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An Insilco approach to bioremediation: Laccase as a case study

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Abstract

Laccase (E.C. 1.10.3.2) is one of the well-studied enzymes used for bioremediation of xenobiotics such as phenols, anilines, etc. Its broad substrate specificity offers a wide opportunity for screening pollutants in order to predict potential targets for degradation. Present study utilizes protein-ligand docking as a tool to achieve the said. For virtual screening, a set of pollutants were selected from five different industries from EPA. X-ray crystal structures of laccase enzymes were taken from the Brookhaven Protein Data Bank (PDB). Two-dimensional structures of pollutants were downloaded from the NCBI Pubchem, which were further converted into three-dimensional structures using CORINA. Protein-ligand docking was carried out using GOLD. Nearly 30 and 17% of the selected datasets showed the best average GOLD fitness score for fungal and bacterial laccase enzyme respectively, suggesting thereby that laccase might be able to oxidize these pollutants. Moreover, in few cases like anthracene, phenanthrene, etc., there is experimental data to support this hypothesis. Similar kind of work would be helpful to find putative pollutants for other biodegradative enzymes.

Keywords: Bioremediation; Laccase; Pollutants; GOLD; ABTS

1. Introduction

One of the most efficient processes to remove pollutants from environment is through bioremediation. It is the process by which living organisms degrade or transform hazardous organic contaminants into less toxic compounds. Screening of indigenous microbes of the pollutant contaminated site for their degradation potential is one way to approach the problem. Thus, microorganisms that can degrade various pollutants (e.g. nitroaromatics, chloroaromatics, polycyclic aromatics, biphenyls and components of oil) have been isolated with the eventual goal of exploiting their metabolic potential for the bioremediation of contaminated sites [1–3].

Oxidoreductases including horseradish peroxidase, lignin peroxidase, manganese peroxidase and laccase have high capability to catalyze oxidation of aromatic compounds. Therefore many researchers have studied both the degradation as well as the removal of environmental pollutants by these enzymes. Among the blue copper oxidases, laccases (benzenediol: oxygen oxidoreductase E.C. 1.10.3.2) are a sub class of comparatively broader substrate specificity enzymes known to degrade several xenobiotics such as phenols, anilines, benzenethiols, etc. [4]. Consequently, laccases have evoked particular interest in biotechnological applications, ranging from biopulping [5] to remediation of wastewater [6]. Laccases have been reported in fungi [7], in plants [8] and in bacteria [9].

The catalytic properties of laccase have had a great impact on the development of biosensors. Advances in research have widened the variety of xenobiotics that can be degraded by laccases from simple phenols, anilines and benzenethiols to polycyclic aromatic hydrocarbons, and organophosphorus insecticides [10]. One of the well-known laccase substrates 2,2′-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) is shown in Fig. 1.

X-ray crystal structure studies over the past decade have enabled the elucidation of a significant number of structural and

Abbreviations: ABTS, 2,2′-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid; AMBER, assisted model building with energy refinement; E.C., enzyme commission number; GOLD, genetic optimisation for ligand docking; NCBI, National Center for Biotechnology Information; PDB, Protein Data Bank; EPA, Environmental Protection Agency; g/mol, gram per mole; RMSD, root mean square deviation

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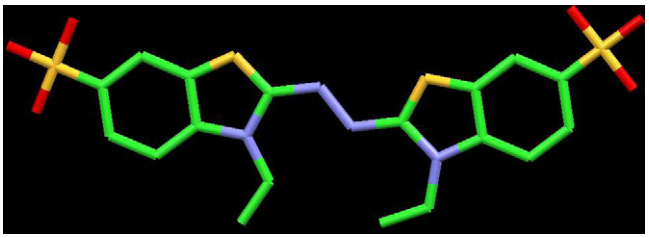

Fig. 1. Two-dimensional structures of 2,2′-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS). This and other structural figures were prepared using Pymol (http://www.pymol.org).

functional aspects of these enzymes. Due to their comparatively broader substrate specificity, Laccases share a sequence pattern that can distinguish them as a specific subgroup of multi-copper oxidase family [11].

Simple bioinformatics searches that integrate multiple sources of data offer a faster and more rapid means of identifying new potential targets for bioremediation as compared to conventional method. Protein-ligand docking tool can be used to screen pollutants for their susceptibility to degradation by already characterized enzyme. Laccase being a broad substrate specific enzyme offers us an opportunity to evaluate our approach. As the substrate specificity differs from one laccase to other, laccase from different sources can be utilized for degrading different pollutants. Although docking has been successfully used for drug screening [12], its utility in predicting the pollutants which can be potential targets for bioremediation has not been acknowledged so far.

2. Materials and methods

A set of 186 compounds (Supplementary Table 1) were selected from the EPA's (U.S. Environmental Protection Agency) Chemical Releases and Transfers List, available for various industries [URL http://www.epa.gov/compliance/resources/publications/assistance/sectors/notebooks/]. Five industries namely textile, pulp and paper, pharmaceutical, organic chemical and agricultural pesticide were selected from EPA using the following criteria.

Only land disposals, water discharges and underground injection chemicals were considered. Metal ions and gases were not included. Only small molecules (substrates and pollutants) with rotatable bonds ranging from 0 to 15 were selected because greater number of rotatable bonds may result in incorrect prediction in docking [13]. Compounds with molecular weights ranging from 50 to 600 g/mol were chosen. Another set of 71 substrates (Supplementary Table 2) of laccase were taken from the Brenda Database [14].

Tyrosine is a known non-substrate for laccase enzyme. In case of *Bacillus subtilis*, in addition to tyrosine, few other non-substrates have also been reported (Table 3) [22]. These non-substrates were thus taken as negative controls.

X-ray crystal structures for laccase enzymes with PDB IDs 1gyc (*Trametes versicolor*) [15] and 1uvw (*Bacillus subtilis*) [16] (resolution 1.7 and 2.5 Å, respectively) were taken from the Brookhaven Protein Data Bank [17]. *Trametes versicolor* and *Bacillus subtilis* laccase structures were co-crystallized with isopropyl alcohol and ABTS, respectively.

The catalytic binding site of laccase enzyme was determined with the help of Insight II (Accelrys Insight II San Diego, CA). In these crystal structures, all amino acids with at least one atom lying within 10 Å distance of any atom bound to either substrate (ABTS or isopropyl alcohol) were considered to be a part of the active site pocket.

Hetero atoms including cofactors and ligands were removed from the protein complex except for the copper ion at the active site. Hydrogens were added at appropriate geometries taking into account the protonation states. Atomic Gasteiger charges were used for the small molecules, and amber charges were used for protein atoms. Water molecules within the active site were considered. Protein-ligand docking was carried out using GOLD v3.0 [Genetic Optimisation for Ligand Docking] [13]. GOLD calculations were performed as previously described [18]. Docking procedure was performed using both scoring functions (Goldscore and Chemscore). Laccase enzyme is a copper metalloprotein, and as there are no copper parameters incorporated in the docking software, copper metal ion geometries were added to the 'gold.parm' file.

Prior to docking, the protein and the ligands were fully minimized using the Discover module of Insight II. Two-dimensional structures of selected datasets were downloaded from the NCBI PubChem Database [URL: http://pubchem.ncbi.nlm.nih.qov/]. Three-dimensional structures were generated using CORINA [URL: http://www2.chemie.uni-erlanqen.de/software/corina/index.html].

3. Results and discussion

Selected laccase enzymes and ligand datasets were docked using GOLD with 30 runs for each approach. The well-docked complexes (RMSD < 2.0 Å) in lowest docked energy with average GOLD fitness score were enumerated. Predicted GOLD average fitness score and chemscore are shown in Figs. 2 and 3 for fungal and bacterial laccase, respectively.

Fig. 4 shows the percentages of docked ligands with good average GOLD fitness score, docked ligands with low GOLD fitness score, and undocked ligands. Nearly 30 and 17% of the

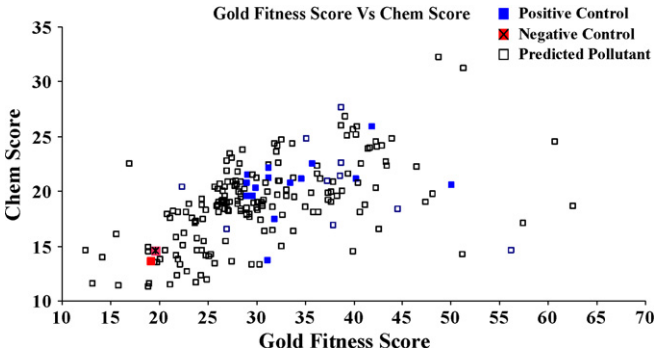

Fig. 2. GOLD fitness score and chemscore of the docked datasets for laccase enzyme in *Trametes versicolor*. Red squares represent negative controls, blue squares represent proved substrates and pollutants and white squares represent selected datasets from EPA and BRENDA databases.

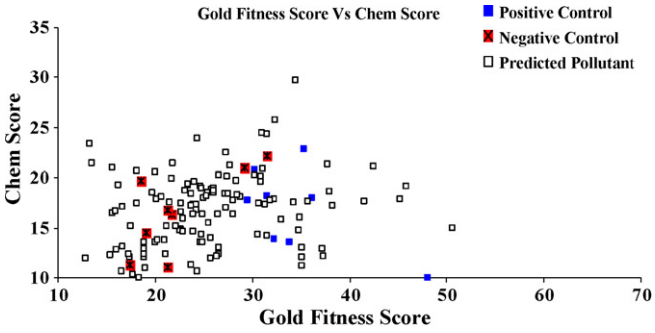

Fig. 3. GOLD fitness score and chemscore of the docked datasets for laccase enzyme in *Bacillus subtilis*. Red squares represent negative controls, blue squares represent proved substrates and pollutants and white squares represent selected datasets from EPA and BRENDA databases.

selected datasets showed the best average GOLD fitness score for fungal and bacterial laccase, respectively, suggesting the broader substrate specificity for fungal laccase.

The lowest RMSD found within the 30 solutions provides an indication whether the experimental structure has been found by docking program. In bacterial laccase, the computed binding mode of ABTS to laccase enzyme is indeed very close to the X-ray pose (1uvw) with RMS deviations of 2.2 Å (Fig. 5).

Our experiments show that the GOLD docking software is capable of selecting well-known substrates and pollutant such as ABTS, syringaldazine, napthol, guaiacol, catechol, phenanthrene, anthracene, etc. (Table 1). Further support to this hypothesis comes from the experimental data that the predicted potential targets (Figs. 2 and 3) can be oxidized by these laccases [19,20]. Thus, it confirms that GOLD can detect well-known binders for an enzyme from a pool of ligands.

GOLD fitness scores for negative controls are shown in Tables 2 and 3. In case of fungal laccase, tyrosine has a low GOLD fitness score supporting that non-substrates and non-binders have a low GOLD fitness score. In contrast, a good average GOLD fitness score for tyrosine in case of bacterial laccase suggests that it might be a substrate, supporting the studies by Shliakhov et al. [23]. Although it has been challenged by Hullo et al. [9]. In addition to this, the other known non-substrates of bacterial laccase have a low GOLD score (Table 3). Thus it can be concluded that known laccase ligands have a good average GOLD fitness score as compared to known non-substrate and hence docking scores can be used as a measure of selecting the preferred ligands for an enzyme. Laccase ligands with good GOLD average fitness scores

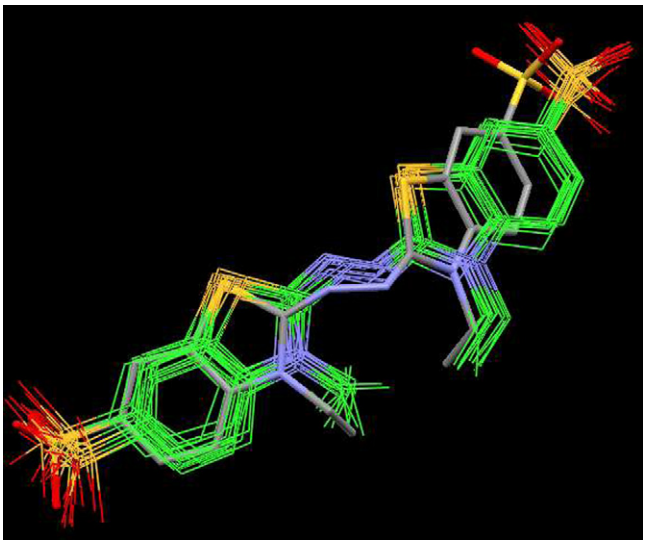

Fig. 5. Representative best poses generated by GOLD in reference to co-crystallized ABTS in 1uvw (*Bacillus subtilis*).

Table 1
GOLD average fitness scores for known substrates and few predicted targets

S.no.	Name	GOLD average fitness score	
		Trametes versicolor	*Bacillus subtilis*
1	ABTS	50.58	48.14
2	Anthracene	40.37	30.22
3	Phenanthrene	42.05	31.62
4	Thiodicarb[a]	59.01	41.61
5	Malathion[a]	57.29	48
6	Captan[a]	44.23	39.27
7	Atrazine[a]	44.24	30.29
8	Indigo[a]	44.6	40.34
9	Remazol Red B[a]	47	33.5
10	Vanillic acid	31.86	–
11	2,4-Dichlorophenol	30.22	30.66
12	*m*-Chlorophenol	30.25	–
13	2,4,6-Trichlorophenol	32.17	31.94
14	Sinapic acid	37.67	–
15	Syringaldazine	33.32	30.3

[a] Newly predicted targets for bioremediation.

predicted in this manner might therefore be potential targets for bioremediation.

Docking gives encouraging results, but not perfect in all the cases. When the library of pollutants was screened for potential targets of fungal laccase, all the well-known substrates were

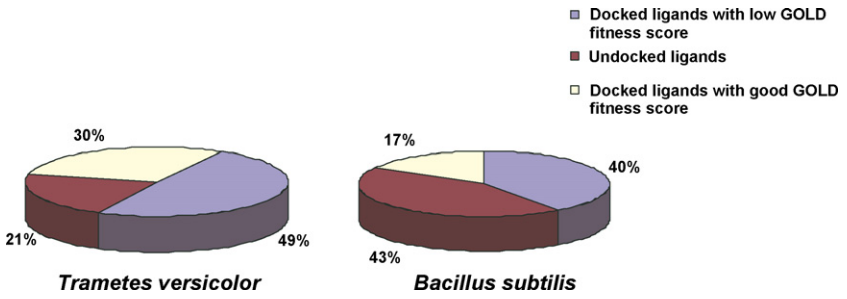

Fig. 4. Percentages of docked ligands with a good average GOLD fitness score, docked ligands with low GOLD fitness score, and undocked ligands.

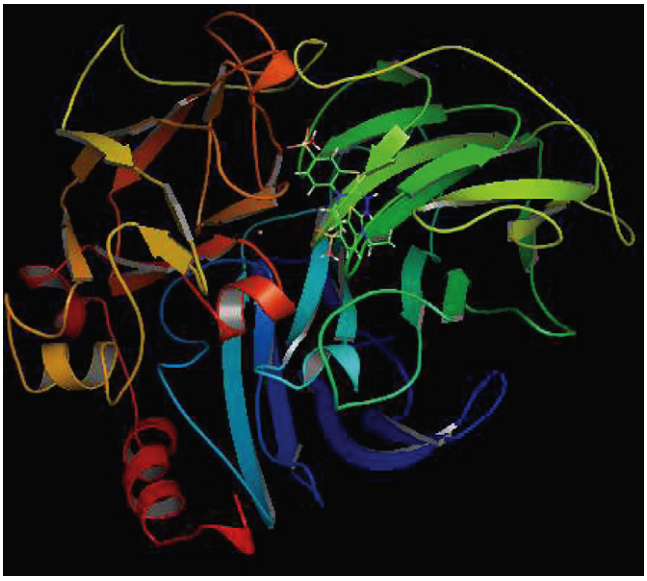

Fig. 6. Docked structure of ABTS with 1gyc (*Trametes versicolor*) laccase enzyme.

Table 2
Comparison of negative control (tyrosine) scores for 1gyc and 1uvw laccase

S. no.	Name	1gyc	1uvw
1	L-Tyrosine	19.07	31.58
2	D-Tyrosine	20.82	29.16

Table 3
GOLD fitness scores for negative controls in *Bacillus subtilis* (1uvw) laccase

S. no.	Name	1uvw GOLD score
1	4-Chlorophenol	21.33
2	2,3-dimethoxyphenol	21.67
3	2,4-dichlorophenol	17.23
4	3,5-dimethoxyphenol	21.36
5	*p*-Cresol	19
6	*o*-Cresol	18.35

ranked at the top positions with very good GOLD average fitness score. Docked ABTS with fungal laccase (1gyc) is shown in Fig. 6. In contrast, when fungal laccase co-crystallized with isopropyl alcohol was separated and re-docked using docking tool, the correct binding pose could not be predicted, possibly due to the small ligand size.

The inaccurate prediction of docked ligand complexes might be due to: (i) drawback in scoring function or (ii) metal-related problems in docking which are further complicated by the difficulty in reproducing the multiple coordination geometries of copper complex [18,21]. Apart from the important factors such as concentration of enzymes, pollutants and its bioavailability, it is important to understand that with all its limitations in docking, it would be very useful to be able to identify binders and non-binders from a pool of ligands and this would be much more cost-effective than the conventional approach.

Finally, we wish to remark that this work can be considered as the first step in determining the putative targets for bioremediation. After the discovery of oxidizing reaction pollutant range of laccases, their biotechnological importance showed a marked increase [20]. Their importance could be further extended by the use of docking. Only two enzymes were considered in this study (1gyc and 1uvw) out of the great number of biodegradative enzymes. Similar kind of work would be helpful to find putative pollutants for other biodegradative enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2007.05.005.

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