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A study of starch hydrolysis by α–amylase from porcine pancreas with deactivation of enzyme

Justyna MIŁEK[1]*, Ireneusz GRUBECKI[2], Wirginia TOMCZAK[2]

[1] Bydgoszcz University of Science and Technology, Department of Chemical and Biochemical Engineering, Faculty of Chemical Technology and Engineering, Semianryjna 3, 85-326 Bydgoszcz, Poland
[2] Cracow University of Technology, Faculty of Chemical Engineering and Technology, Warszawska 24, 31-155 Cracow, Poland

*** Corresponding author, e-mail:** jmilek@pbs.edu.pl

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Abstract
The demand for energy and the search for alternative energy sources are the reasons why scientists are interested in starch hydrolysis. The aim of this work was to experimentally study the hydrolysis of starch by α–amylase from porcine pancreas with α–amylase deactivation. Based on the experimental data, the parameters of starch hydrolysis by α–amylase with deactivation of enzyme were estimated. A mathematical model of temperature impact on the activity of α–amylase from porcine pancreas was used. It was estimated that the activation energy E_a and the deactivation energy E_d were equal to 66 ± 4 kJ/mol and 161 ± 12 kJ/mol, respectively. Additionally, specific constant of starch hydrolysis k_0 and specific constant of α–amylase deactivation k_{d0} were calculated. The optimum temperature T_{opt} equal to 318 ± 0.5 K was obtained from the mathematical model. The obtained values of E_a, E_d, k_0 and k_{d0} parameters were used to conduct the model starch hydrolysis by α–amylase from porcine pancreas at 310 K and 333 K.

Keywords
α–amylase from porcine pancreas, starch hydrolysis, activation energy, deactivation energy

1. INTRODUCTION

Starch is naturally present in vegetables and cereals, which in turn are ones of the most important nutrients. The importance of starch and starch products in the food industry is significant. Amylases are hydrolyzing enzymes that hydrolyze glycosidic bonds present in starch molecules and produce dextrin and oligosaccharides. α-amylases (E.C. 3.2.1.1) are endo–hydrolases which cleave α–1,4 glycosidic bonds of starch, glycogen and other related carbohydrates to low molecular weight products, such as maltose, dextrin and other reduced sugars (Maalej et al., 2021). α–amylase can be isolated from microbial sources, plants and animals and has extensive applications in the food industry, in baking and brewing during which starch hydrolysis occurs (Balakrishnan et al., 2019). Native and modified starches are used as texturizing and stabilizing agents in a variety of processed foods (Zinck et al., 2023). Therefore, enzymatic hydrolysis of starch is environmentally friendly technology, and it is important to understand the processes that occur during starch hydrolysis. Additionally, the constant increase in the demand for energy has driven research into alternative energy source in recent years. Technologies that convert biogenic waste into green fuels and chemicals, such as enzymatic hydrolysis are proving to be viable alternatives. Among the enzymatic hydrolysis, a particularly promising technology for release of monosaccharide and oligosaccharides is the hydrolysis of starch raw materials. Worthy of note, the starch can be a product in itself or can be further transformed into a range of products, including biofuels (Albani, 2007; Ledesma-Amaro et al., 2015; Marques et al., 2018).

In our study, α–amylase obtained from porcine pancreas (*Sus scrofa*) was used. Importantly, it is extremely similar to human pancreatic α–amylase (Gopal and Muralikrishna, 2009) and therefore has several applications in health food medicine (Abd El-latif et al., 2020; Oszmiański et al., 2021) and in medical diagnostics (Ademakinwa et al., 2019; Wang et al. 2022).

It is essential to mention that the kinetic parameters of the hydrolysis process are used in the design of appropriate volume of bioreactors, flow rate and processing time. However, during starch hydrolysis complex processes occur, hence the investigation of a kinetic enzymatic model is a great challenge (Mitchell et al., 2021). Starch used as a substrate is composed of linear amylose molecules and branched amylopectin molecules. Several factors, such as substrate complexity, variability over time, the behavior of intermediate products as well as the production of many final products make it difficult to describe the process of starch hydrolysis. For this reason, a detailed literature review on the kinetics of starch hydrolysis was conducted. Consequently, it has been found that in order to describe starch hydrolysis process many kinetics models may be applied (Mitchell et al., 2021). For instance, Woj-

ciechowski et al. (2001) described both multienzymatic and multisubstrate reactions simulating the "real" concentrations of all components as a function of time. The above-mentioned authors fitted the model to experimental data on the concentration of reducing sugar obtained as a results of starch hydrolysis by an α–amylase. The model adequately predicted starch hydrolysis results beyond the conditions that were used to demonstrate the method. The times for productive attack, non–productive attack and inhibition have been scaled to corresponding real times. In addition, it has been noted that competitive inhibition of enzyme kinetics has not been demonstrated. Besselink et al. (2008) used the same model with modifications. Murthy et al. (2011) described the hydrolysis of starch by α–amylase, which liquefies the starch, and then maltoligosaccharides are saccharified by glucoamylase. The model included effects of temperature, pH and enzyme activity with enzyme dose. Problematic starch heterogeneity, gelation and product inhibition were taken into account. Model predictions for glucose were characterized by low determination of regression coefficients R^2 ranging from 0.69 to 0.80, hence it can be concluded that the model is described as highly empirical. Moreira et al. (2021) analyzed the Michaelis-Menten kinetics with product inhibition and obtained starch hydrolysis time according to an analytical equation. This time corresponded to actual process time, i.e., the model of Moreira et al. (2021) had an advantage over the models of Wojciechowski et al. (2001) and Besselink et al. (2008), which used hypothetical time that is later scaled empirically. It should be noted that the researchers, taking into account the kinetics of starch hydrolysis, did not present the effect of α–amylase deactivation affecting starch hydrolysis. In most cases, deactivation of α–amylase has been presented as a temperature effect without starch hydrolysis (Apar and Özbek, 2004b) or enzyme concentration on starch hydrolysis (Apar and Özbek, 2004a, 2005; Bravo Rodríguez et al., 2006; Koyama et al., 2013; Presečki et al., 2013). The results of studies on starch hydrolysis with α–amylase deactivation, according to the first order deactivation of α–amylase model were presented by Apar and Özbek (2004a, 2005), where α–amylase was derived from *Bacillus* spp. Bravo Rodríguez et al. (2006) described starch hydrolysis using the Michealis–Menten equation with a second-order deactivation model for a commercial α–amylase from *Bacillus licheniformis* (Termamyl 300 L). Presecki et al. (2013) applied the Michealis–Menten equation with the inhibition of non-competitive products (maltose and glucose) to describe hydrolysis of starch by commercial α–amylase from *Bacillus licheniformis* (Liquozyme Supra and Termamyl 120 L). In this work the scientists indicated that the products of the reaction were not strong inhibitors. However, when considering the kinetics of starch hydrolysis, the researchers did not analyze the effect of α–amylase deactivation from porcine pancreas on enzyme kinetics.

It is necessary to mention that in the previously published work (Miłek, 2021a) the values of the activation energy E_a, the deactivation energy E_d and the optimum temperature T_{opt} have been determined for starch hydrolysis by α–amylase from porcine pancreas for results obtained via experimental studies by other researchers (Akhond et al., 2016; Aksoy et al., 1998; Gopal and Muralikrishna, 2009; Guo et al., 2016; Louati et al., 2010). In order to make model calculations and thus to design the process, it necessary to know an additional parameter, which is the specific pre-exponential rate constant k_0. Experimental data allowed to determine the k_0 with parameters E_a, E_d and k_{d0}. Therefore, our own study was carried out, the basis on which the above-mentioned parameters were determined. To be complete, it should be noted that studying starch hydrolysis by porcine pancreas α–amylase with simultaneous deactivation of α–amylase it is important to provide a better understanding the investigated process.

2. EXPERIMENTAL AND MATHEMATICAL MODEL

2.1. Materials

α–amylase from porcine pancreas (EC 3.4.21.4) (Typ VI-B, $>$ 10 U/mg protein), DNS – dinitrosalicylic acid and soluble starch from potato have been purchased from Sigma–Aldrich (Poznań, Poland). Monosodium phosphate (NaH_2PO_4), disodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) were obtained from Avantor Performance Materials Poland S.A. (Gliwice, Poland). All chemicals used were of analytical grade.

2.2. Kinetic rate equations for starch hydrolysis with deactivation of α–amylase

Starch hydrolysis kinetics can be described by Michaelis–Menten kinetic model (Bravo Rodríguez et al., 2006; Gopal and Muralikrishna, 2009; Koyama et al., 2013). The Michaelis–Menten constant K_m assumed was much less than the concentration of starch ($K_m \leq S$) thus, the change of starch concentrations S in time t, an isothermal of in starch hydrolysis by α–amylase can be described by the following equation:

$$\frac{dS}{dt} = -kE \tag{1}$$

where k is enzymatic reaction rate constant (1/min), whereas E is enzyme concentration (M). The change of dimensionless activity α–amylase a (Ademakinwa et al., 2019; Apar and Özbek, 2004a, 2005; Koyama et al., 2013; Miłek, 2021a) in time t in starch hydrolysis by α–amylase can be given by the equation:

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

in which k_d is rate constant of enzyme deactivation (1/min).

The solution to Eq. (2) for initial conditions $a(t = 0) = 1$ is dependence

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

The rate constant of enzymatic reaction k and rate constant of enzyme deactivation k_d depend on temperature T according to the Arrhenius equation:

$$k(T) = k_0 \exp\left(-\frac{E_a}{RT}\right) \tag{4}$$

$$k_d(T) = k_{d0} \exp\left(-\frac{E_d}{RT}\right) \tag{5}$$

in which k_0 and k_{d0} are specific constants for starch hydrolysis and for deactivation α–amylase (1/min), respectively; E_a is activation energy (kJ/mol) whereas E_d is deactivation energy (kJ/mol), R is gas constant 8.314 J/(mol·K) and T is temperature (K).

The solution of Eq. (1)–Eq. (5) has been presented in several previously published papers (Miłek and Wójcik, 2009; Miłek, 2011; Wojcik and Miłek, 2016). It describes the change in dimensionless enzyme activity depending on temperature T

$$a(T) = \frac{\exp\left(\frac{(T_{opt}-T)}{RTT_{opt}} \cdot \frac{E_d\beta}{(\exp\beta-1)}\right)\left\{1-\exp\left[-\beta\exp\left(\frac{E_d(T-T_{opt})}{RTT_{opt}}\right)\right]\right\}}{1-\exp(-\beta)} \tag{6}$$

where T_{opt} (K) is optimum temperature in which activity of α–amylase has the maximum activity and dimensionless parameter β determined by the following relationship

$$\beta = k_{d0} t_a \exp\left(-\frac{E_d}{RT_{opt}}\right) = t_a k_d\,(T_{opt}) \tag{7}$$

in which t_a is time of starch hydrolysis (min).

The value of activation energy E_a is determined by the equation

$$E_a = E_d - \frac{E_d \cdot \beta}{\exp\beta - 1} \tag{8}$$

Equations (6)–(8) were applied to determine the parameters E_a, E_d and T_{opt} for inulin hydrolysis by recombinant exo–inulinase from *Aspergillus niger* (Miłek, 2022), endo–inulinase from *A. niger* no recombinant (Miłek, 2020) and recombinant (Miłek, 2023), as well as for olive oil hydrolysis by lipase from porcine pancreas (Miłek, 2021b).

The software SigmaPlot 15.0 was used to estimate parameters occurring in Eq. (6) by non-linear regression of Levenberg–Margurdt method already used in several previous studies (Miłek (2021b, 2022, 2023)).

2.3. Effect of temperature on activity of α–amylase from porcine pancreas

The activity of α–amylase was assayed according to Miller method (Maalej et al., 2021; Merck, 2023; Miller, 1959) using dinitrosalicylic acid (DNS). The reaction mixture consisted of 1 mL 1% starch solution dissolved in phosphate buffer 0.1 M (pH 6.9) was incubated with 1 mL of α–amylase solution (2.3 U/mL) at various temperature values (298 K, 303 K, 308 K, 313 K, 318 K, 323 K, 328 K and 333 K) for 3 min. The reaction was stopped by adding 1 mL of 0.5% DNS. The mixture was then heated in boiling water bath for 15 min. Next, the contents were cooled and the concentration of reducing sugars, were measured spectrophotomerically at 540 nm using UV-Vis Jasco 530. All assays were performed in triplicate and the obtained results were presented ±SD. One unit of α–amylase was defined as the amount of α–amylase which produced 1 μmol of reducing sugar in 1 min under specified condition.

Subsequently, in order to determine kinetic parameters of α–amylase deactivation, the activity was determined by incubating the reaction mixture at 298 K, 303 K, 308 K, 313 K, 318 K, 323 K, 328 K, and 333 K for 15 min.

2.4. Modeling of starch hydrolysis by α–amylase from porcine pancreas

The mathematical model described by Eq. (1) and Eq. (2) for starch hydrolysis by α–amylase from porcine pancreas was studied. It has been assumed that the enzyme concentration is $E = aE_0$ and knowledge of dimensionless activity of α–amylase described by Eq. (3) allowed to transform Eq. (1) to the following form:

$$\frac{dS}{dt} = -kE_0 \exp(-k_d t) \tag{9}$$

Transformed Eq. (9) as function conversion of starch hydrolysis X is as follows:

$$\frac{dX}{dt} = \frac{kE_0}{S_0} \exp(-k_d t) \tag{10}$$

An important point which should be noted is that in the current study, the modelling of starch hydrolysis was investigated at 310 K and 333 K. Firstly, it is related to the potential use of porcine pancreas α–amylase in health food medicine and medical diagnostics. Secondly, the obtained results would be useful for the process of saccharification of starch.

3. RESULTS AND DISCUSSION

3.1. Kinetic parameters of starch hydrolysis

To determine the enzymatic reaction rate constant of starch hydrolysis, α–amylase activity was determined by incubating the reaction mixture for 3 min at specified temperature in the range from 298 K to 333 K. However, according to the data presented in Figure 1, at 328 K and 333 K the effect of deactivation was significant. Hence, to determine the enzymatic reaction rate constant k, the values of temperature

mentioned above were not taken into account. Indeed, the quantity of reducing sugars were analysed at 298 K, 303 K, 308 K, 313 K, 318 K and 323 K.

Equation (4) was transformed to a linear equation and from Arrhenius plot (Figure 2) the values of E_a and k_0 were estimated. It has been found that the above-mentioned parameters were equal to 50.6 ± 2 kJ/mol and $1.93 \cdot 10^{12}$ 1/min, respectively. The regression coefficient R^2 was higher than 0.99, and the sum of squares error SE was smaller than 0.056. In turn, the value of the Fisher test F was higher than 559 and the value of probability P was smaller than 0.0001.

According to the afore mentioned data it can be clearly indicated that the statistical data are highly significant. The parameters E_a and k_0 were determined using a non-linear regression in software SigmaPlot version 15.0 (Gambit, Poland).

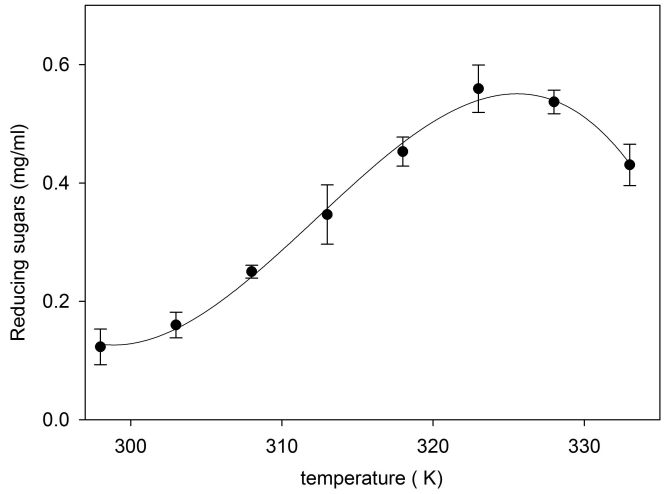

Figure 1. The effect of temperature on the concentration of reducing sugars produced during the starch hydrolysis by α–amylase from porcine pancreas at 3 min.

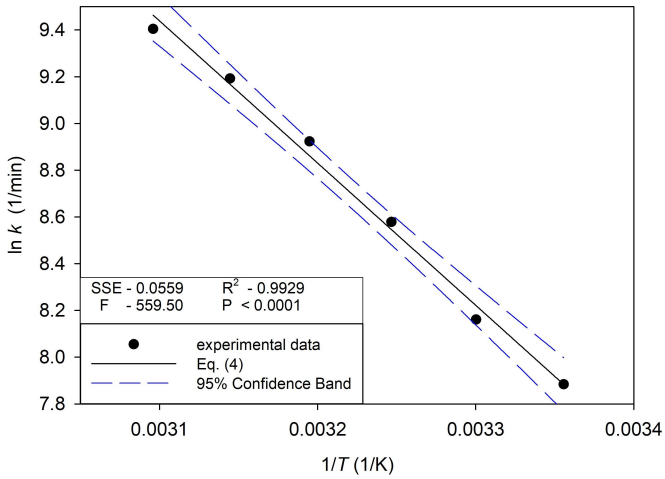

Figure 2. Arrhenius plot to determine values of the activation energy E_a and specific constant k_0.

3.2. Effect of temperature on activity α–amylase from porcine pancreas

The effect of temperature on the activity of α–amylase from porcine pancreas with statistical data is presented in Figure 3.

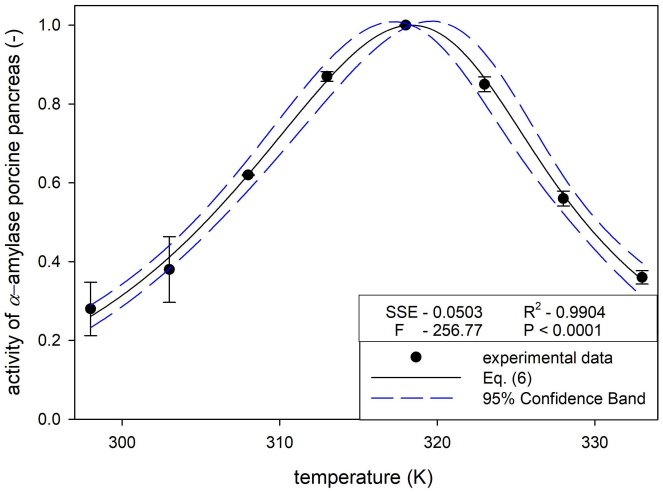

Figure 3. The effect temperature on activity of α–amylase from porcine pancreas with starch hydrolysis for 15 min.

Table 1 shows the values of deactivation energy E_d and optimum temperature T_{opt} as well as parameter β predicted based on Eq. (6). Then, the knowledge of the E_d and βvalues allowed to calculate the value of the activation energy E_a from Eq. (8). Remarkably, E_a was found to be approximately 30% higher when starch hydrolysis was measured after 15 minutes and deactivation of α–amylase occurred, compared to starch hydrolysis which was measured after 3 minutes and no deactivation occurred. The values of regression coefficient R^2 was higher than 0.99 and the sum of squares error SSE was smaller than 0.05. Finally, the value of the Fisher test F was higher than 256 and the value of probability P was smaller than 0.0001. The key highlight is therefore that the obtained statistical data are highly significant. The comparison of the obtained values of E_a, E_d, k_0 and T_{opt} for α–amylase from porcine pancreas with those available in the literature are presented in Table 1.

In previous work (Miłek, 2021a) the values of E_a, E_d and T_{opt} parameters for α–amylase from porcine pancreas were determined from the experimental results of other researchers (Akhond et al., 2016; Aksoy et al., 1998; Gopal and Muralikrishna, 2009; Guo et al., 2016; Louati et al., 2010). It should be emphasized that according to the literature (Table 2), there has been no investigation on the deactivation of α–amylase from porcine pancreas. Notably, determined values of T_{opt} and β and t_a allowed to investigate the rate constant of enzyme deactivation in optimum temperature $k_d(T_{opt})$. Subsequently, Eq. (7) was transformed and specific constant for α–amylase deactivation k_{d0} was obtained (Table 2).

Table 1. Comparison of the obtained values of E_a, E_d, k_0 and T_{opt} parameters for α–amylase from porcine pancreas with literature data.

Source porcine pancreas α–amylase	E_a, kJ/mol	k_0, 1/min	T_{opt}, K	E_d, kJ/mol	References
Sigma–Aldrich	50 ± 2	$1.93 \cdot 10^{12}$	–	–	this study[1)]
Sigma–Aldrich	66 ± 4	$7.80 \cdot 10^{14}$	318 ± 0.5	161 ± 12	this study
Model*	63 ± 0.6	–	–	–	Sočan et al. (2020)
Sigma-Aldrich (St. Louis, MO, USA)	92 ± 23	–	311 ± 1	165 ± 19	Miłek, (2021a)[2)]
Merck AG (Germany)	129 ± 9	–	313 ± 0.6	209 ± 5	Miłek, (2021a)[3)]
Sigma Chemical Company	55 ± 17	–	318 ± 1	153 ± 11	Miłek, (2021a)[4)]
Sigma	54 ± 16	–	321 ± 1	163 ± 19	Miłek, (2021a)[5)]
Shanghai Kaiyang Biological Technology Co., Ltd. (Shanghai, China)	20 ± 7	–	326 ± 2	124 ± 14	Miłek, (2021a)[6)]

*For simulation on basic on structural information from Protein Data Bank.
[1)]From Arrhenius equation (Fig. 2).
Parameters were determined based on the experimental data: [2)]Akhond et al. (2016), [3)]Aksoy et al. (1998), [4)]Gopal and Muralikrishna (2009), [5)]Louati et al. (2010) [6)]Guo et al. (2016).

Table 2. Comparison of the obtained values of E_a, k_0, E_d and k_{d0} parameters for α–amylase from porcine pancreas with literature values for α–amylase from various origin.

Source α–amylase	E_a, kJ/mol	k_0, 1/min	E_d, kJ/mol	k_{d0}, 1/min	References
porcine pancreas Sigma–Aldrich	66 ± 4	$7.80 \cdot 10^{14}$	161 ± 12	$2 \cdot 10^{25}$	this study
salivary human[1)]	–	–	3600	$4.7 \cdot 10^{61}$	Koyama et al. (2013)
salivary human[2)]	–	–	2400	$2.9 \cdot 10^{41}$	
Bacillus licheniformis	42	$1.74 \cdot 10^{8}$	172	$2 \cdot 10^{25}$*	Bravo Rodríguez et al. (2006)

*The thermal deactivation was presented by the second–order equation (L/(g·min)),
[1)]0.1% starch suspension, [2)]3% starch suspension.

3.3. Modeling of starch hydrolysis by α–amylase from porcine pancreas

The obtained values of E_a, k_0, E_d and k_{d0} for starch hydrolysis with deactivation of α–amylase from porcine pancreas were used for modelling first–order conversion of starch for the initial concentration equal to 5%, 10% and 20% at temperature of 310 K and for the initial concentration of 20%, 30% and 40% at 333 K.

Based on the results presented in Figure 4, it was found that the conversion of starch hydrolysis decreased correspondingly with increasing starch concentration for the same quantities of the enzyme. Through detailed studies, we showed that for the model without deactivation of α–amylase at the starch concentration of 5%, 10% and 20%, the values of conversion were higher by 11%, 5.4% and 2.7%, respectively. It must be stressed that the data presented in Fig. 4 can be used in the medical diagnosis of patients with hyperamylasemia. Exceeded norms of amylase levels were observed in a group of patients during hospitalization in Covid 19 pandemic. It should be pointed out that the high values were found in 196 out of 1,515 patients, i.e. in 12.9% (Li et al., 2021).

It must be recognized that the results presented in Figure 5 have confirmed that the conversion of starch hydrolysis decreased correspondingly with increasing starch concentration for the same quantities of the enzyme. However, using the model without deactivation of α–amylase for 20%, 30% and 40 % starch concentration, the values of conversion were higher by 29.4%, 53% and 65%, respectively. In turn, results presented in Figure 6 showed that the conversion of starch hydrolysis could be doubled when the amounts of enzymes were also doubled. Importantly, the data presented in Figures 5 and 6 can be used in the saccharification of starch.

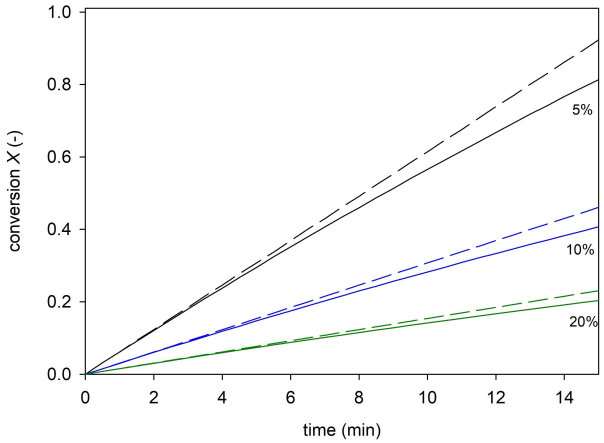

Figure 4. The conversion of starch hydrolysis by α–amylase from porcine pancreas (black line – 5% starch, blue line – 10% starch, green line – 20% starch with deactivation of α–amylase and dash line – without deactivation of α–amylase) at 310 K for 15 min.

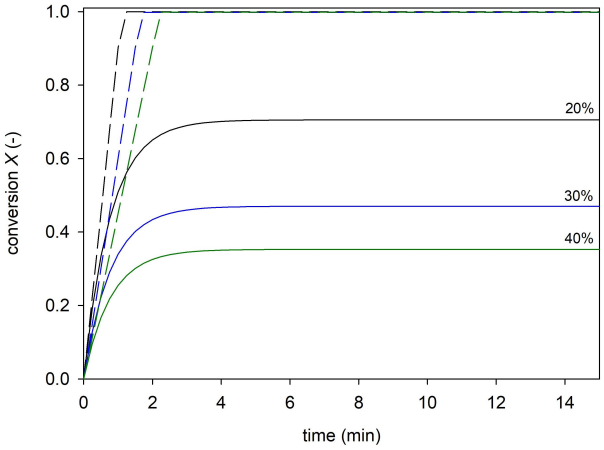

Figure 5. The conversion of starch hydrolysis by α–amylase from porcine pancreas (black line – 20% starch, blue line – 30% starch, green line – 40% starch with deactivation of α–amylase and dash line – without deactivation of α–amylase) at 333 K in 15 min.

What is important to point out is that the obtained parameters for starch hydrolysis with deactivation of α–amylase from porcine pancreas (E_a, k_0 and E_d, k_{d0}) were used to optimize the process along with the optimal amount of enzyme used.

4. CONCLUSIONS

In the current work, the values of E_a, E_d, k_0 and k_{d0} for starch hydrolysis by α–amylase with deactivating enzyme were determined based on the experimental data of the temperature impact on the activity of α–amylase. Additionally, based on the parameters, the starch hydrolysis modeling results for the initial concentration equal to 5%, 10% and 20% at a temperature of 310 K and for the initial concentration of 20%, 30% and 40% at 333 K were presented.

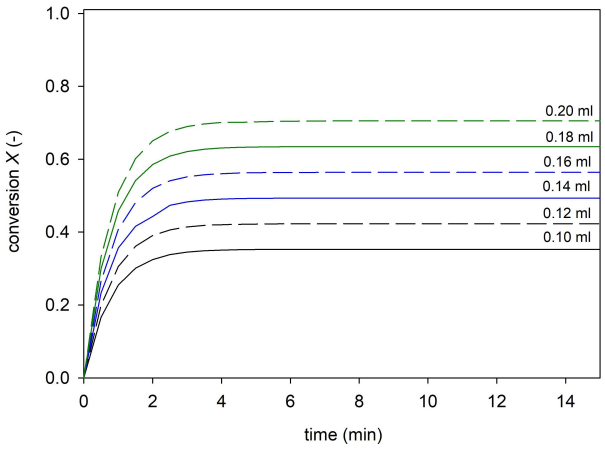

Figure 6. The conversion of starch hydrolysis by α–amylase from porcine pancreas at 333 K, starch concentration 40% and different quantities of the enzyme.

Undoubtedly, the obtained values allowed to better understand starch hydrolysis with deactivation of α–amylase and can be used to design, model and optimize the investigated process.

SYMBOLS

a	dimensionless enzyme activity, –
a_{exp}	α–amylase from porcine pancreas activity determined experimentally, –
$a_{cal}\,(Ed, \beta, T, T_{opt})$	α–amylase from porcine pancreas activity calculated from Eq. (6), –
E	the enzyme concentration, M
E_a	activation energy, J/mol
E_d	activation energy of the deactivation process, J/mol
F	Fisher test values,
k	enzymatic reaction rate constant, (1/min)
k_0	specific constant enzymatic reaction, (1/min)
k_d	rate constant of enzyme deactivation, (1/min)
$k_d(T_{opt})$	rate constant of enzyme deactivation in optimum temperature, (1/min)
k_{d0}	specific constant for α–amylase deactivation, (1/min)
P	probability value, –
R	gas constant, 8.314 (J/(mol K)
R^2	regression coefficient, –
S	the starch concentration, M
SSE	the sum of squares error
t	time of starch hydrolysis, min
T	temperature, K
T_{opt}	optimum temperature, K
Greek symbols	
β	dimensionless parameter, –

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