**BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY**

A N   I N T E R N A T I O N A L   J O U R N A L

Validation of Computationally Predicted Substrates for Laccase

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ABSTRACT

Present study reports the validation (oxidation) of computationally predicted oxidation of xenobiotic contaminants by commercially available pure laccase from Trametes versicolor. Selected contaminants were predicted as potential targets for laccase oxidation by using in-silico docking tool. The oxidation by laccase was measured by change in absorbance at specific λ max of each compound. Sinapic acid and tyrosine were taken as positive and negative controls, respectively. Oxidation was observed in m-chlorophenol, 2,4 di-chlorophenol, 2,4,6 tri-chlorophenol, captan, atrazine and thiodicarb, except malathion, which showed no activity. It could be speculated that the predicted substrates showing oxidation shared homology at structural and chemical level with positive control compounds. In case of malathion, structural non-homology with sinapic acid could be attributed to its inactivity towards laccase that required further structural analysis. Thus, a remediation tool proposing an advanced remediation approach combining the application of theoretical in-silico method and subsequent experimental validation using pure laccase could be proposed. As number and type of xenobiotics increase, the unfeasibility to screen them experimentally for bioremediation also rise. This approach would be useful to reduce the time and cost required in other screening methods.

Key words: biodegradation, In silico screening, laccase, xenobiotics

INTRODUCTION

Industrial and agriculture practices results the release high level of xenobiotic compounds, such as polycyclic aromatic hydrocarbon (PAH), pentachlorophenol (PCP), polychlorinated biphenyls (PCB), DDT, pesticides, benzene, toluene, ethylbenzene, xylene and TNT in the environment (Riva 2006). These are highly toxic and persistent in nature, many of them exert mutagenic and carcinogenic effects on living systems (Samanta 2002; Alcalde et al. 2006). Hence, in view of public health, it becomes necessary to put serious efforts in order to remove them from the environment. Available physico-chemical or mechanical methods require huge chemicals and energy input that enhance process cost and burden on environment. Biodegradation, biostimulation and biotransformation are cost feasible environmental friendly strategies that have wide application in xenobiotics remediation (Rao et al. 2010).

Laccase, a blue copper oxidoreductase [EC:1.10.3.2] is widely used enzyme in bioremediation of xenobiotics (Jurado et al. 2011). Laccases have ability to oxidize both phenolic and non-phenolic lignin related compounds and highly recalcitrant environmental pollutants, which make them attractive and useful tool to serve in biotechnological processes, detoxification of

effluents from pulp and paper industry, textile industry and petrochemical industries (Pinheiro et al. 2004). Laccases have been used as tool for medical diagnostics, as cleaning agents for water purification, as catalysts for the manufacturing of anti-cancer drug and even as ingredients in cosmetics (Shraddha et al. 2011). Their broad substrate specificity makes them able to remove xenobiotic substances (Couto et al. 2006). Since laccases catalyze a wide range of enzymatic reactions, they have also gained considerable biotechnological importance (Durán and Esposito 2000; Leonowicz et al. 2001; Mayer and Staples 2002). They are used as carrier to remove the plant cell wall inhibitors from sugarcane bagasse hydrolysate to obtain desired ethanol yields via microbial reactions (Chandel et al. 2007). A number of biosensors containing laccase have been developed for immunoassays, and for determination of glucose, aromatic amines and phenolic compounds (Kunamneni 2008).

White-rot fungi are main laccase producing organisms in soil, which enable them to grow on various toxic compounds and degrade the pollutants in the soil (Jurado et al. 2011). Enzymes are preferred over the microorganism to work as remediating agent because they can work in harsh environment. Enzyme mediated degradation of pesticides has two advantages; firstly, the degradation reaction proceeds under relatively mild reaction condition and secondly, the reaction rate is faster than that by microorganisms (Couto et al. 2009).

The list of xenobiotics is huge and growing. It is not appropriate to experimentally screen them all to find candidates for which bioremediation can be carried out. Therefore, studies have been carried out to develop *in silico* methods to know the possibility of degradation of a xenobiotic compound by an enzyme like *laccase*. Various bioinformatics methods have been developed to determine the kinetic constant, redox potential of laccase mediated reactions, etc. Some have provided both structural and experimentally determined potentials of laccase for substrate degradation. These theoretical methods may be useful in facilitating *in silico* prediction of substrates for laccase (Librando et al. 2013). Xenobiotic compounds of present study have previously been predicted as substrates for laccase by docking (Suresh et al. 2008). The aim of present study was to validate the prediction strategy applied to screen xenobiotics.

Degradation study has been performed for selected compounds to find out whether they respond similarly towards *laccase* as in the lab. The approach can be useful to develop better remediation strategy.

MATERIALS AND METHODS

Materials

All the chemicals (sinapic acid, L-tyrosine, D-tyrosine, m-chlorophenol, di-chlorophenol, tri-chlorophenol) and pesticides (thiodicarb, malathion, atrazine, captan) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified *laccase* (source organism *Trametes versicolor*) was purchased from Sigma-Aldrich containing 26 U/mg. Laccase-pollutant docking was carried out using GOLD vs.3.0 and docking procedure was performed using both chemscore and goldscore function. The predicted substrates were experimentally evaluated.

Oxidation of xenobiotic compounds by laccase

Enzyme assay of laccase was performed in cuvette in Brittson and Robinson (BR) buffer (pH 4.5). Enzyme stock was prepared in 0.1M BR buffer and stored at 4°C. The 0.1mM BR buffer was prepared by mixing equal volumes of boric acid (0.1 M), orthophosphoric acid (0.1 M) and acetic acid (0.1M). pH was adjusted to 4.5 by using 1N NaOH solution (Xu 1996). Thiodicarb, malathion and atrazine were dissolved in 90% methanol. m-chlorophenol, di-chlorophenol and tri-chlorophenol were dissolved in methanol (50%), while captan was dissolved in acetone (90 %) and tyrosine in water with HCl (0.1 M). All the buffers and solutions were prepared by using double distilled deionized water. Final reaction mixture contained substrate (0.1 mM), enzyme (0.5U) and BR buffer (pH 4.5) in 2.0 mL reaction volume. After the addition of enzyme, absorbance was measured at 0, 15 and 30 min time interval on spectrophotometer (UV-1700 Pharmaspec, SHIMADZU Japan) (Palmieri et al. 1997). The absorbance was measured at λ max of each compound (sinapic acid-315 nm, L-tyrosine-280 nm, D-tyrosine-280 nm, captan-263 nm, thiodicarb-235 nm, malathion-230 nm and atrazine-225 nm, m-chlorophenol-273 nm, di-chlorophenol-28 4nm, tri-chlorophenol-285 nm). Control reactions for all the compounds were performed with inactive enzyme (boiled for 30

min). Absorbance values represented the mean of three independent experiments. Sinapic acid and tyrosine were taken as positive and negative controls, respectively. All the experiments were performed in dark to avoid photo-oxidation of the substrates. Significance was measured for enzymatic activity by paired t test.

RESULTS AND DISCUSSION

Oxidation of predicted substrates by laccase
Enzymatic activity of laccase with positive and negative control (sinapic acid and tyrosine, respectively) and with experimental xenobiotic compounds was examined by measuring the difference in absorbance (at λ_{Max}) at different time intervals. In positive control sample, the enzymatic activity of laccase with sinapic acid was observed as the decrease in absorbance with time, whereas in negative control sample (tyrosine), no enzymatic activity was observed. Chlorophenol compounds (m-chlorophenol, 2,4-dicholorophenol, 2,4,6-trichlorophenol) and pesticides (thiodicarb, captan, and atrazine) were oxidized by laccase. One pesticide (malathion) from predicted compound was not oxidized by laccase. Docking score of predicted compounds indicated the efficiency of their degradation by laccase. Theoretically, higher the gold score,

higher will be the oxidation of compounds by laccase. The score is based on the availability of some reaction promoting chemical features such as H-bonding energy, van-der waals energy, metal interaction, ligand torsion strain in enzyme and compound as well. The results of *in silico* prediction when compared with experimental results showed a similarity. Predicted substrates with high gold score were oxidized efficiently by laccase in comparison to those with low gold score. Although this was not applicable on all the substrates as some xenobiotics were very resistant molecules. The applicability of *in silico* tool to invent environmental remediation strategy could be a practical approach when combined with parallel pure enzyme based remediation. Figure 1 indicates the oxidation pattern of all the compounds. In case of m-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol, higher oxidation by laccase were observed at 15 and 30 min time points as compared to 0 min value. In case of thidocarb, significantly higher (p<0.05) oxidation was observed at 15 and 30 min than 0 min. For captan and atrazine, slight oxidation was observed at 15min that became significantly higher at 30 min time point. Control experiments for each xenobiotic compound with heat inactivated laccase were carried out and no enzymatic activity was observed as indicated in Figure 2.
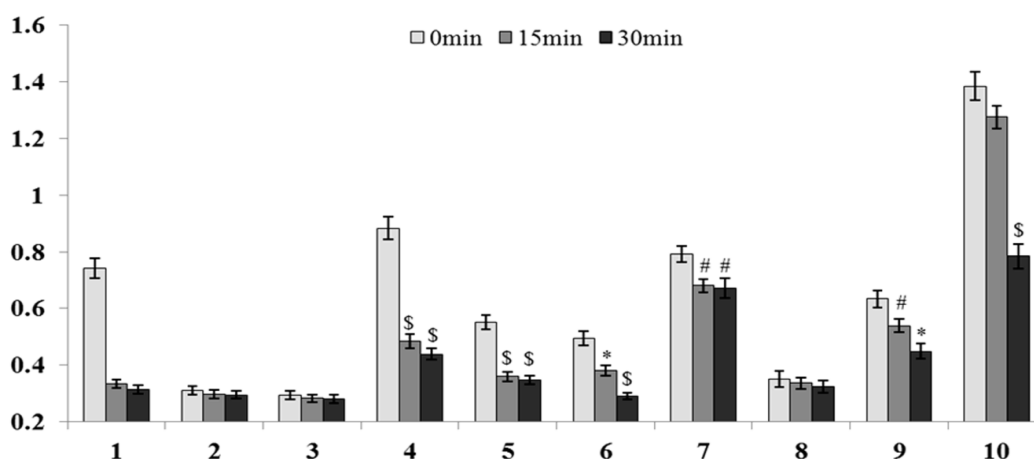

Figure 1 - Oxidation activity of laccase enzyme on various predicted substrates.
N° 1 represents the positive control (sinapic acid), N° 2 and 3 are negative controls (tyrosine D and L, respectively). N° 4, 5, 6, 7, 8, 9 and 10 are representing m-chlorophenol, 2,4-dicholorophenol, 2,4,6-tricholorophenol, thiodicarb, malathion, captan and atrazine, respectively. Decrease in absorbance indicates the decreasing amount of substrate or higher enzymatic activity. Significant changes are denoted by $, for p<0.001, *, for p<0.01 and #, for p<0.05. Error bars (I) indicate mean ± SD from 3 independent experiments.
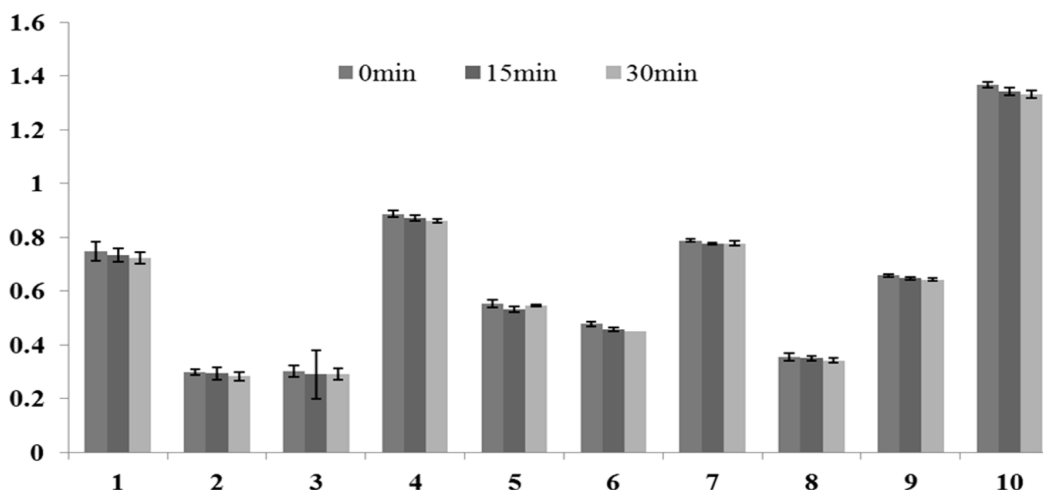
Figure 2- Oxidation activity of heat inactivated laccase enzyme on various predicted substrates. N° 1 represents the positive control (sinapic acid), N° 2 and 3 are negative controls (tyrosine D and L, respectively). N° 4, 5, 6, 7, 8, 9 and 10 are representing m-chlorophenol, 2,4-dicholorophenol, 2,4,6-tricholorophenol, thiodicarb, malathion, captan and atrazine, respectively. Enzyme activity was not observed for any substrate in all the samples as indicated by insignificant differences in absorbance at different time intervals. Error bars (I) indicate mean ± SD from 3 independent experiments.

Biodegradation promoting structures in xenobiotics

Some structural features of xenobiotic compounds and their degradation by laccase have been investigated. Finding of these structural specificities would be important to know the exact mechanism of pesticide degradation by laccase. Structural influence of xenobiotic on the extent of their degradation by laccase was mentioned by Dominguez et al. (2007), particularly for PAH structures (anthracene, benzo pyrene, p-benzoquinone and phenanthrene), indicating the specificity of enzyme towards these substrates. Different laccases differ considerably in their catalytic preferences. Laccases can be grouped according to their preference for ortho-, meta- or para substituted phenols. Ortho-substituted compounds (guaiacol, o-phenylenediamine, caffeic acid, catechol, dihydroxyphenylalanine, protocatechuic acid, gallic acid and pyrogallol) were better substrates than para-substituted compounds (p-phenylenediamine, p-cresol, hydroquinone) and the lowest rates were obtained with meta-substituted compounds (m-phenylenediamine, orcinol, resorcinol and phloroglucinol) with crude laccase (Baldrian et al. 2006). Prediction of substrate degradation by laccase, based on ionization potential has been done by various groups, but no obvious relationship has been established between the ionization potential and degradation percentage. Similar results were reported by other investigators (Pickard et al. 1999; Cho et al. 2002). In an *in vivo* study, (Farnet et al. 2009) showed that six laccase isoforms transformed anthracene and benzopyren. The PAH transformation were linked to the ionization potential (IP) of PAH. It was concluded that molecules with an IP <7.55eV were degraded by laccase. The percentages of anthracene and benzopyrene transformation were higher when ABTS was used as mediator. The presence of redox mediators such as ABTS is required for the oxidation of complex substrates by laccase. Such mediators have been proven efficient system for the degradation of recalcitrant compounds (Piontek et al. 2002). Canas et al. (2010) discussed the application of lignin polymer related phenolic compounds as mediator that promoted the transformation of non-phenolic recalcitrant molecules by laccase. The study emphasized the use of lignocellulosic material to isolate natural, cheap, eco-friendly mediators for industrial uses of laccase to remediate the wastes generated through industrial processes. In another *in silico* study, the role of mediator molecule in the

degradation of large dye molecule was predicted by docking, where the analysis of a dye molecule with enzyme showed that enzyme active site always could not accommodate dye molecule due to constructed nature of active site pocket and steric hindrance of the residues. The mediators, however, were relatively small in size, hence could be accommodated into active site pocket, which together led to the productive binding (Prasad et al. 2012). An interesting study by Reiss et al. (2013) on the oxidation of 91 natural and non-natural industrially relevant compounds by 11 laccasses and LMCOs of bacterial, fungal and plant origin was carried out to classify a newly identified bacterial multi copper enzyme as laccase. Even though the enzymes shared conserved active site residues, the substrate ranges of the individual laccase varied considerably. Such studies could lead designing of desired laccase by enzyme engineering of available 3D structures of laccases according to special requirement like in industrial waste remediation, cosmetic industry, refineries, etc. The main advantage in the approach is the broad substrate specificity of laccase, where enormous possibilities of innovation exist.

Among the predicted substrates, m-chlorophenol, 2,4-dichlorophenol and 2,4,6-tricholorophenol showed a higher degree of structural homology with sinapic acid. Significant decrease in the absorbance of these compounds at 15 min and 30 min time intervals reflected their relatively faster extinction and thereby indicating higher enzymatic activity. Thus, it could be speculated that higher enzymatic activity was attributed to their structural homology to the known substrates such as sinapic acid. They all have aromatic benzene ring and reactive phenol group. However, contradictory results were also obtained in case of thiodicarb and malathione. Despite of higher Gold score (table 1), malathion was not oxidized by laccase, whereas thiodicarb exhibited slow oxidation. The reason of this anomaly, however, lied in the very complex prediction parameters and stringencies of docking calculations (Sousa et al. 2006). The aromatic rings, hydroxyl groups and less ring substitution or chlorination are some biodegradation promoting structures whose presence in xenobiotic compound can lead to their biodegradation by laccase. Librando et al. (2013) have compiled an interesting review on *in silico* methods and their application in bioremediation of pollutants. It was observed that the results of computer simulations used to study laccase agreed well with corresponding experimental data, indicating a high degree of reliability. Delavari et al. (2013) performed docking to characterize the laccase binding pocket of different species in order to establish their common pharmacophoric characteristics. A common km value was also rationalised for a specific substrate oxidised by different laccases. The study was useful in order to understand the oxidation mechanism of laccase for ABTS mediator.

Table 1 - GOLD average fitness score of known and newly predicted substrates for laccase.

Name	GOLD average fitness score
Sinapic Acid	37.32
L-Tyrosine	19.07
D-tyrosine	20.82
m-Chlorophenol	30.25
2,4-Dichlorophenol	30.22
2,4,6- Trichlorophenol	32.17
Captan[a]	44.23
Thiodicarb[a]	59.01
Malathion[a]	57.29
Atrazine[a]	44.24

Microbial degradation of pesticides

Microbes have the ability to degrade the pesticides from the environment. This specialty is contributed by different types of enzymes present in microbes. Laccase is one of them, which has broad substrate specificity, hence is able to degrade wide range of pollutants (Kosehorreck et al. 2009). The simplest case to understand the mechanistic point of reaction catalysed by laccase, is the one in which the substrates are oxidized to corresponding radicals by direct interaction with the copper cluster. This reaction gives rise to the radicals that can spontaneously rearrange, leading to fission of C-C or C-O bonds of the alkyl side chains, or to cleavage of aromatic rings. The substrates of interest cannot be oxidized directly by laccases, either because they are too large to penetrate into the enzyme active site or because they have a particularly high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of so-called 'chemical mediators', which are suitable compounds that act as intermediate substrates for the laccase, whose oxidized radical forms are able to interact with the bulky or high redo potential substrate targets (Kunamneni et al. 2008). The potential of

microbial laccase (*Cerrena unicolor*) to oxidize herbicide, simazine via oxidative coupling reactions has been explored by Sannino et al. (1999). Although Filazzola et al.(1999) showed that simazine behaved as an inhibitor of laccase-mediated-catechol transformation and its degradation occurred as a consequence of the production of the oxidases (lignin peroxidase, manganese peroxidase, laccase) required for the depolymerization of lignin. Although no correlation found between atrazine degradation and laccase activity was studied by Vasilchenko et al. (2002). They studied the decomposition of atrazine by laccase positive and laccase negative clones of *Mycelia sterilia* INB12-26. In another study, *T. versicolor* proved as a strong candidate for bioremediation of atrazine in the soil with low moisture and organic contents (Bastos and Magan 2009). The degradation of organophosphorus compounds by the purified fungal laccase using small molecular weight redox mediator was studied by Leroy et al (2010). LMS were very efficient to promote OP degradation by laccase. Here the importance of the OP nitrogen atom at beta-position and of its substituents was mentioned, though the intimate mechanism of laccase catalysed degradation was not yet known. LMS has been tested in the decolorization of a recalcitrant azo dye Reactive Black 5 (RB5). Both acetosyringone and syringaldehyde demonstrated high mediating ability for RB5 decolorization (Wang et al. 2011).

CONCLUSION

Present study showed the ability of combined approach using laccase as remediation tool for the oxidation of xenobiotic compounds from the environment. Broad substrate specificity of laccase favored its wide spread application in pulp and paper, textile and food processing industry. Predicted compounds were used for the validation of the prediction approach by carrying out their oxidation/degradation. All the compounds were oxidized by laccase, except one. Differences in the structure of xenobiotic compounds were responsible for their extent of oxidation, which could be improved by adding laccase mediator. The approach could be time saving and environmental friendly, as computer based screening and prediction of xenobiotics eliminated

the chemical input demanded in traditional methods.

ACKNOWLEDGMENT

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