Recombinant laccase rPOXA 1B real-time, accelerated and molecular dynamics stability study

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Abstract

Background: Laccases (EC 1.10.3.2) are multi-copper oxidoreductases with great biotechnological importance due to their high oxidative potential and utility for removing synthetic dyes, oxidizing phenolic compounds, and degrading pesticides, among others.

Methods: A real-time stability study (RTS) was conducted for a year, by using enzyme concentrates from 3 batches (L1, L3, and L4). For which, five temperatures 243.15, 277.15, 298.15, 303.15, 308.15, and 313.15 K were assayed. Using RTS data and the Arrhenius equation, we calculated the rPOXA 1B accelerated stability (AS). Molecular dynamics (MD) computational study results were very close to those obtained experimentally at four different temperatures 241, 278, 298, and 314 K.

Results: In the RTS, 101.16, 115.81, 75.23, 46.09, 5.81, and 4.83% of the relative enzyme activity were recovered, at respective assayed temperatures. AS study, showed that rPOXA 1B is stable at 240.98 ± 5.38, 277.40 ± 1.32 or 297.53 ± 3.88 K; with t1/2 values of 230.8, 46.2, and 12.6 months, respectively. Kinetic and thermodynamic parameters supported the high stability of rPOXA 1B, with an $E_d$ value of 41.40 KJ mol$^{-1}$, a low variation of $K_M$ and $V_{max}$, a $t_240.98 ± 5.38$, and $t_297.53 ± 3.88$ K, and $\Delta G$ values showing deactivation reaction does not occur. The MD indicates that fluctuations in loop, coils or loops with hydrophilic or intermediate polarity amino acids as well as in some residues of POXA 1B 3D structure, increases with temperature; changing from three fluctuating residues at 278 K to six residues at 298 K, and nine residues at 314 K.

Conclusions: Laccase rPOXA 1B demonstrated experimentally and computationally to be a stable enzyme, with t1/2 of 230.8, 46.2 or 12.6 months, if it is preserved impure without preservatives at temperatures of 240.98 ± 5.38, 277.40 ± 1.32 or 297.53 ± 3.88 K respectively; this study could be of great utility for large scale producers.

Keywords: rPOXA 1B, Storage conditions, Laccase, Real-time stability, Accelerated stability, Molecular dynamics

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Background

Laccases (EC 1.10.3.2) are blue multi-copper oxidases (MCOs), usually monomeric and distributed in plants, insects, bacteria and fungi [1]. However, laccases from fungi have higher redox potentials ($E_{oc}$) [2, 3], fostering oxidation of chemicals as mono-, di-, poly- and methoxy phenols, aromatic and aliphatic amines, hydroxy indoles, benzenethiols and inorganic/organic metal compounds [4–6]. Due to laccases low specificity [7], they are attractive for different biotechnological applications, including juice clarification [8], biosensor manufacturing (for agri-environmental and biomedical applications) [9], sawdust biotransformation [10], coloured wastewater treatment [11–13], detoxification of cellulose pulp black liquor [14], simultaneous bioconversion of plastics and waste [15], biodeterioration of low-density plastics [16], and oxidation of micropollutants [17].

The laccases catalytic centre contains four copper atoms distributed over CuT1, CuT2 and CuT3 sites, located between cupredoxin-like domains 1 and 3 [1, 18]. Site CuT1 is mononuclear and coordinated with D3 residues. CuT1 is considered as the indicator of the redox potential of the enzyme [7, 19] because it captures electrons from the substrate and transferring them to the trinuclear copper centre (TNC) of the enzyme [20]. The TNC is composed of one copper from the CuT2 site (mononuclear) and two coppers from the CuT3 site (binuclear - CuT3a and CuT3β) [7]. Copper ions coordinate with the D1 and D3 domains located residues, where the reduction of O2 to H2O occurs [1, 19, 21].

Due to water production during catalysis, it has become a promising "green tool" for industrial and environmental applications. Therefore, its production and commercial availability must increase [3].

Knowing the thermal stability and storage conditions of any enzyme is of great importance to determine potential industrial applications [22, 23]. However, each enzyme has self-structure and stability, and it depends for the most part on its chemical environment as pH, ionic force, among others, temperature [24, 25]. Reasons for which, is remarkable to determine the most favourable storage conditions, as a function of the enzyme’s shelf life [24], and to elucidate the necessity of incorporating preservatives agents to the final formulation.

For the study of the environment that favour enzyme and protein stability during storage, different conditions should be assayed (pH, temperature, NaCl concentration, presence of additives, preservatives and enzyme concentration, among others) [23]. Evaluating storage conditions at different temperatures is crucial since an increase in temperature augments the kinetic energy, leading to possible loss of enzyme activity and irreversible enzyme denaturation [26]. Additionally, it could also result in dynamic changes in its 3D structure. These changes could be more or less aggressive, depending on the enzyme’s thermostability [27].

Biological products are largely unstable; thus, it is customary to store them under cold conditions. However, these storage conditions are not always the best as they involve high costs due to the maintenance of the cold chain [28]. Besides, enzyme denaturation can also occur under cold conditions [29]. Therefore, expiration dates are suggested based on collected real-time stability data. Accelerated stability studies are a complementary tool applied to proteins or enzymes with different applications [24].

Protein accelerated stability studies are performed usually at high temperatures (40 °C = 313.15 K), [24] as a tool to assess stability under adverse conditions [30]. Then employing Arrhenius formulas [31], the activation energy ($E_{a}$) and half-life ($t_{1/2}$) can be estimated; it is frequently for estimating protein and enzyme stability for pharmaceutical applications [24].

Accurate predictions of the thermal stability of enzymes and proteins are complex [32]. However, the combination of experimental results and computational analyses are effective for protein stability studies, as they allow rationalisation and interpretation of the experimental results [33]. Molecular dynamics (MD) simulations [34] explain the relationship between dynamics and the molecular mechanisms that stabilise the molecule under study through tools that measure flexibility and correlate it with function [33–35] as flexibility is a key property in protein activity and stability [36].

Some laccase MD analysis has focused on evaluating the influence of environmental conditions or the effect of the degree of glycosylation on structure, function and enzyme dynamics [18, 25]. Moreover, modified laccases by directed mutagenesis in the laboratory have been evaluating by MD [25, 37, 38].

The objectives of this work were, i) to propose the design of a more statistically based stability study, which would allow estimating at each sampling time with the same precision and confidence level the average enzyme activity (UL$^{-1}$) of rPOXA 1B laccase and thus support the stability study of other enzymes and proteins of non-pharmaceutical use [39], ii) use this statistical foundation to determine the real-time and accelerated stability of the recombinant rPOXA 1B enzyme from P. ostreatus produced in P. pastoris and iii) relate the real-time-stability with the in silico behaviour of the enzyme during molecular dynamics simulations.

Materials and methods

Pilot study of enzyme stability

To estimate rPOXA1B enzyme activity (UL$^{-1}$) variability at different temperatures a pilot study was performed.
Fifteen vials were prepared (2 mL polypropylene screw-cap Microtube BIOLOGIX) containing 1 mL rPOXA 1B concentrate with an enzyme activity of 5877.31 ± 278.11 UL\(^{-1}\) (~164.62 Umg\(^{-1}\)) placed at −30, 0, 15, 30, 40 and 50°C, equivalent to absolute theoretical temperatures of 243.15, 277.15, 303.15, 313.15 and 323.15 K, respectively. Temperatures were selected based on a previous thermal stability study performed for 1 h of enzyme exposure [40]. Enzyme activity (UL\(^{-1}\)), [41], was measured at days 0, 15 and 30 (each one considered a sampling time), for each temperature. Sampling was performed at random, using a generator of random numbers with Stata V.14. Pilot study results were used to define the vial population size (N) and from this one, for each sampling time a sample size (n) was calculated. Estimated variances in the pilot study were used to appraise the variability of the process. Moreover, assuming homogeneity of variance, its combined variance was calculated with a similar statistical analysis as is used to estimate variance for a t-test for equal variances.

**rPOXA 1B concentrate batches**

We use *P. pastoris X33/pGAPZaA-LaccPost-Stop Clone I* [42] to produce rPOXA 1B batches on a 10 L bioreactor scale; following the production and concentration methodology previously described [40]. For the study, we used three random selected batches (L1, L3 and L4), with enzyme activity per concentrate batch (preservative-free), were L1: 27,222.2 UL\(^{-1}\); L3: 37,222.2 UL\(^{-1}\) and L4: 34,236.1 UL\(^{-1}\). Every batch was diluted with distilled water to match the initial enzyme activity of 16, 575.50 ± 268.92 UL\(^{-1}\) (758.71 ± 180.18 Umg\(^{-1}\)), time zero of the study without sampling.

**rPOXA 1B stability study: design and conditions**

Based on the pilot stability study results, five temperatures were selected [243.15 K (stored in the freezer), 277.15 K (stored in the refrigerator), 298.15, 303.15 and 313.15 K (stored in independent incubators)]. A thermo-hygrometer placed in each incubation equipment allows daily monitoring of temperature and relative humidity (RH) expressed in %, except for −30 °C (243.15 K) and 4 °C (277.15 K) at which humidity is normally high. Temperature and RH values obtained throughout the study (1 year) were transformed to K and averaged at the end of the stability study to calculate both standard deviation (SD) during the length of the stability study. The stability study has a monthly periodicity (30 calendar days) until the first 6 months and later, every 2 months (60 days) to complete a year. However, the number of samples by temperatures varied according to enzyme activity loss. A relative enzyme activity loss of about ~75% was set up as the limit for sampling. Nonetheless, when enzyme activity loss exceeded before the end of the sampling time assigned; a minimum of five sampling times was established (whenever possible).

A stratified sampling strategy allows estimating the enzyme activity (UL\(^{-1}\)) average at different sampling moments for each temperature. The initial population (time 0) at each temperature, consisted of three batches (L1, L3 and L4) with the same number of vials, containing 2 mL polypropylene screw cap tube (BIOLOGIX) with one millilitre of recombinant enzyme concentrate from each respective batch (L1, L3, L4). Vials of each batch were stored in two square cryopreservation boxes (10 × 10 vials). Each batch constructed constituted a selection stratum for each sampling time.

An Excel spreadsheet was programmed to determine population size (N), varied population sizes were assigned and tested to estimate the mean value in the stratified sampling with proportional allocation, using different levels of precision and confidence, and considering the costs that designs would entail [43]. Finally, the population size calculated was assumed for the three strata (L1, L3 and L4), allowing to estimate the median value of the enzymatic activity of rPOXA1B (UL\(^{-1}\)) for each sampling moment, with a precision of 21.8 (UL\(^{-1}\)) and 95% confidence.

According to stratified sampling, a simple random sample was taken from each batch at each sampling time, to estimate the average enzyme activity of rPOXA 1B (UL\(^{-1}\)) and its corresponding confidence interval (CI). For estimations, we used stratified probability sampling bases with proportional allocation [43].

Sampled vials at each time of the stability study allow to determining enzyme activity (UL\(^{-1}\)). Later, the rest of the sample of the same time point and temperature were pool to determine by triplicate apparent \(V_{max}\) and \(K_M\) with their respective ANOVA analysis using SPSS 19. To determining \(V_{max}\) and significant differences, a 0.05 significance was established for the temperature range assayed.

**Real-time stability of rPOXA 1B**

Real-time stability of rPOXA 1B, was determined by plotting (isotherms) average enzyme activities ± confidence interval (CI) for each sample, at each sampling time. To this object SigmaPlot V11.0., was used. Relative enzyme activity was the percentage of activity recovered at each sampling time, compared to the initial volumetric enzyme activity (100%).

**rPOXA 1B accelerated stability analysis and thermodynamic parameters**

The slope from the Ln \((E/E_0)\) graph as a function of time (months), based on the enzyme activity (UL\(^{-1}\)) analysis of the first-order kinetics for each of the isotherms (243.15, 277.15, 298.15, 303.15, 308.15 and 313.15 K)
allows calculating the inactivation rate constant \((k_d)\). \(E_0\) is the initial enzyme activity \((\text{UL}^{-1})\) and \((E)\) is the average enzyme activity \((\text{UL}^{-1})\) for each sampling time. Deactivation energy \((E_d)\) was calculated by plotting the \(\ln(k_d)\) graph obtained for each isotherm versus the inverse of the temperature \((1/T)\) in K, using Arrhenius (Eq. 1).

The enzyme’s half-life \((t_{1/2})\) is the time required for the enzyme to decrease in half its initial activity [31], and by using (Eq. 2) it was calculated.

Thermodynamic parameters \(\Delta H\) (change in enthalpy of deactivation), (Eq. 3), \(\Delta G\) (change in Gibbs’s free energy of inactivation), (Eq. 4) and \(\Delta S\) (change in entropy of inactivation), (Eq. 5) for an irreversible inactivation were calculated from the following equations.

\[
\ln (k_d) = \ln (A) - \frac{E_d}{RT}\tag{1}
\]

\[
t_{1/2} = \frac{\ln 2}{k_d}\tag{2}
\]

\[
\Delta H = E_d - RT\tag{3}
\]

\[
\Delta G = -RT \ln \left( \frac{k_d h}{k_b T} \right)\tag{4}
\]

\[
\Delta S = \frac{(\Delta H - \Delta G)}{T}\tag{5}
\]

Where: \(k_d\) is the deactivation constant, \(A\) is the frequency factor, \(E_d\) is the deactivation energy, \(R\) is the universal gas constant \((8.314 \text{ J mol}^{-1} \text{ K}^{-1})\), and \(T\) is the absolute temperature (Kelvin) [31, 44], \(h\) is the constant of Planck \((6.626 \times 10^{-34} \text{ J s})\) and \(k_b\) is the constant Boltzmann \((1.38 \times 10^{-23} \text{ JK}^{-1})\), [44, 45].

**Determination of laccase activity**

Laccase enzyme activity was determined using ABTS (2-Azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) as a substrate. Into a 100 \(\mu\L\) spectrophotometric cuvette 20 mM ABTS and centrifuged supernatant (from 2 to 20 \(\mu\L\), depending on the amount of enzyme present in the sample were added). To complete a final volume of 1 \(\text{mL}\) 0.1 M citrate buffer (pH 3.0 ± 0.2) was added. One-minute absorbance change resulting from ABTS oxidation at 420 nm was measured [41]. One unit of the enzyme (U) is defined as the quantity of enzyme capable of transforming 1 \(\mu\mol\) ABTS substrate per minute, per litre, and was calculated based on (Eq. 6).

\[
UL^{-1} = \frac{(\Delta E \times V_t)}{(\varepsilon \times d \times V_s)}\tag{6}
\]

Where: \(\Delta E\) corresponds to the difference between final and initial absorbance after 1 min of reaction, \(V_t\) refers to the total reaction volume \((\text{mL})\), \(E\) refers to the ABTS molar extinction coefficient \((\text{M}^{-1} \text{ cm}^{-1})\) at 420 nm, \(d\) is the length of the cuvette in cm and \(V_s\) is the volume of sample \((\text{mL})\) contained in the reaction.

**Apparent enzyme kinetic constants**

As the real-time stability study progressed and once laccase activity was quantified, at each sampling moment, the remaining volume of samples (n) were pool (distinguishing by batch, by temperature, by sampling time) to determine the kinetics of the enzyme. For the enzyme kinetic assay ABTS dissolved in 0.1 M citrate buffer, was used as a substrate (concentration between 0.1–3 mM), pH 3.0 ± 0.2. An enzyme solution with an activity of 10 UL\(^{-1}\) at 25 °C [46]. All kinetic tests performed in triplicate. The experimental data from each enzyme kinetic were feed to Biomodel software (http://biomodel.uah.es/metab/enzimas/inicio.htm), which allows fitting the experimental data to the Michaelis-Menten equation (Eq. 7) using a non-linear, least-squares based regression. \(K_M\) and \(V_{max}\) values previously estimated by the Hanes-Woolf linear regression (Eq. 8), [47] by using SIMFIT software (V7.6.8), [48] used to start the iteration process at Biomodel.

\[
V_0 = \frac{V_{max}[S]}{K_M + [S]}\tag{7}
\]

\[
\frac{[S]}{V_0} = \frac{[S]}{V_{max}} + \frac{K_M}{V_{max}}\tag{8}
\]

Where: \(V_0\) is the initial velocity, \(V_{max}\) is the maximum reaction rate, \(K_M\) is the Michaelis constant and \([S]\) is the substrate concentration.

**Homology modeling of POXA 1B**

Since there is no crystal structure reported for POXA1B from Pleurotus ostreatus it was necessary to create a 3D model for the enzyme; then the sequence was processed in the SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/) to identify and eliminate peptide signal amino acids.

Laccase 3D structure was predicted by homology modelling [42, 49–51] using the HHpred, Phyre2 and Swissmodel servers. The resulting model was evaluated by QMEAN [52, 53] and for characteristics of the enzyme’s
active site. Saves V 5.0 server allows validating the quality of the modelled structure (https://servicesn.mbi.ucla.edu/SAVES/).

**POXA 1B molecular dynamics simulations at different temperatures**
The CuT1 copper and TNC in laccase’s binding pocket were parametrized, by using the metal centre parameter builder (MCPB.py), [54] included in the Amber18 package [55], the partial charges were obtained from the electrostatic calculation by using Gaussian 16 [56]. A complete set of the parameters generated by mcpb.py for copper ions and their coordination residues are in Supplementary Material S1. All coppers (Cu type I and Cu from TNC) were modelled as Cu²⁺. The hydrogens and protonation state for each residue were carried out with webserver H++ (http://biophysics.cs.vt.edu/) adjusting the PDB file of POXA 1B to pH 3.0. For MD simulations topology files and system, coordinates were prepared using the leap interface. The system was neutralized with Na⁺ and Cl⁻ and was immersed in a TIP3P water box with 10.0 Å between any atom of the protein and the edge of the box. Additionally, the ff14SB force field was used to model all amino acid residues [57].

To compare the stability of the enzyme at different temperatures, four temperatures were set up [241 K (−32 °C); 278 K (5 °C); 298 K (25 °C) and 314 K (41 °C)]. Initial energy minimization was carried out, using 50,000 steps of the steepest descent algorithm steps, followed by 10,000 steps of conjugate gradients. The minimized system was gradually warmed up to four stages from 0 to 241 K (stage 1), from 241 to 278 K (stage 2), from 278 to 298 K (stage 3) and from 298 to 314 K (stage 4); using an NVT canonical ensemble and a Langevin thermostat with a 2.0 ps⁻¹ collision frequency and a 2 fs step size Newton equations were integrated.

All simulations were performed with the same cut-off and electrostatic interactions beyond the cut-off distance are ignored. In all simulations, a cut-off of 8.0 Å was employed for both the electrostatics and the van der Waals interactions. The imposition of periodic boundaries on the system during the calculation was controlled at constant pressure with isotropic position scaling and in the production were kept the coordinate output from overflowing the trajectory. With the results obtained from each of the heating stages, were evaluated several MD stages. The density of each heating step was equilibrated for 400 and 200 ns production and simulated using an isothermal-isobaric (NPT), hydrogen length constrained (SHAKE) assembly. Snapshots of the scenario were taken every 0.2 ns during production (1000 in total). The trajectories obtained for each temperature were analysed using CPPTRAJ [58, 59].

**Principal component analysis**
To evaluate movements of the main chains of the system for the four temperatures studied principal component analysis (PCA) was carried out, according to previously described methodology [60]. The covariance matrix was constructed from MD topology and trajectory obtained files. The root mean square (RMS) was adjusted to eliminate rotational/translational movements and preserve internal dynamics, except for hydrogen atoms. The first five principal components corresponding to the first eigenvectors of the covariance matrix were selected. Structural representations of the different modes were constructed in CPPTRAJ and visualized in Visual Molecular Dynamics (VMD). Additionally, for the PCA a superposition of Cα atoms in the MD trajectories using R’s Bio3D package [61] was constructed from which the contribution of each residue was evaluated for the first five main PCAs [59].

**Results**

**Pilot study of enzyme stability**
The stability pilot assay demonstrated that as the temperature increased, the relative activity of the enzyme decreased. Between 243.15 K (−30 °C) and 277.15 K (4 °C) there were slight enzyme activity variations at day 30 of the study. At 303.15 K (30 °C) relative enzyme activity was maintained (~85%); whereas at 313.15 K (40 °C) and 323.15 K (50 °C), temperatures were destabilizing for the system, with a considerable reduction of the enzyme activity (Table 1).

Assuming equal variances for each temperature, a combined variance estimator (an estimator commonly used in parametric tests) was used to obtain an estimate of enzyme activity variability; with an estimate of 96.6 for rPOXA 1B UL⁻¹ standard deviation.

**Real-time stability of rPOXA 1B concentrate**
Table 2 illustrates population size (N) and sample size (n), at the beginning of the study (month 0) and at each sampling time (months). For the theoretical temperatures of 30 and 4 °C averages of 240.98 ± 5.38 K (−32.55 ± 4.12 °C) and 277.40 ± 1.32 K (4.32 ± 1.22 °C) were

| Temperature °C | Temperature K | Time (days) |
|----------------|---------------|-------------|
| −30            | 243.15        | 95.38       | 96.74        |
| 30             | 303.15        | 84.76       | 85.59        |
| 4              | 277.15        | 88.50       | 95.36        |
| 30             | 303.15        | 84.76       | 85.59        |
| 40             | 313.15        | 74.05       | 61.30        |

Table 1 Percentage of POXA 1B relative activity preserved during 30 days at five theoretical study temperatures
obtained, and the 12 months of the study were completed. For the theoretical temperatures of 25, 30, 35 and 40 °C, averages of 297.53 ± 3.88 K (24.99 ± 0.25 °C), 303.27 ± 1.11 K (30.12 ± 1.11 °C), 309.58 ± 0.23 K (36.43 ± 0.23 °C) and 314.79 ± 0.52 K (41.64 ± 0.52 °C) were obtained.

Figure 1, depicts relative enzyme activity ± confidence interval and in Table 3 rPOXA 1B specific activity (U mg⁻¹) ± confidence interval (CI), as a function of time exposed to different study temperatures. At 240.98 ± 5.38 K, it was observed the activity remained constant throughout the 12 months of the study. At 277.40 ± 1.32 K, a slight increase in enzyme activity percentage was observed after the sixth month of sampling. For the temperatures of 297.53 ± 3.88, 303.27 ± 1.11 and 309.58 ± 0.23 K, a slight gradual reduction in enzyme activity was observed as the temperature increased, maintaining a residual enzyme activity of 75.23, 46.09 and 5.81%, respectively, until the last sample. At 314.79 ± 0.52 K, a considerable loss in relative enzyme activity was observed for the first month of sampling, with a residual enzyme activity of 4.83% (Fig. 1, Table 3).

rPOXA 1B accelerated stability and thermodynamics parameter analysis

Accelerated stability calculations were obtained as a function of time, and rPOXA1B deactivation using different exposure temperatures. First-order deactivation kinetic yielded the enzymatic deactivation constant (kd), (Fig. 2, Table 4) with R² between 0.9710 and 0.8810. Half-life (t₁/₂) and deactivation energy (Eₐ) (Fig. 3, Table 4) were calculated from obtained kd at different temperatures.

The thermodynamic parameters to identify the stability of rPOXA 1B appears in Table 4. ΔH was constant over the study range, as the ΔH values only decreased by approximately 1.5% with increasing temperature by 70 K; this variation is within the experimental uncertainty.

Table 2 Sampling distribution carried out for each lot and sampling time

| Month | Sampling time | N  | n  |
|-------|---------------|----|----|
| 1     | 1             | 200| 16 |
| 2     | 2             | 184| 16 |
| 3     | 3             | 168| 16 |
| 4     | 4             | 152| 16 |
| 5     | 5             | 136| 16 |
| 6     | 6             | 120| 15 |
| 8     | 7             | 105| 15 |
| 10    | 8             | 90 | 15 |
| 12    | 9             | 75 | 14 |

N: Population size, n: sampling size

Fig. 1 rPOXA 1B real-time enzyme stability study. Percentage of relative enzyme activity isotherms of the L1, L3 and L4 batches average ± CI of vs., time in months for each assayed temperature. The three temperatures with the thickest lines (blue, green and red) are those studied computationally. The enzyme activity (U L⁻¹) was averaged, at each sample, batch and storage temperatures. The average enzyme activity allows estimating the percentage of relative enzyme activity concerning the initial enzyme activity.
The \( \Delta G \) showed high and positive values, while \( \Delta S \) exhibited negative values.

**Effect of different temperatures on (apparent) \( V_{\text{max}} \) and \( K_M \)**

Observed apparent \( V_{\text{max}} \) and \( K_M \) appear in Table 5. \( V_{\text{max}} \) for enzymes at different temperatures revealed that between 240.98 ± 5.38 and 297.53 ± 3.88 K no variation was observed, whereas it decreased for higher temperatures (309.58 ± 0.23 and 314.79 ± 0.52 K). \( K_M \) variation was associated with exposure temperature, increasing as exposure temperature augmented. For both analyses, differences between temperatures were significant (Table 5).

**Homology model of POXA 1B**

Evaluated servers generated different predictions for POXA1B 3D’s protein structure from amino acid sequences. However, among servers, the predictions obtained from HHpred and Phyre2 did not result in a model according to the distribution of copper atoms at the site of the enzyme. Therefore, we decided to evaluate different predicted models using an automated comparative 3D protein modelling server SWISS-MODEL [62, 63]. The SWISS-MODEL template library was searched with BLAST [64] and HHBlits [65].

Templates for POXA 1B modelling and models obtained from 5Z1X (Cerrena sp. RSD1), 5E9N (Stecherium murashikinsy) and 1GYC (Trametes versicolor) were evaluated, with identities of 64.12, 62.96 and 62.50%, respectively. The characteristics of the active were evaluated and it was observed the 1GYC template had a more suitable configuration at the active site. The model obtained with the selected template obtain a 0.80 QMEAM and a GMQE of 0.87. Predicted results for the model are in Supplementary Material Figure 1. Validation of the structure was supported by Saves 5.0 model structure scores, which according to Ramachandran’s plot revealed that 88.9% of the residues were in the most favoured region and 10.6% in the additional allowed region (Supplementary Material Figure 2). Verify 3D score was 94.08%, ERRAT score was 88.7734 and the PROVES score was 4.1%.

**Molecular dynamics simulations and PCA analysis**

In the active site parametrization, CuT1 copper was coordinated with His394, His496 and Cys451. Distance between CuT1 and the TNC was approximately 12 Å. CuT2 was coordinated with His64, His397 and a hydroxyl group. CuT3 coppers were coordinated with six histidines (His66, His109, His111, His399, His450, His452) and a hydroxyl group between CuT3 and CuT3 (Supplementary Material Figure 3).

To evaluate the effect temperature (241 K [−32 °C], 278 K [5 °C], 298 [25 °C] K and 314 K [41 °C]) had on the enzyme, MD simulations were performed. Simulated system stability at different temperatures was evaluated by calculating the root-mean-square deviation (RMSD) during the simulation time. It was observed that the conformational stability of the systems was maintained during the simulations since the deviation did not exceed 2 Å for all evaluated temperatures (Supplementary Material Figure 4). Furthermore, it was noticed deviations increased as the simulation temperature was raised. Thus, the higher deviations corresponded to 314 K (40.85 °C), (Supplementary Material Figure 4).

**Table 3** Specific activity (U mg\(^{-1}\) ± CI) during sampling months

| Temperature K | 240.98 ± 5.38 | 277.40 ± 1.32 | 297.53 ± 3.88 | 303.27 ± 1.11 | 309.58 ± 0.23 |
|---------------|---------------|---------------|---------------|---------------|---------------|
| Temperature °C | −32.55 ± 4.12 | 4.32 ± 1.22   | 24.99 ± 0.25  | 30.12 ± 1.11  | 36.43 ± 0.23  |
| Time (Month)  | Specific Activity U mg\(^{-1}\) ± CI |
| 1             | 758.92 ± 8.90 | 857.23 ± 10.09 | 745.57 ± 12.32 | 759.46 ± 16.71 | 236.74 ± 4.07 |
| 2             | 763.62 ± 7.75 | 872.88 ± 9.77  | 737.70 ± 12.11 | 679.41 ± 11.49 | 116.74 ± 2.71 |
| 3             | 752.30 ± 9.54 | 863.89 ± 9.64  | 663.10 ± 11.70 | 627.46 ± 10.27 | 76.87 ± 3.09  |
| 4             | 758.06 ± 9.03 | 860.29 ± 10.13 | 587.68 ± 14.01 | 520.02 ± 10.29 | 54.15 ± 2.86  |
| 5             | 754.66 ± 9.95 | 878.27 ± 9.78  | 567.65 ± 13.30 | 395.92 ± 9.83  | 37.65 ± 1.99  |
| 6             | 763.25 ± 6.38 | 884.82 ± 10.15 | 563.05 ± 0.0   |               |               |
| 8             | 787.08 ± 6.27 | 959.30 ± 10.84 |               |               |               |
| 10            | 759.43 ± 6.30 | 997.01 ± 13.22 |               |               |               |
| 12            | 934.81 ± 9.70 | 1045.58 ± 15.31 |               |               |               |

The \( \Delta G \) showed high and positive values, while \( \Delta S \) exhibited negative values.
function of temperature in the simulation. In Fig. 4A two negative peaks were observed for ΔRMSF at 278, 298 and 314 K corresponding to LYS\textsuperscript{309} and ALA\textsuperscript{363} amino acids (Violet beads - Fig. 5), which indicate the fluctuation was less in comparison with the temperature at 241 K. Moreover, ΔRMSF 298 K an additional negative peak corresponding to residue SER\textsuperscript{412} was observed. On the other hand, it was recognized the number of

![Plot of \(\ln \left(\frac{E}{E_0}\right)\) versus time (month) for the calculation of deactivation constant \((k_d)\). rPOXA 1B first-order deactivation kinetics at different temperatures: A: 240.98 ± 5.38 K (−32.55 ± 4.12 °C), B: 277.40 ± 1.32 K (4.32 ± 1.22 °C), C: 297.53 ± 3.88 K (24.99 ± 0.25 °C), D: 303.27 ± 1.11 K (30.12 ± 1.11 °C), E: 309.58 ± 0.23 K (36.43 ± 0.23 °C). RH is the average of relative humidity (%) at the study temperature.](image)
residues with the biggest fluctuations augmented with increasing temperature.

For Δ RMSF 278 K, in Figs. 4A and 5A, it was observed residues LEU159, ALA391 and ASP429 (Orange beads – Fig. 5A) presented the highest fluctuations, and the residues in proximity to LEU159 and ASP429 generated fluctuating regions. Figures 4A and 5B for Δ RMSF 298 K residues with high fluctuations were THR160, ASP266, GLU293, ALA334, ASP341 and LEU359 (Orange beads – Fig. 5B), only amino acids close to ALA334 conformed a fluctuating region (Yellow region - Fig. 5B). In Figs. 4A and 5C, for Δ RMSF 314 K residues with high fluctuation increased, where ASP101, THR160, GLY265, ASN297, ALA334, GLY370, ALA391, PRO393 and ASP433 were of the greatest fluctuation (Orange beads – Fig. 5).

Likewise, residues close to THR160, ASN297, ALA334 and ASP433 generated fluctuating regions (Yellow region - Fig. 5C).

Residues and regions with Δ RMSF that increased fluctuation in a progressive manner, according to the increase in temperature, were identified as ASP266, with its region in the vicinity between SER264 - PHE269; and ASN297 with its region in the vicinity between GLN293 - PRO298. Standard deviation calculation among Δ RMSF revealed the residues with standard deviations greater than 0.5 Å (cut off point) corresponded to the same previously identified residues, including the additional residues ALA391 and PRO393.

RMSF and PCA supported residue contribution to the system’s atomic fluctuation variations. The PCA was

| Thermodynamic parameters | Temperature K | Temperature °C |
|--------------------------|---------------|----------------|
| 240.98 ± 5.38            | 0.015         | 0.055          |
| 277.40 ± 1.32            | 0.055         | 0.135          |
| 297.53 ± 3.88            | 0.135         | 0.400          |
| 303.27 ± 1.11            | 0.400         | 1.7            |
| 309.58 ± 0.23            | 1.7           | 38.83          |

Fig. 3 Arrhenius plot to get deactivation energy (E_d) calculation of rPOXA 1B exposition temperatures between 240.98 ± 5.38 K (−32.55 ± 4.12 °C) and 309.58 ± 0.23 K (36.43 ± 0.23 °C)
Table 5: Apparent $V_{\text{max}}$ and $K_M$ average variation after X time exposure at different temperatures

| Temperature°C | Time (Month) | Number of (pool) | $V_{\text{max}} \pm \text{SD}$ (mM min$^{-1}$) | $K_M \pm \text{SD}$ (mM) |
|---------------|--------------|------------------|-----------------------------------------------|------------------------|
| −32.55 ± 4.12 | 12           | 27               | 0.0102 ± 0.0004                               | 0.0414 ± 0.0046       |
| 4.32 ± 1.22   | 12           | 27               | 0.0104 ± 0.0005                               | 0.0435 ± 0.0057       |
| 24.99 ± 0.25  | 6            | 18               | 0.0102 ± 0.0004                               | 0.0466 ± 0.0064       |
| 30.12 ± 1.11  | 6            | 18               | 0.0104 ± 0.0004                               | 0.0477 ± 0.0076       |
| 36.43 ± 0.23  | 4            | 12               | 0.0094 ± 0.0005                               | 0.1016 ± 0.0736       |
| 41.64 ± 0.52  | 1            | 3                | 0.0096 ± 0.0006                               | 0.1433 ± 0.1564       |

°Temperatures at which the samples were stored during the real-time stability study

Kinetic enzymatic reaction conditions were ABTS dissolved in 0.1 M citrate buffer at concentration between 0.1–3 mM, pH 3.0 ± 0.2. An enzyme solution with an activity of 10 UL$^{-1}$, reaction temperature 25 °C.

Fig. 4: Comparison of the RMSF differences ($\Delta$ RMSF) calculated between the different study temperatures against 241 K. In the $\Delta$ RMSFs, a positive value indicates that the residual has fluctuations, and a negative value indicates the opposite. A. $\Delta$ RMSF (278–241 K blue; 278–241 K green and 314–241 K red). B. $\Delta$ PC1 (278–241 K blue; 278–241 K green and 314–241 K red). The protein skeleton (C, CA and N), averaged per residue, was used to calculate the RMSF.
performed using C position co-variance to describe the global movements of rPOXA 1B at different temperatures employing the first principal components (PC).

Furthermore, to make a comparison by residue the first $\Delta$PC was calculated considering as reference the lowest temperature; for instance, 278–241 K, 298–241 K and 314–241 K. In Fig. 4B, $\Delta$PC1 are depicted for 278–241 K, 298–241 K and 314–241 K, which included previously observed residues and regions in the $\Delta$RMSF. For $\Delta$PC1 278 K the included residue was ALA 391 and the included regions were PRO 155 - THR 160 and THR 360 - ASP 365. For $\Delta$PC1 298 K the residue was ASP 266 and the regions ALA 330 - ALA 334, THR 360 - ASP 365 and ASP 458 - GLY 460. For $\Delta$PC1 314 the residues were GLU 101, THR 160, PRO 393, ASP 433 and the regions ASN 263 - PRO 267, GLN 293 - PRO 298, THR 360 - ASP 365. PC1 represented 15.4, 15.9, 11.8 and 18.6% variance proportion for 241 K (−32 °C), 278 K (4.85 °C), 298 K (24.85 °C) and 314 K (41 °C), respectively.

$\Delta$PC2 and $\Delta$PC3 were calculated similarly to $\Delta$PC1 (Supplementary Material Figure 7) and included the missing residues that showed fluctuation in the $\Delta$RMSF and were not represented in $\Delta$PC1. The first five components accounted for approximately 41% of the variations at 241 K (−32 °C), 278 K (4.85 °C), 298 K (24.85 °C) and 314 K (41 °C), respectively.

Discussion
Pilot study of rPOXA 1B stability
The pilot study demonstrated temperatures ranging between −30 °C (243.15 K) and 40 °C (313.15 K), were appropriate to perform stability studies since at 40 °C (313.15 K) residual activity was of 61.30% (Table 1). In previous studies [40], rPOXA 1B demonstrated stability between 10 °C (283.15 K) and 50 °C (323.15 K); the temperature at which relative enzyme activity started to decrease. However, in that study, the exposure lasted only one hour. The pilot study demonstrated the negative effect of exposure time to elevated temperatures. The highest activity loss occurred at 50 °C during the first 15 days, with only 25.9% of the residual enzyme activity recovered, and then at 30 days, it further decreased to 13.2% relative enzyme activity (Table 1).

Prolonged exposure to elevated temperatures induces conformational changes that affect enzyme 3D structure [66]. Nevertheless, this is not common; it is more frequent during short time exposures. Li et al., (2017) evaluated thermal stability at 35 and 55 °C using commercial laccase (Lac), (Sigma, China). In this study, the authors found a relative enzyme activity loss of about 20% when the enzyme exposed for nine hours at 35 °C. Activity loss was about 60 and 100%, when the enzyme exposed during 1 and 9 h, respectively, at 55 °C, (323.15 K) [67]. The authors did not specify the origin of the enzyme, which is a crucial fact or, because enzyme stability varies depending on origin and structure.

The sampling design proposed in this work allowed us to construct of a source population from which samples were randomly taken in each of the months (sampling moments). Henceforth in each case, the mean value of enzyme activity was always estimated with the same reliability and precision. The stratified sampling strategy made it possible to control data variations introduced because of using different batches, reducing the sampling error when estimating the enzyme activity mean values.

Analysis of real-time stability of rPOXA 1B concentrate
Proteins are generally more stable at freezing or refrigeration temperatures [24]. Nonetheless, stability depends on the enzyme’s unaltered structure, hence its biological
function. Therefore, non-covalent interactions, such as Van der Waals, hydrophobic-, electrostatic-, and hydrogen-bonds are important [68, 69].

In this work at –32.55 ± 4.12 °C (240.98 ± 5.38 K), we observed that relative enzyme activity was high during the 12 months of the study (101.16%), (Fig. 1, Table 3). Eichlerová et al., (2015) described freezing laccase supernatant obtained from *Trametes versicolor* in liquid nitrogen (–198°C, 75.15 K), previous to –80°C (353.15 K) storage, generated the highest enzyme activity loss in comparison to direct storage at –80°C (353.15 K), [70]. Slow freezing processes produced ice crystals, whereas rapid freezing forms small and slender crystals in the protein’s interfacial area, facilitating its denaturation [71].

Different investigations have analyzed the stability of laccase at freezing temperature, but very few (to the best of our knowledge) have evaluated exposure times longer than 12 months. Zhang et al., (2014) studied the residual activity of freeze-dried laccase from *T. versicolor* (Sigma-Aldrich) during 2 years of storage at –20°C (253.15 K), finding a residual enzyme activity of 14.6% [72].

Refrigeration temperature is frequently to evaluate enzyme stability [31, 73]. At 4.32 ± 1.22 °C (277.40 ± 1.32 K) rPOXA1B displayed a relative residual enzyme activity of 115.81% and a residual specific activity of 1045.58 ± 15.31 Umg⁻¹; which implies it was stable during the 12 months of the study (Fig. 1). An increase in relative residual enzyme activity is infrequent, yet, Has-Schön et al., (2005) studied peroxidase (E.C. 1.11.1.) stability of a crude *Picea abies* extract at 4 °C (277.15 K) for 30 days and found a relative residual enzyme activity of ~190%. These variations are attributable to possible changes in the enzyme’s native environment or the presence of substances that could have acted as activators [74].

In contrast Zhang et al., (2020) obtained a residual enzyme activity of only 10 ± 1.1% after 30-day storage at 4 °C (277.15 K), using *Trametes versicolor* commercial laccase (Sigma-Aldrich), [31]. Bagewadi et al., (2017) monitored *Trichoderma harzianum* HZN10 laccase finding a 45% enzyme activity loss in only 8 days of storage at 4 °C (277.15 K), [75]. Bou-Mitri and Kermasha, (2018) evaluated *Coriolus hirsutus* laccase, finding that after 4 weeks of storage at 4 °C (273.15 K) the residual enzyme activity was 8.5% [73]. rPOXA1B relative residual stability results in this study demonstrated an enzyme with high stability, exceeding that of other laccases, which makes it a promising enzyme for high scale use.

In the present study at 24.99 ± 0.25 °C (240.98 ± 5.38 K), 30.12 ± 1.11°C (303.27 ± 1.11 K) and 36.43 ± 0.23 °C (309.58 ± 0.23 K) a reduced residual activity was observed with increasing temperature and exposure time, obtaining at the end of the study a relative enzyme activity from 76.43, 46.09 and 5.81%, respectively for each temperature (Fig. 1).

Enzyme denaturation at elevated temperatures is associated with an increase in the kinetic energy of the system, which generates conformational fluctuations despite between 25°C (298.15 K) and 100° C (373.15 K) the hydrophobicity appears to remain constant compared to other properties [32]. At 24.99 ± 0.25°C (297.53 ± 3.88 K) and 30.12 ± 1.11°C (303.27 ± 1.11 K) the residual enzyme activities were similar until the third month of exposure (Fig. 1), but in subsequent sampling. At 30.12 ± 1.11°C (303.27 ± 1.11 K) the enzyme activity decreased rapidly; an effect that could be due to irreversible thermal denaturation [76]. However, the stability of rPOXA 1B was higher than that reported for other laccases. Ahn et al., (2007) evaluated commercial laccase from *T. villosa* (Novo Nordisk - Danbury, CT), finding that storage for 30 days at 25°C (298.15 K) generated an exponential deactivation of the enzyme, with a loss of residual relative enzyme activity of 93% [77]. On the other hand, Nadar et al., (2019) evaluated commercial laccase from *T. versicolor* (Sigma-Aldrich), finding that after 18 days at 30°C (303.15 K) only 30% residual activity was obtained [78].

In the present study, we found that at 41.64 ± 0.52 °C (341.79 ± 0.52 K) a considerable decrease in relative enzyme activity was observed, with a recovery of only 4.83% during the month of exposure, whereas in the pilot study relative residual enzyme activity at the same temperature was of 61.30% (Table 1, Fig. 2). In drug stability studies, lower concentrations generally support longer storage times [24]. This concentration effect also applies to biomolecules, since at high concentrations and temperatures enzymes are thermodynamically unstable and prone to form aggregates, due to hydrophobic groups exposure [79]. The difference between specific activity among concentrates used in the pilot study (~164.62 Umg⁻¹) and the stability study (758.71 ± 8.90 Umg⁻¹), demonstrates the number of enzyme units per milligram of protein was highest in the stability study. Tung et al., (2018) evaluated FIP-fve protein stability obtained from *Flammulina velutipes* at high and low concentrations during 1 min at 100°C, recovering 16.4 and 85.0% of protein respectively. However, at 4°C (277.15 K) no changes were observed, recovering 100% of the protein regardless of the concentration used, yet at –30°C (303.15 K) protein loss was slightly lower when the concentration was high [80].

rPOXA 1B accelerated stability and thermodynamics parameter analysis

The $K_d$ for evaluated temperatures ranged between $3 \times 10^{-3}$ and 0.400 months$^{-1}$, with a concomitant increase with the rise in temperature exposure (Table 4). This tendency has been previously observed due to enzyme and protein inactivation [78, 81]. $K_d$ was used to
calculate the half-life (t1/2), revealing that rPOXA 1B at
− 32.55 ± 4.12 °C (240.98 ± 5.38 K) and 4.32 ± 1.22 °C
(277.40 ± 1.32 K) was stable for at least for 230.7 and
46.2 months, respectively (Table 4).

The half-life (t1/2) is an economically important factor
since it allows to establish (according to conditions) ex-
piration and storage dates for industrial or environmen-
tial applications, forasmuch the higher the half-life
(t1/2) the higher the thermostability [82].

Certain reported t1/2 values for other laccases have
been lower. Yadav et al., (2018) evaluated recombinant
small laccase rSLAC from Streptomyces coelicolor A3(2)
produced in P. pastoris and obtained a t1/2 of 60, 32 and
10 h at 60 °C (333.15 K), 70 °C (343.15 K) and 80 °C
(353.15 K), respectively [83]. In contrast, using com-
ter T. versicolor (Sigma–Aldrich) laccase, Zhang et al.,
(2020) obtained a t1/2 of 7.04, 5.63 and 5.38 h at 30 °C
(303.15 K), 35 °C (308.15 K) and 45 °C (318.15 K), re-
spectively [31].

E_d expresses thermal stability [78]. For rPOXA 1B an
E_d of 41 KJ mol−1 was obtained (Fig. 3, Table 4), a value
that is within the deactivation energy ranges reported by
other authors. Nadar and Rathod, (2019) obtained 38.25
kJ mol−1 using commercial T. versicolor (Sigma–Aldrich)
laccase, Maurya et al., (2020) attained 34.97 kJ mol−1
also using commercial T. versicolor laccase (Sigma–Al-
drich), [84] and Ahn et al., (2007) obtained 25 kJ mol−1
using Trametes villosa laccase (Novo Nordisk - Danbury,
CT), [77].

To the best of our knowledge POXA1B from P. ostreu-
tus E_d has not been reported. However, the results
herein obtained (E_d = 41 KJ mol−1) confirm high stability
for rPOXA 1B, because the highest energy is required to
overcome the enzyme’s inactivation barrier. It is known,
changes in protein folding generate less organized and
unstable molecules, due to the disruption of relatively
weaker non-covalent bonds [85].

E_d is directly associated with enthalpy (ΔH), a thermo-
dynamic parameter that expresses the total energy asso-
ciated to enzyme denaturation. Large and positive E_d
and ΔH values represent the high thermostability of the
enzyme [86]. Positive ΔH values indicate that thermal
deactivation is an endothermic reaction [76, 87], where
the higher the ΔH value is, the higher the energy re-
quired to break the stabilizing bonds in a thermal inacti-
vation of the enzyme [83]. Similar tendencies have
reported for laccases and other enzymes [23, 76, 83].
Negative entropy values (ΔS) (Table 4) indicate the sys-

## Effect of exposure to different temperatures on apparent
V_{max} and K_{M}

Table 5 shows that V_{max} estimated from samples previ-
ously preserved between 240.98 K (− 32.55 °C) and
303.27 K (30.12 °C) is constant, suggesting the enzyme’s
reaction time was not affected, although at 303.27 K
(30.12 °C) amplitude in the standard deviation was ob-
erved. At 309.58 K (36.43 °C) and 314.79 K (41.64 °C)
demonstrates data here obtained is in line with other in-
vestigations. ΔG is a more reliable stability indi-
cator than ΔH and ΔS because it includes both
enthalpic and entropic contributions [87, 88].

## Homology modeling of POXA 1B

Sequence similarity is not the only factor determining
3D structure precision generated by homology. The
minimum limit of sequence similarity in homology mod-
eling should be 25% [91]; however, as a general rule,
two sequences are homologous if they are more than
30% identical over their entire length. Notwithstanding,
logomologous sequences that share more than 40% iden-
tity are considered functionally similar [92]. In the three
templates evaluated, the identity value was greater than
60%. QMEAN and GMQE scores were close to 1, sug-
uggesting the proposed model was of high quality and pre-
entified a high functional homology.

Based on the residue percentage in central regions in
Ramachandran’s plot, POXA 1B 3D structure had a high
stereochemical quality (Supplementary Material Figure 1). Verify score value suggested that 94.08% of the 3D modelled atoms were compatible with the sequence. Furthermore, the ERRAT score was adequate. Prognosticated protein models with a quality factor > 50% indicate the models by homology are stable and reliable [93], and the PROVES score was accepted, as it did not exceed 5%.

Our group previously reported a POXA 1B model using as template 1GYC [42]. Nevertheless, it was decided to improve and fine-tune the model using the semiautomatic MCPB.py Amber18 tool for molecules with metallic ion parametrization, since metallic complexes can have various modes of coordination [94], which are important for molecular dynamics analysis.

**Molecular dynamics simulations and principal components analysis**

RMSD determines the difference between the main chains of a protein from its initial to its final structural conformation, through the analysis of the deviations produced during the simulation (the minor deviation suggests higher stability of the system), [95]. RMSD calculation demonstrated the stability of the system at different temperatures was maintained during the simulation. Even though the RMSD deviation was higher with increasing temperatures, it remained low, stabilizing at an average of 0.96, 1.06, 1.26 and 1.20 Å for 241 K (~32.15 °C), 278 K (4.85 °C), 298 (24.85 °C) K and 314 K (40.85 °C), respectively.

Flexibility changes in a protein due to residue fluctuation can destabilize the system [59], where high Ca fluctuations are due to structural changes or free movements in the protein’s backbone [96]. With the MD results, an RMSF and PCA analysis were implemented, to elucidate the effects an increase in temperature would have on the enzyme.

RMSF and PCA analyses complemented each other since PCA represent the residues and identified regions, which are similar in the RMSF analysis. However, to identify the differences in fluctuations among temperatures, Δ RMSF was evaluated using fluctuations of 241 K (~32.15 °C) as the base temperature. The negative peaks observed in Δ RMSF analysis for LYS

as weak interaction sites or regions of the enzyme [98]. Therefore, an increase of the flexible regions demonstrates loss of interactions or bonds that maintain the structure stiff.

ASP

and nearby regions progressively increased the fluctuation with an increase in temperature (Figs. 4A, 5), shifting from a region with low fluctuation to one with high fluctuation between 278 and 314 K. Both residues, (ASP

and ASN

) are characterized by being hydrophilic and the nearby regions are composed of hydrophilic residues and residues of intermediate polarity. Hydrophilic residues are more fluctuating than hydrophobic [99]. Moreover, during MD simulations it is usual to observe an increase in flexibility in the hydrophobic regions. Furthermore, ASP

and ASN

residues are located in regions without a defined secondary structure, such as loops or coils; corresponding to regions of the highest fluctuations in any protein [100, 101]; as was observed during the analyses.

The region between LEU

and VAL

residues (Fig. 4A, 5) showed fluctuations in the three Δ RMSF analysis (278–241, 298–241 and 314–241 K). However, the region with the highest fluctuations was LEU

and THR

, which exhibited a higher fluctuation at 278 and 314 K; demonstrating temperature can increase or decrease residue fluctuations. Residue 162, which is not always VAL, has been studied in computational models for *Aspergillus oryzae* (PM1 and 7D5), [4] and *Coriolopsis trogii* [102] laccases, finding this residue is of great importance because it delimits the substrate-binding pocket and is characterized by having a wide network of hydrophobic interactions near CuT1.

The enzyme kinetic stability depends on the delicate balance between the flexibility and rigidity of its active site. The greater the stiffness of the active site, the greater the kinetic stability is [103]. The residues conserved and coordinated with copper ions obtained very low RMSF values, so they were not reported in the PCA. However, the fluctuation of PRO

adjacent residue to HIS

(CuT1) could have generated instability in the system (at 314 K, 40.85 °C). Weakening of copper coordination or subtle rearrangements in the coordination sphere of CuT1 leads to alterations in the interconnection of residues, which could destabilise the entire active site structure resulting in loss of enzymatic activity [104].

Results demonstrated that experimental data (real-time stability studies) was supported by computational (molecular dynamics) since the MD simulated temperatures had high fluctuation regions that could influence the stability of the system, caused by high-temperature exposure, such as 314 K (40.8 °C). In the same way, the application of this computational approach can be used in other enzymatic systems by applying the same described protocols.
Conclusions
The stratified sampling allows to established population (N) and sample (n) size. The samples at each point in time (months) were random. This approach always allowed us to estimate the median value of the enzyme activity (UL⁻¹) with the same confidence (95%) and precision (21.8 UL⁻¹). Besides, it allows controlling the variation within the results since sampling was carried out from different batches, decreasing sampling error. This statistical approximation done in this research could be considered in other enzymes stability studies. We demonstrated that when rPOXA 1B concentrate (impure and without preservatives agents) stored at −32.55 ± 4.12 °C (240.98 ± 5.38 K), 4.32 ± 1.22 °C (277.40 ± 1.32 K) or 24.99 ± 0.25 °C (297.53 ± 3.88 K) is stable with t₁/2 of 230.8, 46.2 and 12.6 months, respectively, exceeding reported storage time for other laccases. rPOXA 1B kinetic and thermodynamic parameters based on E₅₀ (41.40 KJ mol⁻¹) and low Kₘ and Vₘₐₓ variations between −32.55 ± 4.12 °C (240.98 ± 5.38 K) and 24.99 ± 0.25 °C (297.53 ± 3.88 K); as well as ΔH, ΔG and ΔS values, indicates the enzyme coordinate stability at different temperatures; making it even more attractive for industrial and environmental application. For POXA 1B active centre parametrisation, four copper coordinated with the HIS394, HIS496, CY851, HIS64, HIS397, HIS66, HIS109, HIS111, HIS399, HIS450, HIS452 residues; like the residues reported that coordinates with copper for other laccases. In general, at temperatures between 278 (4.85 °C) and 314 K (40.85 °C) (observed in the MD, using the Δ RMSF), all fluctuating residues were located at undefined secondary structure regions, such as loops or coils. Likewise, the number of residues with high fluctuations increased with temperatures changing from three residues (LEU159, ALA391 and ASP429) at 278 K (4.85 °C) to six residues (THR160, ASP266, GLU293, ALA334, ASP341 and LEU459) at 298 K (24.85 °C) and nine residues (ASP101, THR160, GLY265, ASN297, ALA334, GLY370, ALA391, PRO393 and ASP429) at 314 K (40.85 °C). Real-time stability analyses and MD confirmed that the increase in temperature starkly affects POXA 1B activity. Increase temperature increases the fluctuations in hairpin, coils or loops; regions that mainly contain hydrophilic or intermediate polarity amino acids (ASP266 and ASN297), favouring the long-term exposure of amino acids or hydrophobic zones that maintain the rigidity of the structure, resulting in irreversible inactivation of POXA 1B. At 314 K (40.85 °C), the PRO393 residue next to HIS394 (3.91 Å distance) increased the fluctuation and could result in the active site instability through alteration of the HIS394-CuT1 coordination. This study identified temperatures (under assay conditions) for optimal long-term storage of rPOXA 1B concentrate; allowing the construction of a model based on experimentally assayed temperatures and using the Arrhenius equation to predict the enzyme’s half-life (t₁/2) at temperatures ranging between 240.98 K (−32.55) and 309.58 K (36.43 °C). Our results are crucial to the technology transfer related to rPOXA 1B concentrate production at an industrial scale. Also, our findings help companies to decide to commercialise rPOXA 1B based on its multiple applications. Finally, our research group will continue the studies, using the pure enzyme and the addition of non-toxic preservatives to prolong the t₁/2 further.

Abbreviations
MCOs: Blue multi-copper oxidases; POXA 1B: Pleurotus ostreatus laccase, rPOXA 1B: Laccase recombinantly expressed in P. pastoris; TNC: Trinuclear copper center; SD: Standard deviation; (t₁/2): Half-life; kᵣ: Inactivation rate constant; Eᵣ: Deactivation energy; ΔH: Change in enthalpy of deactivation; ΔG: Change in Gibbs’s free energy of inactivation; ΔS: Change in entropy of inactivation; ABTS: 2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; MCPBPyr: Metal center parameter builder based in python; RTS: Real-time stability; AS: Accelerated stability; MD : Molecular Dynamics; NVT: Canonical ensemble; NPT: Isothermal-isobaric ensemble; PCA: Principal component analysis; PC: Principal Component; VMD: Visual Molecular Dynamics; Vₘₐₓ: Maximum velocity; Kₘ: Michaelis-Menten constant; N: Population size; n: Sample size; RMS: Root mean square; RMSF: Root mean square fluctuation; RMD: Root mean square deviation; ps: Picoseconds; fs: Femtoseconds; ns: Nanoseconds

Supplementary Information
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Additional file 1.

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Availability of data and materials
The raw data supporting the conclusions of this article will be may available by the authors, without undue reservation.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.
Competing interests
All the authors declare no competing interests with the others.

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7Agrawal K, Chaturvedi V, Verma P. Fungal laccase discovered but yet (Chlorella sp.) of a mixed effluent from two treatment sources (immobilized recombinant P. pastoris and rPOXA 18 concentrate) of coloured laboratory wastewater (CLWW). J Biotechnol. 2020;105(5):233.
8Lettera V, Pezzella C, Cicatiello P, Piscitelli A, Giacobelli VG, Galano E, et al. Tertiary treatment of malachite green by Paraconiothyrium variabile MA. Decolorization of some synthetic dyes using optimized culture broth of white rot fungi used as a carrier for plant growth-promoting bacteria. Eur J Foodchem. 2015.10.074. – Efficient immobilization of a fungal laccase and its exploitation in fruit juice catalysis by plant and fungal laccase with respect to lignin biosynthesis and degradation. J Biomol Struct Dyn. 2015;33(9):1835–49. https://doi.org/10.1080/07391102.2014.975282.
9Stanzione I, Pezzella C, Giardina P, Sannia G, Piscitelli A. Beyond natural laccases: extension of their potential applications by protein engineering. Appl Microbiol Biotechnol. 2020;104(3):915–24. https://doi.org/10.1007/s00253-019-11074-5.
10de Salas F, Cañadas R, Santiago G, Vírveda-Jerez A, Vind L, Gentili P, et al. Structural and biochemical insights into an engineered high-redox potential laccase overproduced in Aspergillus. Int J Biol Macromol. 2019;141:855–67. https://doi.org/10.1016/j.ijbiomac.2019.09.052.
11Yang J, Li W, Ng TB, Deng X, Lin J, Ye X. Laccases: production, expression and regulation, and applications in pharmaceutical biodegradation. Front Microbiol. 2017;8:632.
12Sharma A, Jain KK, Jain A, Kidwal M, Kuhad RC. Bi-functional in vivo role of laccase overproduced in Aspergillus. Int J Biol Macromol. 2019;141:855–67. https://doi.org/10.1016/j.ijbiomac.2019.09.052.
13Agrawal K, Catravides K, Verma P. Fungal laccase discovered but yet undiscovered. Bioreis Bioop. 2015(5):1(4). https://doi.org/10.1186/s40463-018-0110-z.
14Leterra V, Pezzella C, Cicatiello P, Piscitelli A, Giacobelli VG, Galano E, et al. Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification. Food Chem. 2016;196:1272–8. https://doi.org/10.1016/j.foodchem.2015.10.074.
15Castronvi MC, Bolognesi P, Chiarinelli J, Avaldi L, Calandra P, Antonacci A, et al. The convergence of forefront technologies in the design of laccase-based biosensors: An update. Trends Anal Chem. 2019;115:115615. https://doi.org/10.1016/j.trac.2019.07.026.
16Rojas-Higuera NS, Pava-Sánchez AM, Pinzón Rangel DL, Díaz-Anza LA, Quevedo-Hidalgo B, Pedraza-Rodríguez AM. Transgenic mustard intermediate plant developed for biochar production, enriched with phosphate solubilizing bacteria for agricultural use. PLoS One. 2019;14(9):e0217100. https://doi.org/10.1371/journal.pone.0217100.
17Moreno-Bayona DA, Gómez-Méndez LD, Blanco-Vargas A, Castillo-Toro A, Herrera-Carlosama L, Poutou-Piñales RA, et al. Simultaneous bioconversion of lignocellulosic residues and oxodegradable polyethylene by Pleurotus ostreatus for biochar production, enriched with phosphate solubilizing bacteria for agricultural use. PLoS One. 2019;14(9):e0217100. https://doi.org/10.1371/journal.pone.0217100.
18Pardo I, Camarero S. Laccase engineering by rational and evolutionary design. CMLS. 2015;72(5):897–910. https://doi.org/10.1007/s41598-018-0142-8.
19Srivasthavam TK, Kanagaraj J, Panda RC. Recent trends in fungal laccase for various industrial applications: An eco-friendly approach – a review. Biotechnol Bioprocess Eng. 2016;21(1):19–38. https://doi.org/10.1007/s12257-015-0278-7.
20Maestre-Reyna M, Liu W-C, Jeng W-Y, Lee C-C, Hsu C-A, Wen T-N, et al. Structural and functional roles of glycosylation in fungal laccase from Lentinus sp. PLoS One. 2015;10(4):e0120601. https://doi.org/10.1371/journal.pone.0120601.
21Moreno-Bayona DA, Poutou-Piñales RA, Salcedos-Reyes JC, Pedraza-Rodriguez AM, Vargas A, et al. Biodeterioration of plasma liquor with Pleurotus ostreatus for biochar production, enriched with phosphate solubilizing bacteria for agricultural use. PLoS One. 2019;14(9):e0217100. https://doi.org/10.1371/journal.pone.0217100.
22Michel CA, Mota MM, Moreira MM, Stochaj S, Romaniuk B, et al. Thermoresistance of fungal laccase from Pleurotus ostreatus sp. PLoS One. 2015;10(12):e0145638. https://doi.org/10.1371/journal.pone.0145638.
23Sanfelice D, Temussi PA. Cold denaturation as a tool to measure protein stability and cold adaptation. Curr Opin Struct Biol. 2017;42:117–24. https://doi.org/10.1016/j.sbi.2017.01.005.
24Huynh-Ba K. Handbook of stability testing in pharmaceutical development. Springer Science+Business Media: Newark, Delaware; 2009. https://doi.org/10.1007/978-0-387-85627-8.
25Christensen NJ, Kepp KP. Stability mechanisms of a thermophilic laccase probed by molecular dynamics. PLoS One. 2013;8(4):e61985. https://doi.org/10.1371/journal.pone.0061985.
26Poonkuzhali K, Palvannan T. Thermostabilization of laccase by polysaccharide additives: enhancement using central composite design of RSM. Carb Polym. 2019;200:106960. https://doi.org/10.1016/j.carbpol.2019.105.028.
27Elias M, Wieczorek G, Rosenne S, Tawfik DS. The universality of enzymatic rate temperature dependency. Trends Biochem Sci. 2014;39(1):1–7. https://doi.org/10.1016/j.tibs.2013.11.001.
28Chen Y-C, Smith T, Hicks RH, Doekieh A, Kooman A, Wells SA, et al. Thermal stability, storage and release of proteins with tailored fit in silica. Sci Rep. 2017;7(1):46568. https://doi.org/10.1038/srep46568.
29Sanfelice D, Temussi PA. Cold denaturation as a tool to measure protein stability. Biophys Chem. 2016;208:4–8.
30Rauk AP, Guo K, Hu Y, Caiya S, Weiss WF IV. Arrhenius Time-Scaled Least Squares: A simple, Robust Approach to Accelerated Stability Data Analysis for Biopharmaceuticals. J Pharm Sci. 2014;103(8):2278–86. https://doi.org/10.1002/jps.24063.
31Zhang R, Wang L, Han J, Wu J, Li C, N L, et al. Improving laccase activity and stability by HKUST-1 with cofactor via onepot encapsulation and its application for degradation of bisphenol A. J Hazard Mat. 2020;383:121130. https://doi.org/10.1016/j.jhazmat.2019.121130.
32Pucci F, Rooman M. Physical and molecular bases of protein thermal stability and cold adaptation. Curr Opin Struct Biol. 2017;42:117–28. https://doi.org/10.1016/j.sbi.2016.12.007.
33. Papaleo E, Riccardi L, Villa C, Fantucci P, De Goia L. Flexibility and enzymatic cold-adaptation: a comparative molecular dynamics investigation of the elastase family. Biochim Biophys Acta. 2006;1761:397–406.

34. Quezada AG, Díaz-Salazar AJ, Cabrera N, Pérez-Monfort R, Piñeiro Á, Costas M. Interplay between protein thermal flexibility and kinetic stability. Struct. 2017;25(5):167–79. https://doi.org/10.1016/j.str.2016.11.018.

35. Pikkarainen MG, Linsen ABM, Berendzen HJC, Janssen DB. Molecular dynamics simulations as a tool for improving protein stability. Protein Eng. 2002;15(3):185–92. https://doi.org/10.1093/protel/15.3.185.

36. Mohitashami M, Fooladi J, Haddad-Mashadzehi A, Housaindokht MR, Monhemi H. Molecular mechanism of enzyme tolerance against organic solvents: insights from molecular dynamics simulation. Int J Biol Macromol. 2015;122:94–23. https://doi.org/10.1016/j.jbiomac.2018.10.172.

37. Festa G, Autore F, Fraternali F, Giordano P. Insights into laccase engineering from molecular simulations: toward a binding–interfacing strategy. J Phys Chem Lett. 2015;6(8):1447–53. https://doi.org/10.1021/acs.jpclett.5b00825.

38. Hartmann J, Asch F. Extraction, storage duration, and storage temperature affect the activity of ascorbate peroxidase, glutathione reductase, and sucraseidum sucrose in Ripe rice. Bioch. 2019;8:70.

39. Ardila-Leal LD, Albarracín-Pardo DA, Rivera-Hoyos CM, Morales-Álvarez ED, Poutou-Piñales RA, et al. Computational modeling and preliminary Sampaio GE, Poutou-Piñales RA, et al. Computational modeling and preliminary characterization of the laccase activity in Pleurotus ostreatus. Bioresour. 2014;9(4):5801–11. https://doi.org/10.1016/j.biombioe.2014.01.023.

40. Lohr SL. Sampling, design and analysis, 2nd edition edn. USA: Chapman and Hall/CRC; 2019. https://doi.org/10.1007/978-0-203-96628-4.

41. Melkioiou M, Ki Lin CS, Webb C. Kinetic studies on the multi-enzyme solution produced via solid state fermentation of waste bread by Aspergillus niger. Biochem Eng J. 2013;18(15):76–82. https://doi.org/10.1016/j.bej.2013.09.016.

42. Gupta V, Sahai V, Gupta RK. Thermal stability and thermodynamics of xylanase from Melanocarpus albomyces in presence of polyls and salts. Bioresources.com. 2014;9(4):5801–11. https://doi.org/10.17172/biores.9.4.5801.

43. Morales-Álvarez ED, Rivero-Hoyos CM, Morales-Álvarez ED, Poutou-Piñales RA, Reyes-Montaño EA, et al. Computational analysis and low-scale constitutive expression of laccases synthetic genes (GCLC1) from Ganoderma lucidum and PPOA 1B from Pleurotus ostreatus in Pichia pastoris. PLoS One. 2015;10(1):e0116524. https://doi.org/10.1371/journal.pone.0116524.

44. Niaer JA, Martinez C, Casavahala K, Wickstrom L, Hausser K, Simmerling C. fH1ASB: improving the accuracy of protein side chain and backbone parameters from r9958. J Chem Theory Comput. 2015;11(8):3696–713. https://doi.org/10.1021/acs.jctc.5b00255.

45. Roe DR. Cheatham TE III. PTRAJ and CPPTRAJ: software for processing and analysis of molecular dynamics trajectory data. J Chem Theory Comput. 2013;9(7):3084–95. https://doi.org/10.1021/ct400347p.

46. Acosta-Tapia N, Galindo J, Baldis R. Insights into the effect of Lowe syndrome-causing mutation p.Asn591Lys of OCR1- through protein–protein interaction networks and molecular dynamics simulations. J Chem Inf Model. 2020;60(2):1019–27. https://doi.org/10.1021/acs.jcim.9b00777.

47. Galindo-Murillo R, Roe DR. Cheatham TE III. The absence of intracellular DNA dynamics on the ms to ts timescale. Nat Commun. 2014;5(1):1512. https://doi.org/10.1038/ncomms6152.

48. Grant RJ, Rodrigues APC, Elsayw KM, McCallum JA, Leo S, Caves D, Biodio: An R Package for the comparative analysis of protein structures. Bioinformatics. 2006;22(21):2695–6. https://doi.org/10.1093/bioinformatics/btl147.

49. Pecht M. ProMod and Swiss-model: internet-based tools for automated comparative protein modeling. Biochem Soc Trans. 1996;24(1):274–9. https://doi.org/10.1042/bst960274.

50. Farzaneh H, Ghaffari M, Rostami S, Asgari S, Funke G. A new method for model quality assessment. Prot. 2008;71(1):26–71. https://doi.org/10.1016/j.prot.2014.04.105.
73. Bou-Mitri C, Kermasha S. Lyoprotection and stabilization of laccase extract from Coniochaeta hirsuta using selected additives. AMB Exp. 2018;8(1):152. https://doi.org/10.1007/s13268-018-0683-3.

74. Has-Schön E, Lepedu H, Jerabek L, Cesar V. Influence of storage temperature on total peroxidase activity in crude extracts from Pisces abies L. Karst. Needles. Croatica Chem Acta. 2005;78(3):349–53.

75. Bagewadi ZK, Mulla SJ, Ninnirke HZ. Purification and immobilization of laccase from Trichoderma harzianum strain HZ10 and its application in dye decolorization. J Gen Eng Biotechnol. 2017;15(1):39–50. https://doi.org/10.1016/j.jgeb.2017.01.001.

76. Agbo KU, Okwuenu PC, Ezugwu AL, Eze SOO, Chilaka FC. Thermostability and thermodynamic characterization of sprouted pearl millet alpha-amylases for its biotechnological applications. Bangladesh J Sci Ind Res. 2017;52(3):159–66. https://doi.org/10.3329/bjcsr.v52i3.34146.

77. Ahn M-Y, Zimmerman AR, Martínez CE, Archibald DD, Bollag J-M, Dec J. Characteristics of Trametes villosa laccase adsorbed on aluminum hydroxide. Enz Microb Technol. 2007;41(1-2):141–8. https://doi.org/10.1016/j.enmic.2006.12.014.

78. Nadar SS, Rathod VK. Amino acid induced hyper activation of laccase and its application in dye degradation. Biocatal Agric Biotechnol. 2015;4:10164. https://doi.org/10.1016/j.bcab.2019.10.064.

79. Vendruscolo M, Knowles TPJ, Dobson CM. Protein solubility and protein homeostasis: a generic view of protein misfolding disorders. Cold Spring Harb Perspect Biol. 2011;3(12):a010454.

80. Tung C-H, Lin C-C, Wang H-J, Chen S-F, Sheu F, Lu T-J. Application of thermal stability difference to remove flavinmutoxin in fungal immunomodulatory protein, FIP-Fve, extract from Flavocultus velutipes. J Food Drug Anal. 2018;26(3):1005–14. https://doi.org/10.1016/j.jfda.2017.12.010.

81. Tavares APM, Silva CG, Dratic G, Silva AMT, Loureiro JM, Dec J. Laccase immobilization over multi-walled carbon nanotubes: kinetic, thermodynamic and stability studies. J Colloid Interf Sci. 2015;454:52–60. https://doi.org/10.1016/j.jcis.2015.10.051.

82. Ahmed SA, Salah SAA, Abdel-Hameed SAM, Fayad AM. Catalytic, kinetic and thermodynamic properties of free and immobilized casematin on mica glass-ceramics. Helvych. 2019;55(5):01674. https://doi.org/10.1002/hely.201901674.

83. Yadav D, Ranjan B, Mchunu N, Le Roes-Hill M, Kudanga T. Secretory expression of recombinant small laccase from Streptomyces coelicolor A3(2) in Pichia pastoris. Int J Mol Sci. 2018;19(1):664–9. https://doi.org/10.3390/ijms19010664.

84. Maurya SS, Nadar SS, Rathod WK. Dual activity of laccase-lysine hybrid organic–inorganic nanoflowers for dye decolourisation. Environ Technol Innov. 2020;19:100798. https://doi.org/10.1016/j.eti.2020.10.079.

85. Sant’Anna V, Cladera-Olivera F, Brandelli A. Kinetic and thermodynamic study of thermal inactivation of the antimicrobial peptide P34 in milk. Food Res Int. 2012;35(10):1884–9. https://doi.org/10.1016/j.foodres.2011.07.001.

86. de Oliveira RL, da Silva OS, Converti A, Porto TS. Thermodynamic and kinetic studies on pectinase extracted from Aspergillus aculeatus: free and immobilized enzyme entrapped in alginate beads. Int J Biol Macromol. 2018;115:1088–93. https://doi.org/10.1016/j.ijbiomac.2018.04.154.

87. Melek J. Thermodynamics and kinetics of thermal deactivation of catalase Aspergillus niger: Polish J Chem Technol. 2020;22(2):667–72.

88. Monteiro Souza PM, Allakbarian B, Filho EX, Oliveira Maquilhas P, Junior AP, Converti A, et al. Kinetic and thermodynamic studies of a novel acid protease from Aspergillus foetidus. Int J Biol Macromol. 2015;81:17–21. https://doi.org/10.1016/j.ijbiomac.2015.07.043.

89. Kutchefaptani SNR, Yeole N, Jana T. Urease immobilized polymer hydrogel: long-term stability and enhancement of enzymatic activity. J Colloid Interf Sci. 2016;463(1):64–72. https://doi.org/10.1016/j.jcis.2015.05.031.

90. Razavi BS, Bagdatakaya E, Kuzaykov Y. Non linear temperature sensitivity of enzyme kinetics explains canceling effect—a case study on loamy haplic Luvisol. Front Microbiol. 2015;6:1126.

91. Muhammed MT, Aki-Yalcin E. Homology modeling in drug discovery: overview, current applications, and future perspectives. Chem Biol Drug Des. 2019;93(1):1–20. https://doi.org/10.1111/cbdi.13388.

92. Pearson WR. An Introduction to sequence similarity (“Homology”) searching. Curr Prot Bioinform. 2013 Chapter 3 Unit 3.1.

93. Kameshwar AKS, Barber R, Qin W. Comparative modeling and molecular docking analysis of white, brown and soft rot fungal laccases using lignin model compounds for understanding the structural and functional properties of laccases. J Mol Graph Model. 2018;79:15–26. https://doi.org/10.1016/j.jmgm.2017.10.019.

94. Mentz JF, Cho MK. What are gene patents and why are people worried about them? Commun Genet. 2005;8(4):203–8.

95. Aier I, Varadaraj P, Raj U. Structural insights into conformational stability of both wild-type and mutant E2H2 receptor. Sci Rep. 2016(6):34984. https://doi.org/10.1038/srep34984.

96. Singh SP, Gupta DK. A comparative study of structural and conformational properties of casein kinase-1 isozymes: insights from molecular dynamics and principal component analysis. J Theoret Biol. 2015;371:59–68. https://doi.org/10.1016/j.jtbi.2015.01.032.

97. Karsihkoff A, Nilsson L, Ladenstein R. Rigidity versus flexibility: the dilemma of understanding protein thermal stability. FEBS J. 2015;282(20):3899–917. https://doi.org/10.1111/febs.13343.

98. Yu H, Huang H. Engineering proteins for thermostability through rigidifying flexible sites. Biotechnol Adv. 2014;32(2):308–15. https://doi.org/10.1016/j.biotechadv.2013.10.012.

99. Zhang T, Faraggi E, Zhou Y. Fluctuations of backbone torsion angles obtained from NMR-determined structures and their prediction. Proteomics. 2010;78:3533–62.

100. Avelar M, Pastor N, Ramirez-Ramirez J, Ayala M. Replacement of oxidizable residues predicted by QM-MM simulation of a fungal laccase generates variants with higher operational stability. J Inorg Biochem. 2018;178:125–33. https://doi.org/10.1016/j.jinorgbio.2017.10.007.

101. Herrera-Zúñiga LD, Millán-Pacheco C, Viniegra-González G, Villegas E, Arequi L, Rojo-Domínguez A. Molecular dynamics on laccase from Trametes versicolor to examine thermal stability induced by salt bridges. Chem Phys. 2019;572:253–64. https://doi.org/10.1016/j.chemicalphys.2018.10.019.

102. Mateljakić I, Monza E, Lucas MF, Guallar V, Aleksejeva O, Ludwig R, et al. Increasing redox potential, redox mediator activity, and stability in a fungal laccase by computer-guided mutagenesis and directed evolution. ACS Catal. 2019;9(5):4561–72. https://doi.org/10.1021/acscatal.9b00531.

103. Xie Y, An J, Yang G, Wu G, Zhang Y, Cui L, et al. Enhanced enzyme kinetic stability by increasing rigidity within the active site. J Biol Chem. 2014;289(11):7994–8006. https://doi.org/10.1074/jbc.M113.536045.

104. Durao P, Bento I, Fernandes AT, Melo EP, Lindley PF, Martins LO. Perturbations of the T1 copper site in the CotA laccase from Bacillus subtilis: structural, biochemical, enzymatic and stability studies. J Biol Inorg Chem. 2006;11(4):514–26. https://doi.org/10.1007/s00775-006-0102-0.

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