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Thermodynamics and kinetics of thermal deactivation of catalase *Aspergillus niger*

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The thermal stability of enzyme-based biosensors is crucial in economic feasibility. In this study, thermal deactivation profiles of catalase *Aspergillus niger* were obtained at different temperatures in the range of 35°C to 70°C. It has been shown that the thermal deactivation of catalase *Aspergillus niger* follows the first-order model. The half-life time $t_{1/2}$ of catalase *Aspergillus niger* at pH 7.0 and the temperature of 35°C and 70°C were 197 h and 1.3 h respectively. Additionally, $t_{1/2}$ of catalase *Aspergillus niger* at the temperature of 5°C was calculated 58 months. Thermodynamic parameters the change in enthalpy ΔH^*, the change in entropy ΔS^* and the change Gibbs free energy ΔG^* for the deactivation of catalase at different temperatures in the range of 35°C to 70°C were estimated. Catalase *Aspergillus niger* is predisposed to be used in biosensors by thermodynamics parameters obtained.

Keywords: catalase *Aspergillus niger*, thermal deactivation, thermodynamics parameters, hydrogen peroxide.

INTRODUCTION

Temperature is one of the causes of enzyme inactivation in addition to pH, heavy metals, salts, solvents, detergents and mechanical agitation pressure[1, 2]. The thermostability of an enzyme is one of the most important parameters in determining its industrial applications in the long term processes carried out in biosensors. This stability of enzyme-based biosensors may change over the lifetime; particularly if storage conditions such as temperature are not controlled. The residual activity, deactivation rate constants and half-life time are the basic parameters to be known before designing the process.

Catalase is one of the first enzymes to be purified and has been the subject of intense study. Catalase catalyzes the breakdown of H_2O_2 into water and oxygen. The catalase in different industrial applications such as the food industry[3], the textile industry[4] and immobilized catalase[2–6] is used. Moreover, catalase is used to decompose the hydrogen peroxide, which being formed in the reaction with the use of oxidases[7]. Several various methods of measuring the activity of catalase, such as spectrophotometry[3–9], oxygen electrode method[1, 10, 11] and, electrochemistry[12, 13] are used. Most of the presented other's authors studies were prepared with the use catalase bovine liver[5].

One of them was known and was used with catalase *Aspergillus niger*[13]. Microbial catalase from fungus *Aspergillus niger* is used for commercial purposes due to the higher stability in conditions of extreme pH and temperature values as well as H_2O_2 content[3, 8, 9].

Cantemir et al.[14] determined the thermal deactivation of kinetics parameters for catalase bovine liver. The kinetic parameters of thermal deactivation for Terminox Ultra were analyzed in the previous work[10] . This is the first report providing information on the thermal deactivation kinetics and the thermodynamic parameters of catalase *Aspergillus niger*. The studies and the obtained results can be used for modelling the process with use catalase *Aspergillus niger*.

EXPERIMENTAL

Materials

Catalase (E.C.1.11.1.6) *Aspergillus niger* (activity > 4 000 U/mg protein) was purchased from Sigma-Aldrich. All other chemicals were of analytical quality.

Thermal deactivation

The thermal deactivation of catalase *Aspergillus niger* was determined at temperatures 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C.

The solution of catalase *Aspergillus niger*, adjusted to pH 7.0 with phosphate buffer ($2 \cdot 10^{-2}$ µl enzyme/ml buffer), was used in all experiments. The enzyme solution was divided into 5 ml samples and these were immersed in a water bath at a temperature of 35°C. After a specified storage time of the catalase at a special temperature, one sample of the enzyme was taken away and the activity of the catalase was investigated. The data were collected within 150 sec. The activity of the catalase was examined using the oxygen electrode method[1, 10, 11].

Method of measuring the activity of catalase

The catalase activity was determined by measuring the quantity oxygen production. In flask fitted with a dissolved oxygen sensor for the quantification of dissolved oxygen the 100 ml 0.01 M hydrogen peroxide solutions in 0.02 M phosphate buffer (pH 7) was inserted. Measurements were carried out at 20°C, in a jacketed vessel. The hydrogen peroxide was degassed with N_2. Measurements of catalase activity were initiated by injecting the 0.1 ml catalase solution into the reaction vessel and the data were collected. On the basis of the results of the measurements at specific temperatures, a quantity of oxygen was calculated in relation to the initial quantity of oxygen at time $t = 0$. The activity of catalase was defined as a quantity of O_2 in percent's, in relation to the keeping time at a specific temperature. The catalase activity was normalized in relation to the initial activity. The thermal deactivation was studied by taking the measurements over 150 s. The catalase activity was assayed in a temperature range of 35°C

to 70°C after an incubation time between 0 and 30 h. Temperatures and value pH has been standardized by used the certificated thermometer and pH-meter was calibrated by standard's buffers.

Kinetic model

The thermal deactivation of catalase was described by the following the first-order model[10, 11, 14, 15]. The thermal deactivation of the catalase was assumed to follow first-order kinetics $E \rightarrow D$, which can be represented as follows

$$\frac{dE}{dt} = -k_D E \tag{1}$$

where E is a concentration of the active enzyme, and k_D is a deactivation rate constant (h^{-1}).

Solution of Eq. (1) with an initial condition $E(t = 0) = E_0$ (E_0 is initial enzyme concentration), yields

$$E = E_0 exp(-k_D t) \tag{2}$$

Usually, a dimensionless enzyme activity, a, is expressed by the following equation $a = E/E_0$. Substituting into Eq. (2) gives

$$a = exp(-k_D t) \tag{3}$$

that expresses the change in the activity of the enzyme as a function of the time.

A dependence between a deactivation constant k_D and a temperature T is also given by the Arrhenius equation

$$k_D = k_{D0} exp\left(-\frac{E_D}{RT}\right) \tag{4}$$

where k_{D0} is a pre-exponential factor and E_D is activation energy for the enzyme deactivation.

One of the important practical problems is to determine the occurring constants in Eq. (4).

Usually from the plot of ln (a) versus t by Eq. (3) slope gives the value of the deactivation rate constant k_D[16]. Other scientists used the reference temperature T_{ref}[10, 17, 18] to decrease the correlation among the parameters in Eq. (4), then the equation takes the following form

$$k_D = k_{D,Tref} exp\left[\frac{E_D}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \tag{5}$$

Taking into account Eq. (5), the change of the enzyme activity can be described with the following expression

$$a_i = exp\left[-k_{D,Tref} exp\left[\frac{E_D(T_j - T_{ref})}{RT_j T_{ref}}\right] t_i\right] \tag{6}$$

where: i – number of measurements (0,1,2...n), T_j – temperature at which the measurement was executed (K), T_{ref} – reference temperature.

Based on Eq. (6) the values of $k_{D,Tref}$ and E_D were found using nonlinear regression of the Levenberg-Marquardt procedure[8–10, 19].

A standard technique used to solve a nonlinear equation by least squares method of finding the minimum of the function is a sum of squares estimate of errors *SSE*

$$SSE(E_D, k_{D,Tref}) = \sum_{i=0}^{n} \frac{1}{(a_{exp})_i^2}\left((a_{exp})_i - a_{cal}(E_D, k_{D,Tref}, t_i, T_j, T_{ref})\right)^2 \tag{7}$$

where: $(a_{exp})_i$ – experimentally determined enzyme activity, $a_{cal}(E_D, k_{D,Tref}, t_i, T_j, T_{ref})$ – enzyme activity calculated based on Eq. (6) in which, the time t_i, the temperature T_j and the reference temperature T_{ref} are known.

Equation (7) allows finding the solution of the objective function with a given set of parameters. The global minimum of the objective function was determined by many local minima in the parameter estimation process.

The enthalpy ΔH^* and entropy of activation ΔS^* and the Gibbs free energy ΔG^* values can provide some clues about enzymes and thermostability[20]. Low enthalpy values ΔH^* indicate the effectiveness of the transition state. The changes in entropy ΔS^* indicate the stability of the transition state and the affinity of the substrate for the enzyme. The entropy ΔS^* for the enzymatic reaction decreases with increasing enzyme stability. The change in the ΔG^* is the energy barrier for the catalase deactivation. This parameter is a measure of the spontaneity of the deactivation process and can reflect the effect of temperature on enzyme activity. The higher ΔG^* is, the more stable is the enzyme.

The thermodynamic parameters such as energy, entropy and enthalpy of the catalase deactivation can be estimated by making use of absolute reaction rates. The thermodynamic parameters were calculated by the rearranged equation in which the temperature dependence on the deactivation rate constant can be expressed as[16, 20]

$$\ln\left(\frac{k_D}{T}\right) = \ln\left(\frac{k}{h}\right) + \left(\frac{\Delta S^*}{R}\right) - \left(\frac{\Delta H^*}{RT}\right) \tag{8}$$

where k_D is the deactivation rate constant (h^{-1}), T is the absolute temperature (K), k is the Boltzmann constant ($1.3806 \cdot 10^{-23}$ J K^{-1}), h is the Planck's constant ($2.3854 \cdot 10^{-30}$ J h), R is the gas constant ($8.314 \cdot 10^{-3}$ kJ $mole^{-1}$ K^{-1}), ΔS^* is the change in entropy (kJ $mole^{-1}$ K^{-1}) and ΔH^* is the change in enthalpy (kJ $mole^{-1}$).

The values of ΔH^* and ΔS^* can be calculated from the slope and intercept of the plot of $\ln(k_D/T)$ versus $(1/T)$ respectively. The change Gibbs free energy ΔG^* of the catalase can be found by the following formula

$$\Delta G^* = \Delta H^* - T\Delta S^* \tag{9}$$

The half-life is defined as the time required for the enzyme to lose half of its initial activity, expressed in hours and is given by

$$t_{1/2} = \frac{ln2}{k_D} \tag{10}$$

Statistical analysis

Statistical analysis is a very useful technique to analyze, interpret, and summarize the experimental data. All the experiments were carried out in triplicate. Results were expressed as average values with error bars indicating the standard deviations. Deactivation rate constant k_D values were determined by fitting the data points to first-order kinetics according to Eq. (6) by performing linear regression. The trials with $R^2 \geq 0.994$ were selected for plotting the graphs. The data is reported as mean ± SD. p values less than 0.05 are considered to be significant. Data obtained from the results were analyzed using nonlinear regression with the help of SigmaPlot 12.3.

RESULTS AND DISCUSSION

Thermal deactivation studies and estimation of thermal deactivation constants

The obtained values of the parameters in Eq. (6) are calculated using nonlinear regression with SigmaPlot 12.3 and represented in Table 1. The Pearson correlation coefficient R for the obtained parameters was 0.9970 and the determination coefficient (correlation coefficient squared) R^2 was 0.9940, however, the standard error of the estimate was equal to 0.0693, with the statistical probability of parameters $P < 0.0001$.

Table 1. Parameters of thermal deactivation for catalase

T_{ref} [K]	$k_{D,Tref}$ [h^{-1}]	E_D [kJ mol^{-1}]
328.9	0.082 ± 0.0011	126.94 ± 1.65

The energy of thermal deactivation E_D catalase *Aspergillus niger* indicated good stability of the enzyme at higher temperatures, and requirement of comparatively high energy to thermally deactivate the catalase *Aspergillus niger*.

Having at our disposal the constants of the thermal deactivation of the catalase $k_{D,Tref}$ and E_D at the reference temperature calculated with the transformed Arrhenius equation it is possible to calculate the value of the pre-exponential thermal deactivation rate constant k_{D0} for catalase, which equals $1.18 \cdot 10^{19}$ (h^{-1}). Calculated by Eq. (4) the thermal deactivation constants k_D were presented in Table 2.

Fig. 1 presents the experimental data of the activity change of catalase for temperatures in the range of 35°C to 70°C enabling us to compare them with the values of the activity change of catalase calculated using the value of parameters from Table 1.

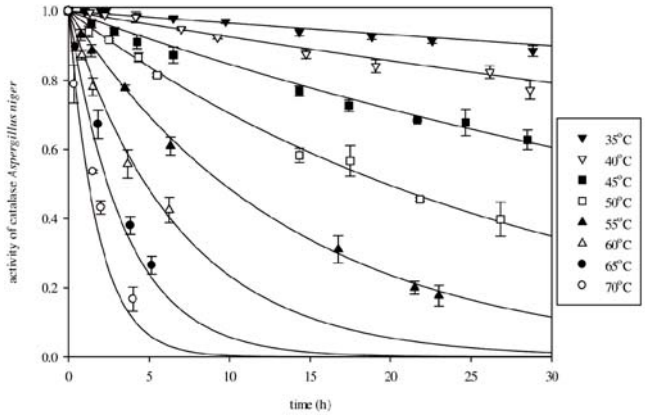

Figure 1. The activity catalase *Aspergillus niger* vs time in the thermal deactivation

The first-order model can be adequately fitted at all the temperatures by results analysis. At a temperature of 70°C, the catalase *Aspergillus niger* is rapidly deactivated to less than 20% of the initial activity after the incubation time of 4 h. While almost 90% of the initial catalase activity remains when it was kept at 30 h at 35°C. Akertek and Tarhan[3] presented thermal stabilities of catalase *Aspergillus niger* after incubation for 15 h in the temperatures of 32°C, 40°C and 50°C only; and in the same buffers. The retained activities were 98%, 70% and 25% respectively. The presented in Fig. 1 results of the thermal stability of catalase *Aspergillus niger* activity are higher than those presented earlier.

The activity of catalase *Aspergillus niger* at specific times and temperatures was calculated using Eq. (6) with the estimated parameters from Table 1. The residual analysis of catalase activity results for temperatures in the range of 35°C to 70°C was shown in Fig. 2.

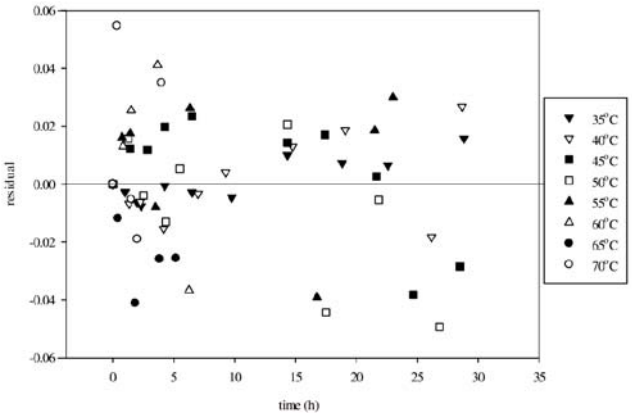

Figure 2. Residual analysis of catalase activity results at temperatures in the range of 35°C to 70°C

The regular distribution of residuals without patterns is observed, indicating the adequacy of the estimated parameters. The residual analysis of results presented in Fig. 2 was in the range ± 0.06 but the value of average absolute residuals was equal to $3.05 \cdot 10^{-3}$. The accomplished statistical analyses at specific temperatures in the range of 35°C to 70°C for catalase *Aspergillus niger* confirm that the assumption of first-order kinetics is well-founded.

Half-life time of the catalase *Aspergillus niger*

The effect of temperatures on the half-life times $t_{1/2}$ and Gibbs free energies ΔG^* of the catalase *Aspergillus niger* was calculated from Eq. (10) and Eq. (9), respectively and were presented in Table 2.

Table 2. Deactivation rate constants, half-time and Gibbs free energies for the thermal deactivation of catalase *Aspergillus niger*

Temperature (°C)	k_D (h^{-1})	$t_{1/2}$ (h)	ΔG^* (kJ mol^{-1})
35	0.0035	197	69.57
40	0.0078	89	68.55
45	0.0167	41	67.53
50	0.0351	19.8	66.51
55	0.0721	9.6	65.48
60	0.1451	4.8	64.48
65	0.2859	2.4	63.44
70	0.5522	1.3	62.42

The value of the half-life time at a temperature of 35°C is approximately 150 times higher than the values of the half-life time at a temperature of 70°C. The half-life time activity for catalase *Aspergillus niger* was presented by Kaddour et al.[2] and Hooda[29] but only at temperatures of 55°C and 75°C, respectively. Figure 3 shows the comparison of the half-life time $t_{1/2}$ for catalase

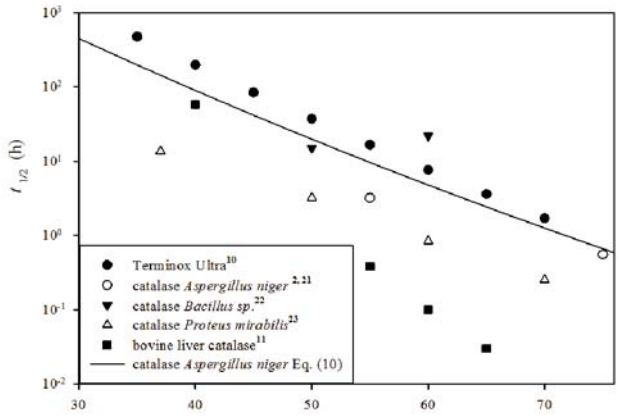

Figure 3. Comparison between the half-life times $t_{1/2}$ for catalase of various origins and calculated $t_{1/2}$ for catalase *Aspergillus niger* according to Eq. (10)

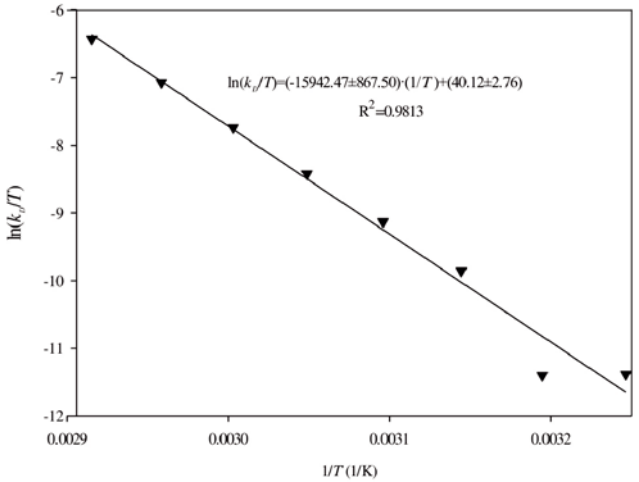

Figure 4. Plot *ln* (k_D/T) versus ($1/T$) for determining thermodynamic parameters

of various origins[10, 11, 21, 22] and calculated for catalase *Aspergillus niger* from Eq. (10).

As can be seen in Fig. 3, the values of the half-life time of the decrease in activity, calculated for catalase *Aspergillus niger*, are situated in the range of values for $t_{1/2}$ determined by other researchers. The results are different when the enzymes of various origins are used. Although catalases, which are produced by most aerobic microorganisms, are very well studied enzymes, there are only a few reports on the half-life time $t_{1/2}$ thermostable catalase[22, 23]. Fig. 3 also shows the half-life time $t_{1/2}$ for catalases from thermostable bacterium: *Bacillus sp.*[22] and *Proteus mirabilis*[23]. At pH 7.0 and temperatures of 37°C, 50°C, 60°C and 70°C, the half-life time $t_{1/2}$ for catalase from *Aspergillus niger* was approximately five to ten times longer than the half-life time $t_{1/2}$ for catalase *Proteus mirabilis*[23]. The reported value of the half-life time $t_{1/2}$ at a temperature of 55°C for catalase from *Aspergillus niger*[2] was equal to about 3.2 h, which was three times smaller than the value in Table 2. Hooda[21] reported that values $t_{1/2}$ at a temperature of 75°C for catalase from *Aspergillus niger*, were similar to those presented and were equal to about 0.55 h.

Jürgen-Lohmanna and Legge[11] presented that the constant thermal deactivation rates for catalase from the bovine liver can be established at temperatures in the range of 40°C to 65°C. However, the calculated values of the half-life time $t_{1/2}$ for catalase from *Aspergillus niger* at temperatures of 40°C and 60°C were, respectively, 150 and 80 times the longer than the values of the half-life time for bovine catalase.

Thermodynamic parameters

The change in enthalpy ΔH* and the entropy ΔS* during the thermal deactivation of catalase *Aspergillus niger* were calculated within the temperature range between 35°C and 70°C. The calculated values of ΔH^* as well as ΔS^* were enumerated from the slope and from the intercept of Fig. 4 to be 132.56 kJ mol^{-1} and 0.2045 kJ K^{-1} mol^{-1}, respectively.

Table 3 presents calculated values of ΔH^*, ΔS^*, ΔG^* for catalase *Aspergillus niger.* They have been compared with thermodynamic parameters of catalase of various origins.

The values of the activation energy for the thermal deactivation reaction for catalase *Aspergillus niger* presented in Table 3 are over 1.48 times higher than the values of the activation energy for the thermal deactivation reaction determined for bovine catalase[11]. The positive values of ΔH^* indicate that the thermal deactivation of catalase *Aspergillus niger* is an endothermic reaction. Positive values of ΔH^* have also been reported for catalase *Aspergillus niger*[24] and catalase bovine liver[25]. The value thermodynamic parameters catalase *A. niger*[24] was calculated by measurements at only two different temperatures 27°C and 37°C. The ΔG^* values were calculated at different temperatures according to the Eq. (9) and included in Table 2. Since the Gibbs free values energy ΔG^* catalase *Aspergillus niger* decreases with increasing temperature whereas ΔH^* and ΔS^* are constants values, one could point out that entropy changes ΔG^* are the result of the thermal deactivation of proteins in catalase[26]. The Gibbs free values energy ΔG^* catalase bovine liver was also decreasing; in the range of 6 kJ/mol to − 9 kJ/mol for temperatures in the range of 55°C to 75°C. Catalase bovine liver is less stable than catalase *Aspergillus niger*. There is not much information available about the energy of the thermal deactivation of E_D of catalase. It has been reported that the energy of the thermal deactivation E_D of catalase should be between 86.00 kJ/mol and 116.00 kJ/mol during the deactivation (Table 3). Therefore, the obtained value of E_D of catalase *Aspergillus niger* was

Table 3. The thermodynamic parameters characterizing the thermal deactivation of catalases of various origins

Catalase	ΔH^* (kJ mol^{-1})	ΔS^*(kJ mol^{-1}K^{-1})	ΔG^*(kJ mol^{-1})	E_D (kJ mol^{-1})
Aspergillus niger	132.56 ± 7.21	0.2045 ± 0.1061	69.57 ÷ 62.42	126.94 ± 1.65
Aspergillus niger[24]	121.10	–	10.80	–
bovine liver[14]	–	22.90	–	110 ± 16
bovine liver[11]	–	–	–	86.00
bovine liver[25]	437.38	1.33	6÷(–9)	–
Terminox Ultra [10]	–	–	–	116 ± 0.63

equal to 126.94 ± 1.65 kJ mol^{-1} and was the highest value among the designated ones.

The knowledge of the thermal deactivation constants allows for the design, modelling and optimization of work of sensors within a short period. The long-term stability of catalase in biosensors when it comes to the low and medium temperature range will take months or years. Based on the parameters presented in Table 1 it is possible to calculate, from Eq. (6), the thermal stability of the catalase activity for the temperature in the range of 5°C to 30°C as presented in Fig. 5.

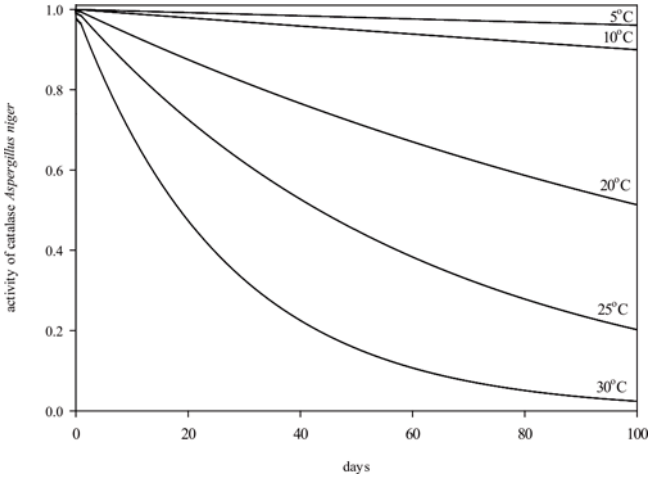

Figure 5. The thermal stability of catalase activity *Aspergillus niger* at the temperature in the range of 5°C to 30°

Fig. 5 clearly shows a significant advantage of the presented method. The stability of the bovine catalase biosensor[13] was 91.3% of its initial activity after storing it for two months at 4°C. The thermal stability catalase *A. niger* presented in Fig. 5 was 97.5% of its initial activity after storing it for two months at 5°C.

The half-time for catalases at the temperature in the range of 5°C to 30°C was calculated and presented in Table 4. Literatures information about the half-time for catalases of various origins in low temperatures was presented in the same Table.

The half-life of catalase *Aspergillus niger* at 30°C is about 19 days. It would take about 58 months to determine the half-life times for catalase *Aspergillus niger* in the temperature of 5°C using common isothermal techniques. The thermal stability of catalase *Aspergillus niger* at 5°C is considerably higher than catalase from *Aspergillus terreus*[29]. The obtained results confirm the universally acceptable opinion, that the catalase of microbiological origins is more stable than bovine catalase.

Catalase *Aspergillus niger* is a suitable enzyme for its use in biosensors (Fig. 4, Table 4). The value of the half-life time at room temperature equals about 3.5 months. The obtained parameters for this enzyme can be used in mathematical models for predicting the influence of the temperature on biosensors and optimization of the batch bioreactors[30].

CONCLUSIONS

The presented work includes determination of thermodynamic parameters for catalase *Aspergillus niger*, based on tests in the range of from 35°C to70°C. The kinetic parameters of thermal deactivation were determined on the basis of Eq. (6), taking into account the results of measurements of catalases activity over time, taking into account changes in the catalases activity under the effect of the temperature.

It was found that the thermal deactivation of catalase proceeded according to first-order kinetics. This consideration is validated by the accordance between the measured and the calculated values. Thermal deactivation rate constants varied depending on the temperature, following the Arrhenius equation. The activation energy for the thermal deactivation process for catalase *Aspergillus niger* was 116.00 ± 0.63 kJ mol^{-1}.

Change in entropy ΔS^* and change in enthalpy ΔH^* of the thermal deactivation of catalase *Aspergillus niger* were 0.2045 ± 0.1061 kJ mol^{-1}K^{-1} and 132.56 ± 7.21 kJ mol^{-1}, respectively. The change in Gibbs free energies ΔG^* were from 69.57 kJ mol^{-1} to 62.42 kJ mol^{-1} with increasing temperature. The stability of catalase *Aspergillus niger* and the half-life time in low temperatures were also calculated.

The presented methodology of measurement can be used to determine the half-life time of commonly used catalases, which are less stable than catalase *Aspergillus niger*.

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NOTATION

$(a_{exp})_i$ – enzyme activity determined experimentally,–
a_{cal} – enzyme activity calculated on based on Eq. (8)., –
E – concentration of the enzyme, M
E_0 – concentration of enzyme in initial time $t = 0$, M
E_D – the activation energy for the enzyme thermal deactivation, kJ mol^{-1}
h – the Planck's constant, $2.3854 \cdot 10^{-30}$ J h
k – the Boltzmann constant, $1.3806 \cdot 10^{-23}$ J K^{-1}
k_D – the thermal deactivation rate constant, h^{-1}
$k_{D,Tref}$ – thermal deactivation rate constant in reference temperature, h^{-1}
k_{D0} – pre-exponential thermal deactivation rate constant, M^{-1}h^{-1}
P – the statistical probability, –

Table 4. The half-life time for catalases

Catalase	$t_{1/2}$ (h)				
	5ºC	10ºC	20ºC	25ºC	30ºC
bovine liver[27]	21 days	–	5 days	–	–
bovine liver[28]	4 days	–	2 days	–	–
Aspergillus terreus[29]	30 months	–	–	–	–
Aspergillus niger (in study)	58 months	22 months	3.5 months	1.5 months	19 days

R – the gas constant, $8.314 \cdot 10^{-3}$ J mol^{-1}K^{-1}
T – temperature, K
T_j – temperature at which the measurement was executed, K
T_{ref} – the reference temperature, K
t – time, h
$t_{1/2}$ – the time of half-life activity of enzyme, h
SSE – the sum of squares estimate of errors, –
ΔS^* – the change in entropy, kJ mol^{-1} K^{-1}
ΔH^* – the change in enthalpy, kJ mol^{-1}
ΔG^* – the change Gibbs free energy, kJ mol^{-1}

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