To explore the substrate specificity and catalysis preference of this enzyme, seven biphenyl derivatives used in this study represent chloro- and hydroxyl-substituent at different positions on the aromatic bicyclic molecules: the single substituent (4-Chlorobiphenol and 4-Hydroxybiphenyl), double substituents on the same ring (4-Hydroxy-2-chlorobiphenyl and 4-Hydroxy-3-chlorobiphenyl), and double substituents on the different rings (4,4'-Dichlorobiphenyl, 4,4'-Dihydroxybiphenyl and 4-Hydroxy-4'-chlorobiphenyl). 2,4-DCP hydroxylase shows a broad substrate specificity and satisfactory activities to certain biphenyl and its derivative at 25^{o}C (Fig. 2). The relative enzymatic activity (expressed as a percentage of the maximum enzyme activity against its regarded natural substrate 2,4-DCP at 25^{o}C without FAD) to 4-Hydroxy-2-chlorobiphenyl, 4-Chlorobiphenol, 4-Hydroxybiphenyl and 4,4'-Dichlorobiphenyl was 273%, 235%, 131% and 96%, respectively at 25^{o}C with FAD. Superior to laccase which do not accept nonhydroxylated biphenyl substrates, the enzymatic activities for 4-Chlorobiphenol (235%) and 4,4'-Dichlorobiphenyl (96%)

were satisfactory. Temperature has been found to have greatly effect on enzymatic activities. Fig. 2 shows that the relative enzymatic activities against the detected substrate at 0^{o}C were in general significantly lower than those at 25^{o}C. The differences between these enzymatic activities were statistically significant ($p < 0.05$).

It is very interesting that the substrate specificity to different substrates was quite different. The specificity pattern of the enzyme for biphenyl derivatives was correlated with both the relative positions of the chlorine or hydroxyl substituent on the biphenyl rings and with the number of chlorine or hydroxyl substituent on the rings. Thus, we would like to propose a preliminary assumption on the metabolic pathways for degradation of biphenyl and its derivatives in the enzymatic hydroxylazation step prior to the tedious and precise detection. Data analysis was conducted on the basis of the enzymatic activities against biphenyl derivatives to estimate the position preference of this enzyme. The substrate specificities at 25 and 0^{o}C were similar, as such, the assumption was proposed on the basis of the results at 25^{o}C (Table 1). Enzymatic activities observed for the biphenyl and its derivatives were quite different. Almost no enzymatic activity was observed when biphenyl was used as substrate (Table 1). However, the enzymatic activities were greatly improved when biphenyl derivatives with substituent group were used as substrates, which suggested that suitable substitution on the biphenyl is of significant for stimulating the enzymatic activity. The result also shows that the activities of the enzyme were related to the substitution type and patterns of specific biphenyl derivatives. The enzyme activities against single substitute substrates (235% for 4-Chlorobiphenol and 131% for 4-Hydroxybiphenyl) are

in generally higher than those of double substitute substrates with the exception for that of 4-Hydroxy-2-chlorobiphenyl (273%), which exhibited the highest activity in the detected substrates. The higher enzyme activity of 4-Chlorobiphenol (235%) compared to that of 4-Hydroxybiphenyl (131%), as well as 4,4'-Dichlorobiphenyl (96%) compared to those of 4-Hydroxy-4'-chlorobiphenyl (76%) and 4,4'-Dihydroxybiphenyl (45%) suggested that enzyme activities against chloro-substituted substrates are higher than those of hydroxyl-substituted substrates when the substituent groups are on the same positions on the aromatic ring of biphenyl. Significant enzymatic activity differences were observed when the substrates with double substituent on one aromatic ring (273% for 4-Hydroxy-2-chlorobiphenyl and 12% for 4-Hydroxy-3-chlorobiphenyl). This result suggested that the enzyme might have a strict specificity for attacking at position 3 (*ortho*-position to 4 hydroxyl group) on one aromatic ring of biphenyl during the hydroxylation reaction since when this position was occupied, only few activities were left. Similar as that of cytochrome P450-catalyzed aromatic hydroxylation, the result was generally consistent with the rules of electrophilic aromatic substitution (EAS) effect.[25] Hydroxylazation of biphenyl and its derivatives is a typical EAS reaction. Hydroxyl and chloro are important substituents for EAS. And these two substituents will have different effects on the electron distributions in the biphenyl ring system.

It is well known that both the speed and the regioselectivity of EAS are affected by the substituents already attached to the aromatic ring.[25] In terms of speed, some groups promote the reaction rate of hydroxylazation, while other groups decrease it. Substituents can generally be divided into two classes regarding electrophilic

227 substitution: activating and deactivating towards the aromatic ring. Activating
228 substituents or activating groups such as hydroxyl will stabilize the cationic
229 intermediate formed during the substitution by donating electrons into the ring system,
230 by either inductive effect or resonance effects. This well explained the promotion of
231 enzymatic activities against OH-biphenyl compared to that of biphenyl. On the other
232 hand, deactivating substituents such as chloro would destabilize the intermediate cation
233 and thus decrease the reaction rate. They do so by withdrawing electron density from
234 the aromatic ring. The increase of enzymatic activities against chloro-substitute
235 biphenol derevitives compared to that of biphenyl is surprising since chloro is a
236 deactivating group for aromatic ring. The deactivating effect might be offset by other
237 factors. In the enzymatic reaction, regioselectivity of EAS and substrate might also play
238 an important role in influencing the reaction rate. It might be that there is some
239 interaction between chloro-substituent on the biphenyl derivatives and the active site of
240 enzyme, which help the hydroxylase to direct the substrates more efficiency during
241 hydroxylazation. Interaction with enzyme might also change the balance of resonance
242 and polar effects, strengthen the weak rate-enhancing resonance effect, or weaken the
243 strong rate-retarding polar effect. The in general higher activities of single substitute
244 substrates than those of double substitute substrates might be due to that the enzyme has
245 a sterically permissive active site that is not overly restrictive to the motion of single
246 substitute substrates.[26]

247 This result was coincident with the previous reports in terms of preferred
248 hydroxylation position since most of the other favoprotein hydroxylases that

hydroxylating the primary substrate either *ortho* or *para* to the existing hydroxyl groups. The slightly activities of 4-Hydroxy-3-chlorobiphenyl suggested that other positions on the phenol ring might also be hydroxylated. Despite the certainty of product formation, hydroxylation might not be the only pathway for the reaction, further study to identify the product is needed to be done.

Moreover, introducing the second substituent on the other aromatic ring of biphenyl derivative resulted in decreasing the enzyme activity compared to those with substituent only on one aromatic ring. For example, the enzymatic activity to 4-Hydroxybiphenyl was 131%. However, only 76% (for 4-Hydroxy-4'-chlorobiphenyl) and 45% (for 4,4'-Dihydroxybiphenyl) activity was remained, respectively after introducing the other substituent on the other aromatic ring of 4-Hydroxybiphenyl. Similarly, the enzymatic activity to 4-Chlorobiphenol was 235%. And only 96% activity was remained for 4,4'-Dichlorobiphenyl, after introducing the other chlorine on the other aromatic ring of 4-Chlorobiphenol. As far as the enzymatic activities of OH-PCBs were concerned, the enzymatic activities preference of OH-PCBs is in the order of 4-Hydroxy-2-chlorobiphenyl > 4-Hydroxybiphenyl > 4-Hydroxy-4'-chlorobiphenyl > 4-Hydroxy-3-chlorobiphenyl. The reactivity order result suggested that the secondary substituent groups might be very important for the substrate orientation when acting on the active site of the enzyme. When the biphenyl ring has two substituent groups, each exerts an influence on subsequent substitution reactions. Both chloro and hydroxyl-substituents are *ortho-para* director for aromatic compounds. The highest enzymatic activity against 4-Hydroxy-2-chlorobiphenyl might be that the two

271 substituents (hydroxyl and chloro) on the one ring of biphenyl have the same directing
272 effect for hydroxylazation reaction, and thus greatly improve the reaction rate. The final
273 result of the electrophilic aromatic substitution seemed hard to predict. The lowest
274 enzymatic activity against 4-Hydroxy-3-chlorobiphenyl might be caused by steric
275 hindrance between substituent and electrophile.

276 To sum up, three hypotheses on the catalysis preference of the enzyme on the
277 aromatic ring was concluded based on the presence results: (1) position 3 in one ring of
278 biphenyl is the preferred position for hydroxylation; (2) the presence of substituent in
279 *para*-position in one ring of biphenyl greatly improve the enzyme activity; (3) the
280 activity of chloride substituent is better than that of hydroxyl group. Our assumption
281 needs to be further confirmed by other experiments. Notably, the premise of our
282 assumption is that the main reaction is hydroxylation reaction. Moreover, hydroxylation
283 only occurs on the free position of the biphenyl structure instead of dechlorination.[27]
284 Many recent studies have shown the multiple functions of oxygenase.[28,29] As such, the
285 oxygen consumption[30] and products derived from each substrate should be investigated
286 to determine whether other side reactions occur or not.

287 Apparent kinetic parameters (Michaelis-Menten constant, K_m; catalytic constant,
288 k_{cat}, and catalytic efficiency, k_{cat}/K_m) for the hydroxylation were calculated from
289 Lineweaver-Burk and Eadie-Hofstee plots. Our kinetic results shown in Table 2 fit well
290 with our enzyme specificity result. The higher the substrate activity, the lower the
291 corresponding K_m, suggesting that 2,4-DCP hydroxylase exhibits high affinity against
292 its favourable biphenyl derivatives. The K_m of 2,4-DCP hydroxylase against

4-Hydroxy-2-chlorobiphenyl (4.2 μM) and 4-Chlorobiphenol (5 μM) are even comparable with that of its preferred nature substrate 2,4-DCP (5 μM). Also k_{cat} and k_{cat}/K_m values were in the order of 4-Hydroxy-2-chlorobiphenyl > 4-Chlorobiphenol > 4-Hydroxybiphenyl > 4,4'-Dichlorobiphenyl > 4-Hydroxy-4'-chlorobiphenyl > 4,4'-Dihydroxybiphenyl > Biphenyl > 4-Hydroxy-3-chlorobiphenyl.

3.2. Enzymatic removal of biphenyl and its derivatives

The removal of biphenyl and its derivatives should be double-checked by using high-performance liquid chromatography to confirm the removal of biphenyl and its derivatives, not just by measuring NADPH consumption detected by UV detection. The product derived from each substrate was not determined in this study. The results indicated that the high enzymatic activities for biphenyl and its derivatives generally resulted in corresponding high biphenyl and its derivatives removal. For example, the high activities of 4-Hydroxy-2-chlorobiphenyl (273%), 4-Chlorobiphenol (235%) and 4-Hydroxybiphenyl (131%) resulted in corresponding high substrate removal which was 81.92%, 73.08% and 84.69%, respectively at 25^{o}C. Notably, although the enzymatic activities for certain biphenyl and its derivatives were similar (235% relative activity for 4-Chlorobiphenol) or even higher (273% relative activity for 4-Hydroxy-2-chlorobiphenyl) than that of 2,4-DCP (235% relative activity) at 25^{o}C, their removal were lower (73.08% removal for 4-Chlorobiphenol and 81.92% removal for 4-Hydroxy-2-chlorobiphenyl) than that of 2,4-DCP (92.38% removal), as shown in Fig. 3. As far as the enzymatic removal of double substitutions biphenyl derivatives

were concerned, most of the detected derivatives were more resistant to 2,4-DCP hydroxylase degradation than 4-Hydroxy-2-chlorobiphenyl. The results in Fig. 3 also indicated that the removal of biphenyl and its derivatives were less efficient at 0°C than that at 25°C. Since the removal of biphenyl and its derivatives was not as good as that of 2,4-DCP in one hour. We intended to prolong the reaction time of enzymatic removal of biphenyl and its derivatives to see if these contaminates could be further degradated. Fig. 4 shows that further increasing the reaction time to 24 h resulted in remarkable improvement of the biphenyl and its derivatives removal at 25°C. However, no obvious increase of biphenyl and its derivatives removal was observed when further increasing the reaction time at 0°C (data not shown). The differences between these removals were statistically significant ($p < 0.03$). Although the enzymatic removal rates of biphenyl and its derivatives was lower than those of CPs which required only one hour to achieve their maximum removal,[23] this enzymatic process is still attractive for industrial use.

3.3. Cofactor requirement for hydroxylase activity and removal of biphenyl and its derivatives

Previous studies reported that the reactions catalyzed by specific hydroxylases require FAD as a cofactor to stimulate their substrates.[31] However, hydroxylases, such as the hydroxylase from *Arthrobacter*, do not exhibit any demonstrable FAD requirement.[31] TfdBs display high sequence and structural similarity to FAD-dependent hydroxylases and contain FAD as a prosthetic group.[32] The FAD requirement for hydroxylase activities and removal of biphenyl and its derivatives was investigated to

335 specify if FAD is the essential cofactor for this enzyme.

336 Our previous results showed that FAD is very important in improving 2,4-DCP
337 hydroxylase activity against 2,4-DCP and the optimum FAD concentration is 5 μM.[23]
338 So 5 μM was selected as the final FAD concentration for the following FAD
339 requirement investigation. The result shows that addition of FAD resulted in a general
340 significant increase in the hydroxylase activity in the range of 1.05-fold to 2.63-fold
341 (Fig. 5a), and 1.12-fold to 8.80-fold (Fig. 5b) at 25 and 0^{o}C, respectively, for different
342 biphenyl and its derivatives with the exception of 4-Hydroxy-3-chlorobiphenyl.
343 Moreover, the enzymatic activity incremental effects were substrate dependent. Notably,
344 the FAD requirement for hydroxylase activity at 0^{o}C seemed to be higher than that at
345 25^{o}C because the enzymatic activity improvements were in general higher at 0^{o}C.

346 The result of cofactor requirement for removal of biphenyl and its derivatives are
347 shown in Fig. 6. The addition of FAD also resulted in a general improvement of
348 biphenyl and its derivatives removal in the range of 1.24-fold to 3.74-fold (Fig. 6a), and
349 1.10-fold to 6.21-fold (Fig. 6b) at 25 and 0^{o}C, respectively. Notably, the result in Fig. 6
350 also demonstrated that the removal improvement with the addition of FAD for
351 4-Hydroxy-2-chlorobiphenyl (3.74-fold at 25^{o}C and 3.2-fold at 0^{o}C) was fairly high
352 than those of other biphenyl derivatives, suggesting the high FAD requirement for this
353 substrate. The differences between the enzymatic activities shown in Fig. 6 and the
354 removal shown in Fig. 5 were statistically significant ($p < 0.05$).

355 The bright yellow color and its visible absorption spectrum (Fig. S1) suggested that
356 certain amount of FAD bound to the enzyme after the protein purification. Also FAD

concentration in the supernatant after heat-denaturing of protein was determined according to method in the literature.[33] The concentration of the free FAD released was assumed to be equivalent to the concentration of the FAD-bound enzyme. The free FAD released from the enzyme and its concentration was calculated on the basis of the free FAD molar absorption coefficient (ε450 of 11.3 mM^{-1} cm^{-1}).[33] And the FAD concentration measured after the heat-denature experiment was 0.59 μM. This result well explained the existence of activity and substrate transformation ability of the enzyme. Cofactor requirement results also showed that further addition of FAD in the reaction mixture, led to in general improvement of enzymatic activities as well as substrates transformation ability. In general, one flavin per enzyme active site is required. All flavoprotein aromatic hydroxylases contain one molecule of FAD per subunit and that the 2,4-DCP hydroxylase is a tetrameric protein. So the molar ratio of FAD/protein should be 4:1. It is notable that the enzyme concentration in the reaction mixture is 0.19 μM, and the molar ratio of FAD/protein ratio without FAD addition was approximately 3:1, which is lower than 4:1. Our result might suggest that supplementing the flavin cofactor FAD in the reaction mixture may be possible to reconstitute the flavoprotein.

4. Conclusions

In the present study, substrate promiscuity of 2,4-DCP hydroxylase against biphenyl derivatives was explored. The enzyme activities of certain biphenyl derivatives are comparable with that of its regarded natural substrate 2,4-DCP. The high removal

ability of this enzyme against certain biphenyl derivatives as well as CPs would make it a potentially catalyst in the bioremediation of aromatic contaminants. This enzyme would also be a promising template candidate for PAHs bioremediation-catalyst reconstruction through directed evolution and protein engineering. The preliminary assumption we proposed on the metabolic pathways for degradation of biphenyl and its derivatives in the enzymatic hydroxylazation step would provide a good reference value for screening new potential substrates and enzyme reconstruction. Further enzymatic and reaction mechanism studies may improve our understanding of biphenyl derivatives degradation pathway and help optimize our efforts to remediate biphenyl derivatives-contaminated environment.

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Table 1

Catalysis preference analysis of the enzyme against biphenyl derivatives.

Substrate	Substituent group	Occupied position	Relative activity (%)[a]
Biphenyl	NA[b]	–	16 ± 1
4-Chlorobiphenol	Cl	4	235 ± 9
4-Hydroxybiphenyl	OH	4	131 ± 7
4,4'-Dichlorobiphenyl	Cl	4,4'	96 ± 8
4,4'-Dihydroxybiphenyl	OH	4,4'	45 ± 3
4-Hydroxy-4'-chlorobiphenyl	OH, Cl	4,4'	76 ± 6
4-Hydroxy-2-chlorobiphenyl	OH, Cl	4,2	273 ± 15
4-Hydroxy-3-chlorobiphenyl	OH, Cl	4,3	12 ± 1

[a] Relative activity is expressed as a percentage of the maximum enzyme activity towards its regarded natural substrate 2,4-DCP at 25^{o}C without addition of FAD. The specific activity is given as percentage of the activity towards 2,4-DCP, which corresponded to 1.55 U (mg protein)$^{-1}$ at 25^{o}C.

[b] –, Not determined.

Table 2

Kinetic parameters of 2,4-DCP hydroxylase towards biphenyl and its derivatives.

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Biphenyl	73.6 ± 3.2	7.0 ± 0.3	0.095 ± 0.006
4-Chlorobiphenol	5.0 ± 0.2	102.3 ± 5.7	20.5 ± 0.9
4-Hydroxybiphenyl	9.2 ± 0.5	56.9 ± 4.2	6.2 ± 0.2
4,4'-Dichlorobiphenyl	12.3 ± 0.7	41.8 ± 2.9	3.4 ± 0.1
4,4'-Dihydroxybiphenyl	26.1 ± 1.4	19.5 ± 0.8	0.75 ± 0.03
4-Hydroxy-4'-chlorobiphenyl	15.5 ± 0.7	33.0 ± 1.1	2.1 ± 0.2
4-Hydroxy-2-chlorobiphenyl	4.2 ± 0.1	118.5 ± 5.2	28.2 ± 1.8
4-Hydroxy-3-chlorobiphenyl	98.7 ± 4.8	5.2 ± 0.2	0.053 ± 0.004

Figure legend

Fig. 1. Structures and names of the biphenyl derivatives investigated in the study.

Fig. 2. Specific activity of 2,4-DCP hydroxylase against biphenyl and its derivatives at 25^{o}C (black column) and 0^{o}C (gray column). Relative activity is expressed as a percentage of the maximum enzyme activity against its regarded natural substrate 2,4-DCP at 25^{o}C, which corresponded to 1.55 U (mg protein)$^{-1}$.

Fig. 3. 2,4-DCP hydroxylase removal of biphenyl and its derivatives at 25^{o}C (black column) and 0^{o}C (gray column). The removal of biphenyl and its derivatives after 1 h was calculated by dividing the concentration of the amount of reduction of NADPH by the amount of the initial NADPH.

Fig. 4. Effect of reaction time on biphenyl and its derivatives removal. Gray columns stand for the biphenyl and its derivatives removal, respectively after 1 h reaction at 25^{o}C with 5 μM FAD. Black column stand for the improvement of biphenyl and its derivatives removal, respectively after 24 h reaction.

Fig. 5. Cofactor requirement for hydroxylase activity against biphenyl and its derivatives (a) at 25^{o}C and (b) at 0^{o}C. Black and gray column stand for the reaction without 5 μM FAD and with FAD, respectively. Digits with underline above the column stand for the growth factors of hydroxylase activity against different biphenyl and its derivatives by addition of FAD, respectively.

Fig. 6. Cofactor requirement for biphenyl and its derivatives removal (a) at 25^{o}C and (b) at 0^{o}C. Black and gray column stand for the reaction without 5 μM FAD and with FAD, respectively. Digits with underline above the column stand for the growth factors against different biphenyl and its derivatives removal by addition of FAD, respectively.

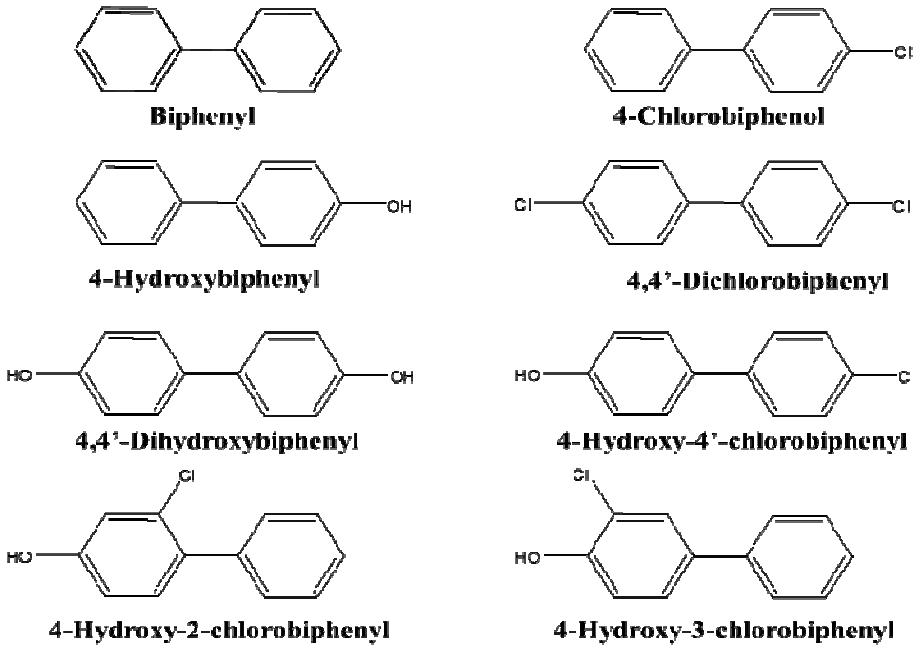


Fig. 1

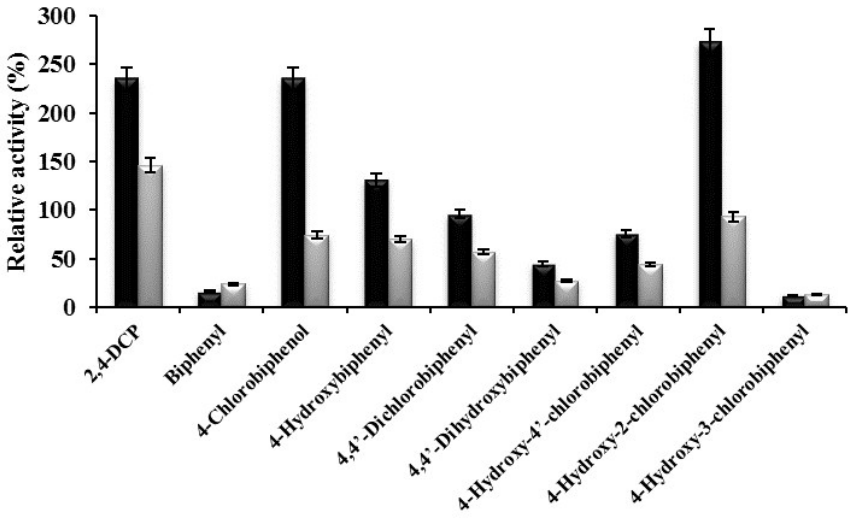
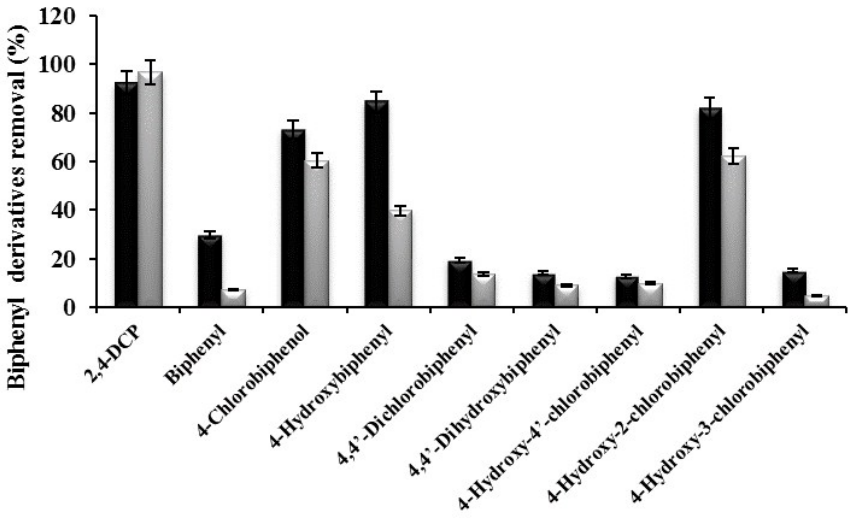


Fig. 2



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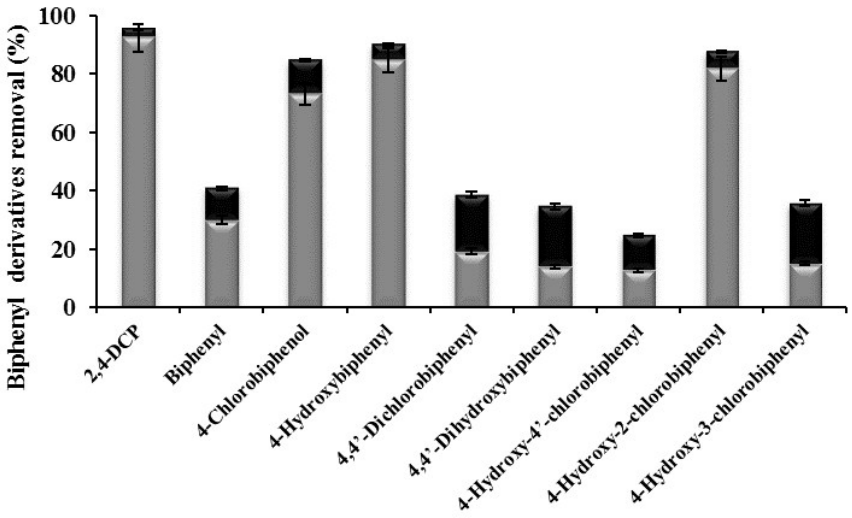
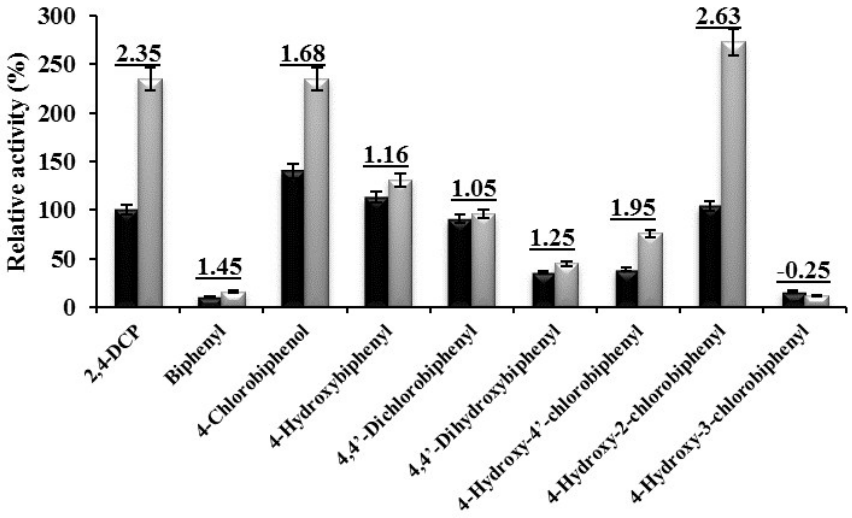


Fig. 4

a



b

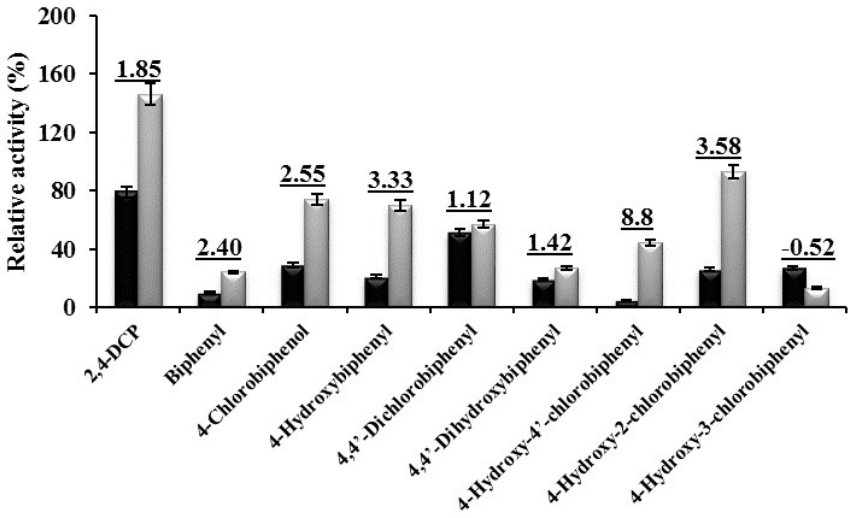
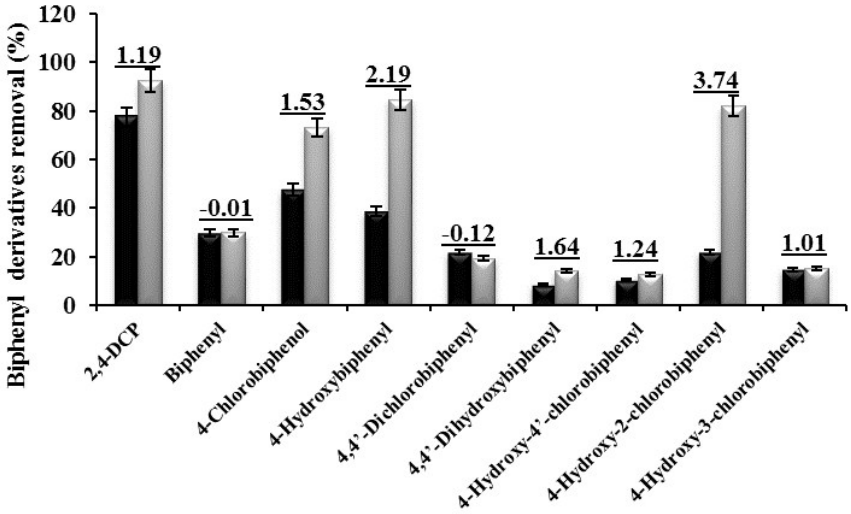


Fig. 5

a



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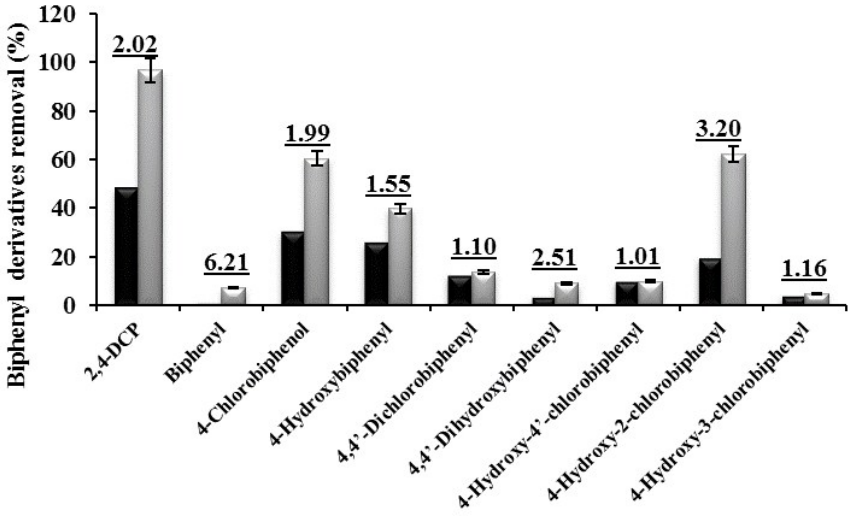

Fig. 6