Article

The Energies of Activation and Deactivation of 2,4-Dichlorophenol Degradation by Horseradish Peroxidase Immobilized on the Modified Nanofibrous Membrane

Justyna Miłek [ID]

Department of Chemical and Biochemical Engineering, Faculty of Chemical Technology and Engineering, Bydgoszcz University of Science and Technology, Seminaryjna 3, 85-324 Bydgoszcz, Poland; jmilek@pbs.edu.pl

Featured Application: The obtained results can be used in the design and modeling of industrial degradation of 2,4-dichlorophenol when using an enzyme membrane with horseradish peroxidase.

Abstract: Chlorophenol compounds pose a health risk to many organisms due to their toxicity. The present paper presents the estimation of the activation and deactivation energies and the optimum temperatures of 2,4-dichlorophenol degradation by horseradish peroxidase (HRP). The activities of horseradish peroxidase depending on temperature were analyzed. In a mathematical model, describing 2,4-dichlorophenol degradation by HRP was assumed that both the 2,4-dichlorophenol degradation and the deactivation of HRP were first-order reactions by the enzyme concentration. The parameters of the optimum temperatures T_{opt}, the activation energies E_r, and the deactivation energies E_d in the process of 2,4-dichlorophenol degradation by HRP immobilized on a modified nanofibrous membrane were determined k_d and $t_{1/2}$ were determined for HRP immobilized at temperatures in the range of 25 °C to 75 °C. Likewise, thermodynamic parameters such as the change in the enthalpy $\Delta H^{\#}$, change in entropy $\Delta S^{\#}$, the change in Gibbs free energy $\Delta G^{\#}$ for native HPR and the change in the enthalpy $\Delta H_d^{\#}$, change in entropy $\Delta S_d^{\#}$, and the change in Gibbs free energy $\Delta G_d^{\#}$ for deactivated HRP were determined at 25 °C.

Keywords: horseradish peroxidase; 2,4-dichlorophenol; nanofibrous membrane; deactivation energy; thermodynamic parameters
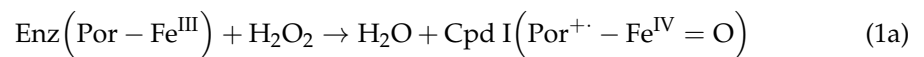
1. Introduction

Chlorophenols permanently pollute aquatic ecosystems mainly due to their harmful effects on organisms even at much lower concentrations [1,2]. Chlorophenols were widely used in the production of insecticides, herbicides, plasticizers, and woodworking agents, as well as medical and domestic activities [3]. 2,4-Dichlorophenol (2,4–DCP) is a priority pollutant specified by the European Union and the US Environmental Protection Agency [4]. The herbicides 2,4-dichlorophenoxyacetic acids and 2,4,5-trichlorophenoxyacetic acid often used on food crops can generate 2,4-dichlorophenol and 2,4,5-tricholorphenol [4]. Additionally, 2,4-DCP is one of the significant chlorinated phenols formed as a by-product of water chlorination [5]. It has been documented that people who consume water containing 2,4-DCP for a long time may suffer from headaches, fever, dryness, anemia, and even death [6].

It was noticed that immobilized bacterial strains remove much higher concentrations of phenol than free microorganisms. Hence, phenol is a biodegradable compound. Therefore, phenol was removed in an attempt to use microorganisms and enzymes both native and immobilized [7–10]. Biomembrane processes are the most commonly used organic pollutant purification processes in water.

Horseradish peroxidase (HRP) can catalyze oxidation phenol and also anilines, bisphenols, benzidines, and related heteroaromatic unions. It is necessary to mention that HRP can be used for waste treatment because it maintains its activity in a wide range of temperature

and pH [11]. The peroxidase catalytic reaction occurs in three stages. In the first step, oxidation with peroxidase takes place to form an unstable intermediate compound Cpd I (1a). Next, in the second stage, an appropriate electron donor reduces Cpd I to Cpd II, and also the free radical was produced (1b). In the final stage, the electron donor substrate reacts with Cpd II, restoring the enzyme to its original form, as well as to the free radical (1c) [12].

$$\text{Enz}\left(\text{Por} - \text{Fe}^{\text{III}}\right) + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{Cpd I}\left(\text{Por}^{+\cdot} - \text{Fe}^{\text{IV}} = \text{O}\right) \tag{1a}$$

$$\text{Cpd I}\left(\text{Por}^{+\cdot} - \text{Fe}^{\text{IV}} = \text{O}\right) + \text{AH}_2 \rightarrow \text{AH}^{\cdot} + \text{Cpd II}\left(\text{Por}^{+} - \text{Fe}^{\text{IV}}\text{OH}\right) \tag{1b}$$

$$\text{Cpd II}\left(\text{Por}^{+} - \text{Fe}^{\text{IV}}\text{OH}\right) + \text{AH}_2 \rightarrow \text{AH}^{\cdot} + \text{Enz}\left(\text{Por} - \text{Fe}^{\text{III}}\right) \tag{1c}$$

When horseradish peroxidase on the membrane was used to remove the chlorophenols, it is necessary to determine the optimum temperature T_{opt}, the activation energy E_{r}, and the deactivation energy E_{d} for horseradish peroxidase.

The study determined parameters such as the optimum temperatures T_{opt}, the activation energies E_{r}, and the deactivation energies E_{d} in the process of 2,4-dichlorophenol degradation by horseradish peroxidase immobilized on a modified nanofibrous membrane. The obtained values can be used in the industrial design process and mathematical modeling of 2,4-dichlorophenol degradation by horseradish peroxidase in an enzymatic membrane reactor [13].

2. Materials and Methods

2.1. Preparation of PAN/PVdF Nanofibrous Membranes by Electrospinning

The membrane prepared by using 1.5 g of 10% polyvinylidene fluoride (PVdF) solution in *N*,*N*-dimethylformamide was added into 4.5 g of 8% polyacrylonitrile solution (PAN) and stirred for 2 h at 60 °C, as prepared by Wei et al. [13]. After this process, a spinning solution was obtained. The electrospinning condition was a high voltage of 16 kV and a flow rate of 1.5 cm³/h.

2.2. Immobilization of Horseradish Peroxidase on the PAN/PVdF Nanofibrous Membranes

The membrane prepared with the immobilization of horseradish peroxidase [13] by using 10 mg PAN/PVdF nanofibrous membrane was immersed into the mixture solution of 10 cm³, 0.8 M NaOH, and 2 cm³ ethanol for 2 h to convert part of the cyano groups into carboxylate. Next, the membrane was taken out and washed with anhydrous tetrahydrofuran. The reaction of carboxyl groups with 1,1′-carbonyldiimidazole was controlled under non-aqueous conditions by 12 h at 25 °C. Then, the activated nanofibers were washed three times. The activated membranes were immersed in a horseradish peroxidase solution in citrate phosphate buffer at pH 6.0, at 25 °C for 12 h. After enzyme immobilization, the membranes were removed from the enzyme solution and rinsed with solution citrate phosphate buffer. Under the optimum immobilization conditions (after 8 h at pH 8.0 and 25 °C), the maximum enzyme loading of PAN/PVdF nanofibrous membranes was 440 mg/g.

The horseradish peroxidase activity is measured by the change in 2,2′-azinobis-(3-ethylbenzthizaoline-6-sulphonate) (ABTS) at 420 nm spectrophotometrically [13,14]. The assay activities of the immobilized horseradish peroxidase and free horseradish peroxidase were measured under optimal conditions (pH, temperature). Also, 2,4-DCP removal kinetics by both types of enzyme were measured under optimal conditions [13]. The measurement conditions were to reflect the enzymatic productivity as a factor as accurately as possible. Productivity is a factor that depends on, among others, enzyme activity, half-time, and the type of using substrate [15].

The parameters such as optimal temperatures T_{opt}, activation E_{r}, and deactivation E_{d} energies of 2,4-dichlorophenol degradation by horseradish peroxidase on the modified nanofibrous membrane were determined based on the activity change curves depending on temperature [10].

2.3. The Horseradish Peroxidase Activity Depending on Temperature

In more cases, the values of activation energy E_r and E_d are determined from the transformed Arrhenius equation. Based on this equation, curves are prepared depending on the logarithm of the rate constant (ln k) or deactivation rate constant (ln k_d) on the reciprocal of temperature (1/T) [12,16]. However, earlier work showed that the determined values of E_r and E_d from the Arrhenius relationship are subject to error [17].

Determining the kinetics parameters of 2,4-dichlorophenol degradation by horseradish peroxidase on the modified nanofibrous membrane is possible based on the built model.

The kinetics of the decomposition of phenol and its derivative compounds can be described using the Michaelis–Menten kinetic model [18]. According to Xu et al. [19], first-order kinetics can also be used to describe the change in paracetamol concentration as the substrate, which is removed by immobilized horseradish peroxidase on nanofibrous membranes. If in the Michaelis–Menten kinetic model, the Michaelis–Menten constant K_M is much smaller than the substrate concentration ($K_M << C_S$), which can be assumed in this case [19], then the change in substrate concentrations C_S in time t with the enzyme deactivation process is presented by the following equation:

$$\frac{dC_S}{dt} = -kC_E, \tag{2a}$$

Likewise, it was assumed that the enzyme activity a [13,20,21] was described by first-order kinetics as shown in Equation (2b):

$$\frac{da}{dt} = -k_d a, \tag{2b}$$

where k and k_d are kinetic constants (1/min) for enzymatic and deactivation processes, respectively, and C_E is the concentration of active enzyme (M).

The kinetic constant k is dependent on temperature T as described in the Arrhenius equations in general form:

$$k = k_0 \exp\left(-\frac{E_r}{RT}\right), \tag{3}$$

where k_0 is a pre-exponential factor of the kinetic constant (1/min), E_r is activation energy (kJ/mol), and R is the gas constant 8.315 J/(mol·K).

The deactivation kinetic constant of horseradish peroxidase k_d are dependent on temperature T as described by the Arrhenius equations:

$$k_d = k_{d0} \exp\left(-\frac{E_d}{RT}\right), \tag{4}$$

where k_{d0} is a pre-exponential factor of the kinetic constant (1/min) for the deactivation process and E_d is activation energy (kJ/mol) for the deactivation process.

The analysis of Equations (2a) and (2b) that describe the concentration changes and the changes in the enzyme activity over time, respectively, was presented in the previous work [21]. Finally, the equation that describes the effect of temperature on the dimensionless activity a of the enzymes is presented in Equation (5), which is described as follows:

$$a(T) = \frac{\left\{1 - \exp\left[-\theta \exp\left(\frac{E_d(T-T_{opt})}{RTT_{opt}}\right)\right]\right\} \exp\left(\frac{(T_{opt}-T)}{RTT_{opt}} \cdot \frac{E_d\theta}{(\exp\theta-1)}\right)}{1 - \exp(-\theta)}, \tag{5}$$

where T_{opt} is the optimum temperature for horseradish peroxidase (K). The parameter θ is dimensionless, described by the equation:

$$\theta = k_{d0} \exp\left(-\frac{E_d}{RT_{opt}}\right) t = k_d(T_{opt})t, \tag{6}$$

where t is the measurement time of horseradish peroxidase activity, which is immobilized on the modified nanofibrous membrane (min).

When the values of the dimensionless parameter θ and the deactivation process energy E_d were estimated, it is possible to calculate the value of the activation energy E_r relationship.

$$E_r = E_d - \frac{E_d \cdot \theta}{\exp \theta - 1}. \tag{7}$$

Based on Equation (5), the parameters T_{opt}, θ, and E_d were estimated by the Levenberg–Marquardt procedure [17,22–26] to calculate the minimum sum of squared errors (*SSE*).

The optimum temperatures and activation energies were determined for other bioprocesses by using enzymes. Processes such as inter alia starch hydrolysis by α-amylase from the porcine pancreas [25], inulin hydrolysis by recombinant exo-inulinases [26], and olive oil hydrolysis by porcine pancreas lipase [17] were analyzed.

2.4. The Thermodynamic Parameters of Active and Deactivated Horseradish Peroxidase

The thermodynamic parameters such as the change in enthalpy $\Delta H^{\#}$ and the change in entropy $\Delta S^{\#}$ provide some information about the enzyme's thermostability [27]. The thermodynamic parameters were calculated from equations [28]:

$$\Delta H^{\#} = E_r - RT \tag{8}$$

$$\Delta G^{\#} = -RT \frac{kh}{k_B T} \tag{9}$$

$$\Delta S^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \tag{10}$$

where $\Delta H^{\#}$ is the change in enthalpy (kJ/mol), E_r is activation energy (kJ/mol), R is the gas constant (8.314×10^{-3} kJ/(mol K)), T is the temperature (K), $\Delta G^{\#}$ the change in Gibbs free energy (kJ/mol), k is the rate constant (1/h), k_B is the Boltzmann constant (1.3806×10^{-26} kJ/K), h is the Planck's constant (2.3854×10^{-33} kJ h), $\Delta S^{\#}$ is the change in entropy (kJ/(mol K)), and $\Delta G^{\#}$ the change in Gibbs free energy (kJ/mol).

When the deactivation process energy E_d was used in Equation (8), the thermodynamic parameters of the deactivation process can be calculated from Equations (8)–(10) and described by symbols $\Delta H_d^{\#}$, $\Delta S_d^{\#}$, and $\Delta G_d^{\#}$ for the deactivation of horseradish peroxidase of 2,4-dichlorophenol degradation, as depicted in Equations (11)–(13):

$$\Delta H_d^{\#} = E_d - RT \tag{11}$$

$$\Delta G_d^{\#} = -RT \frac{k_d h}{k_B T} \tag{12}$$

$$\Delta S^{\#} = \frac{\Delta H_d^{\#} - \Delta G_d^{\#}}{T} \tag{13}$$

where the symbols were explained as mentioned above.

2.5. Modeling of 2,4-Dichlorophenol Degradation by Horseradish Peroxidase

The mathematical models represented by Equations (2a) and (2b) were analyzed. The first of them presented the change in 2,4-dichlorophenol degradation by horseradish peroxidase and the next one was the change in activity of the enzyme over time. It has been assumed that the enzyme concentration is equaled $C_E = aC_{E0}$ and knowledge of dimensionless activity of horseradish peroxidase a described by Equation (2b), allowed for the transformation of Equation (2a) to the following form:

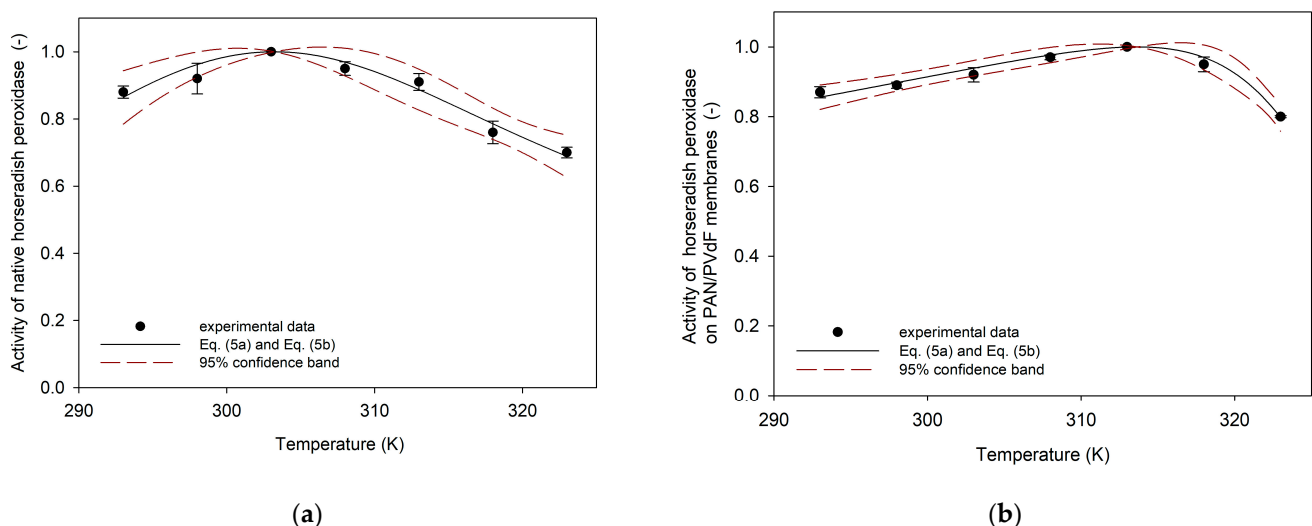$$\frac{dC_S}{dt} = -kC_{E0} exp(-k_d t). \tag{14}$$

The important point to note is that, in the current study, the modeling of 2,4-dichlorophenol degradation was investigated at 298 K. It is related to the potential use of horseradish peroxidase in the environmental process of removing phenol from water at ambient temperature.

3. Results

Literature data [13] for horseradish peroxidase immobilized on the modified nanofibrous membrane were analyzed. Horseradish peroxidase activity was monitored by the absorbance change in ABTS (2,2′-azinobis-(3-ethylbenzthizaoline-6-sulphonate)) at 420 nm. The assay reaction contained 20 cm^3 1 mM ABTS in 0.1 M phosphate buffer, 0.8 mM H_2O_2, and 1 mg free or immobilized enzyme. The activity of horseradish peroxidase native and immobilized on the modified nanofibrous membrane at a specified temperature was determined and the reaction time was at pH 4.0 [19]. One unit of horseradish peroxides activity was the amount of HPR that oxidized 1 μmol of substrate per minute.

3.1. Parameters T_{opt}, E_d, E_r

Equation (5) was used to analyze experimental data regarding changes in the activity of horseradish peroxidase under the influence of temperature allowing for the determination of parameters that occur in Equation (5), such as the optimal temperature T_{opt}, deactivation energy E_d, and the parameter θ. Figure 1 shows experimental data on horseradish peroxidase activity as a function of temperature and activity curves plotted based on Equation (5).

Figure 1. The activity of horseradish peroxidase: (**a**) native and (**b**) immobilized on the modified nanofibrous membrane.

The values of the parameters T_{opt}, E_d, and θ for horseradish peroxidase were determined using the non-linear methods using SigmaPlot 15.0 software, and the values of these parameters are presented in Table 1. Also, they are calculated based on Equation (7), and the E_r values are depicted in Table 1.

Table 1. The value of parameters estimated for horseradish peroxidase immobilized on the modified nanofibrous membrane.

Horseradish Peroxidase	T_{opt} (K)	θ	E_d (kJ/mol)	E_r (kJ/mol)
Native	303.21 ± 1.07	1.77 ± 0.52	63.35 ± 5.45	40.33 ± 10.72
Nanofibrous membrane	313.64 ± 1.15	0.06 ± 0.03	215.00 ± 55.04	6.39 ± 4.00

The statistical data obtained during the determination of the parameters of horseradish peroxidase are given in Table 2.

Table 2. The statistical data of obtained parameters T_{opt}, E_d, and θ values of horseradish peroxidase.

Horseradish Peroxidase	SSE	R^2	p			F	P
			E_d (kJ/mol)	T_{opt} (K)	θ (−)		
Native	0.0348	0.9538	0.0003	<0.0001	0.0276	41.27	0.0021
Nanofibrous membrane	0.0177	0.9642	0.0174	<0.0001	0.0776	53.92	0.0013

SSE—the sum of squared errors; R^2—regression coefficients; *F*—Fisher test value; *p*-value—probability values for each parameter separately; *p*-value—probability value for all value: parameters E_d, T_{opt}, and θ.

The value of the regression coefficient R^2 was high, above 0.95. Simultaneously, the *SSE* sum of squared error values below 0.035 is satisfactory. Additionally, the *F*-Fisher test values were above 41.00, and the probability for the T_{opt} and E_d parameters was below 0.0175. The statistical data of obtained parameters T_{opt}, E_d, and θ of horseradish peroxidase were satisfactory when the commonly used probability value is much lower and equal to $p < 0.05$ [26,29]. Additionally, Figure 1 represents standard deviation errors for experimental points, while the 95% confidence limits were marked for the obtained curves. Therefore, the statistical data confirmed that the application of Equation (5) when determining parameters is justified.

3.2. The Deactivation Constant k_d and Half-Time $t_{1/2}$

The transformation of Equation (6) allowed us to calculate the values of the deactivation constant at optimum temperature $k_d(T_{opt})$ when the parameter θ and the reaction time of horseradish peroxidase assay t were known. Next, from Equation (4), knowing the optimum temperature T_{opt}, the values of a pre-exponential factor of the kinetic constant for the deactivation process k_{d0} were determined. Then, based on calculated k_{d0} and E_d values (from Table 2), the values of deactivation constant k_d at temperatures in the range of 25 °C to 75 °C for horseradish peroxidase immobilized on the modified nanofibrous membrane were analyzed. For the calculations, the time of activity measurement was assumed to be 10 min. The half-time $t_{1/2}$ was calculated as follows:

$$t_{1/2} = \frac{ln2}{k_d} \tag{15}$$
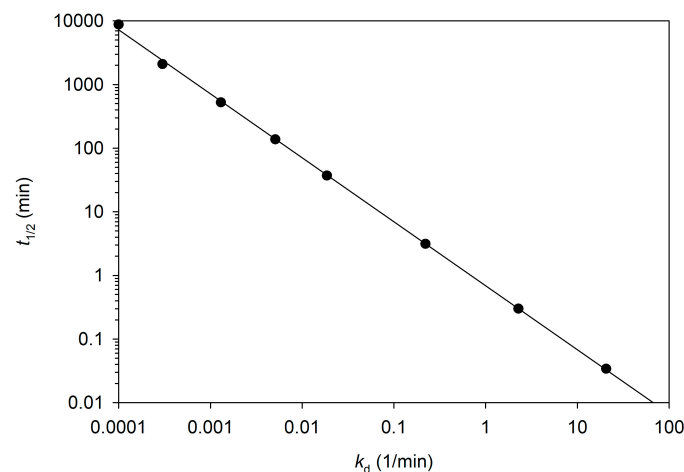
The values k_d and $t_{1/2}$ are given in Table 3.

Table 3. The values k_d for horseradish peroxidase immobilized on a nanofibrous membrane.

Temperature (°C)	25	30	35	40	45	55	65	75
k_d (1/min)	0.0001	0.0003	0.0013	0.0051	0.0186	0.2216	2.28	20.6
$t_{1/2}$ (min)	8745	2089	523	137	37	3.13	0.30	0.034

The data presented in Table 3 were used to create a double-logarithmic plot for the half-time $t_{1/2}$ as a function of the deactivation constant k_d (Figure 2).

The obtained kinetic constants of the process deactivation k_d values were used in calculating the thermodynamic parameters for the deactivation of horseradish peroxidase on the nanofibrous membrane.

Figure 2. The double-logarithmic plot for the half-time $t_{1/2}$ as a function of the deactivation constant k_d.

3.3. The Thermodynamic Parameters of Active and Deactivated Horseradish Peroxidase

The values of thermodynamic parameters (enthalpy $\Delta H^\#$ and entropy of activation $\Delta S^\#$ and $\Delta G^\#$ the changes in Gibbs free energy) were calculated for the degradation process of 2,4-dichlorophenol by horseradish peroxidase at a temperature of 25 °C. The values k are equal for native horseradish peroxidase 7.32 (1/h) and horseradish peroxidase on modified nanofibrous membrane 6.72 (1/h), and these values were determined by Wei et al. [13].

To determine the thermodynamic parameters of horseradish peroxidase deactivation (enthalpy $\Delta H_d^\#$ and entropy of activation $\Delta S_d^\#$ and $\Delta G_d^\#$ the change in Gibbs free energy) in the degradation process of 2,4-dichlorophenol, it is necessary to know the value of horseradish peroxidase constant rate deactivation k_d. The values k_d (1/min) for horseradish peroxidase immobilized on a nanofibrous membrane at a temperature of 25 °C were given in Table 3. The value k_d for native horseradish peroxidase was calculated. It was determined in the same way. To determine the thermodynamic parameters of horseradish peroxidase deactivation, the values of k_d were used, which were 6.86 (1/h) for native and 0.048 (1/h) for horseradish peroxidase on the modified nanofibrous membrane. The obtained values of thermodynamic parameters for the degradation process of 2,4-dichlorophenol by horseradish peroxidase and also horseradish peroxidase deactivation at 25 °C are given in Table 4.

Table 4. The values of thermodynamic parameters of horseradish peroxidase at 25 °C.

E_r		$\Delta H^\#$	$\Delta G^\#$	$\Delta S^\#$
	(kJ/mol)			
Native	40.33	37.85	47.77	−0.03
Nanofibrous membrane	63.35	60.87	47.98	0.04
E_d		$\Delta H_d^\#$	$\Delta G_d^\#$	$\Delta S_d^\#$
	(kJ/mol)			
Native	6.39	3.91	47.93	−0.15
Nanofibrous membrane	215.00	212.52	64.11	0.55

The obtained values of thermodynamic parameters provide information on the thermostability of native and immobilized horseradish peroxidase.

3.4. Modeling of 2,4-Dichlorophenol Degradation by Horseradish Peroxidase

The obtained values of E_d and k_d for 2,4-dichlorophenol degradation with the deactivation of horseradish peroxidase were used for modeling first-order degradation of 2,4-dichlorophenol at an initial concentration of 20 mg/dm^3 at a temperature of 298 K.

4. Discussion

4.1. The Values of the Optimum Temperature T_{opt}

The determined values of the optimum temperatures T_{opt} for 2,4-dichlorophenol degradation by native horseradish peroxidase and on the nanofibrous membrane were 303.21 ± 1.07 K and 313.64 ± 1.15 K, respectively (Table 1). The HRP on the nanofibrous membrane exhibited a higher T_{opt} about 10 °C higher in comparison to native horseradish peroxidase.

4.2. The Values of the Activation Energy E_r and the Deactivation Energy E_d

The determined values of the activation energies E_r of 2,4-dichlorophenol degradation by horseradish peroxidase on the nanofibrous membrane were 40.33 ± 10.72 kJ/mol and 6.39 ± 4.00 kJ/mol, respectively. It should be noted that the lowest value was obtained for the horseradish peroxidase on the nanofibrous membrane. The lower activation energy corresponds to higher reaction rates. The value of the activation energy E_r determined from the Arrhenius equation was 14.63 kJ/mol [30] and 17.7 kJ/mol [16]. Hewson and Dunford [30] obtained the value of E_r for the reaction of horseradish peroxidase with hydrogen peroxide, as shown by Equation (1a). On the other hand, Ostojic et al. [16] obtained the value of E_r for horseradish peroxidase, the activity of which was determined using guaiacol as a substrate.

The obtained values of the deactivation energies E_d for native horseradish peroxidase and on the nanofibrous membrane were 63.35 ± 5.45 kJ/mol and 215.00 ± 55.04 kJ/mol, respectively (Table 1). The immobilized horseradish peroxidase on the nanofibrous membrane has a high activation energy of deactivation E_d due to thermal stability. The E_d values were not found in the literature for horseradish peroxidase immobilized on the membrane. The lower E_r and higher E_d values for the enzyme on the membrane compared to the E_r and E_d values for the native enzyme may indicate an effective immobilization process on the membrane. The activation energy of deactivation E_d of horseradish peroxidase determined from the half-time $t_{1/2}$ value at 50 °C was 64.10 kJ/mol [31]. None of E_r and E_d values was found in the literature for horseradish peroxidase immobilized on the membrane.

Therefore, the estimated E_r and E_d parameters can be used in the design and modeling of the biotransformation process when using an enzyme membrane with horseradish peroxidase. In the literature, fixed bed flow bioreactors were analyzed [32], in which the value of deactivation energy E_d obtained in a classical reactor study [33] was used.

4.3. The Values of the Deactivation Constant k_d

The determined values of the deactivation contents k_d for horseradish peroxidase on the nanofibrous membrane (at temperature 25 °C–75 °C) were in the range from 0.0001 1/min to 20.6 1/min. The half-time $t_{1/2}$ values for horseradish peroxidase on this nanofibrous membrane were in the range from 8745 min to 0.034 min. With an increase in temperature from 25 °C to 35 °C, the half-time $t_{1/2}$ decreases about 15-fold, with subsequent temperature increases about 10 °C.

Comparing the removal of paracetamol by native horseradish peroxidase (60 min at 25 °C with pH 4.0 and 0.8 mM H_2O_2 initially) and the poly(vinyl alcohol)/poly(acrylic acid)/SiO_2 electrospinning nanofibrous membrane (60 min at 25 °C with pH 6.0 and 0.8 mM H_2O_2 initially) characterized by $t_{1/2}$ values of 27.7 (min) and 385.1 (min), respectively [19]. So, the half-time $t_{1/2}$ value for horseradish peroxidase on this nanofibrous membrane was almost 14-fold higher than native horseradish peroxidase.

4.4. The Values of Thermodynamic Parameters of Active and Deactivated Horseradish Peroxidase

To obtain further insights into the stability of horseradish peroxidase immobilized on the membrane, their thermodynamic activation parameters were determined at 25 °C. Relative to native HRP, the immobilized HRP on the PAN/PVdF nanofibrous membrane variant was stabilized enthalpically. A higher $\Delta H^{\#}$ when the process of horseradish peroxidase deactivation occurs implies that more bonds need to be broken to reach the partially unfolded transition state [34]. The enthalpy values $\Delta H^{\#}$ and the entropy $\Delta S^{\#}$ are lower for HRP
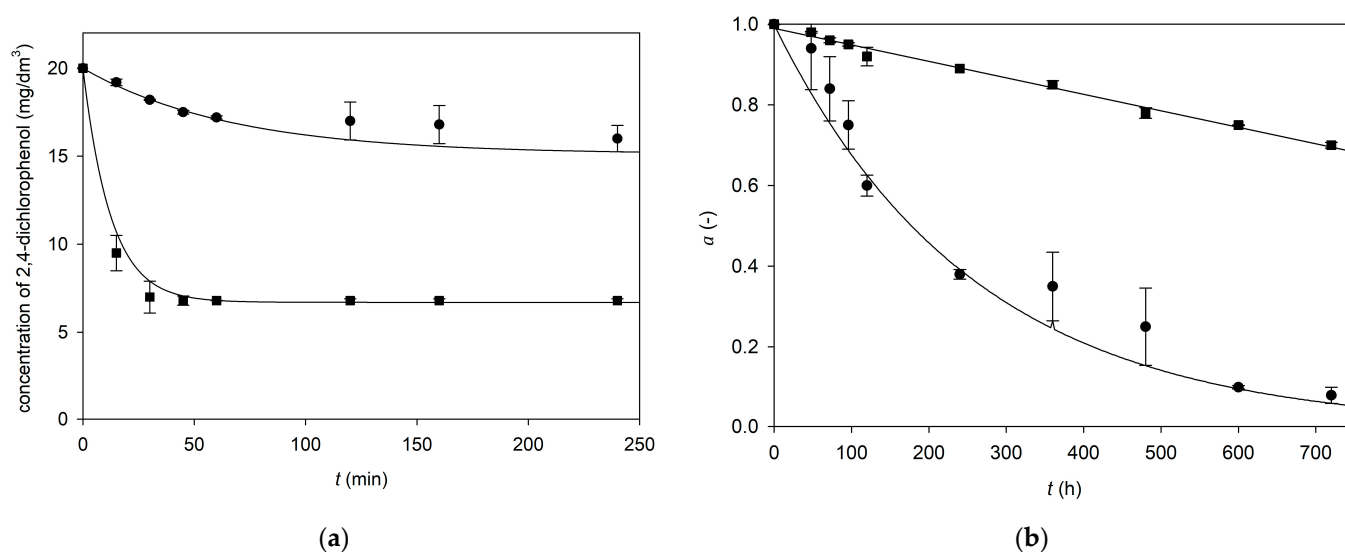
nanofibrous membrane than the values $\Delta H^{\#}$ for native horseradish peroxidase (Table 4). Low enthalpy values $\Delta H^{\#}$ indicate the effectiveness of the transition state. The changes in entropy $\Delta S^{\#}$ indicate the stability of the transition state and the affinity of the substrate for the enzyme. The entropy $\Delta S^{\#}$ for the enzymatic reaction decreases with increasing enzyme stability. The values of $\Delta G^{\#}$ for the degradation process of 2,4-dichlorophenol by HRP were approximately 47.77 kJ/mol and 47.98 kJ/mol for native horseradish peroxidase and horseradish peroxidase on the nanofibrous membrane, respectively. When the process of horseradish peroxidase deactivation occurred, the higher values of $\Delta G_{d}^{\#}$ were observed for horseradish peroxidase on the nanofibrous membrane (64.11 kJ/mol) compared to native horseradish peroxidase (47.93 kJ/mol). This confirms that the change in the $\Delta G_{d}^{\#}$ is the energy barrier for the enzyme deactivation. The higher the $\Delta G_{d}^{\#}$ is, the more stable the enzyme [27,35]. The increase in the stability of horseradish peroxidase immobilized on the membrane relative to native horseradish peroxidase is accompanied by a decrease of $\Delta H^{\#}$ and $\Delta S^{\#}$ values according to an enthalpy–entropy [34]. The thermodynamic parameters were shown and allowed to explain the thermal stabilization of immobilized horseradish peroxidase on the nanofibrous membrane, resulting from a decrease in entropy when the enzyme deactivation process does not occur. When the enzyme deactivation process occurs, the thermal stabilization results from an increase in enthalpy.

It can be concluded that thermokinetic studies are an important issue of "energy evaluation" in the industry during process scale-up. Both the process of horseradish peroxidase deactivation and the degradation process of 2,4-dichlorophenol require knowledge of the activation energies E_{r} and E_{d} values [27]. It is necessary to determine the values of the deactivation constants k_{d} for horseradish peroxidase on the nanofibrous membrane.

4.5. Modeling of 2,4-Dichlorophenol Degradation by Horseradish Peroxidase

Based on the results presented in Figure 3a, it was found that the 2,4-dichlorophenol degradation by horseradish peroxidase was higher when the enzyme was immobilized on the modified nanofibrous membrane. The values of 2,4-dichlorophenol degradation were approximately 17.5% and 66%, respectively. The modeling of the change in horseradish peroxidase activity (Figure 3b) shows that within 30 days (720 h), the enzyme activity decreased by 90% and 30% for native horseradish peroxidase and the enzyme immobilized on the modified nanofibrous membrane, respectively.

Figure 3. The numerical modeling of (**a**) the change in concentration of 2,4-dichlorophenol as the substrate C_{S} and (**b**) the activity of the horseradish peroxidase a compared with experimental data, (●) native and (■) immobilized on the modified nanofibrous membrane.

5. Conclusions

The parameters E_r, E_d, and T_{opt} values of native horseradish peroxidase and the enzyme immobilized on the modified nanofibrous membrane were estimated. The 3.5-fold higher value of deactivation energy E_d was obtained for horseradish peroxidase immobilized on the modified nanofibrous membrane than the E_d value for native horseradish peroxidase. The lower E_r and higher E_d values for horseradish peroxidase on the membrane compared to the native horseradish peroxidase may indicate an effective immobilization process on the membrane.

The presented thermodynamic parameters of active and deactivated horseradish peroxidase are noteworthy. Additionally, based on the obtained parameters, the concentration of 2,4-dichlorophenol degradation and horseradish peroxidase activity were modeled for an initial concentration of 20 mg/dm^3 at a temperature of 298 K.

The obtained values of E_r, E_d, and T_{opt} could be used to design and model the enzymatic process of removing the 2,4-dichlorophenol from water in a membrane bioreactor with immobilized horseradish peroxidase.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All research data are available in the manuscript.

Conflicts of Interest: The author declares no conflicts of interest.

References

1. Antizar-Ladislao, B.; Galil, N.I. Biosorption of phenol and chlorophenols by acclimated residential biomass under bioremediation conditions in a sandy aquifer. *Water Res.* **2004**, *38*, 267–276. [CrossRef]
2. Khenifi, A.; Zohra, B.; Kahina, B.; Houari, H.; Zoubir, D. Removal of 2,4–DCP from wastewater by CTAB/bentonite using one-step and two-step methods: A comparative study. *Chem. Eng. J.* **2009**, *146*, 345–354. [CrossRef]
3. Olaniran, A.O.; Igbinosa, E.O. Chlorophenols and other related derivatives of environmental concern: Properties, distribution and microbial degradation processes. *Chemosphere* **2011**, *83*, 1297–1306. [CrossRef]
4. ATSDR. Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Priority List of Hazardous Substances. 2007. Available online: https://www.atsdr.cdc.gov/spl/previous/07list (accessed on 2 January 2024).
5. Czaplicka, M. Sources and transformations of chlorophenols in the natural environment. *Sci. Total Environ.* **2004**, *322*, 21–39. [CrossRef]
6. EPA. Chemical Advisory and Notice of Potential Risk: Skin Exposure to Molten 2,4-Dichlorophenol Can Cause Rapid Death. 2000. Available online: https://www.epa.gov/archive/epapages/newsroom_archive/newsreleases/f4543d90482f4d8a85256 88d005624d9.html (accessed on 2 January 2024).
7. Aruotu, J.O.; Chikere, C.B.; Okafor, C.P.; Edamkue, I. Microbial consortium for polycyclic aromatic hydrocarbons degradation from petroleum hydrocarbon polluted soils in Rivers State, Nigeria. *Appl. Sci.* **2023**, *13*, 9335. [CrossRef]
8. Liang, Y.; Chen, X.; Zeng, J.; Ye, J.; He, B.; Li, W.; Sun, J. Mesoporous polymeric ionic liquid via confined polymerization for *Laccase* immobilization towards efficient degradation of phenolic pollutants. *Molecules* **2023**, *28*, 2569. [CrossRef]
9. Costa, R.A.; Cunha, A.S.; Peres, J.C.G.; Azzoni, A.R.; Laurenti, E.; Vianna, A.S., Jr. Enzymatic degradation of 2,4,6-trichlorophenol in a microreactor using soybean peroxidase. *Symmetry* **2020**, *12*, 1129. [CrossRef]
10. Liu, R.; Zhang, W.; Wang, S.; Xu, H.; Hu, Y. Magnetic polyethyleneimine nanoparticles fabricated via ionic liquid as bridging agents for *Laccase* immobilization and its application in phenolic pollutants removal. *Molecules* **2022**, *27*, 8522. [CrossRef]
11. Vasileva, N.; Godjevargova, T.; Ivanova, D.; Gabrovska, K. Application of immobilized horseradish peroxidase onto modified acrylonitrile copolymer membrane in removing of phenol from water. *Int. J. Biol. Macromol.* **2009**, *44*, 190–194. [CrossRef]
12. Zeyadi, M.; Almulaiky, Y.Q. A novel peroxidase from *Ziziphus jujuba* fruit: Purification, thermodynamics and biochemical characterization properties. *Sci. Rep.* **2020**, *10*, 8007. [CrossRef]
13. Wei, N.; Xu, R.; Tang, R. Immobilization of horseradish peroxidase on modified electrospun nanofibrous membrane for 2,4-dichlorophenol removal. In Proceedings of the 2nd International Conference on Materials Chemistry and Environmental Protection, Sanya City, China, 23–25 November 2018; pp. 283–292. Available online: https://www.scitepress.org/Papers/2018/8 1889/81889.pdf (accessed on 2 January 2024).
14. Terres, J.; Battisti, R.; Andreaus, J.; de Jesus, P.C. Decolorization and degradation of Indigo Carmine dye from aqueous solution catalyzed by horseradish peroxidase. *Biocatal. Biotransform.* **2014**, *32*, 64–73. [CrossRef]

15. Siddiqui, K.S.; Ertan, H.; Poljak, A.; Bridge, W.J. Evaluating enzymatic productivity—The missing link to enzyme utility. *Int. J. Mol. Sci.* **2022**, *23*, 6908. [CrossRef]

16. Ostojic, J.; Herenda, S.; Galijasevic, S.; Galic, B.; Milos, M. Inhibition of horseradish peroxidase activity by boroxine derivative, dipotassium-trioxohydroxytetrafluoro-triborate $K_2[B_3O_3F_4OH]$. *J. Chem.* **2017**, *2017*, 8134350. [CrossRef]

17. Miłek, J. The activation energies and optimum temperatures of olive oil hydrolysis by lipase porcine pancreas. *Ecol. Chem. Eng. S* **2021**, *28*, 389–398. [CrossRef]

18. Liu, C.; Tan, L.; Zhang, K.; Wang, W.; Ma, L. Immobilization of horseradish peroxidase for phenol degradation. *ACS Omega* **2023**, *8*, 26906–26915. [CrossRef]

19. Xu, R.; Si, Y.; Li, F.; Zhang, B. Enzymatic removal of paracetamol from aqueous phase: Horseradish peroxidase immobilized on nanofibrous membranes. *Environ. Sci. Pollut. Res.* **2015**, *22*, 3838–3846. [CrossRef]

20. Malomo, S.O.; Adeoye, R.I.; Babatunde, L.; Saheed, I.A.; Iniaghe, M.O.; Olorunniji, F.J. Suicide inactivation of horseradish peroxidase by excess hydrogen peroxide: The effects of reaction pH, buffer ion concentration, and redox mediation. *Biokemistri* **2011**, *23*, 124–128. Available online: https://www.ajol.info/index.php/biokem/article/view/77688 (accessed on 2 January 2024).

21. Carvalho, C.L.S.; Gomes, F.M.; Pereira, F.M. Mathematical modeling of estrogen degradation in enzymatic membrane reactor. *Ind. Biotechnol.* **2020**, *16*, 50–55. [CrossRef]

22. Miłek, J. Recombinant endo–inulinases: Determination of the activation, deactivation energies and optimum temperatures in inulin hydrolysis. *J. Therm. Anal. Calorim.* **2023**, *148*, 859–866. [CrossRef]

23. Miłek, J. Determination the optimum temperatures and activation energies of inulin hydrolysis by endo-inulinase *Aspergillus niger*. *Chem. Proc. Eng.* **2020**, *41*, 229–236. [CrossRef]

24. Miłek, J. Thermodynamics and kinetics of thermal deactivation of catalase *Aspergillus niger*. *Pol. J. Chem. Technol.* **2020**, *22*, 67–72. [CrossRef]

25. Miłek, J. Determination of activation energies and the optimum temperatures of starch hydrolysis by α-amylase from porcine pancreas. *Molecules* **2021**, *26*, 4117. [CrossRef]

26. Miłek, J. The inulin hydrolysis by recombinant exo-inulinase: Determination the optimum temperatures and activation energies. *J. Therm. Anal. Calorim.* **2022**, *147*, 8061–8067. [CrossRef]

27. Kikani, B.A.; Singh, S.P. The stability and thermodynamic parameters of a very thermostable and calcium-independent α-amylase from a newly isolated bacterium, *Anoxybacillus beppuensis* TSSC-1. *Process Biochem.* **2012**, *47*, 1791–1798. [CrossRef]

28. Jayawardena, M.B.; Yee, L.H.; Poljak, A.; Cavicchioli, R.; Kjelleberg, S.J.; Siddiqui, K.S. Enhancement of lipase stability and productivity through chemical modification and its application to latex-based polymer emulsions. *Process Biochem.* **2017**, *57*, 131–140. [CrossRef]

29. Onaizi, S.A. Statistical analyses of the effect of rhamnolipid biosurfactant addition on the enzymatic removal of Bisphenol A from wastewater. *Biocatal. Agric. Biotechnol.* **2021**, *32*, 101929. [CrossRef]

30. Hewson, W.D.; Dunford, H.B. Horseradish peroxidase. XVIII. The Arrhenius activation energy for the formation of compound I. *Can. J. Chem.* **1975**, *53*, 1928–1932. [CrossRef]

31. Giannakopoulou, A.; Patila, M.; Spyrou, K.; Chalmpes, N.; Zarafeta, D.; Skretas, G.; Gournis, D.; Stamatis, H. Development of a four-enzyme magnetic nanobiocatalyst for multi-step cascade reactions. *Catalysts* **2019**, *9*, 995. [CrossRef]

32. Grubecki, I. Optimal feed temperature for hydrogen peroxide decomposition process occurring in a bioreactor with fixed-bed of commercial catalase. A case study on thermal deactivation of the enzyme. *Chem. Process Eng.* **2018**, *39*, 491–501. [CrossRef]

33. Miłek, J.; Wójcik, M.; Verschelde, W. Thermal stability for the effective use of commercial catalase. *Pol. J. Chem. Technol.* **2014**, *16*, 75–79. [CrossRef]

34. Siddiqui, K.S.; Poljak, A.; De Francisci, D.; Guerriero, G.; Pilak, O.; Burg, D.; Raftery, M.J.; Parkin, D.M.; Trewhella, J.; Cavicchioli, R. A chemically modified α-amylase with a molten-globule state has entropically driven enhanced thermal stability. *Protein Eng. Des. Sel.* **2010**, *23*, 769–780. [CrossRef] [PubMed]

35. De Borba, T.M.; Machado, T.B.; Brandelli, A.; Kalil, S.J. Thermal stability and catalytic properties of protease from *Bacillus* sp. P45 active in organic solvents and ionic liquid. *Biotechnol. Prog.* **2018**, *34*, 1102–1108. [CrossRef] [PubMed]