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Thermal inactivation kinetics and thermodynamic properties of immobilised *Aspergillus niger* pectinase in rigid polyurethane foam

[1]Bustamante-Vargas, C. E., [2]Junges, A., [3]Venquiaruto, L. D., [2]Oro, C. E. D., [4]Julio- Orozco, D. J., [5]Zabot, G. L., [5*]Tres, M. V., [2]Paroul, N., [2]Backes, G. T. and [2]Dallago, R. M.

[1]*Food Engineering Department, Universidad de Córdoba, PO Box 230002, Berástegui, Córdoba, Colombia*

[2]*Department of Agricultural Sciences, Universidade Regional do Alto Uruguai e das Missões, URI Erechim, PO Box 743, CEP 99709-910, Erechim-RS, Brazil*

[3]*Department of Exact and Earth Sciences, Universidade Regional do Alto Uruguai e das Missões, URI Erechim, PO Box 743, CEP 99709-910, Erechim-RS, Brazil, Erechim-RS, Brazil*

[4]*Department of Engineering, Universidade de Passo Fundo, BR 285, São José CEP 99052-900, Passo Fundo-RS, Brazil*

[5]*Laboratory of Agroindustrial Processes Engineering (LAPE), Federal University of Santa Maria (UFSM), 1040, Sete de Setembro St., Center DC, Cachoeira do Sul - RS, 96508-010, Brazil*

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Abstract

The present work was performed in order to investigate the effect of in situ immobilisation of commercial *Aspergillus niger* pectinase in rigid polyurethane foam (RFPU) on thermal stability. Pectinase (liquid, lyophilised, and immobilised) was incubated from 50 to 80°C. Residual activity data were fit to various kinetic models, which were rigorously evaluated by statistical and physical criteria. The first-order model provided the best fit to describe the thermal deactivation of pectinase. Immobilised pectinase exhibited higher thermal stability, presenting lower K_d-values (0.0015 - 0.0157 h^{-1}) and higher half-life ($t_{1/2}$; 456.89 - 44.01 h), D (1517.86 - 146.22 h) and Z (29.85°C) values. Thermodynamic parameters (ΔG*, ΔH* and ΔS*) corroborate that immobilised pectinase is thermodynamically more stable if compared to its free and non-immobilised forms (liquid and lyophilized), suggesting that their functionally active conformation was more conserved during the heat treatment. The feasibility of immobilisation is presented towards improving the thermal stability and the resistance to irreversible deactivation of pectinase.

Introduction

Pectin is polysaccharide acid with high molecular weight and composed of residues of D-galacturonic acid linked by glycosidic linkages of α-D-(1,4) type. Pectin is located in the primary cell wall and the middle lamella of the plant cell (Kohli and Gupta, 2015; Müller-Maatsch *et al.*, 2016). Pectinases are a versatile group of enzymes that degrade or modify pectin substances by hydrolysis, trans-elimination and de-esterification reactions (Rehman *et al.*, 2016). Recently, the use of pectinases as a catalyst in various biotechnological processes has attracted the attention of different industrial sectors (e.g., food, textile, wastewater treatment) (Fang *et al.*, 2016). However, its large-scale applications are still limited. The action of pectinases dissolved in homogeneous catalysis systems can be easily affected by various agents. One of these agents is temperature which causes the loss of catalytic activity over time and promotes changes in the spatial configuration, thereby causing denaturation (Ladero *et al.*, 2006a).

To overcome such limitations, one of the main objectives of the enzymatic immobilisation is to develop strategies to improve activity, stability and heat resistance of enzymes under reaction conditions. The strategies can protect the enzymes against denaturation, thus enabling the use of immobilised enzymes in biocatalytic processes (Lei *et al.*, 2015). Additionally, it is desirable that some enzymatic

*Corresponding author.
Email: marcus.tres@ufsm.br

reactions are conducted at high temperatures to improve the productivity of the catalytic process by increasing reaction rates and reducing microbial contamination. Consequently, heat resistant enzymes could be of high interest at an industrial scale (Ortega *et al.*, 2004a; Kumar *et al.*, 2006). In our previous work, the thermal stability (40 to 80°C) of immobilised pectinase was evaluated in rigid foam polyurethane (RFPU) (Bustamante-Vargas *et al.*, 2015). In such case, immobilised pectinase was significantly more thermally stable than free pectinase. However, thermal stability studies are still insufficient to elucidate if the catalytic ability of the enzyme during the technological application will be affected. In this sense, the knowledge of kinetics and thermodynamic properties of enzymes is fundamental to understand this progress of deactivation reactions. Thermodynamic data determine the spontaneity of the reaction and indicate changes in entropy and energy of systems under various environmental conditions (Gummadi, 2003). Furthermore, thermal deactivation kinetic studies provide information necessary to comprehend the mechanisms involved in enzymatic deactivation by heat effect and the rate at which this phenomenon occurs (Ortega *et al.*, 2004b; Aguiar-Oliveira and Maugeri, 2011).

From a scientific and technological perspective, knowledge of the kinetics of thermal deactivation of enzymes is useful for predicting the performance of enzymes during processing and, in this context, for developing and optimising the bioprocesses (Iyer and Ananthanarayan, 2008; Sant'Anna *et al.*, 2013). Enzymatic deactivation is a very complex phenomenon that includes reactions such as dissociation of the multimeric enzyme into subunits, aggregation, denaturation, chemical decomposition and coagulation (Iyer and Ananthanarayan, 2008). Several mathematical models are available in the literature to represent the kinetics of enzyme deactivation in relation to time and isothermal conditions. Kinetic modelling plays the key role to understand and predict the enzymatic deactivation mechanism under the lethal effect of heat in reaction conditions (Sant'Anna *et al.*, 2012).

Based on the aforementioned, it is evident that the thermal deactivation of enzymes is a problem in biocatalytic processes. Therefore, in-depth studies are needed in order to evaluate the thermal effect on enzyme activity. The lack of available information about thermal behaviour and thermodynamic properties of immobilised pectinases makes our study a new and important contribution to this field. In the present work, the effect of *in situ* immobilisation of commercial pectinase (Rohapect® DA6L) in RFPU on thermal deactivation kinetics and thermodynamic properties was evaluated. This investigation is considered to be the first one in which the data of thermal deactivation (residual activity) of free, non-immobilised and immobilised pectinase were fit to some mathematical models in order to select one model that best describes the heat deactivation kinetics of enzymatic derivatives. Furthermore, a detailed analysis of the kinetics and thermodynamic parameters was presented for thermal deactivation of immobilised and free-form pectinases.

Materials and methods

Materials

Commercial pectinase (Rohapect® DA6L) from *Aspergillus niger* was purchased from AB Enzymes (Germany) and was previously characterised (Bustamante-Vargas *et al.*, 2015). Polyol polyether and toluene isocyanate were the monomer precursors used for the synthesis of RFPU, and were kindly donated by Mannes (Brazil). The esterified citric pectin used for hydrolysis reactions and the α-D-galacturonic monohydrated acid used for building the standard curve were purchased from Sigma Aldrich (Switzerland). All other reactants used in the present work were of analytical grade and used without previous treatment.

Lyophilisation of pectinas

Commercial pectinase (30 mL) was stored at -80°C for 24 h. Afterward, the samples were lyophilised in a lyophiliser (Edwards®, module 4 K, United Kingdom) for 72 h at -50°C and vacuum pressure of 10 Pa. The lyophilised material was stored in glass flasks at 4°C until further use. The enzyme was lyophilised to increase its stability.

Pectinase immobilisation

Pectinase was immobilised *in situ* in RFPU as reported previously. Immobilised derivatives were prepared during the synthesis of RFPU by the polymerisation reaction of the monomer polyol polyether and toluene isocyanate. Initially, 0.2 g of the lyophilised enzyme (approximately 84 units) was dissolved in 3 mL of polyol polyether until complete solubilisation. After that, 2 mL of toluene isocyanate was added, and the polymerisation reaction was initiated. The foam containing the enzyme was allowed to stand for 24 h to complete solidification. Then, the foam was fractionated, washed with acetate buffer (50 mM, pH 4.5), dried using vacuum pump (Tecnal, TE-085, Brazil) for 20 min, and stored in a desiccator at 4°C.

Determination of kinetic parameters

The kinetic parameters (V_{max} and K_m) of free (liquid), non-immobilised (lyophilised) and immobilised pectinases were determined by incubating a fixed amount of enzyme with different concentrations of citric pectin as a substrate ranging from 0.5 to 17 mg.mL^{-1}. The V_{max} and K_m values were calculated from the Michaelis-Menten model with the aid of Statistica 7.0 (StatSoft Inc., EUA).

Thermal inactivation experiments

Isothermal deactivation process of commercial pectinases (free and immobilised in RFPU) was evaluated at temperatures ranging from 50 to 80°C. Samples were placed in closed glass flasks (sterilised) in the absence of substrate and incubated in a thermostatically controlled dry bath at a specified temperature. Periodically, samples were withdrawn at different times during the incubation assays, rapidly cooled in an ice bath to stop the thermal heating and their pectinolytic activity was assayed. This procedure was performed until a residual activity value lower than 50% was reached. After 1 min of heating, the activity was considered to be the initial activity. In this way, the effects of heating are eliminated, and the isothermal process is evaluated (Malheiros *et al.*, 2015). For each evaluated time, new samples were used, which were discarded after the activity dosage. Residual activity in each sample was determined as the ratio between the initial activity and the activity during the deactivation process at different times. The assays were done in triplicate.

Mathematical modelling

In the literature, several mathematical models explaining the catalyst thermal deactivation have been proposed as a result of the complexity of processes involving enzymatic deactivation, which may occur by denaturing reactions, aggregation, coagulation or chemical decomposition (Sant'Anna *et al.*, 2013; Malheiros *et al.*, 2015). The models can be based on first-order mechanisms, in consecutive and parallel reactions (Ladero *et al.*, 2006b). Residual activity data for free, non-immobilised and immobilised pectinases in RFPU at several temperatures were fit to kinetic models through non-linear regression based on the Quasi-Newton algorithm with the help of Statistica 7.0 (StatSoft Inc., USA). The following models were used: First-order (FO) (Labus *et al.*, 2015), Henley-Sanada (HS) (Henley and Sadana, 1985), Series (SE) (Tavares *et al.*, 2015), Two-fraction (TF) (Chen and Wu, 1998), Distinct isoenzymes (DI) (Terefe *et al.*, 2009), Multi-component first-order (MFO) (Fujikawa and Itoh, 1996) and Fractional conversion (FC) (Fachin *et al.*, 2003).

Discrimination of kinetic models

The kinetic model selection was carried out according to statistical and physical criteria (Malheiros *et al.*, 2015). Chi-square (X^2) (Equation 1), coefficient of determination (R^2) and standard error of means (SEM) (Equation 2) have been applied for comparison between the kinetic models tested.

$$X^2 = \frac{\sum(\propto observed - \propto predicted)^2}{m-p} \quad \text{(Equation 1)}$$

$$SME = \frac{\sum(\propto observed - \propto predicted)^2}{\sqrt{m}} \quad \text{(Equation 2)}$$

where m = number of observations, p = number of parameters, and $\alpha_{observed}$ and $\alpha_{predicted}$ = residual activities observed and predicted by the models, respectively.

The model with higher values of R^2 and lowest values of X^2 and SEM was chosen to explain the variation in the experimental deactivation data. Negative or equal parameters, or both, at a given temperature, are the physical criteria to reject the model.

Estimation of thermal stability parameters

Deactivation energy (E_d), half-life ($t_{1/2}$), decimal reduction time (D) and Z-value were estimated for explicating the irreversible thermal deactivation of free and immobilised pectinases. The temperature dependence of deactivation rate constants (K_d) at several temperatures (50 - 80°C) was analysed based on Arrhenius law. E_d-value was calculated considering the Arrhenius equation (Equation 3) by non-linear regression analysis using Statistica 7.0 (StatSoft Inc., USA).

$$K_d = Ae^{\frac{-E_d}{RT}} \quad \text{(Equation 3)}$$

where A = pre-exponential factor, E_d = deactivation energy, R = universal gas constant (8.314 × 10^{-3} kJ mol^{-1} K^{-1}), and T = absolute temperature (K).

The $t_{1/2}$ and D-values of an enzyme are defined, respectively, as the times needed for reduction of 50 and 90% of initial activity at a given temperature. Such parameters are given by Equations 4 and 5.

$$t_{1/2} = \frac{Ln(2)}{K_d} \quad \text{(Equation 4)}$$

$$D = \frac{Ln\,(10)}{K_d} \quad \text{(Equation 5)}$$

The Z-value is defined as the temperature difference required to change the D-value by one log cycle, which was estimated by plotting the log (D-values) *versus* temperature (°C).

Estimation of thermodynamic parameters

The thermodynamic parameters of free and immobilised pectinases for irreversible thermal deactivation were estimated by Equations 6, 7 and 8 (Abdel-Naby *et al.*, 2015).

$$\Delta H^* = E_d - RT \quad \text{(Equation 6)}$$

$$\Delta G^* = -RTLn\left(\frac{K_d h}{K_b T}\right) \quad \text{(Equation 7)}$$

$$\Delta S^* = \frac{(\Delta H^* - \Delta G^*)}{T} \quad \text{(Equation 8)}$$

where ΔH* (kJ mol^{-1}), ΔG* (kJ mol^{-1}) and ΔS* (kJ mol-1 K-1) = changes of enthalpy, free energy of Gibbs and entropy, respectively, between the denatured and active forms of free and immobilised pectinase; h = Planck constant (1.84×10^{-40} kJ h) and Kb = Boltzmann constant (1.38×10^{-26} kJ K^{-1}).

Recycling efficiency

The recycling efficiency was determined by repeatedly reusing the immobilised pectinase in successive batch reactions. A content of 0.2 g of immobilised pectinase (26 U g^{-1}) was incubated with 3 mL of citric pectin (0.7% w/v in sodium acetate buffer 100 mM, pH 4.5). The reaction mixture was incubated at 55°C for 6 min. After completing each batch, the immobilised pectinase was collected, washed with distilled water and sodium acetate buffer (100 mM, pH 4.5), and fresh citric pectin aliquot was introduced to start a new cycle. The supernatant was assayed for pectinolytic activity (Miller, 1959).

Determination of enzyme activity

The pectinolytic activity was determined by the estimation of reducing sugars produced by the hydrolysis of citric pectin (1% w/v in sodium acetate buffer 50 mM) for 3 min for liquid pectinase and 6 min for lyophilised and immobilised pectinases at the optimal conditions (pH 3.4 and 37°C for free and non-immobilised enzymes (liquid and lyophilised) or pH 4.5 and 55°C for immobilised enzyme) (Bustamante-Vargas *et al.*, 2015). The concentration of reducing sugars was determined for the method of 3.5-dinitrosalicylic acid (Miller, 1959) and a calibration curve established with α-D-galacturonic monohydrated acid. The absorbance was measured in a spectrophotometer (Beckman Coutler, model DU640, USA) at 540 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 μmol of galacturonic acid per minute (U = μmol min^{-1}) at the studied conditions. Pectinolytic activities were expressed in units of activity per millilitre (U mL^{-1}) of liquid pectinase and gram (U g^{-1}) of lyophilised enzyme or support, respectively.

Results and discussion

Kinetics modelling to thermal inactivation of free and immobilized pectinases

Considering the complexity of the structural conformation of the enzymes and the different phenomena involved during the enzymatic thermal inactivation, different mathematical models have been proposed to describe the inactivation kinetics and predict the performance of biocatalysts when subjected to heat treatment. Therefore, the first-order kinetic model (FO) is based on the assumption that the breaking of a single bond or a structure is sufficient for the transition from native to the denatured state of the enzyme without the presence of an intermediate. The models represented by HS and SE are based on two irreversible first-order reactions. In the first reaction, the enzyme is converted towards an intermediate with a low specific activity. In the second reaction, this intermediate is irreversibly converted to an inactive form. The models TF, DI and MFO correspond to kinetic models, which suggest that the enzyme is constituted by isoenzymes with different thermal stabilities. The loss of enzyme activity during the inactivation process can be described by the sum of two exponential decays: one corresponding to the labile fraction and the other to heat resistant (Fujikawa and Itoh, 1996; Chen and Wu, 1998; Terefe *et al.*, 2009). Fractional conversion model (FC) describes a process of first-order inactivation. After prolonged heating of the enzyme, it is not totally inactivated due to the presence of a fraction of the highly heat resistant enzyme (Fachin *et al.*, 2003).

Residual activity of the enzymatic derivatives in isothermal treatments at temperatures from 50 to 80°C was evaluated. A summary of the performance of selected models to describe thermal inactivation of pectinase (immobilised and free) is given in Table 1.

For free and non-immobilised pectinases (liquid and lyophilised, respectively), negative (P_n) or equal (P_i) parameters (Table 1) were estimated for all models tested, except for the kinetic model of the first-order. Consequently, these models (HS, SE, TF, DI, MFO, and FC) are inappropriate for understanding the thermal inactivation mechanism and were rejected for

Table 1. Performance of evaluated kinetic models to describe thermal inactivation of free and immobilised pectinase. Pn and Pi represent negative and equal parameters, respectively.

Model	R^2	X^2	SME	Remarks
Liquid pectinase				
FO	[0.91; 0.98]	[2.1×10^{-3}; 9.3×10^{-3}]	[3.2×10^{-3}; 1.9×10^{-2}]	High R^2, and low X^2 and SEM; accepted
HS	[0.91; 0.99]	[1.1×10^{-4}; 9.9×10^{-3}]	[1.1×10^{-4}; 1.6×10^{-2}]	P_n; rejected
SE	[0.92; 0.99]	[9.2×10^{-4}; 1.8×10^{-2}]	[6.3 × 10-5; 1.4×10^{-2}]	P_n; rejected
TF	[0.92; 0.99]	[2.0×10^{-4}; 1.2×10^{-2}]	[9.9 × 10-5; 1.5×10^{-2}]	P_i; rejected
DI	[0.93; 0.99]	[1.3×10^{-3}; 1.7×10^{-2}]	[5.7×10^{-4}; 1.3×10^{-2}]	P_i; rejected
MFO	[0.92; 0.99]	[2.0×10^{-4}; 1.2×10^{-2}]	[9.9 × 10-5; 1.5×10^{-2}]	P_n; rejected
FC	[0.92; 0.98]	[2.0×10^{-4}; 1.2×10^{-2}]	[1.0×10^{-4}; 1.5×10^{-2}]	P_n; rejected
Lyophilised pectinase				
FO	[0.91; 0.99]	[5.0×10^{-4}; 1.8×10^{-2}]	[1.2×10^{-3}; 3.2×10^{-2}]	High R^2, and low X^2 and SEM; accepted
HS	[0.90; 0.98]	[5.0×10^{-4}; 2.9×10^{-2}]	[1.2×10^{-3}; 4.7×10^{-2}]	P_n; rejected
SE	[0.87; 0.99]	[4.4×10^{-4}; 2.7×10^{-2}]	[6.2×10^{-4}; 4.4×10^{-2}]	P_n; rejected
TF	[0.87; 0.99]	[5.0×10^{-4}; 1.2×10^{-2}]	[8.9×10^{-4}; 1.2×10^{-2}]	P_i; rejected
DI	[0.85; 0.99]	[7.4×10^{-4}; 6.4×10^{-2}]	[7.6×10^{-4}; 1.4×10^{-3}]	Pi; rejected
MFO	[0.87; 0.99]	[5.0×10^{-4}; 2.8×10^{-2}]	[1.2×10^{-3}; 1.9×10^{-2}]	P_n; rejected
FC	[0.87; 0.98]	[5.8×10^{-4}; 3.6×10^{-2}]	[8.3×10^{-3}; 4.5×10^{-2}]	P_n; rejected
Immobilised pectinase				
FO	[0.93; 0.95]	[2.8×10^{-3}; 1.0×10^{-2}]	[1.1×10^{-3}; 3.2×10^{-2}]	Accepted
HS	[0.93; 0.99]	[4.6×10^{-4}; 4.3×10^{-3}]	[1.4×10^{-3}; 6.7×10^{-3}]	P_n; rejected
SE	[0.96; 0.99]	[3.2×10^{-4}; 1.4×10^{-3}]	[8.0×10^{-4}; 3.5×10^{-3}]	P_n; rejected
TF	[0.95; 0.99]	[2.0×10^{-4}; 4.0×10^{-3}]	[4.3×10^{-4}; 7.0×10^{-3}]	Rejected
DI	[0.92; 0.99]	[2.3×10^{-4}; 5.0×10^{-3}]	[4.3×10^{-4}; 1.2×10^{-2}]	P_i; rejected
MFO	[0.95; 0.99]	[2.0×10^{-4}; 4.0×10^{-3}]	[4.3×10^{-4}; 7.0×10^{-3}]	Rejected
FC	[0.92; 0.99]	[3.7×10^{-4}; 4.2×10^{-3}]	[1.0×10^{-3}; 1.2×10^{-2}]	P_n; rejected

Data within the square brackets represent the minimum and maximum values of statistical criteria estimated for each inactivation kinetic model.

mathematical modelling. Equal parameters suggest that the commercial pectinase Rohapect® DA6L is constituted only by one type or several isoenzymes with similar characteristics of thermal inactivation. Considering this behaviour, the first-order model was selected to explain the mechanism of thermal inactivation of free and non-immobilised pectinases (liquid and lyophilised), since this model yielded a good fit of experimental data with higher coefficients of determination ($R^2 > 0.91$) and low values of X^2(< 1.8×10^{-2}) and SEM (< 1.9×10^{-2}) (Table 1).

Similar results were reported by Ortega *et al.* (2004a). When evaluating the kinetics of thermal inactivation of the pectin lyase in commercial pectinase preparations, the enzyme was thermally inactivated by first-order kinetics. For immobilised pectinase in RFPU (Table 1), the mathematical models of FO, TF and MFO showed a good fit. High coefficients of determination (0.93 to 0.99) and low X^2 and SEM, without any physical criteria that would allow the rejection of models, were reported. Consequently, these models were pre-selected to explain the thermal inactivation of the immobilised derivative. The other models (HS, SE, DI, and FC) tested in the present work were rejected for modelling the thermal inactivation process of immobilised pectinase. The values of the parameters were negative or equal, which are a physical criterion for model rejection. The values of R^2, X^2 and SME were unsuitable and were rejected.

A final selection of the model was performed considering the temperature as a variable (Ladero *et al.*, 2006a). Temperature dependence of inactivation rate constants (K_d, K_L, and K_R) obtained for FO, TF and MFO models were analysed by fitting to Arrhenius model (Equation 3). Figure 1 illustrates the results of Arrhenius plots for thermal inactivation at 50 - 80°C of immobilised, liquid and lyophilised pectinases.

The MFO (Figures 1a and 1b) and TF (Figures 1c and 1d) models demonstrated an inadequate fit to inactivation rate constant for the labile fraction

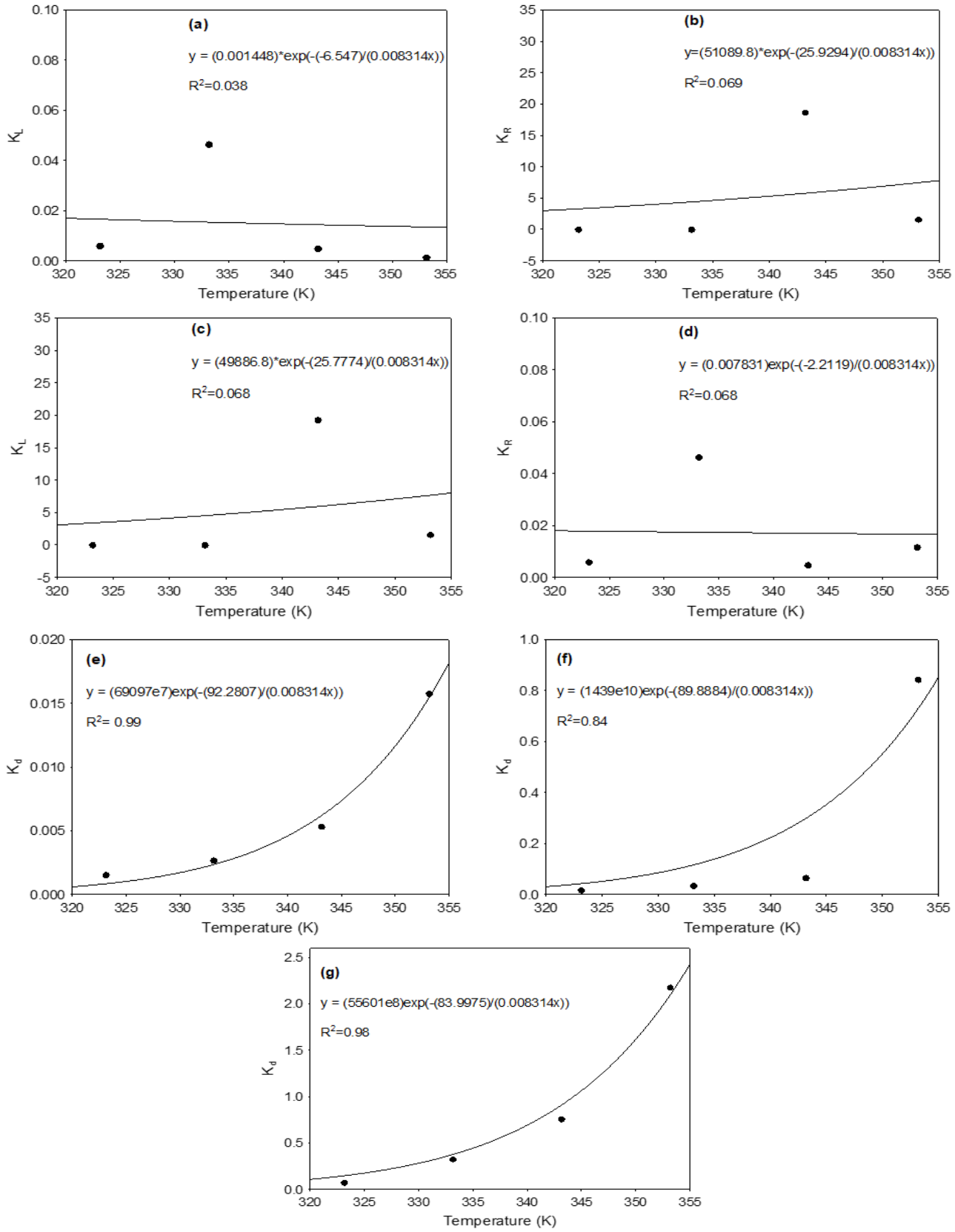

Figure 1. Arrhenius plots for first-order rate constants for labile (KL) and resistant (KR) fraction of the MFO (a and b) and TF (c and d) models of immobilised pectinase, and rate constant of first-order model for immobilised (e), lyophilised (f) and liquid (g) pectinase.

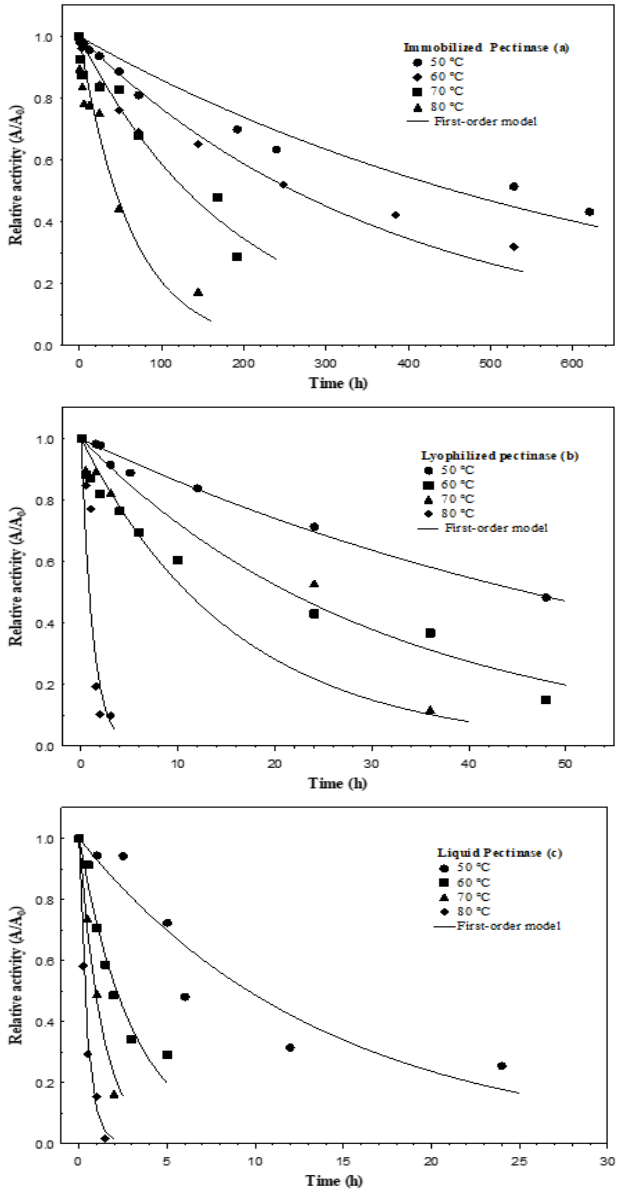

Figure 2. Thermal inactivation plots of immobilized (a), lyophilised (b) and liquid (c) pectinase at temperatures of 50 - 80°C. Symbols represent the mean of three independent assays ($n = 3$) of enzyme activity and the solid line is the first-order model (FO).

(MFO K_L; $R^2 > 0.038$ and TF K_L; $R^2 > 0.068$) and heat resistant fraction (MFO K_R; $R^2 > 0.068$ and, TF K_R; $R^2 > 0.068$). Consequently, these models were rejected. On the other hand, for the FO model (Figure 1e), a high coefficient of determination ($R^2 > 0.99$) was found, indicating that the first-order model satisfactorily described the thermal inactivation of immobilised pectinase in the range of temperatures tested. Therefore, it was selected to represent the kinetic inactivation of the immobilised derivative.

Thermal inactivation of most enzymes, including pectinases, follows an irreversible first-order mechanism. The active enzyme is directly converted to an inactive state without the need of intermediaries, and the decrease of pectinolytic activity can be described as a function of time at constant processing conditions (Ortega *et al.*, 2004a; Basu *et al.*, 2008). Figure 2 shows the graphical representation of the fit for the first-order kinetic model of the residual activity data of free and non-immobilised (liquid and lyophilised) and immobilised pectinases.

The thermal inactivation kinetics for free and immobilised pectinases at the temperatures evaluated were suitably represented by the FO model. The free pectinase was more rapidly inactivated when compared with immobilised pectinase in RFPU (Figure 2). These results could be attributed to an increase in vibrational movements of atoms of the protein due to enzyme incubation at high temperatures. It causes the breaking of chemical bonds that keep stable the protein three-dimensional structure. Therefore, the enzyme may acquire a new conformation with low catalytic ability (Tavares *et al.*, 2015).

As a consequence of the immobilisation process, thermal stability is improved as a result of a higher rigidity of the tertiary structure, which increases the biocatalyst's ability to resist thermal breakdown. The enzyme is protected from temperature by the support and the vibratory movements affect its structural conformation to a lesser extent. Overall, the results summarised in Table 2 show that the temperature affects the process of heat inactivation of the enzymatic derivatives. However, the kinetic parameters estimated of isothermal evaluation demonstrate that the thermal stability of immobilised pectinase was much higher when compared with free pectinase. Improvement of thermal stability of the

Table 2. Kinetic parameters for irreversible thermal inactivation of free and immobilised pectinase.

Temperature (°C)	K_d (h^{-1})	R^2	$t_{1/2}$ (h)	D (h)	Z (°C)
Liquid pectinase					
50	0.0720	0.90	9.63	31.98	20.8
60	0.3241	0.96	2.14	7.11	
70	0.7521	0.98	0.92	3.06	
80	2.1773	0.96	0.32	1.06	
Lyophilised pectinase					
50	0.0150	0.98	46.09	153.12	18.03
60	0.0323	0.91	21.44	71.24	
70	0.0633	0.99	10.95	36.38	
80	0.8431	0.93	0.82	2.73	
Immobilised pectinase					
50	0.0015	0.95	456.89	1517.86	29.85
60	0.0027	0.90	260.96	866.94	
70	0.0053	0.93	130.43	433.31	
80	0.0157	0.91	44.01	146.22	

immobilised enzyme indicates the efficiency of the immobilisation method and differentiates the balance between the acquired conformational stability and the surrounding microenvironment of the enzyme (Ramirez *et al.*, 2013).

Inactivation rate constant (K_d), which is proportional to the concentration of active enzyme, demonstrated that the rate at which the immobilised catalyst is inactivated is much lower when compared with the free and non-immobilised pectinase (liquid and lyophilised) (Table 2). Consequently, comparing the K_d-values at 80°C, such K_d-value of immobilised pectinase were 145.3 and 53.7 times lower than those given for liquid and lyophilised pectinases, respectively. This same tendency was observed in the other evaluated temperatures. The lower K_d-values of immobilised pectinase suggest that the immobilisation process makes the enzyme more thermostable, thereby protecting it from thermal inactivation in the temperature range of 50 - 80°C.

The half-lives ($t_{1/2}$) and decimal reduction (D) are values representing the time required for the enzyme activity to drop down to 50 and 10% of the initial value, respectively. Comparison of $t_{1/2}$ and D-values for free and immobilised pectinases (Table 2) showed substantial differences between the enzymatic derivatives. The values of $t_{1/2}$ (456.89 to 44.01 h) and D (1517.86 to 146.22 h) estimated for immobilised pectinase corroborated that it was more stable to thermal inactivation than the free enzyme. High values of these parameters are desirable for industrial operations, as they ensure that the enzyme is more resistant to inactivation caused by the effect of temperature (Castro *et al.*, 2015).

The Z-values of 20.18, 18.03 and 29.03°C (Table 2) indicate that a change in the temperature ranges would generate a log cycle reduction in the enzymatic activity of liquid, lyophilised and immobilised pectinase in the same order. The Z value of immobilised pectinase in RFPU (29.03°C) corroborates with increasing thermal stability observed for the enzyme after immobilisation.

Thermodynamic parameters

In addition to the thermal inactivation kinetics, the determination of the thermodynamic parameters is also necessary to understand the behaviour of enzyme systems during the process of thermal inactivation (Maisuria and Nerurkar, 2012). Thermodynamic parameters of free and immobilised pectinases were investigated in the range of 50 - 80°C and the results are presented in Table 3.

The inactivation energy (E_d) provides information regarding the minimum energy needed to inactivate the enzyme. The higher value of E_d of the immobilised pectinase in RFPU (91.63 kJ mol^{-1}) as compared to the free enzyme (lyophilised pectinase, 88.61 kJ mol^{-1}; liquid pectinase, 83.55 kJ mol^{-1}) indicates that the immobilised pectinase requires a larger amount of energy before protein unfolding takes place and begins the process of thermal inactivation (Tran and Chang, 2014).

Table 3. Thermodynamic parameter for irreversible thermal inactivation of free and immobilised pectinase.

Temperature (°C)	E_d (kJ mol^{-1})	ΔG* (kJ mol^{-1})	ΔH* (kJ mol^{-1})	ΔS* (kJ mol^{-1} K^{-1})
Liquid pectinase				
50	83.55	108.43	80.86	-0.085
60		107.70	80.78	-0.081
70		108.62	80.69	-0.081
80		108.75	80.61	-0.080
Lyophilised pectinase				
50	88.61	112.64	85.93	-0.083
60		114.09	85.84	-0.085
70		115.68	85.76	-0.087
80		111.53	85.63	-0.073
Immobilised pectinase				
50	91.63	118.80	88.95	-0.092
60		121.01	88.86	-0.096
70		122.75	88.78	-0.099
80		123.22	88.70	-0.098

The enthalpy of activation of thermal unfolding (ΔH*) and the entropy of activation for unfolding of transition state (ΔS*) represent the number of non-covalent bonds broken and the degree of disorder in the enzyme structure associated with the formation of the transition state for the enzyme inactivation during the heat treatment, respectively (Ortega *et al.*, 2004a; Sant'Anna *et al.*, 2012). In terms of stability, there is a relationship between ΔH* and ΔS* values. High levels of ΔH* and low levels of ΔS* are associated with an increase in thermal stability of the protein (Olusesan *et al.*, 2011).

As can be seen in Table 3, there was a decreasing trend in ΔH* values of the enzymatic derivatives with the increase of temperature. Nevertheless, ΔH* values for the immobilised pectinase were higher when compared to liquid and lyophilised pectinases. For example, the ΔH* at 70°C was 88.78 kJ mol^{-1} for the immobilised pectinase, which was higher than for the lyophilised pectinase (85.76 kJ mol^{-1}) used during immobilisation. Values of enthalpy represent a measure of the energy barrier that must be overcome for the molecules which begin to react (Olusesan *et al.*, 2011; Ramos *et al.*, 2018). Therefore, the

stabilisation of pectinase after immobilisation could be corroborated, since the immobilised enzyme requires more energy for stretching, squeezing or breaking of a chemical bond to reach the transition state of the native form to denatured state (Yan *et al.*, 2010). When compared to that of free pectinase, higher and positive E_d and ΔH* values of immobilised pectinase may be attributed to more resistance to thermal denaturation and, consequently, to the thermostabilisation of the immobilised enzyme (Souza *et al.*, 2015).

The negative value of ΔS* is a unique characteristic of biocatalytic processes, which is most likely caused by the aggregation of partially unfolded enzymatic molecules (Maisuria and Nerurkar, 2012). Lower ΔS* values of immobilised pectinase (Table 3) confirmed that immobilised pectinase in RFPU was thermodynamically more stable than the free enzyme. It suggests that the structural conformation of the immobilised derivative was changed in the direction of the partially unfolded transition state (more orderly than the free enzyme) with higher enzyme stability (Yan *et al.*, 2010; Abdel-Naby *et al.*, 2015; Romero-Fernández *et al.*, 2018). Positive ΔG* values for all enzymatic derivatives studied showed that thermal inactivation process of the pectinase is not thermodynamically spontaneous (Saleem *et al.*, 2005; Tavares *et al.*, 2015).

In addition, higher ΔG* values (> 118 kJ mol^{-1}) recorded for the immobilised pectinase demonstrated that the state assumed of enzyme immobilised, upon thermal treatment, has higher available energy. Besides, the original structural conformation remained more active as compared to free enzyme. The unfolding of the protein structure is accompanied by disruption of non-covalent linkages. It creates a disorganised system with an increase in entropy and reduced Gibbs free energy, and makes the enzymatic denaturation more feasible (Saleem *et al.*, 2005). Subsequently, an enzyme with a higher energy state and lower disorder retains its original structure and, hence, it is less susceptible to enzymatic inactivation caused by the heat treatment (Aguiar-Oliveira and Maugeri, 2011; Wallace *et al.*, 2016).

Recycling efficiency

The recycling efficiency was evaluated in order to find the operational thermal stability of immobilised pectinase when reused in batch reaction of hydrolysis of citric pectin at high temperature (55°C). The results confirmed the increase in the thermal stability of the pectinase after the immobilisation process. The immobilised biocatalyst can be used continuously for three catalytic cycles, preserving almost 100% of its initial activity and demonstrating its good performance in the catalytic reaction.

Our results provide evidence that immobilised pectinase in RFPU is more thermally stable than free pectinase. This behaviour could be attributed to conformational changes of the immobilised enzymes at high temperatures, indicating that the structure of the immobilised pectinase was changed towards a partially unfolded transition state. These results suggest that polyurethane support protected the pectinase against thermal inactivation and increases their thermal stability.

Conclusion

In summary, commercial pectinase was efficiently immobilised *in situ* in RFPU. Kinetic and thermodynamic arguments were used to demonstrate that the resistance of pectinase against thermal inactivation was improved by the immobilisation process. The superior performance of immobilised pectinase is most likely associated with its improved ability to resist unfolding of the protein structure under high temperatures. It permits the preservation of its unique structural conformation more active than the soluble enzyme. The results confirmed the superiority of immobilised enzyme and can be a point of reference to research aimed at optimisation, as well as the scale-up of the immobilisation process and its subsequent implementation.

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