The starch hydrolysis by α-amylase Bacillus spp.: an estimation of the optimum temperatures, the activation and deactivation energies

Justyna Miłek1 · Jan Lamkiewicz1

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Abstract
Amylases have potential application, inter alia, in processes with starch hydrolysis. The present paper reports the estimation of the optimum temperatures, the activation and deactivation energies of starch hydrolysis by α-amylase Bacillus spp. The literature activity of α-amylase Bacillus spp. versus temperature curves was analyzed. The mathematical model presented the activity of α-amylase Bacillus spp. and the starch hydrolysis. Both the starch hydrolysis and the deactivation process of α-amylase were analyzed by the first-order equations according to the enzyme concentration. Determined optimum temperatures $T_{\text{opt}}$ were in the range from 323.67 ± 1.48 K to 354.00 ± 2.27 K, activation energies $E_r$ were in the range from 18.01 ± 7.22 kJ mol$^{-1}$ to 102.85 ± 20.53 kJ mol$^{-1}$, and the values of deactivation energies $E_d$ were in the range from 79.76 ± 8.77 kJ mol$^{-1}$ to 162.85 ± 32.23 kJ mol$^{-1}$. The present study is related to the starch hydrolysis by α-amylase Bacillus spp. The obtained results might find application in the industry hydrolysis of starch.

Keywords Deactivation energy · Activation energy · Optimum temperature · α-amylase Bacillus spp

Introduction
Amylases are hydrolytic enzymes that hydrolyze the glycosidic bonds present in starch molecules and produce dextrins and oligosaccharides [1]. Generally, amylases are classified into the following three subtypes: α, β and γ. The enzyme α-amylase (E.C. 3.2.1.1) catalyzes the hydrolysis (biodegradation) of α-1,4-glycosidic bonds present in starch, glycogen and other related carbohydrates to low molecular weight products, such as glucose, maltose and maltotriose [2]. The optimum pH for α-amylase is found to be 7.0. β-Amylase (EC 3.2.1.2) catalyzes the hydrolysis of the non-reducing α-1,4-glycosidic linkages to yield successive maltose units. β-Amylase has a maximally active range of 4.0–5.5 pH. In turn, γ-amylase (EC 3.2.1.3) catalyzes the hydrolysis α-1,6-glycosidic bonds, unlike other amylases and also hydrolyze the amylose and amylpectin non-reducing α-1,4-glycosidic linkages and produces glucose [1, 2]. The optimum pH of γ-amylase is equal to 3 [3]. α-Amylase will be discussed later in this paper.

α-Amylase can be isolated from microorganisms, plants and animals [4] and has extensive applications in industry in textiles, detergent, fermentation and the food industry. Moreover, it is used in baking, brewing [4–13] and medicine [14–16]. The activity of α-amylase is important in each of the mentioned branches and particular in the industrial hydrolysis of starch by α-amylase.

Bacillus spp. are a source of enzymes characterized by wide availability, work safety and ease of cultivation, obtaining an economic enzyme in production. Among the bacterial species, the most widely used source for commercial production of α-amylases are B. amyloliquefaciens and B. licheniformis. It has been reported that these α-amylases are stable at extreme thermal conditions [1].

An important point which should be noted is that with the discovery of new bacterial strains, it is necessary to determine the optimum temperature $T_{\text{opt}}$, the activation energy $E_r$ and the deactivation energy $E_d$ for α-amylase Bacillus spp. Importantly, based on literature review, it can be concluded...
that activation energy $E_r$ and the deactivation energy $E_d$ for $\alpha$-amylase Bacillus spp. were presented in previous studies [17–19] for $\alpha$-amylase Bacillus licheniformis.

Starch hydrolysis by $\alpha$-amylase Bacillus spp. is usually carried out at optimum temperatures higher than 50 °C [5–12] and even 100 °C [20, 21]; thus, a significant deactivation of the enzyme may occur.

The study aimed to determine parameters of the optimum temperatures $T_{opt}$, the activation energies $E_r$ and the deactivation energies $E_d$ of starch hydrolysis by $\alpha$-amylase Bacillus spp. such as $\alpha$-amylases from B. subtilis, B. amyloliqufaciens and B. licheniformis. The obtained values can be used in industrial design process and modeling of starch hydrolysis.

**Methods**

**Measurement of $\alpha$-amylase Bacillus spp. activity**

Literature data [5–12] for $\alpha$-amylase Bacillus spp. from different origins were analyzed. $\alpha$-Amylase Bacillus spp. activity is most often determined by Bernfeld [5, 7–10, 12, 22]. According to this method determination of the $\alpha$-amylase activity, the reaction mixture containing 1% (v/v) starch and buffer solutions was prepared. After adding the appropriate amount of enzyme, the reaction solution should be incubated for different times (min) at 90 °C. The reaction was stopped by the addition of a 3, 5-dinitrosalicylate acid (DNS). During the breakdown of starch by $\alpha$-amylase, maltose is formed, the amount of which was determined spectrophotometrically. The unit of $\alpha$-amylase was defined as the amount of enzyme which produced 1 μmol of reducing sugar as glucose in 1 min under specified conditions. The quantity of reducing sugar was measured spectrophotometrically at 540 nm. Also to determination of activity $\alpha$-amylase is used Fuwa’s colorimetric method [23] of iodine-starch color reaction [6, 11].

**Parameters:** Optimum temperatures $T_{opt}$, activation energies $E_r$ and the deactivation energies $E_d$ of starch hydrolysis by $\alpha$-amylase Bacillus spp. were estimated from the activity change curves at temperature effect [5–12].

**$\alpha$-amylase Bacillus spp. activity versus temperature**

The values of activation energies $E_r$ and $E_d$ can be determined of the dependence of the logarithm of the reaction rate (ln ν) on the reciprocal of temperature (1/T), the so-called Arrhenius dependence [17, 18]. It has been shown that the determined values of $E_r$ and $E_d$ by application of the Arrhenius relationship is burdened with an error [19, 24–26].

When studying the starch hydrolysis by $\alpha$-amylase Bacillus spp., it is assumed that the change a substrate concentration $C_S$ during reaction time $t$ and change dimensionless activity $a$ [17, 19] are described by the first-order equations

$$\frac{dC_S}{dt} = -k_1C_E$$

(1)

$$\frac{da}{dt} = -k_2a$$

(2)

where $k_1$, $k_2$ are the enzymatic reaction and deactivation process kinetic constants, respectively (min$^{-1}$) and $C_E$ is the concentration of the active enzyme (M). Dimensionless activity of enzyme $a$ is expressed by the equation

$$a = \frac{C_E}{C_{E0}}$$

(3)

where $C_{E0}$ is the active enzyme initial concentration (M).

Considering equation describing the dimensionless activity of enzyme $a$ and Eq. (1) in Eq. (2), it was obtained

$$\frac{dC_S}{dt} = -k_{i0} \exp\left(\frac{-E_i}{RT}\right)$$

(4)

Kinetic constants $k_1$ and $k_2$ are dependent on temperature $T$ according to the Arrhenius equations in general form

$$k = k_{i0} \exp\left(\frac{-E_i}{RT}\right)$$

(5)

where $i$ is equal to $r$ or $d$, depending on whether the enzymatic reaction or the deactivation process is analyzed, $E_i$ is the activation energy for the enzymatic reaction (kJ mol$^{-1}$), while $E_d$ is the activation energy of the deactivation process (kJ mol$^{-1}$), $R$ is the gas constant equals (8.315 J mol$^{-1}$ K$^{-1}$), and $T$ is the temperature (K).

Substituting Eq. (5) into Eq. (4) leads to

$$\frac{dC_S}{dt} = -k_{i0} \exp\left(\frac{-E_i}{RT}\right) C_{E0} \exp\left(-k_{i0} \exp\left(\frac{-E_d}{RT}\right) t\right)$$

(6)

Integration of Eq. (6) leads to the following relation

$$\int_0^{C_S} dC_S = -k_{i0} \exp\left(\frac{-E_i}{RT}\right) C_{E0} \left[ \exp\left(-k_{i0} \exp\left(\frac{-E_d}{RT}\right) t\right) - 1 \right]$$

(7)

for the bonds condition $C_S(t = 0) = 0$ and $C_S(t = 0) = C_{S0}$.

The substrate concentration $C_S$ is calculated after integrating Eq. (7)

$$C_S = -k_{i0} \frac{E_d - E_r}{k_{d0}} C_{E0} \left( \exp\left(-k_{i0} \exp\left(\frac{-E_d}{RT}\right) t\right) - 1 \right)$$

(8)
It is well known that the activity of the enzyme changes with temperature. In the first stage, the activity of the enzyme increases with increasing temperature. At a certain temperature, referred to as \( T_{\text{opt}} \), the activity of the enzyme is maximal. When the \( T_{\text{opt}} \) is exceeded, the activity of the enzyme decreases. The dimensionless enzyme activity \( a \) can be described as follows:

\[
a(T) = \frac{C_d(T)}{C_S(T_{\text{opt}})} \tag{9}
\]

Dependence of the change in the dimensionless activity of the enzyme versus the temperature measurement \( T \) is presented in the following

\[
a(T) = \exp \left( \frac{(T_{\text{opt}} - T)(E_d - E_{\text{a}})}{RT_{\text{opt}}} \right) \cdot \left( \frac{\exp \left(-k_{\text{d}} \exp \left(\frac{E_d}{RT_{\text{opt}}}\right)T\right) - 1}{\exp \left(-k_{\text{d}} \exp \left(\frac{E_d}{RT_{\text{opt}}}\right)\right) - 1} \right) \tag{10}
\]

The maximum activity is determined by calculate the necessary condition, i.e.

\[
\frac{da(T)}{dT} = 0 \tag{11}
\]

Considering account the described assumption Eq. (11), the effect of temperature on the dimensionless activity \( a \) of the enzymes describes the equation:

\[
a = \frac{\exp \left(\frac{(T_{\text{opt}} - T)E_d}{RT_{\text{opt}}}\right) \cdot \left(1 - \exp \left(-\beta \exp \left(\frac{(T_{\text{opt}} - T)E_d}{RT_{\text{opt}}}\right)\right)\right) - 1}{\exp \left(-\beta \right) - 1} \tag{12}
\]

where \( T_{\text{opt}} \) is the optimum temperature for \( \alpha \)-amylase Bacillus spp. and dimensionless parameter \( \beta \) is determined by the equation

\[
\beta = t_a k_{\text{d0}} \exp \left(\frac{E_d}{RT_{\text{opt}}}\right) = t_a k_{\text{d}} (T_{\text{opt}}) \tag{13}
\]

where \( t_a \) is time of assay \( \alpha \)-amylase Bacillus spp. activity (min).

The transformation of Eq. (13) allows to determine the value of the parameter deactivation constant \( k_{\text{d}} \) at optimum temperature \( T_{\text{opt}} \)

\[
k_{\text{d}} (T_{\text{opt}}) = \frac{\beta}{t_a} \tag{14}
\]

With the values of the dimensionless parameter \( \beta \) and the deactivation process energy \( E_d \), it is possible to calculate the value of the activation energy \( E_r \) with the following relationship

\[
E_r = E_d - \frac{\beta E_d}{\exp \beta - 1}. \tag{15}
\]

Based on Eq. (12), the \( T_{\text{opt}} \), \( \beta \) and \( E_d \) parameters were estimated by the Levenberg-Marquardt procedure [26–30], calculated in SigmaPlot 14.5 the minimum sum of squared errors SSE defined by the equation

\[
\text{SSE}(T_{\text{opt}}, E_d, \beta) = \sum_{i=0}^{n} \left( \frac{1}{a_{\exp}^2} \cdot \left( a_{\exp} - a(T_{\text{opt}}, E_d, \beta) \right) \right)^2 = \min
\]

where \( a_{\exp} \) is \( \alpha \)-amylase Bacillus spp. dimensionless activity determined experimentally and \( a(T_{\text{opt}}, E_d, \beta) \) is \( \alpha \)-amylase Bacillus spp. activity calculated from Eq. (12).

Equations from Eq. (12) to Eq. (15) were used to determine optimum temperatures and the activation energies inter alia of starch hydrolysis by \( \alpha \)-amylase Bacillus licheniformis [23], \( \alpha \)-amylase from porcine pancreas [27], inulin hydrolysis by exo-inulinases Aspergillus niger [28] and recombinant exo-inulinases [29] and olive oil hydrolysis by porcine pancreas lipase [26].

**Results**

Literature data [5–12] for \( \alpha \)-amylase Bacillus spp. from different origins were analyzed. Table 1 presents the conditions for measuring \( \alpha \)-amylase activity during the hydrolysis of starch with the various buffer pH and the various measurement times [5–12]. The activity of \( \alpha \)-amylase Bacillus spp. at a specified temperature was determined in the pH range from 6.5 to 7.2.

\( \alpha \)-Amylase Bacillus sp. B-10 used by Singh et al. [5] was purified from bacterial strains isolated from soil samples. These were collected from different agricultural farms, with

| **Source** | **pH phosphate buffer** | **t/min** | **References** |
|------------|-------------------------|----------|----------------|
| Bacillus sp. B-10 | 7.2 | 30 | [5] |
| Bacillus sp. PS-7 | 6.5 | 10<sup>6</sup> | [6] |
| B. subtilis | 7.0 | 3 | [7] |
| B. amyloliquiﬁcans BH072 | 7.0 | 3 | [8] |
| B. amyloliquiﬁcans TSWK1 − 1 | 7.0 | 20 | [9] |
| B. licheniformis SKB4 | 6.5 | 5 | [10] |
| B. licheniformis A120 | 7.0 | 10<sup>6</sup> | [11] |
| Bacillus sp. 12B | 7.0 | 30 | [12] |

<sup>*</sup> \( t \) is the reaction time of \( \alpha \)-amylase Bacillus spp. activity

<sup>a</sup> method of iodine-starch (\( \lambda \) equals 660 nm)
kitchen waste and compost from Bijnor (U.P.), India, and which were mixed properly. The next amylolytic bacterial strains named Bacillus sp. PS-7 was isolated from a hot spring of Manikaran, HP, India [6]. B. subtilis isolated from fermented banana waste was selected by Shula and Kar for α-amylase production [7]. B. amyloliquefaciens BH072 was isolated from honey [8]. B. amyloliquifaciens TSWK1 − 1 was collected from the hot water reservoir at Tulsi Shyam, Gujarat, India [9]. α-Amylase B. licheniformis SKB4 studied by Samanta et al. [10] was purified from bacterial strains isolated from soil isolate. The bacterial strain used in the work Abdel-Fattah et al. [11] named B. licheniformis Al20 was isolated from garden soil samples collected from Indonesia. In turn, α-amylase Bacillus sp. 12B presented by Božić et al. [12] was isolated from wild-type strains of Bacillus sp. from some regions of Serbia.

Based on experimental data showing the change in the activity of α-amylase Bacillus spp. [5–12] in function of temperature, values of the optimum temperatures $T_{opt}$, deactivation energies $E_d$ and β parameters were determined from Eq. (12). Figures 1–8 present the experimental data of α-amylase activity as a function of temperature and the activity curves plotted by Eq. (12) for the values estimated parameters $T_{opt}$, $E_d$ and β presented in Table 2.

Table 2 presents the value of parameters $T_{opt}$, $E_d$ and β for α-amylase Bacillus spp. by the increasing value of optimum temperatures. The next step was to calculate deactivation constants $k_d$ at optimum temperature $T_{opt}$ and the activation energy parameter $E_r$ values based on Eq. (14) and Eq. (15), respectively. The calculated $k_d(T_{opt})$ and $E_r$ values are placed in Table 2.

Table 3 presents statistical data calculated for the estimated the parameters of α-amylase Bacillus spp. High values of regression coefficient ($R^2$ above 0.95) in most of the
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analyzed cases were noted. The sum of squared errors SSE below 0.20 was obtained. The F-Fisher test values and low probability value were calculated. The statistical data confirmed the accuracy of the estimated values parameters.

Additionally, Figs. 1–8 present standard deviation errors for experimental data with the 95% confidence bands. The statistical data confirmed that the application Eq. (12) when determining parameters is justified.

Discussion

This work aimed to identify the values of the activation energy $E_a$ and the deactivation energy $E_d$ and the optimum temperature of starch hydrolysis by $T_{opt}$ of starch hydrolysis α-amylase Bacillus spp. based on the literature activity versus temperature. The obtained values can be used in works focused on industrial designed and modeling of the process starch hydrolysis by α-amylase Bacillus spp.

The values of optimum temperature $T_{opt}$

The determined values of the optimum temperature $T_{opt}$ of starch hydrolysis by α-amylase Bacillus spp. were in the range from $323.67 \pm 1.48$ K to $354.00 \pm 2.27$ K (Table 2) and are different by about thirty degrees. The highest value of $T_{opt}$ was calculated for α-amylase Bacillus sp. 12B, with a long 30 min measurement time. It is worth noting that in an earlier work [19], a $T_{opt}$ of starch hydrolysis (pH 8.9) by α-amylase Bacillus licheniformis EMS-6 was determined and equal to $339.76 \pm 0.95$ K for the measurements presented by Haq et al. [17]. The presented values of the optimum temperature $T_{opt}$ in Table 2 are acceptable, when we know that optimum temperatures could be even 100 °C [20, 21].
The values of activation energy $E_r$

Results obtained in this work have demonstrated that the values of the activation energy $E_r$ of starch hydrolysis by $\alpha$-amylase Bacillus spp. are in the range from $18.01 \pm 7.22$ kJ mol$^{-1}$ to $102.85 \pm 20.53$ kJ mol$^{-1}$. It should be noted that the lowest value was obtained for the $\alpha$-amylase Bacillus sp. 12B, with a long 30 min measurement time [12]. This fact, together with a high $T_{opt}$ value, proves the very good parameters of $\alpha$-amylase Bacillus spp. 12B.

The value of the activation energy $E_r$ determined in an earlier paper [19] for the hydrolysis of starch by an $\alpha$-amylase Bacillus licheniformis EMS-6 was within the range of values reported in Table 2 and amounted to $27.16 \pm 6.89$ kJ mol$^{-1}$. In turn, the value of the activation energy $E_r$ determined by Samanta et. al. [10] for the hydrolysis of starch (pH 8.9) by $\alpha$-amylase Bacillus sp. was $31.53$ kJ mol$^{-1}$ and this value is over two twice lower than the calculated value from Eq. (15) and shown in Table 2. According to the calculations for the measurement of Božić et al. [12], the energy activation value $E_r$ for Bacillus sp. 12B was four times lower compared to the $E_r$ values obtained for $\alpha$-amylase B. licheniformis SKB4 by Samanta et al. [10]. The observed difference may be due to the different times in which the $\alpha$-amylase activity is determined.

Comparing the value of activation energy $E_r$ for $\alpha$-amylase Bacillus spp. of different origins for the starch hydrolysis time and equal to 30 minutes, the value of $E_r$ for $\alpha$-amylase Bacillus sp. B-10 is higher about $60\%$ than the value of $E_r$ for $\alpha$-amylase Bacillus sp. 12B. On the other hand, when comparing the values of $E_r$ for $\alpha$-amylase B. licheniformis of different origins, the value of $E_r$ for $\alpha$-amylase B. licheniformis A120 [11] is lower about $60\%$ than the value of $E_r$ for $\alpha$-amylase B. licheniformis SKB4 [10].

The values of deactivation energy $E_d$

The obtained values of the deactivation energy were in the range from $79.76 \pm 8.77$ kJ mol$^{-1}$ to $162.85 \pm 32.23$ kJ mol$^{-1}$
(Table 2). Short measuring times result in lower $E_d$ values. In an earlier work [19], the $E_d$ value of hydrolysis of starch by $\alpha$-amylase Bacillus licheniformis EMS-6 was found as equal to $143.54 \pm 13.31$ kJ mol$^{-1}$.

The difference in the obtained the activation energy of the deactivation process $E_d$ can be used by the different times in which the $\alpha$-amylase activity is determined.

Comparing the value of deactivation energy $E_d$ for $\alpha$-amylase Bacillus spp. of different origins and thus the starch hydrolysis time of 30 minutes, the value of $E_d$ for $\alpha$-amylase Bacillus sp. B-10 is lower about 30% than the value of $E_d$ for $\alpha$-amylase Bacillus sp. 12B. On the other hand, when comparing the values of $E_d$ for $\alpha$-amylase B. licheniformis of different origins, the value of $E_d$ for $\alpha$-amylase B. licheniformis AI20 [11] is higher about 40% than the value of $E_d$ for $\alpha$-amylase B. licheniformis SKB4 [10]. The reason for the differences in the obtained values $E_d$ may be due to the longer measurement time for B. licheniformis AI20 [11], and then process deactivation was apparent.

### The values of deactivation constant $k_d(T_{opt})$

The calculated from Eq. (14) values of the deactivation constant $k_d$ at optimum temperature $T_{opt}$ were in the range from $0.01 \pm 0.003$ min$^{-1}$ to $1.37 \pm 0.16$ min$^{-1}$ (Table 2). The highest $k_d(T_{opt})$ value, proving the thermostability of $\alpha$-amylase, was obtained for $\alpha$-amylase Bacillus subtilis at a temperature equal to $335.85 \pm 2.46$ K, while the lowest $k_d(T_{opt})$ value was obtained for $\alpha$-amylase Bacillus sp. 12B at a temperature equal to $354.00 \pm 2.27$ K.

The knowledge the values $k_d(T_{opt})$ and transform Eq. (5) allows to calculate the values of $k_d0$.

### Conclusions

The study aimed to identify a parameter for $\alpha$-amylase Bacillus spp., which has never been determined by other researchers before, i.e., the energy deactivation $E_d$. Additionally, the parameters of the optimum temperatures $T_{opt}$ and activation energies $E_r$ of starch hydrolysis by $\alpha$-amylase from the different origins of Bacillus spp. the family were determined.

The lower deactivation energy values $E_d$ were obtained for those $\alpha$-amylases for which the measurement time was shorter, i.e., up to 10 minutes. The exception is amylase $\alpha$-amylase Bacillus sp. 12B. Also, shorter measurement times resulted in higher values of $T_{opt}$ in $\alpha$-amylases Bacillus from a given genus.

The differences in the obtained values $E_r$, $E_d$ and $T_{opt}$ are, above all, different origins of Bacillus spp. The noted differences in values of parameters can be caused by the various duration of the $\alpha$-amylase Bacillus spp. activity assay.

To sum up, it should be pointed out that the obtained values of the $E_r$, $E_d$ and $T_{opt}$ can be used to design and optimize starch hydrolysis by $\alpha$-amylase Bacillus spp. in the industry where the saccharification of the processed starch was used.

### Authors’ contributions

JM was involved in the conception and design of the study. JM and JL were involved in the review literature. JM and JL were involved in analysis and interpretation of data. JM was involved in drafting the article. JM was involved in the final approval of the version to be submitted. All authors read and approved the final manuscript.

### Declarations

#### Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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