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Original Article

Thermostability and functionality evaluation of laccases from *Pleurotus ostreatus* and *Pycnoporus cinnabarinus: An In-silico assay*

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Abstract

Laccase enzymes are widely used in industrials and therefore achievement to the resources of this enzyme with high thermostability is obligatory. Accordingly, a deeper investigation for understanding the structure and function of PoxA1b from *Pleurotus ostreatus*, as a fungal enzyme with the possible desired conditions, was accomplished by using *in-silico* methods. Our study led to modeling a tertiary structure of the enzyme with 72% identity to the laccase from *Trametes sp. AH28-2*, with high quality. Moreover, structural stability of modeled enzyme compared to laccase from *Pycnoporus cinnabarinus* (LPC), were proved during 20 ns at 300 and 333K. Interestingly, this data showed that the modeled enzyme is more stable than LPC at 333 K. On the other hand, interaction assay of PoxA1b and LPC with benzo[a]pyrene (BaP), as a Polycyclic aromatic hydrocarbons (PAHs), revealed suitable affinity for both of them with -9.1 and -8.8 of binding energy, respectively. Taken together, these data show that both laccase from *Pleurotus ostreatus* and *Pycnoporus cinnabarinus* are stable until 60 °C with suitable affinity to substrate. Bearing in mind, PoxA1b is a favorable candidate for industrial and environmental applications, especially in PAH detoxification.

Keywords: Enzyme activity; PoxA1b; Stability; PAHs; Molecular simulation.

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1. Introduction

Laccases (*p*-diphenol oxygen oxidoreductases, EC: 1.10.3.2) are glycosylated phenol oxidases which catalyze the oxidation of substrates, with the concomitant four-electron reduction of oxygen to water [1]. According to their spectroscopic properties, laccases contain four

copper ions distributed into three domains including D1, D2 and D3 [2]. Blue-copper T1 site in D1 sequentially abstracts one electron from each of the four substrate molecules and mediates their transfer to a tri-nuclear center comprised of T2 and T3 copper sites in D2 and D3, where the molecular oxygen is reduced [2, 3]. These enzymes with huge catalytic capabilities, have gained much attention in both scientific community and industrial context in recent years, because of their very basic requirements [4, 5]. Laccase which is found in a wide range of organisms such as higher plants, insects and bacteria, secreted by a broad range of fungi, such as white rot basidiomycetes in amounts which not found in other fungi [6-9]. Accordingly fungal laccases have been applied in many areas [10], such as detoxification of a wide range of phenolic and aromatic compounds, which are presumed toxic to living organisms and/or carcinogenic to humans [11]. For example benzopyrene with two isomeric types, benzo[a]pyrene and benzo[*e*]pyrene, is a polycyclic aromatic hydrocarbon with the formula $C_{20}H_{12}$, and carcinogenic for humans [12]. Bearing in mind, achieve to a suitable isoenzyme of laccases, in accordance with industrial conditions such as thermostability could be provide opportunity for PAHs metabolism via several approaches such as probiotic and prebiotics development in diets strategy. In the industrial context, thermotolerance (i.e. keeping activity at least at 60 °C for an adequate period of time) is a general enzyme requirement [13], as thermostable enzymes allow proceeding high-temperature processes with associated higher reaction rates and less risk of microbial contamination [14]. In this regard, it has been demonstrated that laccase isoenzymes produced by *Pleurotus ostreatus* and *Pycnoporus cinnabarinus,* two white rot basidiomycete fungi, have been studied extensively [15, 16]. *Pycnoporus cinnabarinus* has a simple ligninolytic system, with neither lignin peroxidase nor manganese peroxidase activity, but laccase just is produced [17, 18]. *Pleurotus ostreatus* produces five laccase isoenzymes under different growth conditions, including PoxA1b, PoxA1w, PoxA3a, PoxA3b, and PoxC [17, 19]. PoxA1b isoform has great redox potential (+650 mV) and is greatly stable at high temperature and in the pH range of 7-10 [20, 21]. Consequently, PoxA1b and other similar laccase can be employed in industrial context for removal the various environmental pollutants such as PAH compounds [21, 22]. Nonetheless, a comprehensive vision of the structures, functions and activities of these enzymes could be provided opportunity to achieve a competent enzyme with suitable features for industries requests, and/or opening ways for enzymes optimization with favorable visions.

Currently, in silico approaches have become a key part of the drug discovery route [23, 24]. In this regard, Molecular dynamics (MD) is an appropriate option for enzyme characterization, which was considered in this study for characterize the laccase of *Pycnoporus cinnabarinus* and *Pleurotus ostreatus*. MD is a computational method for obtaining structural and dynamical information about bimolecular [25]. There is little MD study focused on the effect of single parameters on the stability of laccases. In the present research, MD simulations were carried out to compare the thermostability of laccase from *Pycnoporus cinnabarinus*, as a reference, and PoxA1b from

Pleurotus ostreatus, at temperatures 27 °C and 60 °C. In addition, docking approach has been applied to analyze the interaction between laccases and BaP as the ligand, to compare the substrate binding between the two functional enzymes.

2. Materials and Methods

2.1. Achievement to three-dimensional structures of desired enzymes

The crystal structure of laccase from *Pycnoporus cinnabarinus* was attained from the Protein Data Bank with PDB ID: 2XYB [26]. However, there is no laccase structure crystallographically determined for *Pleurotus ostreatus*. Thus, the structure of PoxA1b was achieved using homology modeling from the crystal structure of *Trametes sp. AH28-2* (PDB ID: 3KW7) [27]. The template and query structures share 72% sequence identity. It is well known that a 30% identity threshold sequence is a reasonable choice for obtaining model [28]. Homology modeling was implemented by the software Modeller version 9.15 [29].The obtained model was validated in terms of structure quality by verifying the location of amino acids in the Ramachandran plot, based on Rampage tool [30].

2.2. MD simulations of Pleurotus ostreatus PoxA1b and laccase from Pycnoporus cinnabarinus

In order to simulation, the ligands were deleted from *Pycnoporus cinnabarinus* laccase, and hydrogen atoms were added to both structural models. The final system included protein within solvent molecules characterized using the SPC216 model, a 3-point model for water. The solvated model was contained in a rectangular box (5.8 × 6.7 × 7.9 nm) using periodic boundary conditions. The MD simulations were performed with the GROMACS suite of programs, version 5.0.7. The GROMOS96 43a1 force field was employed to define the atomic interacts. For the accurate treatment of long-range electrostatics, the Particle Mesh Ewald summation algorithm was used. The high frequency degrees of freedom from the covalent bonds of hydrogen atoms are constrained by the LINCS algorithm employed on entirely bonds [31]. Simulations were carried out at temperatures of 300 and 333 K. The V-rescale algorithm was applied for the temperature coupling and Parrinello-Rahman method was used for pressure coupling [32] . Data on the trajectories were saved every 10 ps. The initial step was a minimization using the steepest descent way for 100 steps, until the energy grade was lesser than 1000.0 kJ/mol/nm. After minimization, NVT (constant number, volume, and temperature) equilibration was performed, with constant temperature of 300 K for 100 ps, followed by NPT (constant number, pressure and temperature) equilibration, with constant pressure of 1 atm and constant temperature of 300K for 100 ps. After that, the system to the desired temperature and pressure, MD simulation was continued under NPT conditions for 20 ns. To study the thermostability of laccases, the MD simulations were implemented again under similar conditions, but the temperature was set on 333 K in all NVT, NPT and production runs. Thermal stability and conformational changes in different temperatures were evaluated by the examination of root mean square deviation (RMSD), root mean square fluctuation (RMSF) and radius of gyration (R_g).

2.3. Molecular docking studies

In the present work, BaP (PubChem CID: 2336) was used as a ligand molecule, retrieved from NCBI PubChem in SDF format and converted into 3D structure using Discovery Studio 3.0, a software developed by Accelrys (Studio D. Version 3.0, Accelrys Software Inc. San Diego, CA, 2010). Laccases were opened using Discovery Studio, followed by removing water molecules and other bound ligands, and adding hydrogens to the protein structures. Laccase and BaP molecules were saved as PDB files, and then opened in the AutoDockTools portion of MGL-tools 1.5.6 (The Scripps Research Institute) in order to prepare the receptor and ligand molecule for docking. The binding site was defined in order to describe the receptor cavity. Docking was performed using AutoDock Vina, analyzed using UCSF Chimera.

3. Results and Discussion

3.1. Assessment the thermostability of laccases

3.1.1. RMSD analysis based on the Cα atoms

Homology modeling analysis indicated the location of 92.6% of residues in favored regions of plot, including all functionally important residues identified in subsequent steps of the study. Residues located in allowed and outlier regions of Ramachandran plot composed 4.8% and 2.6% of all amino acids, respectively. To study thermostability, the structural changes in laccase from *Pycnoporus cinnabarinus* and PoxA1b from *Pleurotus ostreatus* at 300 K at neutral pH as control trajectories and 333K at neutral pH were studied by root mean square deviation (RMSD) plots. The simulations performed on the system have reached equilibration after 20 ns. In **Figure 1a**, the RMSD of Cα atoms from the starting position are shown for LPC and PoxA1b in 300 K. As can be seen, both enzymes at 300 K remain close to their respective starting structures throughout the simulations with a final RMSD close to 0.26 Å. They have reached equilibration after 19160 ps. similar final RMSD value of both enzymes at 300 K, as control trajectories, strongly suggests that, at the fold level, there is no alteration between the two enzymes. This indicated that there was no significant difference in terms of the structural behavior between final conformations of the two enzymes. In **Figure 1b**, the RMSD based on the Cα atoms of PoxA1b and LPC in 333 K have been plotted. At this temperature, we observe that both simulations have similar rates of enhance in RMSD for the first 170 ps, during which the simulation reaches a value of 5.6 Å. However, after this point, the laccase from *Pycnoporus cinnabarinus* exhibits an enhance in RMSD compared to that of the PoxA1b from *Pleurotus ostreatus*. The result of our long MD simulation studies demonstrated slightly higher stability of PoxA1b at 333 K, compared to the LPC.

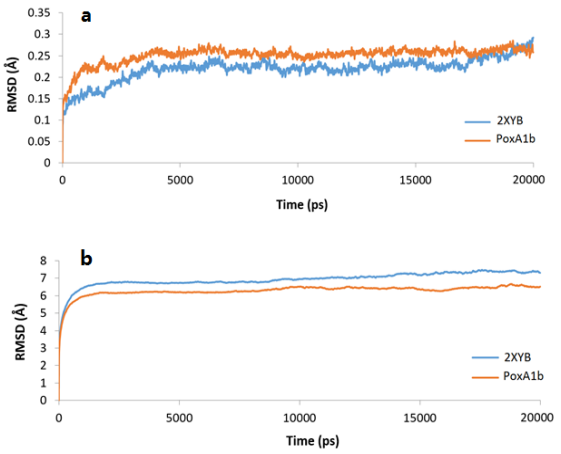

Figure 1. The RMSD plot for laccase from *Pycnoporus cinnabarinus* (2XYB), and PoxA1b from *Pleurotus ostreatus* at (a): 300 K (NPT) simulations and (b): at 333 K (NPT) simulations.

3.1.2. Calculation of RMSF for all individual residues

The root means square fluctuation (RMSF) calculated for all individual residues at 300 K are shown in **Figure 2a**. LPC shows more fluctuation than PoxA1b, demonstrating the higher stability of PoxA1b at 300 K. **Figure 2b** illustrates the RMSF plot at 333 K. As expected differences in the RMSF values are very small at this temperature. The highest fluctuation is evidenced around residue 343 of PoxA1b, which is placed on a flexible loop on the protein surface.

Analyzing the radius of gyration (R_g) of the sampled conformations in two temperature trajectories showed that it was close to 24.3 Å for both enzymes across all simulations, indicating invariable compactness, hence the structural stability, of studied laccases.

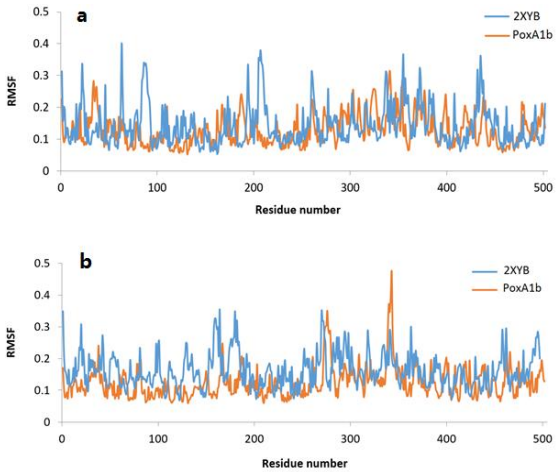

Figure 2. RMSF plot calculated for all individual residues of *Pycnoporus cinnabarinus* and PoxA1b from *Pleurotus ostreatus* at (a): 300 K temperature and (b): at 333 K temperature.

3.2. Molecular docking studies

Interaction assay of laccases with BaP, generating nine different poses for both enzymes with different binding energy (**Table 1** and **2**). The best pose was selected based on having the lowest binding interactions energy with the substrate molecule. As showed in these tables, binding affinity of PoxA1b and LPC to substrate reveal -9.1 kcal/mol -8.8 kcal/mol energy at the best interaction poses, respectively. The best docking poses have been illustrated in **Figure 3**.

Table 1: Poses with energy calculation and RMSD values for docking of *Pycnoporus cinnabarinus* laccase with BaP.

Pose ranking	Affinity (kcal/mol)	Distance from best mode	
		RMSD lower bound	RMSD upper bound
1	-8.8	0.000	0.000
2	-8.6	32.818	35.356
3	-8.6	32.822	32.254
4	-8.5	33.224	35.819
5	-8.4	0.918	5.809
6	-8.2	33.359	36.184
7	-7.8	28.001	31.199
8	-7.8	32.908	35.756
9	-7.7	3.184	4.855

Table 2: Poses with energy calculation and RMSD values for docking of *Pleurotus ostreatus* PoxA1b with BaP.

Pose ranking	Affinity (kcal/mol)	Distance from best mode	
		RMSD lower bound	RMSD upper bound
1	-9.1	0.000	0.000
2	-9.0	0.294	3.820
3	-7.6	24.114	27.448
4	-7.2	33.285	35.220
5	-7.1	32.311	33.405
6	-7.0	33.548	35.693
7	-6.9	31.995	33.236
8	-6.8	32.073	33.051
9	-6.8	32.094	33.017

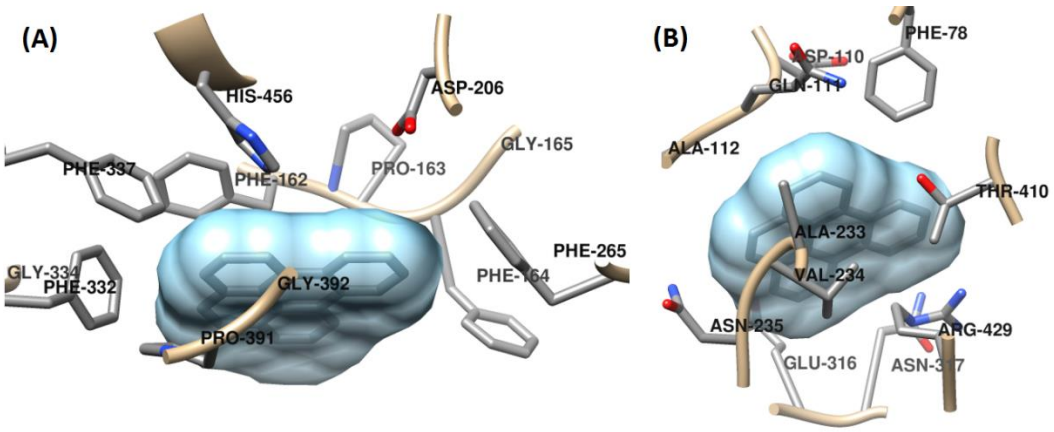

Figure 3. Docking of BaP with (A) laccase from *Pycnoporus cinnabarinus*, and (B) PoxA1b from *Pleurotus ostreatus.*

3.3. Enzyme active site evaluation

BaP binding cavity sites in LPC and PoxA1b were found to be composed of 12 and 11 residues, respectively. The residues have been shown in **Table 3** and **Figure 3**. In addition, regiospecificity of binding of substrate at the active site of *Pycnoporus cinnabarinus* laccase and PoxA1b was analyzed and illustrated in **Figures 4a** and **4b**, respectively. As can be seen, a good geometrical fit exists between the protein and ligand, however the physicochemical environment of the cavity site in PoxA1b is less hydrophobic, despite the energetically more favorable enzyme-substrate interaction in this type of laccase.

Table 3: Key amino acid residues involved in formation of the receptor cavity.

Pycnoporus cinnabarinus laccase	*Pleurotus ostreatus* PoxA1b
Phe162	Phe78
Pro163	Asp110
Phe164	Gln111
Gly165	Ala112
Asp206	Ala233
Phe265	Val234
Phe332	Asn235
Gly334	Glu316
Phe337	Asn317
Pro391	Thr410
Gly392	Arg429
His456	-

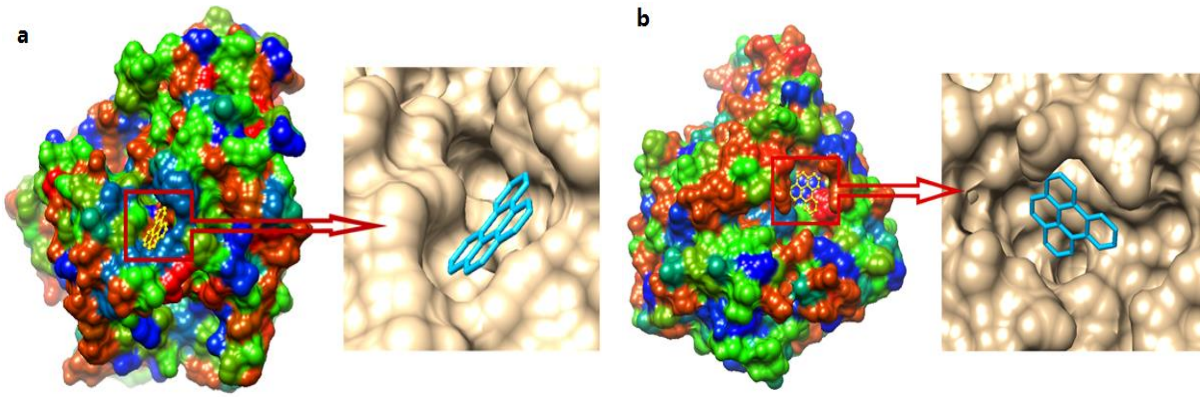

Figure 4. Receptor cavity in (a): laccase and (b): PoxA1b, from *Pycnoporus cinnabarinus*. Left panel: Protein coloring indicates the hydrophobicity as a spectrum, increasing from red through green to blue. Right panel: BaP docked into its binding cavity.

Increasing evidences have shown that industrial wastes result in environmental pollution which is toxic and hazardous for human health [33-35]. PAHs are in the middle of the most public industrial pollutants in the world, generated during such manners as coal gasification, coking and wood preservation, and may be found in air, water, soils, sediments and foods [36]. PAHs show highly lipophilic properties and may cause carcinogenic and mutagenic and cytotoxic effects on human [37] Thus, degradation of these pollutants is necessary for reducing the environmental pollution as well as the risks for human health. Studies have shown that a large number of microbial enzymes can be effectively used for biodegradation of the contaminants [38]. The use of laccases, a type of oxidative enzymes, has received great attention for removing the contaminants because of their capabilities against a wide range of phenolic compounds [39]. Because of harsh industrial process conditions that may comprise high temperature, resistant enzymes are necessary [13]. In fact, thermostability is one of the common properties for an enzyme to be usable in industrial conditions. In general, thermostable enzymes have a capacity to keep activity at high temperatures and resist irreversible inactivation at least at 60 °C for a prolonged period of time [40, 41]. It has been reported that temperature stability of laccases varies significantly depending on the source organism [42, 43]. The most thermostable laccases have been isolated from bacteria but their practical application is still limited due to low redox potential [44], and the narrow range of substrates they are capable to oxidize [45]. In addition, most bacterial laccases studied so far are located intracellularly, which is a disadvantage for micro pollutant degradation [46, 47]. Fungal laccases have been considered widely, due to their strong catalytic abilities and greater redox potential that permits a broader array of substrates to be oxidized [48]. In the previous studies, the laccase from basidiomycete *Pycnoporus cinnabarinus* has been defined as a model for laccase study, because of its high redox potential (around 0.75 V) among the redox potentials described for laccase (0.4-0.8 V) [49], and high stability against temperature and mediator inactivation [50]. *Pleurotus ostreatus*, is the second most edible universal mushroom, cultivated on straw, sawdust, waste of cereal etc [51]. It requires a short growth time and produces the laccase enzyme [13]. In addition to, laccase *Pleurotus ostreatus*, Pox1b, displayed an good stability in wide PH ranges of 5 to 9 [52]. All these features make it an appropriate candidate for larger-scale production of laccase enzyme and application in biotechnology. Accordingly, in this study, the 3D structure of *Pycnoporus cinnabarinus* laccase was used as a reference to compare the features of *Pleurotus ostreatus* PoxA1b. So, PoxA1b was modeled and subjected to 20 ns molecular dynamics simulations to investigate and compare its thermal stability with the reference model. Our results for RMSD of PoxA1b from *Pleurotus ostreatus* and laccase from *Pycnoporus cinnabarinus* indicated that the difference in their RMSD values was not significant (**Figure 1**), suggesting stability of both enzymes in 60 °C without obvious loss of activity. Although no published data are available regarding comparison of thermal stability of PoxA1b from *Pleurotus ostreatus* and laccase from *Pycnoporus cinnabarinus*, several works have assessed the thermal stability of each

enzyme separately. In this area, Eggert *et al.* reported that laccases from *Pycnoporus cinnabarinus* was completely stable until 50 °C; whereas, at 80 °C the half-time of the enzyme was 60 min [53]. in another study, it has been reported that the laccase of *Pycnoporus cinnabarinus* was stable at temperature range of 50-60 °C [44]. The activity of the same enzyme at 60 °C for 1 hour have been demonstrated [46]. Moreover, Garcia *et al.* demonstrated that the purified laccase from *Pycnoporus sanguineus* retained 80% of its initial activity at 60 °C for 2 h [45]. In other hand, it has been indicated that PoxA1b from *Pleurotus ostreatus* was stable in the temperature range of 45 - 65 °C [49]. In another study, it has also been reported that PoxA1b from *Pleurotus ostreatus* maintained its optimum activity at 55-65 °C [50]. In addition to, PoxA1w from *Pleurotus ostreatus* was reported to be stable at 60 °C for 3.3 hours [54]. ARC280 and HP-1 laccases of *Pleurotus ostreatus* maintained 60% and 50% of their initial activity after 120 [21], and 15 min [55] incubation at 60 °C respectively. On the other hand, catalytic activity of laccase was markedly associated with the properties of substrates and enzyme include molecular interaction properties between it and its substrate [56, 57]. It remains unclear detailed molecular interaction between BaP and lacase. Characterization of binding properties between BaP and laccase is important for its application in BaP degradation. Thus we, analyzed, for the first time, the molecular base of lacase- BaP interaction using molecular docking in both representative classes of laccases. The results of binding energy revealed lower values for PoxA1b from *Pleurotus ostreatus* than laccase from *Pycnoporus cinnabarinus*, suggesting the better fit and the more stable interaction between BaP and PoxA1b. The residues composing the binding site cavity of each laccase type were also investigated (**Table 1** and **2**). As expected, the ligand binding site of laccase from *Pycnoporus cinnabarinus* is composed of several hydrophobic residues, mainly phenyl alanine and proline (**Table 3**). An interesting observation is the dominance of amino acids with polar and charged side chains in the binding site of PoxA1b, while the ligand, BaP, is a non-polar molecule (**Figure 4**). Despite this apparently unfavorable interaction, BaP shows a lower energy of binding with PoxA1b, than with laccase from *Pycnoporus cinnabarinus* (Table1 and 2). As can be seen in visual inspection (**Figure 3B**), one possible explanation for the stability of this docking is that the polar/charged residues contribute to the binding of BaP through hydrophilic parts of their side chains.

4. Conclusion

The aim of study was to evaluate the structure and mechanisms of action PoxA1b from Pleurotus ostreatus, as a fungal enzyme with the possible desired conditions. The consequences of this work provide a model of POXA1 from *Pleurotus ostreatus* with quality in structure and function via simulation and docking study. Based these data, POXA1 from *Pleurotus ostreatus* is more stable from LPC in 60 °C, without loss activity and interaction after 20 ns simulation. Therefore, based on these data POXA1 could be propound as a suitable candidate for industry application.

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Conflict of interest

The authors declare to have no conflict of interest.

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