Kinetics And Thermodynamic Properties of Trametes Polyzona WRF03 Laccase

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Kinetics and thermodynamic properties of *Trametes polyzona* WRF03 laccase

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Abstract

The effect of thermal treatment on the activity of laccase from *Trametes polyzona* WRF03 was studied at pH and temperature ranges of 3.0 to 6.5 and of 40 to 70 ºC respectively. Kinetic data revealed that the heat inactivation of *Trametes polyzona* WRF03 laccase (*Tp*L) was pH dependent and followed first-order kinetics. There was a positive correlation between activation energy (*Ea*) for thermal inactivation of *Tp*L and the reaction pH. Highest activation energy, *Ea*, value of 175.49 kJ/mol was obtained at pH 6.0. On the contrary, the z-value decreased with a lowest value of 12.37 ºC at pH 6.0. The high *Ea* value and low z-value were indicative of the thermo-stable nature of *Tp*L which suggests that pH 6.0 had a compensatory stabilizing effect on *Tp*L against its thermal denaturation. There was a gradual decrease in the enthalpy of denaturation (ΔH^o) and Gibb's free-energy with every 10 % rise in temperature within the investigated pH range, suggesting that *Tp*L was more stable at 40 ºC. Positive values of entropy of inactivation (ΔS^o) at each temperature indicated that there was no aggregation during the inactivation processes. Thus, these results provided useful information about the behaviour of *Tp*L under certain pH and temperature combination with respect to biotechnological application. Thus, the kinetic and thermodynamic data could be used to design a model to predict the thermal inactivation of *Tp*L during industrial application.

Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a multi-copper oxidase that catalyzes a four-electron oxidation of phenolic and certain aromatic compounds with concomitant reduction of molecular oxygen to water molecule[1]. Yoshida was the first to isolate laccase from Japanese lacquer tree, *Rhus vernicifera* in 1883 from where the name was derived. In addition, it was first characterised by Bertrand and Lamborde in 1895 as a metal-containing oxidase[2]. Subsequently, laccases have been identified in many organisms such as fungi, bacteria, and insects where they play diverse roles[3]. . Laccase is glycosylated monomer or homodimer protein. Fungal and bacterial laccases contain fewer saccharides per molecule (10-25 %) than plant laccases[4]. Glycosylation of fungal laccase was reported to determine enzyme secretion, susceptibility, activity, copper-retention, and thermal stability[2,5]. Removal of the carbohydrate moiety from *Ganoderma lucidum* laccase completely inactivated the protein[6]. Glycosylation has also been reported to protect the enzyme molecule against proteolytic degradation and inactivation by free radicals[7,8].

Laccase from white rot fungi has drawn much research and industrial interests over the past few years due to its broad substrate specificity and sole requirement of molecular oxygen (electron acceptor) as a co-substrate, which is abundant in nature. As a result, the enzyme has great potential for many environmental and industrial applications[9], such as pulp-delignification, pesticide or insecticide degradation, organic synthesis, waste detoxification, textile dye transformation, food technology and development of biosensor[10]. The commercial applications of fungal laccases are usually at extreme conditions of temperature, pH, and high salt concentration, which greatly affect the catalytic efficiency of the enzyme[11,12]. However, to be viable for commercial applications, laccases with unique properties such as thermal

stability and resistance to harsh reaction conditions are required. This has therefore, intensified the search for laccases with robust properties for biotechnological applications.

Since the fungal laccases have relevance in biotechnology and industrial food processing, investigations of their pH and thermal stabilities are vital strategies towards activity optimization. The inactivation kinetics and the determination of thermodynamic parameters may not only help in the appreciation of the structure-function relationship of the enzyme, but also offer insight into the probable mechanism of its inactivation[13]. The knowledge of the enzyme stability can also facilitate an economical production design by optimizing the profitability of enzymatic processes[14].

Although many fungal laccases have been reported to be heat stable, their thermal stability properties vary considerably even within the same fungal strain/specie because they are coded by gene families that allow multiple expression of isoforms [15,16]. Several studies have determined the properties and kinetic parameters of laccase, which include the inactivation rate constants (k_d), half-life ($t_{1/2}$), activation energy, *E*a, for thermal inactivation and the kinetic constants K_m and V_{max}[17-20]. However, data on thermodynamic parameters are scarce. Moreover, many researchers reported thermo-stability of laccase only in the context of optimum pH, without considering other pH. In view of this, the present study provided information on thermal stability of *Tp*L at different pH and the interactive effect of pH and temperature on the enzyme stability.

Materials and methods

Chemicals and Reagents

The standard substrate ABTS (2, 2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was purchased directly from Sigma- Aldrich, Germany. All other chemical used for buffer preparation, enzyme production and purification were of analytical grade and were obtained from local vendors. The white rot fungi, *Trametes polyzona* WRF03, was collected from the Microbial Culture Collection of Enzymology and Protein Chemistry Unit, Department of Biochemistry, University of Nigeria, Nsukka (MCCEPU).

Laccase production and purification

The fermentation of *Trametes polyzona* WRF03 for laccase production and the enzyme purification were carried out as previously described by Ezike *et al*[21].

Laccase assay

Laccase activity was determined by monitoring the oxidation of ABTS radical (ABTS-azine) by the enzyme at 420 nm (ε_{420}= 36 $mM^{-1}cm^{-1}$). The intensity of the deep blue colour developed was directly proportional to the enzyme activity, which was expressed in international units (IU). One IU of enzyme is defined as the amount of enzyme forming one micromole of product per minute under standard assay condition[22].

Thermal inactivation of *Tp*L

Thermal inactivation of *Tp*L was investigated by incubating the enzyme at pH range of 3.0 to 6.5 and temperature range of 40 to 70 ºC for a period of 120 min in a temperature-controlled water bath (Model: DK 420, China). At each pH, the enzyme solution was incubated at temperature range of 40 - 70 ºC for 120 min. During the heat treatment, the tubes containing the enzyme solution were sealed to prevent changes in volume and concentration due to evaporation. After heat treatment, aliquots of the enzyme were taken at regular time intervals, allowed to cool on ice and the residual activity determined. The residual activity was expressed in percentage relationship with the control (% RA). The data obtained was used to analyse both the kinetic and thermodynamic parameters for thermal inactivation of *Tp*L.

Determination of kinetic parameters for thermal inactivation of *Tp*L

The experimental data were fitted to a simple first-order exponential decay model

$$E \xrightarrow{k} E_{inact} \quad (1)$$

where E and E_{inact} symbolize the active-enzyme and heat-inactivated enzyme, respectively. The first-order denaturation rate constants (k_{inact}) for the thermal inactivation of laccase was calculated as the gradients of the plot of the inactivation time courses of log (% RA) against time of exposure to heat according to following equation 2:

$$\log\left(\frac{At}{A0}\right) = -\left(\frac{k_{inact}}{2.303}\right)t \quad (2)$$

Where, A_0 and A_t represent the enzyme activity at time zero and t respectively. The gradients of the plots of the natural log of the first-order rate constants, ln k_{inact}, against 1/T (Arrhenius plots), was calculated as the activation energies (Ea) for thermal inactivation of *Tp*L at different pH according to equation 3.

$$\ln k_{inact} = -\frac{Ea}{RT} + c, \quad (3)$$

where R is the molar gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature in Kelvin. The inactivation data, k_{inact} and Ea, were used to express $D-$ and $z-$values according to Anthon *et al*[23]. D-value is the time required to reduce the enzyme activity to 10 % of its initial value which has an inverse relationship with the first-order inactivation rate constant k_{inact} as written below in equation 4:

$$k_{inact} = \frac{2.303}{D} \quad (4)$$

The log D is linearly related to the inverse of temperature (ºC). The change in temperature needed to cause one logarithmic cycle decline in the D-value was evaluated from the gradient of the plot of log D against T (ºC) according to equation 5. This is known as the z-value.

$$Slope = \frac{-1}{z} \quad (5)$$

The half-life of laccase ($t_{1/2}$, min^{-1}) was evaluated using equation 6.

$$t_{1/2} = \frac{lin2}{(k_{inact})} \quad (6)$$

Estimation of the thermodynamic parameters

The enthalpy change (ΔH^o, J mol^{-1}), Gibb's free energy (ΔG^o, J mol^{-1}) and change in entropy (ΔS^o, J mol^{-1} K^{-1}) for thermal inactivation of laccase were calculated as described by Pal and Khanum[24] using the following equations.

$$\Delta H^o = Ea - RT \quad (7)$$

$$\Delta G^o = -RTln\frac{(k_{inact}.h)}{k_B.T} \quad (8)$$

$$\Delta S^o = \frac{\Delta H^0 - \Delta G^0}{T} \quad (9)$$

Where Ea is the activation energy for thermal inactivation, T is the corresponding absolute temperature (K), R is the molar gas constant (8.314 J mol-1 K^{-1}), h is the Planck's constant

(11.04 X 10^{-36} J min), k_B is the Boltzman constant (1.38 X 10^{-23} J K^{-1}) and (k_{inact})is the inactivation rate constant (min^{-1}).

Results

Thermal inactivation of *Tp*L

The effect of heat treatment on *Tp*L was investigated at various temperature and pH combinations (Figure 1 – 8). Thermal inactivation of *Tp*L was observed to be pH-dependent. The residual activity of *Tp*L was observed to decrease at different rates as pH, temperature, and time increased. At pH 3.0, the enzyme lost 100 % of its activity after incubation at 40 ºC for 30 min, 50 ºC for 20 min, 60 ºC and 70 ºC for 10 and 5 min respectively. However, as pH decreased from 3.0 to 6.0, the residual activity gradually increased. It was observed that *Tp*L characteristically exhibited higher residual activity when incubated at pH 6.0 than other pH at all the investigated temperatures.

Kinetic parameters for thermal inactivation of *Tp*L

A plot of log (% residual activity) against time of heat treatment for different combination of temperature and pH fitted into a straight line graph with good R^2-value over most of the investigated time courses. This indicated that the heat inactivation of laccase is a first-order kinetics. Generally, as the temperature increased, the *D*-value and $t_{1/2}$ decreased whereas the rate of enzyme inactivation (k_{inact}) increased (Table 1). Also, *D*-value and $t_{1/2}$ at all temperature increased with decrease in pH from 3.0 to 6.0. Conversely, the rate of enzyme inactivation (k_{inact}) at all temperature decreased with decrease in pH. This result suggests thermo-stabilizing effect of pH on *Tp*L since a lower inactivation rate constant translates to a more thermo-stable enzyme. The z- value was evaluated from the gradient of log *D* against temperature (Figure 9). The result showed a gradual decrease in z-value as the pH increased from 3.0 – 6.5. The z-value for laccase at different pH was presented in Table 1. The activation energy, *E*a, for thermal inactivation of *Tp*L as deduced from the gradients of the plot of ln k_{inact} against 1/*T* (Figure 10) showed a significant increase with decreasing values of pH, with the highest at 6.0 (175.49 kJ/mol) and the lowest value 3.0 (80.90 kJ/mol), respectively. The result in Table 10 implies a higher resistance of *Tp*L to heat inactivation at pH 6.0.

Thermodynamic parameters for thermal inactivation of *Tp*L

The *Tp*L showed a gradual decrease in enthalpy of denaturation (ΔH^o) with every 10 % rise in temperature at all tested pH (Table 2). The decrease in ΔH^o value indicates lesser incremental energies required for denaturation of the enzyme as temperature, *T*, increased. Change in pH was also observed to affect the ΔH^o. At 40 ºC and pH 4.0, the enthalpy change, ΔH^o, was 78.29 kJ/mol, whereas at pH 6.0, ΔH^o was 172.89 kJ/mol indicating the requirement for higher energy in the thermal inactivation of *Tp*L at higher pH values. The Gibb's free energy for thermal denaturation (ΔG^o) of the enzyme was observed to increase with decrease in pH. At 40 ºC, the Gibb's free energy, ΔG^o, increased from 50.31 kJ/mol at pH 3.0 to 63.97 kJ/mol at pH 6.0. Conversely, ΔG^o decreased with increase in temperature at each investigated pH. The values of entropy of inactivation (ΔS^o) were positive over the investigated temperature and pH ranges.

Discussion

Enzyme denaturation is found to be one of the major problems associated with rapid development of biotechnological processes. It occurs when there is a conformational change in the three-dimensional structure of proteins without the breakage of covalent bonds leading to an inactive state[13]. Therefore, understanding the effects of different environmental factors

on enzyme activity and molecular structure is essential for its industrial applications. The temperature stability of fungal laccases varies considerably with the producing organism and the pH of the reaction environment[25]. Whereas many researchers have reported on the thermo-stability of laccase only at the optimum pH, the present study provided further information on the interactive effect of pH and temperature on the stability of *Trametes polyzona* WRF03 laccase. Results of this investigation showed a marked dependence of enzyme's thermo-stability on pH of the reaction medium. *Tp*L was stable over a range of pH and temperature but more stable at pH 6.0 and 40 ºC. This was supported by the observed increase in *D*-value and half-life at higher pH values and the decrease in inactivation rate constant (k_{inact}), since lower k_{inact} means that the enzyme is more thermostable. The dependency of the inactivation rate constants on temperature fitted well to Arrhenius equation. The linear nature of the curve suggests a distinctive inactivation mechanism such as protein-unfolding that is uniquely temperature-dependent[26].

A half-life of 2.9 h at 50 °C, 1.5 h at 60 °C and 3.7 min at 70 °C was reported for laccase from *Trametes pubescens*[17]. In contrast, *Tp*L exhibited a greater stability with half-life of 12 h at 50 °C, 1.5 h at 60 °C and 7.5 min at 70 °C. Stability studies of *Tp*L at 40 °C, 50 °C, 60 °C and 70 °C showed better results than the findings of Galhaup *et al*[17]. Laccase from *Pleurotus ostreatus* was almost fully active within the temperature range of 40 - 60 °C and showed half-life of 30 min at 60 °C[27]. Also, Jaouani *et al*[28] reported a half-life of 8 h and 2 h at 50 ^{0}C and 60 ºC, respectively for laccase from *Pycnoporous coccineus* whereas laccase from *Pleurotus florida* showed a half-life of 60 min at 60 °C[29]. The half-life of laccase B isoenzyme from *Trametes sp.* was reported to be 14 min at 60 ºC and was found to be stable at 50 ºC for more than 50 min.

Higher z-value means greater sensitivity of an enzyme to the time interval of heat treatment while lower z-value means greater sensitivity to temperature rise. In this study, the higher z-value noted at the acidic region compared to neutral/alkaline region indicates that increase in pH makes the enzyme more sensitive to the duration of heat treatment than the increase in temperature and *vice versa*. Inferentially, neutral/alkaline media rather than acidic media confer greater thermo-stability on *Tp*L. There was dearth of information pertaining to this aspect of laccase characterisation with which to compare and discuss our findings, though Gouzi *et al*[30] reported similar type of studies with polyphenol oxidase. The activation energy, (*E*a), for thermal inactivation, which is the minimum amount of energy required to denature the enzyme was higher at higher pH values suggesting that *Tp*L is more stable at neutral/alkaline pH. Eze *et al*[31] posited that high Ea is an indication that the rate of the thermal inactivation is temperature dependent. However, this rate will be insignificant at lower temperature.

Thermodynamic parameters such as enthalpy changes (ΔH^o), entropy changes (ΔS^o), and Gibbs free energy (ΔG^o) were used to characterize the spontaneity of the inactivation process. These parameters provided useful data on the thermo-stability of *Tp*L regarding the step by step thermal denaturation process. Generally, enthalpy of inactivation (ΔH^o) is quantified as the discrete number of non-covalent linkages on a protein surface that must be broken to form the inactivated enzyme (the transition state). Conversely, thermo-stability of enzyme increases with ΔH^o value which translates to a larger number of non-covalent bonds present in the enzyme structure[30]. Results show that enthalpy change, ΔH^o, decreased with increasing temperature values but increased with increasing pH values. This implies that there are low energy requirements for the inactivation of the enzyme at high temperatures, while there are high-energy requirements at high pH values for the inactivation of the enzyme. However, Bruins *et al*.[32] highlighted the multi-factorial basis of protein stability as component parts of the delicate balance between the stabilizing and destabilizing interactions, which may include the number of hydrogen bond and disulphide bridges, the folding degree and hydrophobicity

of the enzyme molecule and the amount of ionic and other interactions. Although enthalpy changes, ΔH^o, provided information about the non-covalent forces broken during the inactivation process, its sole use as indicator of enzyme stability may not be suitable[26].

Unlike enthalpy change, ΔH^o, the energy barrier to inactivation, ΔG^o, has a direct relationship to protein stability. There is a parallel increase in ΔG^o and enzyme stability. In the present study, Gibb's free energy, ΔG^o, increased with decrease in pH at all temperature investigated but decreased as the temperature was elevated from 40 °C to 70 °C. This result indicates that destabilisation of this protein structure was a consequence of temperature increase. Since the ΔG^o is much lower that the ΔH^o one would expect a significantly positive entropic contribution to the thermodynamics properties. This also confirms that the enzyme was more stable at 40 °C and at higher pH value (pH 6.0). Entropy changes (ΔS^o) indicate the net disorder in enzyme and solvent mixture during the inactivation process. All entropy change, ΔS^o, for thermal inactivation of *Tp*L was positive, implying incremental disorderliness or randomness in the enzyme/solvent system upon denaturation. Klibanov[33] observed the loss of native 3-D structure, also known as thermo-denaturation as the basic driving factor in heat inactivation of an enzyme, which arises from heightened molecular mobility associated with high temperatures.

Positive entropy change, ΔS^o, indicate that there was no protein aggregation while negative ΔS^o indicates that there was protein aggregation during the inactivation process in which a few inter- and intra-molecular bonds developed. The foregoing also suggests that for the irreversible inactivation of laccase, the unfolding of the native 3-D structure is the rate-determining step. In general, if an enzyme is incubated at high temperature for certain period, it will exhibit denaturation in two steps. The native enzyme is first converted to the inactive and unfolded conformer, which on cooling can re-gain its native conformation thereby regaining its activity. In the second step, the inactive and unfolded protein can be converted into a denatured enzyme, which is unable to re-gain its native conformation and activity on cooling. The denaturation occurs due to continuous disruption of non-covalent linkages that cumulatively elevate the global heat content (ΔH^o) of inactivation[34]. Being a metallo-enzyme however, the thermo-inactivation effect on laccase as observed at higher temperatures may also be a result of conformational changes in the type-1 and type-2 copper centres. Higher temperatures might result in the loss of the type-2 copper ion, which is completely absent at 70 °C. Beyond 70 °C, the type-1 and type-3 copper sites were also completely disintegrated[35].

Conclusion

A study on the thermal inactivation of an enzyme is important in assessing its suitability for biotechnological applications. The thermal inactivation of laccase from *Trametes polyzona* WRF03 followed first-order kinetics and was dependent on the pH of the reaction medium. The kinetics and thermodynamic parameters used to analyse the thermal inactivation of *Tp*L showed that the enzyme was more stable at pH 6.0 and temperature of 40 °C. The result of the present study provided valuable information about the thermal behaviour of *Tp*L at different pH conditions in view of its biotechnological application. Thus, the kinetic and thermodynamic data could be used to design a model to predict the thermal inactivation of *Tp*L during industrial application

Author Contributions

Ezike, T.C.: Investigation, Data curation, Writing- Original Draft, Visualization
Ezugwu, A. L.: Writing- Review and Editing
Udeh, J.O. and Ugwuoke K.C.: Validation and Data analysis
Eze, S.O.O: Conceptualization and Methodology
Chilaka, F.C.: Supervision

Competing Interests Statement

The authors declare no competing interests.

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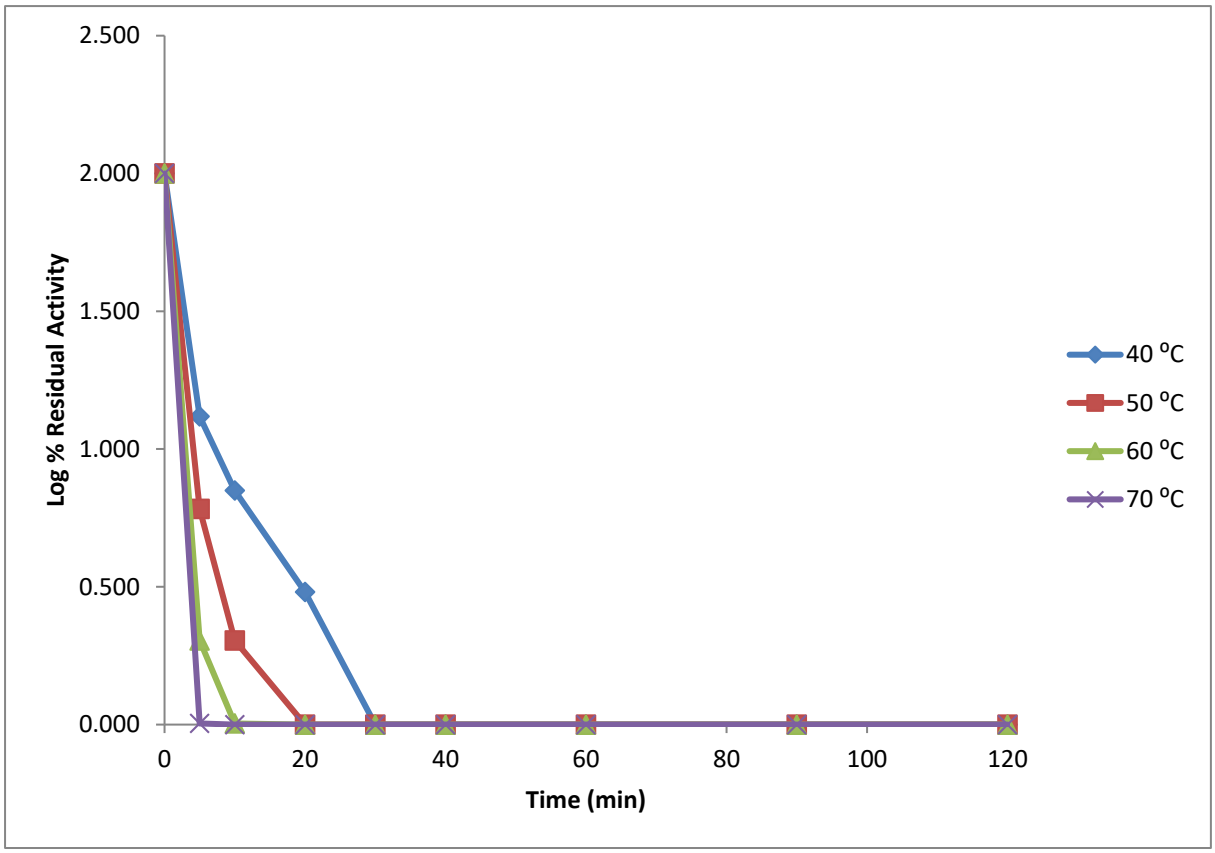

Figure 1: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 3.0 (0.1 M sodium acetate buffer) and temperature of 40 to 70 °C.

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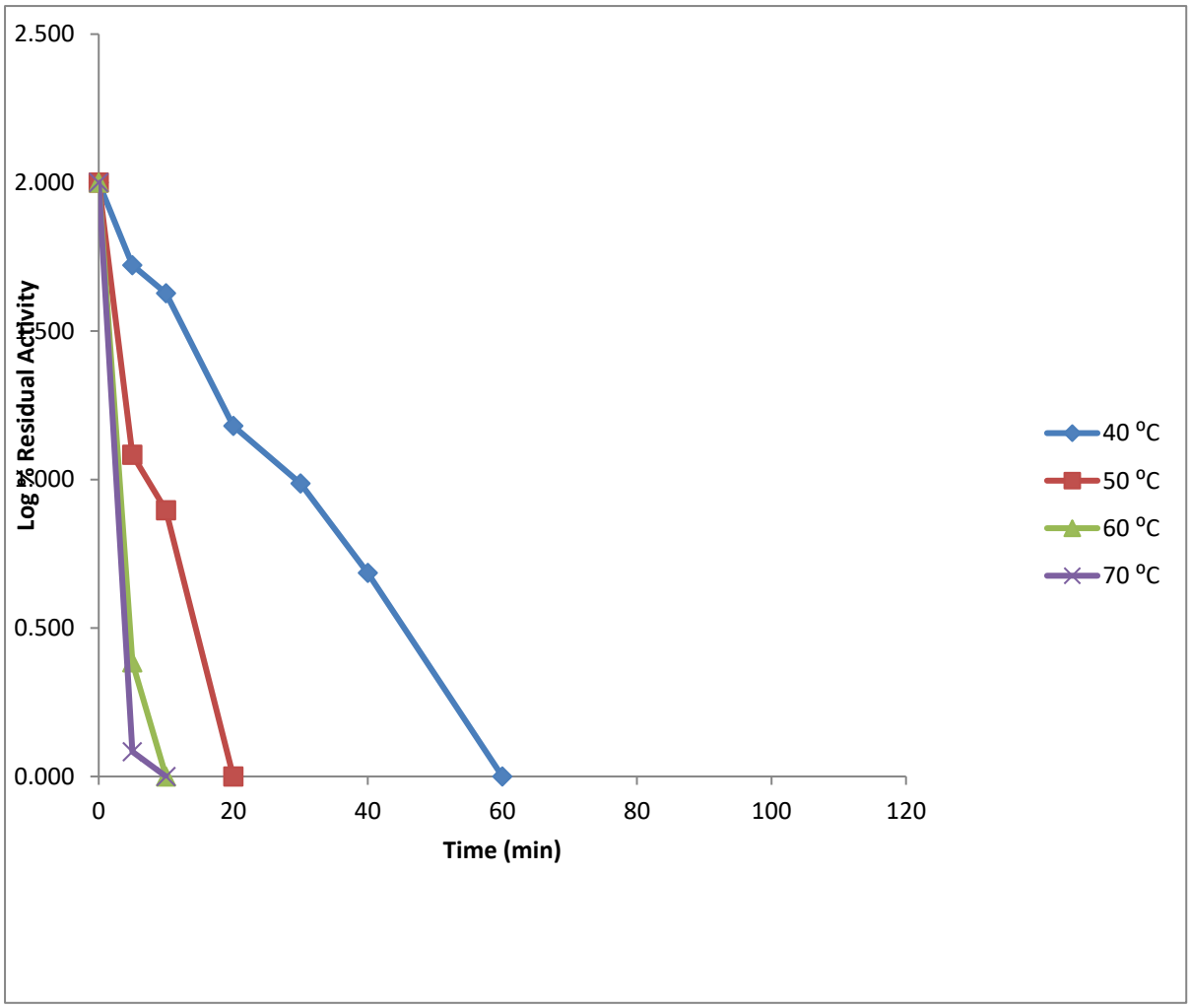

Figure 2: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 3.5 (0.1 M sodium acetate buffer) and temperature range of 40 to 70 °C.

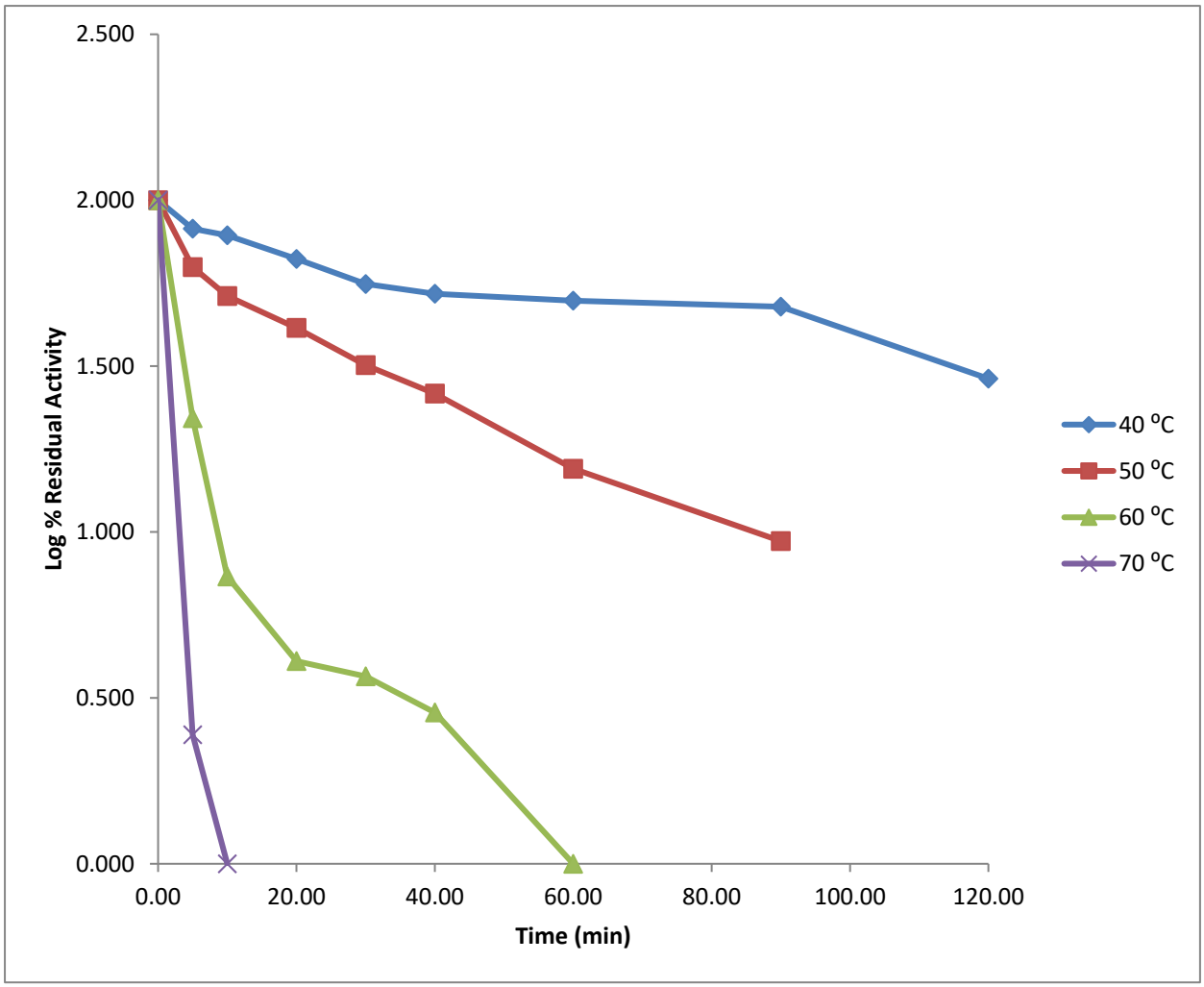

Figure 3: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 4.0 (0.1 M sodium acetate buffer) and temperature range of 40 to 70 °C.

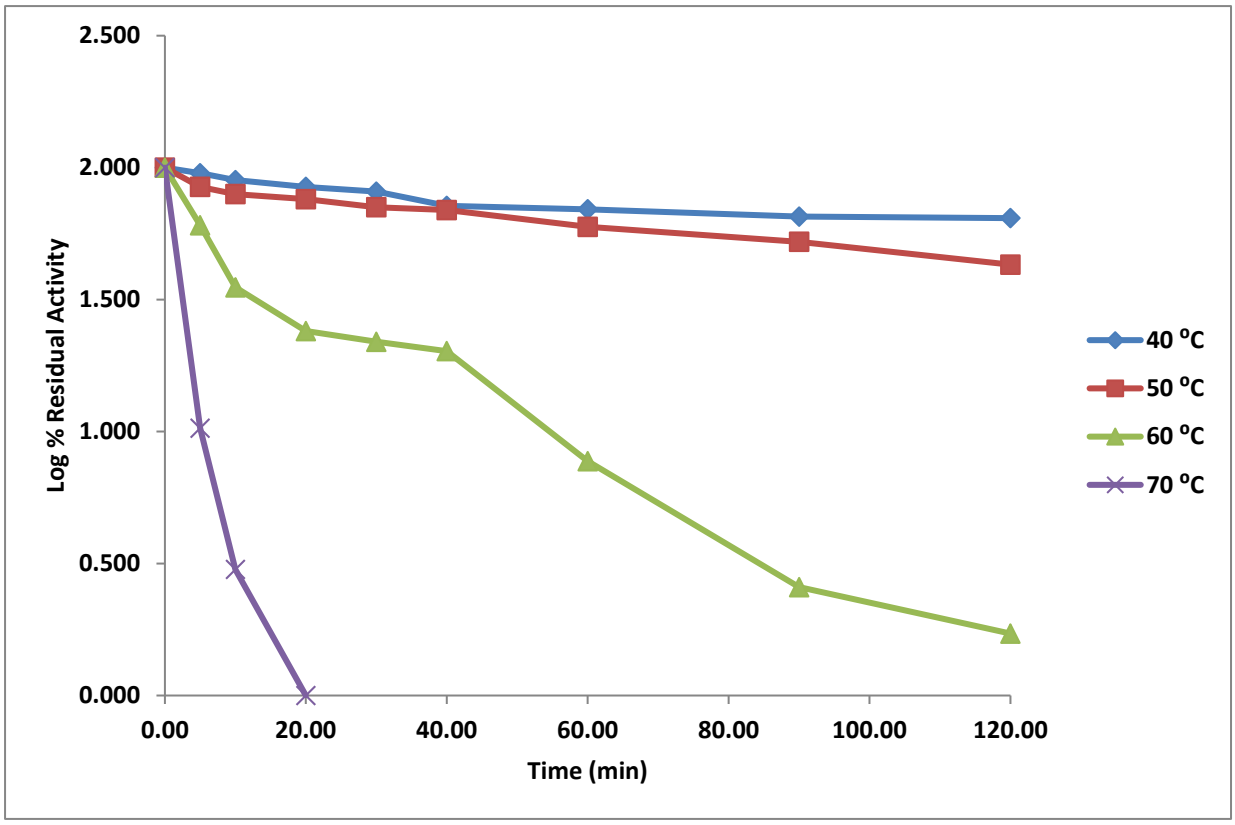

Figure 4: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 4.5 (0.1 M sodium acetate buffer) and temperature range of 40 to 70 °C.

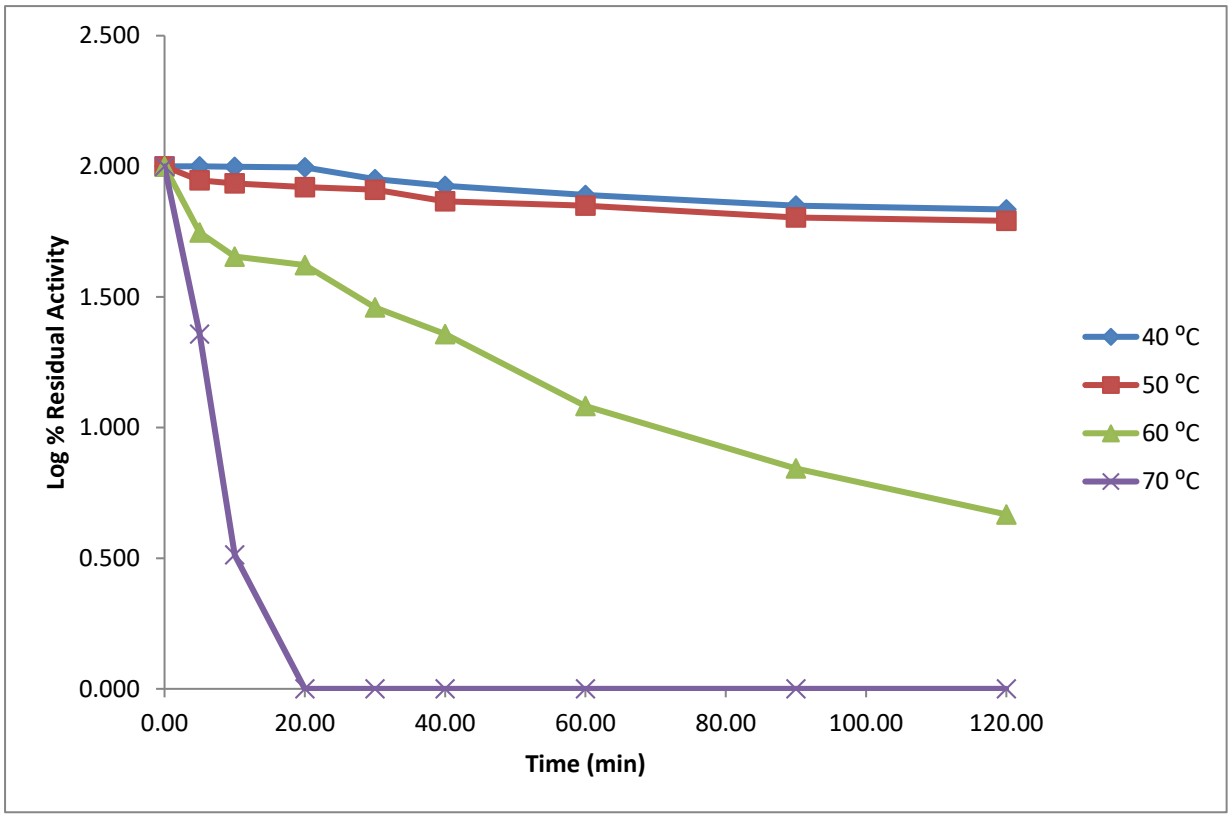

Figure 5: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 5.0 (0.1 M sodium acetate buffer) and temperature of 40 to 70 °C.

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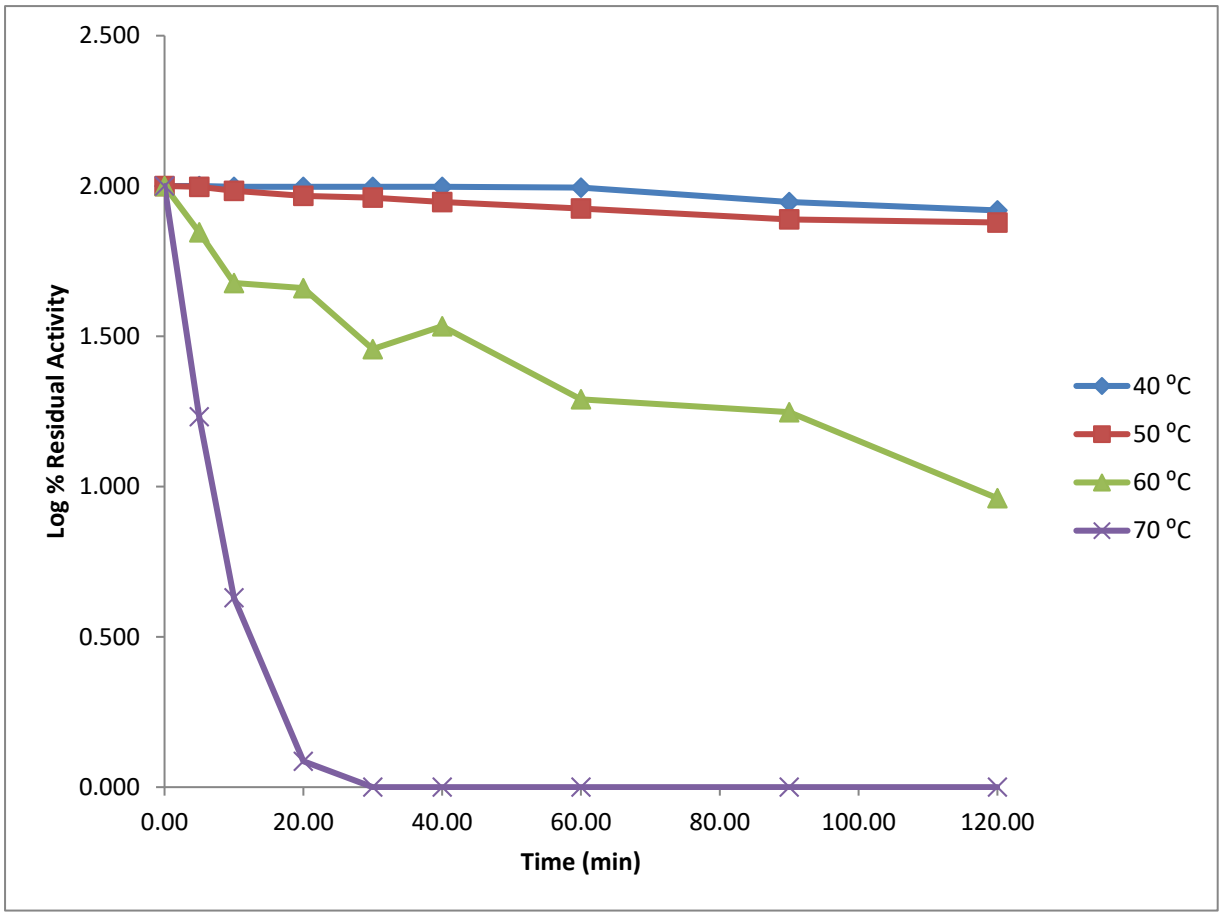

Figure 6: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 5.5 (0.1 M sodium acetate buffer) and temperature of 40 to 70 °C.

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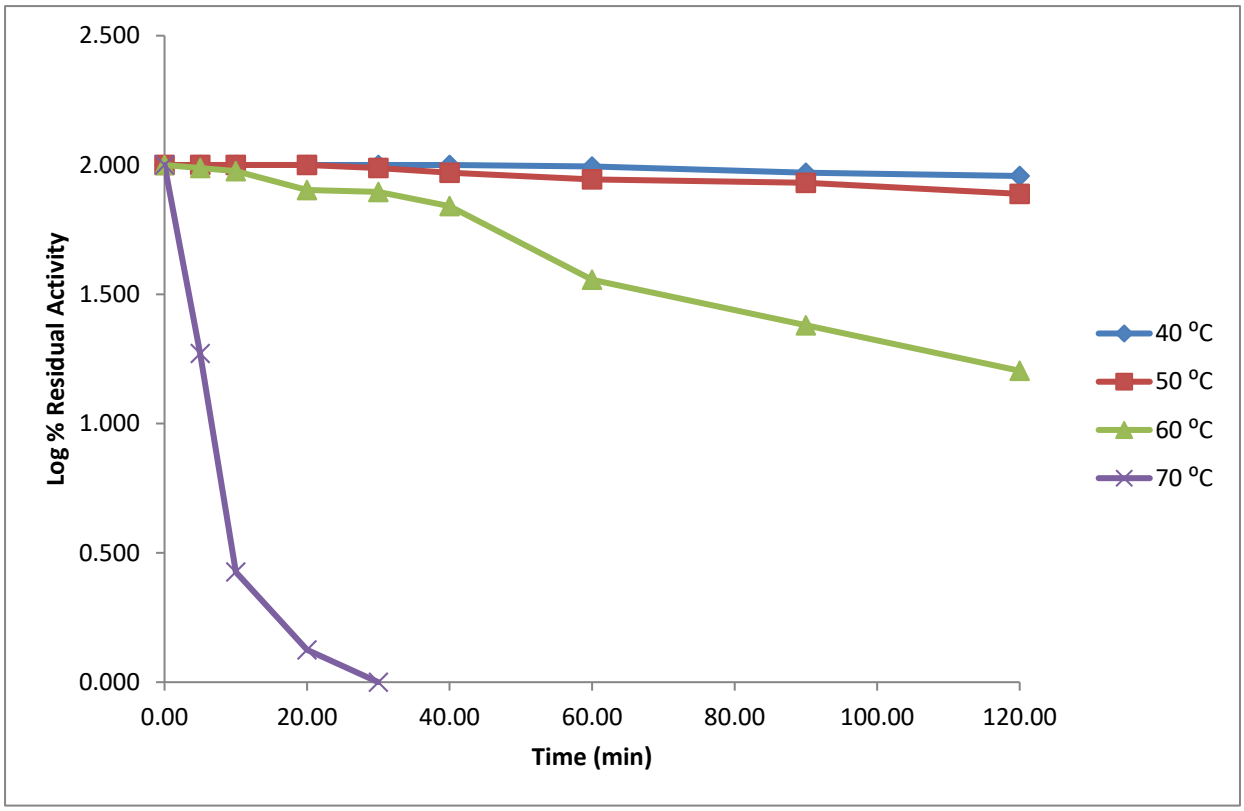

Figure 7: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 6.0 (0.1 M sodium phosphate buffer) and temperature range of 40 to 70 °C.

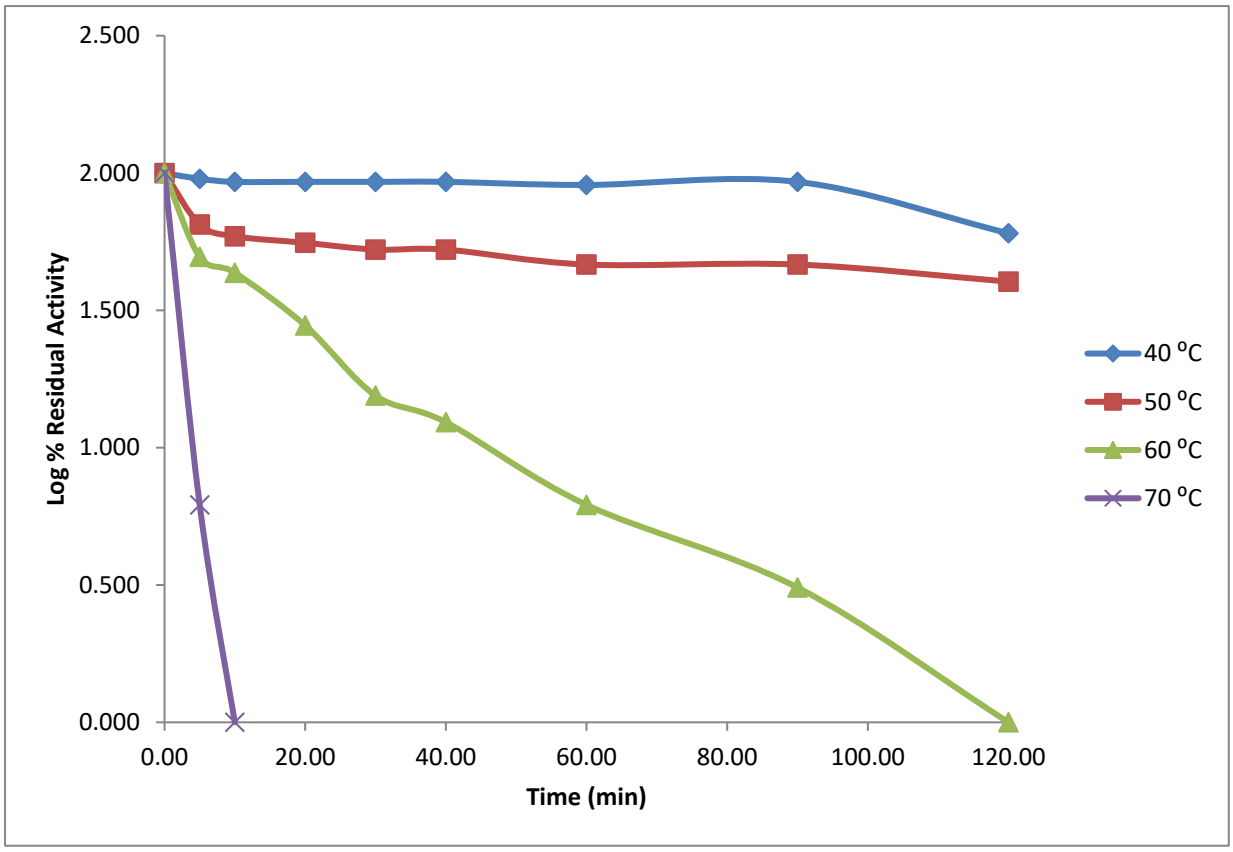

Figure 8: Thermal inactivation of *Trametes polyzona* WRF03 laccase at pH 6.5 (0.1 M sodium phosphate buffer) and temperature range of 40 to 70 °C.

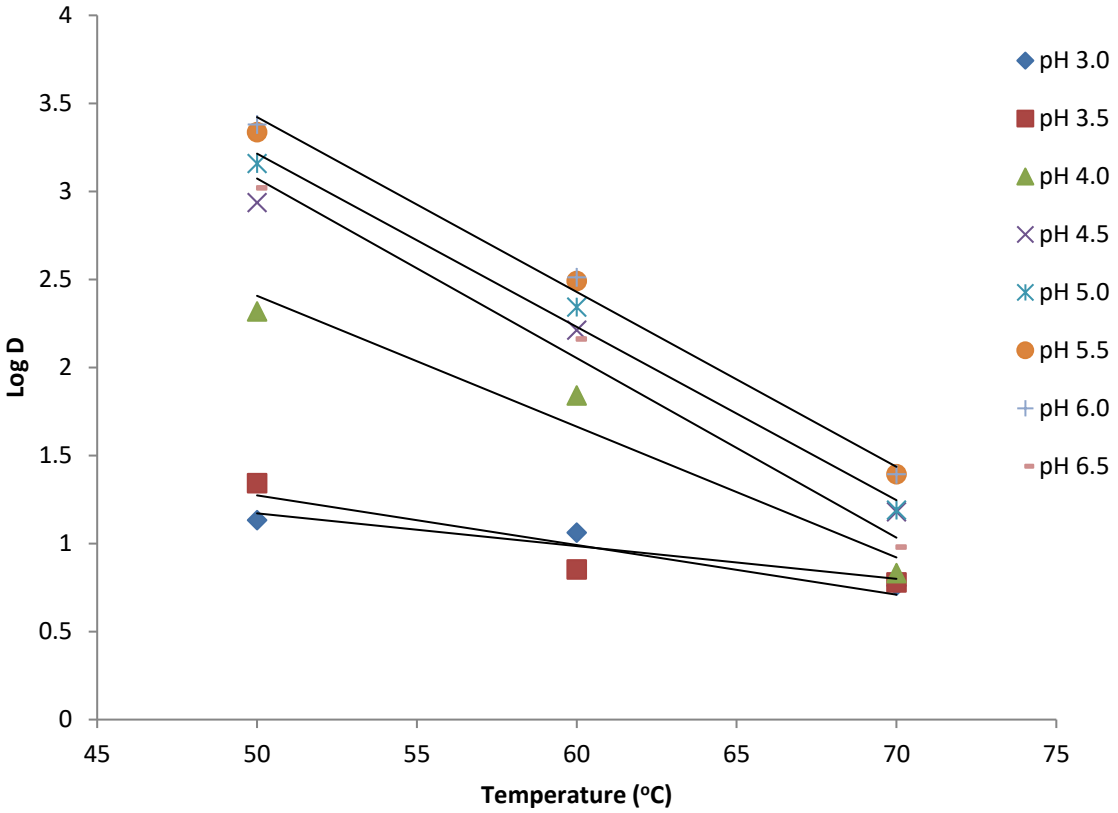

Figure 9: Temperature dependence of the decimal reduction of *Trametes polyzona* WRF03 laccase to calculate z-values

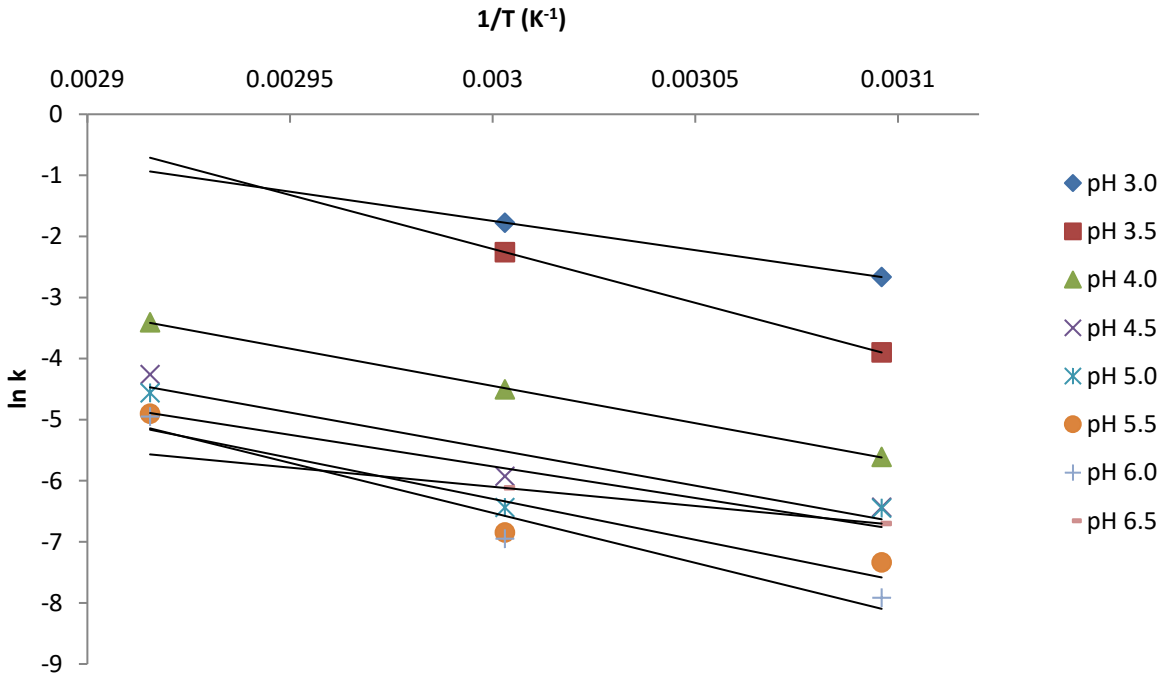

Figure 10. Arrhenius plot for the thermal inactivation of *Trametes polyzona* WRF03 laccase.

Table 1: Kinetic parameters for thermal inactivation of *Trametes polyzona* WRF03 laccase

pH	Temp (°C)	k_{inact} (min-1)	D value (min)	$t_{1/2}$ (min)	*E*a (kJ/mol)	z-value (°C)
3	**40**	0.070	33.10	9.96	80.90	42.61
	50	0.170	13.59	4.09		
	60	0.200	11.54	3.47		
	70	0.399	5.77	1.74		
3.5	40	0.020	113.72	34.23	95.10	23.14
	50	0.105	22.02	6.63		
	60	0.323	7.13	2.15		
	70	0.383	6.01	1.81		
4	40	0.004	628.55	189.18	136.65	15.68
	50	0.011	208.23	62.67		
	60	0.033	69.35	20.87		
	70	0.339	6.80	2.05		
4.5	40	0.002	1432.21	431.06	141.72	15.03
	50	0.003	863.84	260.00		
	60	0.014	162.99	49.06		
	70	0.152	15.13	4.55		
5	40	0.002	1453.00	437.32	142.83	14.85
	50	0.002	1440.28	433.49		
	60	0.010	220.17	66.27		
	70	0.149	15.49	4.66		
5.5	40	0.001	3533.83	1063.60	155.61	13.68
	50	0.001	2168.55	652.68		
	60	0.007	309.67	93.20		
	70	0.093	24.74	7.45		
6	40	0.000	6307.86	1898.51	175.49	12.37
	50	0.001	2401.71	722.86		
	60	0.007	324.96	97.81		
	70	0.093	24.78	7.46		
6.5	40	0.001	1873.88	563.99	164.84	12.92
	50	0.002	1045.39	314.64		
	60	0.016	144.93	43.62		
	70	0.242	9.53	2.87		

Table 2: Thermodynamic parameters for thermal inactivation of *Trametes polyzona* WRF03 laccase

Temperature (°C)	pH	ΔH^o kJ mol^{-1}	ΔG^o kJ mol^{-1}	ΔS^o kJ mol^{-1}
40	3.0	78.29	50.31	102.51
	3.5	92.49	53.52	142.76
	4.0	134.05	57.97	278.68
	4.5	139.12	60.11	289.40
	5.0	140.23	60.15	293.35
	5.5	153.01	62.46	331.68
	6.0	172.89	63.97	398.97
	6.5	162.24	60.81	371.53
50	3.0	78.21	49.61	104.77
	3.5	92.41	50.90	152.04
	4.0	133.96	56.94	282.14
	4.5	139.04	60.76	286.73
	5.0	140.15	62.13	285.78
	5.5	152.93	63.23	328.56
	6.0	172.81	63.23	401.38
	6.5	162.16	61.27	369.54
60	3.0	78.13	50.78	100.19
	3.5	92.33	49.44	157.09
	4.0	133.88	55.74	286.22
	4.5	138.95	58.11	296.14
	5.0	140.07	58.94	297.17
	5.5	152.84	59.88	340.52
	6.0	172.72	60.02	412.84
	6.5	162.07	57.78	382.02
70	3.0	78.04	50.41	101.23
	3.5	92.24	50.52	152.82
	4.0	133.80	50.88	303.74
	4.5	138.87	53.16	313.96
	5.0	139.98	53.22	317.80
	5.5	152.76	54.56	359.71
	6.0	172.64	54.57	432.51
	6.5	161.99	51.84	403.48