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## Modeling of the thermal behaviour of free $\beta$ -galactosidase from palm weevil, *Rhynchophorus palmarum* Linn. (Coleoptera: Curculionidae) larvae using Equilibrium model

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### ABSTRACT

$\beta$ -galactosidases are a class of enzyme widely used as biocatalysts in the food, dairy, and fermentation industries. However, due to their biological origin, enhancement of these enzymes is generally necessary. The effect of temperature upon enzymes is a mandatory stage in rational enzyme engineering. The present work was devoted to *Rhynchophorus palmarum* Linn.  $\beta$ -galactosidase (*Rpbgal*) as part of the investigation of insect-derived enzymes for biotechnological applications. The thermal behaviour of *Rpbgal* has been studied in the temperature range 303-353 K by measuring enzymatic activities in presence of *o*NPG as substrate. Equilibrium model which gives complete and quantitative description of the effect of temperature on enzyme activity has been used to analyze experimental data. A satisfactorily agreement between the calculated results and the experimental data was obtained. The thermodynamic parameters provided by this model were given. Results showed that *Rpbgal* is relatively stable and active at 323 K. Temperatures over 330 K produce a significant decrease in the enzyme activity. In the temperature range 331 - 339 K, *Rpbgal* showed the best thermal stability compared to a commercial  $\beta$ -galactosidase from *Aspergillus oryzae*.

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**Keywords:**  $\beta$ -galactosidase, *Rhynchophorus palmarum*, thermal behaviour, Equilibrium model.

### INTRODUCTION

The use of enzymes in various industries such as food industry is increasing rapidly due to reduced processing time, low energy input, cost effectiveness, nontoxic and eco-friendly characteristics (Singh et al., 2016). Enzymes are ubiquitous in nature (plants, animal organs, bacteria, yeasts, fungi, etc.) (Dué et al., 2008; Akpalo et al., 2021; Elian et al., 2021; Olakusehin and Oyedeji,

2021). It has been pointed out that enzymes from insects or insect-associated microorganisms are highly demanded by the food industry to reduce food incompatibilities such as celiac disease or to eliminate potential anti-nutritive factors (Mika et al., 2013). *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae) is an insect pest widely distributed in the tropical regions and in agrosystems exploiting oil palms (EPP0

Bulletin, 2005; EPPO Bulletin, 2007). The larvae of *R. palmarum* feed on the growing tissue in the crown of the palm, during which it makes a gallery, often destroying the apical growth area and causing eventual death of the palm. *R. palmarum* produces various hydrolases including  $\beta$ -galactosidase which are involved in digestive processes (Yapi et al., 2007).

$\beta$ -galactosidases have interesting applications in the food, dairy and fermentation industries (Panesar et al., 2006). They specifically catalyze two reactions such as hydrolysis and transglycosylation reaction (Alikkunju, 2016). During hydrolysis they cleave the disaccharide to monosaccharides which enter into glycolysis and transgalactosylation reaction. The reactions products are called galactooligosaccharides (GOSs), which are useful in human health care as prebiotic food constituents (Anisha, 2017; Saqib et al., 2017). In addition to their clinical applications,  $\beta$ -galactosidases also play a vital role in food processing and biosensor for specific lactose determination in milk, treatment of lactose malabsorption, and production of lactosehydrolyzed milk (Asraf and Gunasekaran, 2010; Chanalia et al., 2018).

As a part of investigation of insect-derived enzymes for biotechnological applications, the present work is devoted to *R. palmarum* Linn.  $\beta$ -galactosidase (*Rpbgal*). To the best of our knowledge, the only data relating to *Rpbgal* are from Yapi et al. (2007). They presented the influence of temperature on enzyme activity which is among the important factors in the control of bioprocesses in biotechnology (Najafpour, 2015; Goswami and Stewart, 2016). Yapi et al. (2007) used the classic approach based on application of the well-known Arrhenius equation to describe the temperature-dependence of enzyme activity. In this approach so called "classical model", parameters defining enzyme thermal behavior are activation energy ( $E_a$ ) and thermal inactivation rate constant ( $k_{inact}$ ) (Peterson et al., 2004). The weaknesses of this classical model have been mentioned several times in literature and highlighted by 3D plot of rate

versus temperature versus time: apparent optimum temperature decreases with increasing time during the assay, very shallow maxima throughout the region, and an inexplicable abrupt rise in the rate at high temperatures (Patnaik, 2002; Peterson et al., 2004; Eisenthal et al., 2006; Daniel et al., 2008; Daniel and Danson, 2010). The Equilibrium model (EM) (Daniel and Danson, 2001; Peterson et al., 2004; Lee et al. 2007; Peterson et al., 2007; Daniel et al., 2008; Weinberg, 2008; Daniel and Danson, 2010; Daniel and Danson, 2013; Lee et al., 2013) has been formulated to correct these weaknesses in order to give complete and quantitative description of the effect of temperature on enzyme. In our recent work (Kambiré et al., 2021), this model has been used satisfactorily to analyze the thermal inactivation data of the  $\beta$ -glucosidase from *Rhynchophorus palmarum* larvae.

In the present work, the effect of temperature on enzymatic activity of *Rpbgal* in presence of *o*-nitrophenyl- $\beta$ -D-galactopyranoside (*o*NPG) is investigated. Then, experimental data are analyzed using Equilibrium model. The main purpose was to provide a set of reliable thermodynamic parameters which could be used for the enzyme optimization.

## MATERIALS AND METHODS

### Enzyme samples

Extraction, purification and biochemical characterization of  $\beta$ -galactosidase from the digestive juice of the palm weevil *Rhynchophorus palmarum* larvae (*Rpbgal*) targeted in this work, have been already performed and presented in details (Yapi et al., 2007).

### Influence of temperature on enzymatic activity

#### Experimental

The effect of temperature on enzymatic activity of *Rpbgal* is studied using *o*NPG as substrate. 50  $\mu$ L of conveniently diluted enzyme and 75  $\mu$ L of 5 mM *o*NPG are dissolved in 150  $\mu$ L of 100 mM sodium acetate buffer (*pH* = 5.6). The reaction medium is incubated over a period of 60 min

with a sampling rate of 5 min and at 5 K intervals from 303.15 K to 353.15 K using a thermostatically controlled water bath (Thermomix BM-S, B. Braun Biotech International, Melsungen, Germany). Enzyme concentration in the reaction mixture is 9.09 nM. The reaction is stopped by adding 3 mL of 1 M sodium carbonate and the absorbance is measured at 410 nm. One unit is defined as the amount of enzyme that hydrolyzes 1 μmole per min of *o*NPG to *o*-nitrophenol at 310.15 K. All chemicals used in this study (*o*NPG, buffers, etc.) are analytical grade and are purchased from Merck KGaA® (Darmstadt, Germany).

**Modeling**

Data modeling is performed using Equilibrium model (EM) extensively described in literature (Lee et al., 2007; Peterson et al., 2007; Daniel et al., 2008; Daniel and Danson, 2010). This model yields the following expression (Eq. (1)) for the variation of enzyme activity with temperature, expressed as  $V_{max}$  (maximum velocity of enzyme):

$$V_{max} = \frac{k_{cat} E_0 e^{-\frac{k_{inact} K_{eq} t}{1+K_{eq}}}}{1 + K_{eq}} \quad (1)$$

Where  $k_{cat}$  is the enzyme catalytic rate constant,  $k_{inact}$  is the thermal inactivation rate constant,  $t$  is the assay duration,  $E_0$  is the total enzyme concentration,  $K_{eq}$  is the equilibrium constant.

$k_{cat}$ ,  $k_{inact}$  and  $K_{eq}$  at a given temperature  $T$  are expressed by Eqs. (2), (3) and (4), respectively.

$$k_{cat} = \frac{k_B T}{h} e^{-\frac{\Delta G_{cat}^*}{RT}} \quad (2)$$

$$k_{inact} = \frac{k_B T}{h} e^{-\frac{\Delta G_{inact}^*}{RT}} \quad (3)$$

$$K_{eq} = e^{\frac{\Delta H_{eq}}{R} \left( \frac{1}{T_{eq}} - \frac{1}{T} \right)} \quad (4)$$

Where  $k_B$  is Boltzmann constant ( $1.3805 \times 10^{-23} \text{ J K}^{-1}$ ),  $h$  is Planck constant ( $6.6256 \times 10^{-34} \text{ J s}$ ),  $T$  is the absolute temperature,  $R$  is universal gaz constant ( $8.314 \text{ J K}^{-1} \text{ mol}^{-1}$ ),  $\Delta G_{cat}^*$  is the Gibbs free enthalpy of the

catalytic reaction,  $\Delta G_{inact}^*$  is the Gibbs free enthalpy of the thermal inactivation process,  $\Delta H_{eq}$  is the enthalpy of the equilibrium and  $T_{eq}$  is the equilibrium temperature.

The quantitative expression of the dependence of rate on temperature and time can be described by the relationship presented below (Peterson et al., 2007; Daniel et al., 2008; Daniel and Danson, 2010).

$$V_{max} = \frac{k_B \times T \times E_0 \times e^{-\left( \frac{-\Delta G_{cat}^*}{RT} \right)} e^{\left( \frac{\Delta H_{eq}}{T_{eq}} \left( \frac{1}{T_{eq}} - \frac{1}{T} \right) / R \right) t}}{h \left( 1 + e^{\left( \frac{\Delta H_{eq}}{T_{eq}} \left( \frac{1}{T_{eq}} - \frac{1}{T} \right) / R \right)} \right)} \quad (5)$$

Using Eq. (5), it is possible to fit the experimental data for “zero time” (*i.e.*, initial rates) to the Equilibrium model (Eq. (6)). In these conditions, the time-dependent thermal denaturation parameter,  $\Delta G_{inact}^*$ , cannot be determined.

At  $t = 0$ ,

$$V_{max} = \frac{k_B \times T \times E_0 \times e^{-\left( \frac{-\Delta G_{cat}^*}{RT} \right)}}{h \left( 1 + e^{\left( \frac{\Delta H_{eq}}{T_{eq}} \left( \frac{1}{T_{eq}} - \frac{1}{T} \right) / R \right)} \right)} \quad (6)$$

From Eq. (6), the “classical” optimum temperature ( $T_{opt}$ ) can be deduced (Peterson et al, 2004; Daniel et al., 2008):

$$T_{opt} \approx T_{eq} (1 - \alpha T_{eq}) \quad (7)$$

Where,

$$\alpha \approx \frac{R}{\Delta H_{eq}} \ln \left( \frac{\Delta H_{eq} - \Delta G_{cat}^*}{\Delta G_{cat}^*} \right)$$

$T_{opt}$  is the graphical optimum temperature of the enzyme at time zero (Lee et al., 2007; Daniel et al., 2008). Although, this parameter does not reflect an intrinsic enzyme property since it depends on assay conditions (Almeida and Marana, 2019), it is still considered in most enzyme characterization studies.

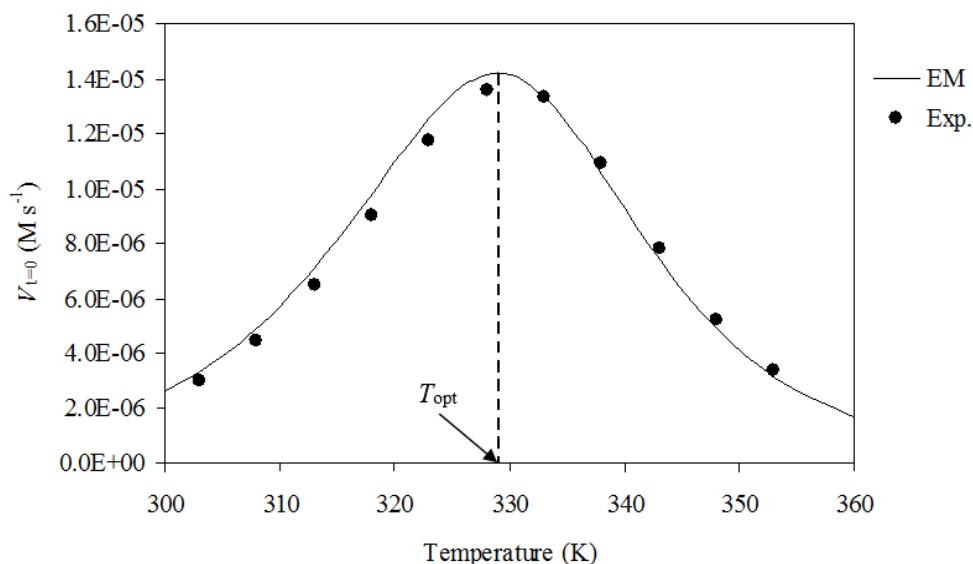
In order to access to the EM parameters ( $\Delta G_{cat}^*$ ,  $\Delta G_{inact}^*$ ,  $\Delta H_{eq}$  and  $T_{eq}$ ), experimental data are analyzed using SigmaPlot version 14.0 software with iterations number of 200, step size and tolerance equal to 1 and  $10^{-12}$ , respectively. Firstly, values of these parameters (except for  $\Delta G_{inact}^*$ ) are estimated from initial rate vs. temperature (Eq. (6)). Then, the obtained values are used as initial values during the complete optimization (rate vs. time vs. temperature). 3D plot has been also done by the same software.

## RESULTS

The initial rate versus temperature (Eq. (6)) is fitted satisfactorily using the equilibrium model ( $R^2 = 0.972$ ) as shown in Figure 1. Thermodynamic parameters ( $\Delta H_{eq}$ ,  $\Delta G_{cat}^*$  and  $T_{eq}$ ) deduced from this plot are presented in Table 1 altogether with

the final optimized parameters obtained from 3D plot of velocity vs. time vs. temperature. The 3D plot with a determination coefficient of  $R^2 = 0.977$  is shown in Figure 2. As already mentioned (Klein et al., 2018), this representation is an innovative approach in the field of thermal inactivation of enzymes, offering the possibility to evaluate the interaction of time and temperature on enzyme activity. For a better analyze of the thermal stability of *Rpbgal*, EM parameters are used to plot activity versus time (Figure 3).

In order to assess the possibilities that *Rpbgal* could offer in food processing industries from the thermal properties point of view, it is compared to a commercial  $\beta$ -galactosidase from *Aspergillus oryzae*. Recently, the thermal inactivation of this enzyme (CAS Number: 9031-11-2) was studied by Klein et al. (2018) in the temperature range 331 - 339 K using *o*NPG as substrate in an acidic buffer. The temperature dependence of  $t_R$  obtained for *Rpbgal* is compared with that of the commercial  $\beta$ -galactosidase in Figure 4.  $t_R$  is defined as the necessary time to the enzyme activity decays 90% of its initial activity.

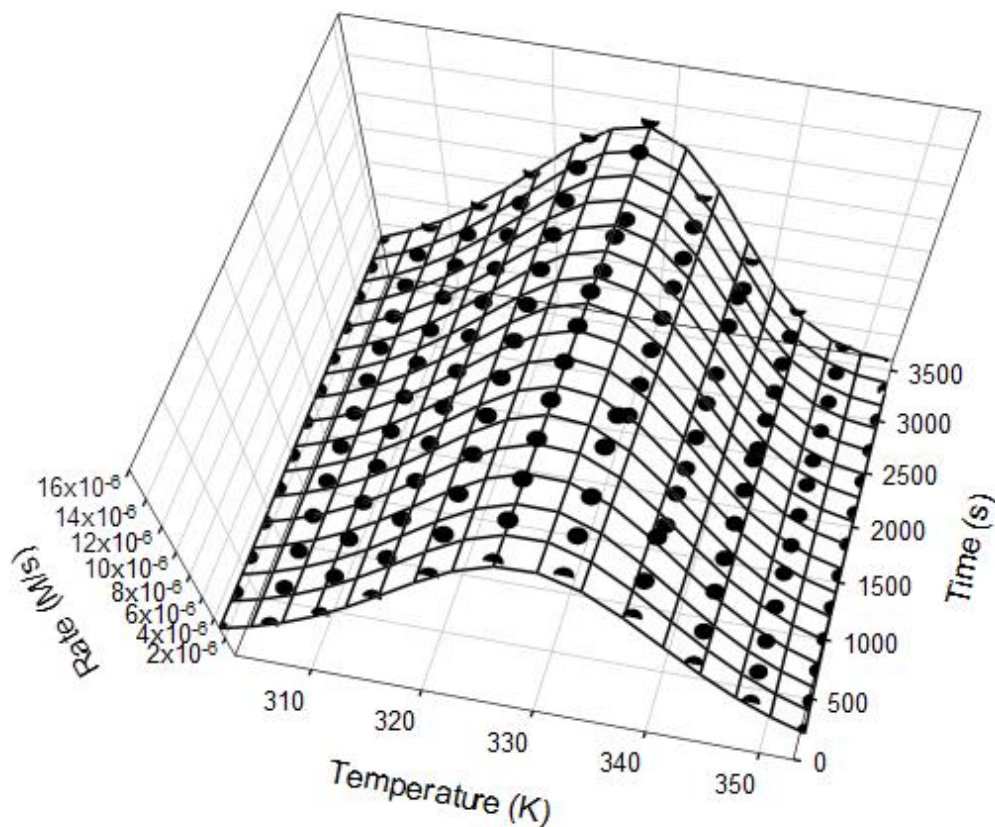


**Figure 1:** The effect of temperature on the initial (zero-time) rate of reaction of *Rpbgal*: Equilibrium model (Eq. (6)) compared with experimental data.

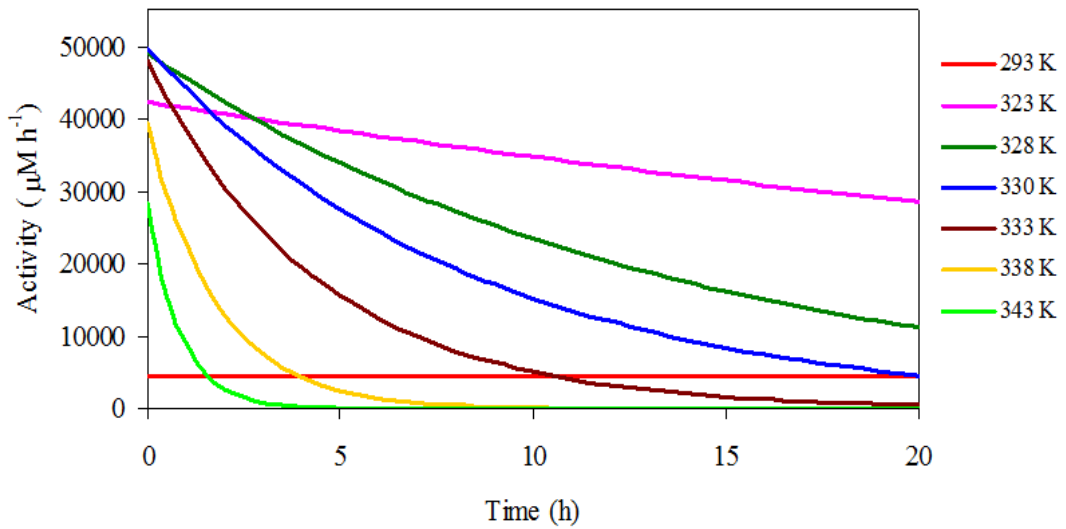
**Table 1:** EM parameters for *Rpbgal* ( $[E_0] = 9.09 \text{ nM}$ ).

Parameters	Initial rate (Eq. (6))	Entire time course (Eq.(5))
$\Delta G_{cat}^*$ (kJ mol <sup>-1</sup> )	59.4 ± 0.2	59.62 ± 0.04
$\Delta G_{inact}^*$ (kJ mol <sup>-1</sup> )	---	106.9 ± 0.2
$\Delta H_{eq}$ (kJ mol <sup>-1</sup> )	159.5 ± 0.9	159 ± 3
$T_{eq}$ (K)	331.5 ± 0.8	332.4 ± 0.2
$T_{opt}$ (K) <sup>a</sup>	328.9	---
$\Delta S_{eq}$ (J K <sup>-1</sup> mol <sup>-1</sup> ) <sup>b</sup>	---	479 ± 10

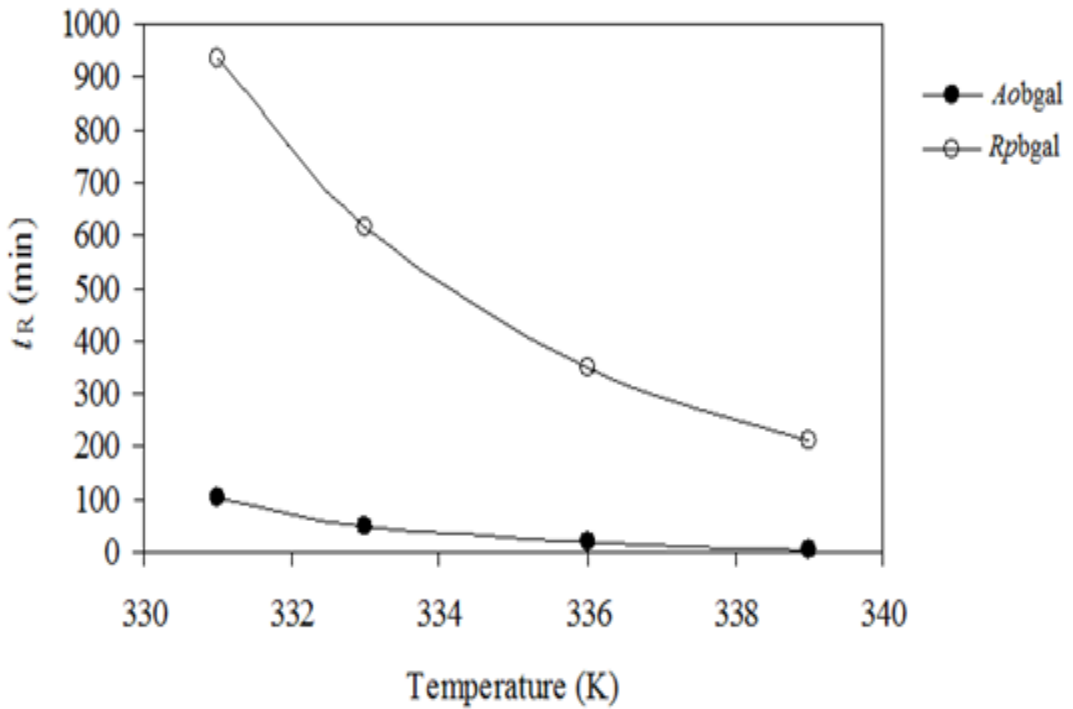
<sup>a</sup>: Graphical optimum temperature of the enzyme at time zero. <sup>b</sup>: Equilibrium entropy deduced from  $T_{eq}$  and  $\Delta H_{eq}$ .



**Figure 2:** 3D plots of rate versus time versus temperature obtained by Equilibrium model (solid lines) for  $\beta$ -galactosidase from larvae of *R. palmarum* surperimposed with experimental data (full black circles) ( $pH = 5.6$ , substrate: *o*NPG).



**Figure 3:** Temperature stability of *Rpbgal* simulated from Equilibrium model.



**Figure 4:** Evolution of the necessary time to the enzyme activity decays 90% of its initial activity ( $t_R$ ) with temperature.

*Aobgal*: commercial  $\beta$ -galactosidase from *Aspergillus oryzae* (CAS Number: 9031-11-2) (Klein et al., 2018); *Rpbgal*: *Rhynchophorus palmarum*  $\beta$ -galactosidase (this work).

## DISCUSSION

From the plot of *Rpbgal* activity vs. time (Figure 3), it can be observed that, the smallest variation in activity with temperature is obtained for 323 K. Only a change of 18% is noted after 10 h. The highest activity is exhibited for 328 K during about 150 min (2.5 h). *Rpbgal* keeps about 50% of its initial activity at 338 K for up to 75 min (1.25 h). Over this temperature, the enzyme becomes very sensitive to heat. For the same time (75 min) at 343 K, the enzyme loses more than 76% of its catalytic capacity and it is completely inactivated after 3.5 hours. At 293 K (predicted values), only 9% of the highest activity of *Rpbgal* (328 K) is obtained. Therefore, it can be concluded that *Rpbgal* is not active at low temperatures. Accordingly, this enzyme is not a psychrophilic  $\beta$ -galactosidase.

On the other hand, from the high value of the Gibbs free enthalpy of the thermal inactivation process ( $\Delta G_{inact}^*$ ) of *Rpbgal* ( $106.9 \pm 0.2$  kJ mol<sup>-1</sup>) compared to other enzymes from different sources under different assay conditions (type of substrate, medium pH, etc.) (Peterson et al., 2004; Lee et al. 2007; Daniel et al., 2008; Lee and Peterson, 2008; Daniel and Danson, 2010; Kambiré et al., 2021), it can be deduced that this enzyme exhibits a high thermal stability. In addition, the relatively low value of the enthalpic change associated with the conversion of active to inactive enzyme ( $\Delta H_{eq}$ ) of *Rpbgal* ( $159 \pm 3$  kJ mol<sup>-1</sup>) suggests eurythermal behavior, i.e. the ability of the enzyme to function at relatively high activity in an environment with large temperature fluctuations (Lee et al., 2007). Taking into account the value of equilibrium temperature ( $T_{eq}$ ) ( $332.4 \pm 0.2$  K), *Rpbgal* could be considered as mesophilic enzyme (Lee et al., 2007; Ansari and Satar, 2012). This kind of enzyme is of great biotechnological interest due to its ability to function under conditions

that normally denature thermophilic enzymes (Ansari and Satar, 2012).

The values of the necessary time to the enzyme decays 90% of its initial activity ( $t_R$ ) for *Rpbgal* are at least nine times higher than those of the commercial  $\beta$ -galactosidase (Figure 4). Therefore, *Rpbgal* has higher thermal tolerance than the commercial  $\beta$ -galactosidase and it could be a potential candidate for biotechnological applications as a safe enzyme in food industry (lactose hydrolysis whey, formation of oligosaccharides, etc.). Indeed, stability at high temperature is very important for controlling and preventing potential microbial risk during the hydrolysis process (Daniel and Danson, 2010; Zolnere and Ciprovica, 2017).

## Conclusion

The thermal behaviour of  $\beta$ -galactosidase from larvae of *Rhynchophorus palmarum* (*Rpbgal*) has been performed using *o*NPG as substrate in the temperature range 303 - 353 K. Experimental results have been analyzed using Equilibrium model (EM). A satisfactorily agreement between the calculated results and the experimental data was obtained. Thus, once again, EM showed its ability to account for thermal behavior of enzymes. It is worthy to notice that, this is the first application of EM to a  $\beta$ -galactosidase. This study revealed that  $\beta$ -galactosidase extracted from the digestive juice of the larvae of *Rhynchophorus palmarum* (*Rpbgal*) is more resistant to thermal inactivation at high temperatures as compared to a commercial  $\beta$ -galactosidase from *Aspergillus oryzae*. The highest thermal stability exhibited by *Rpbgal* is obtained at 323 K. The present results are encouraging and open new perspectives for development of safe  $\beta$ -galactosidases in food applications. Due to its biological origin, *Rpbgal* could have different operational characteristics from those required for conventional industrial processes. Therefore, its enhancement should be necessary. In these conditions, the thermodynamic parameters

obtained in this work, which provide useful thermal information, will be very important in assessing structure-stability relationships.

### COMPETING INTERESTS

The authors declare that there is no competing interests in relation to this article.

### AUTHORS' CONTRIBUTIONS

The investigation and the writing-original draft were done by MSK. The formal analysis, the writing-review and the experimental results validation were done by JMG. The conceptualization, the methodology, the formal analysis and the supervision were done by DB and EJPK. The revision of the manuscript was done by LPK.

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