Determination of optimum temperatures and activation energies of inulin hydrolysis by endo-inulinase *Aspergillus niger*

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The paper presents a comparative analysis to determine the optimal temperatures and the activation energies for various origin endo-inulinases from *Aspergillus niger*. The parameters were estimated based on the literature of the activity curves vs. temperature for hydrolysis of inulin. It was assumed that both the hydrolysis reaction process and the deactivation process of endo-inulinase were first-order reactions by the enzyme concentration. A mathematical model describing the effect of temperature on endo-inulinases from *Aspergillus niger* activity was used. Based on the comparison analysis, values of the activation energies E_a were in the range from 23.53 ± 3.20 kJ/mol to 50.66 ± 3.61 kJ/mol, the deactivation energies E_d were in the range from 88.42 ± 5.03 kJ/mol to 142.87 ± 2.75 kJ/mol and the optimum temperatures T_{opt} were obtained in the range from 317.12 ± 0.83 K to 332.55 ± 0.72 for endo-inulinase *A. niger*.

Keywords: endo-inulinase *Aspergillus niger*, optimum temperature, activation energy, deactivation energy

1. INTRODUCTION

Inulin consists of linear chains of 2,1-β-D-fructofuranose molecules terminated with a glucose residue at the reducing end. Inulin is a reserve carbohydrate (0.5–22%) in plant tubers like Jerusalem artichoke, chicory, garlic and onion, leek, rye, barley, dandelion, burdock, banana (Chi et al., 2011; Xu et al., 2016). Inulin can be used for industrial purposes in the production of high-fructose syrups, bioethanol, fructose and glucose inulo-oligosaccharides, unicellular oils and production of a unicellular protein in the production of citric acid, butanediol, alcohols and lactic acid (Chi et al., 2009; 2011).

Inulinases (2,1-β-D-fructan fructanohydrolase) are biocatalysts, catalyze the hydrolysis of inulin to obtain fructose with a yield of about 90–95% (Singh and Gill, 2006). Inulinases can be divided into endo-inulinases (EC 3.2.1.7) and exo-inulinases (EC 3.2.1.80). Exo-inulinases remove terminal fructose residues from the non-reducing end of inulin, and endo-inulinases act on the internal bonds of the inulin molecule (Singh and Gill, 2006; Chi et al., 2009).

Inulinases occur in plants, fungi, yeasts and bacteria. Strains belonging to *Aspergillus sp.* and *Kluyveromyces sp.* are the most common and preferred for the production of inulinase. Inulinases obtained from them

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are characterized by high activity and high thermostability (Singh and Chauhan, 2018). The optimum value of pH for inulinase from *Aspergillus niger* is the same for both exo- and endo-inulinase and equals 5.0. However, the optimal temperature for *A. niger* exo-inulinase is above 55 °C and for endo-inulinase it is lower and amounts to about 40 °C (Singh and Gill, 2006) or higher for commercial endo-inulinases (Megazyme, 2020; Nguyen et al. 2011).

There have been many publications in the literature on *A. niger* endo-inulinase (Karimi et al., 2014; Megazyme, 2020; Nguyen et al. 2011; Zaita et al., 2000) used for immobilization process. However, processes involving endo-inulinase *A. niger* for industrial purposes cannot be designed and optimized without knowing the kinetic parameters of the process and thus the effect of temperature on activity endo-inulinase (Ricca et al., 2009). Hydrolysis with endo-inulinase *A. niger* is usually carried out at temperatures higher than 45 °C (Ohta et al., 2004), when a significant inactivation of the enzyme may occur. Therefore, it is necessary to determine the optimum temperature T_{opt}, activation energy E_a and activation energy of the deactivation process E_d for endo-inulinase *A. niger*. The work was aimed at modelling and determining parameters based on the available literature data. The method of determining the optimum temperatures and activation energies based on experimental data on the effect of temperature on the activity of endo-inulinase *A. niger* has been presented by Karimi et al. (2014), Megazyme, 2020, Nguyen et al. (2011) or Zaita et al. (2000).

2. DETERMINATION THE PARAMETERS

2.1. The effect of temperature on the endo-inulinase activity

The endo-inulinase activity of the inulin hydrolysis reaction changes under the influence of temperature. At temperatures below the optimum temperature (T_{opt}), the endo-inulinase activity increases with increasing temperature. After exceeding the optimum temperature (T_{opt}), the enzyme activity decreases. Both enzyme activity increase or decrease are described by the first-order equations due to the enzyme concentration (Karimi et al., 2014).

To determine the value of the optimal temperature (T_{opt}), the activation energy E_a and the deactivation energy E_d are used from the previously presented equation (Miłek, 2018; Miłek, 2020a; Wójcik and Miłek, 2016) describing the change in the absolute activity of the enzyme a vs. temperature T as follows:

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

The activation energy E_a is determined by the equation

$$E_a = E_d - \frac{E_d \cdot \beta}{\exp \beta - 1} \quad (2)$$

Dimensionless parameter β determines the relationship

$$\beta = k_{d0} t_a \exp\left(-\frac{E_d}{RT_{opt}}\right) \quad (3)$$

where k_{d0} is reaction time of the hydrolysis of inulin by endo-inulinase from *A. niger* and t_a is reaction time of the hydrolysis of inulin by endo-inulinase from *A. niger*.

The full analysis of the solution of Eq. (1) was presented in an earlier publication of Wojcik and Miłek (2016). Based on Eqs. (1) and (2), the kinetic parameters E_d, β and T_{opt} were determined by a non-linear

regression according to the Levenberg–Marquardt procedure (Miłek, 2018; 2020a; 2020b; Wójcik and Miłek, 2016), determining the minimum sum of squared errors defined by Eq. (3)

$$ SSE\left(E_d, \beta, T_{opt}\right) = \sum_{i=0}^{n} \frac{1}{a_{\exp}^2} \left(a_{\exp} - a_{cal}\left(E_d, \beta, T, T_{opt}\right)\right)^2 \tag{4} $$

2.2. Conditions for measuring endo-inulinase activity

Experimental literature data (Karimi et al., 2014; Megazyme, 2020; Nguyen et al., 2011; Zaita et al., 2000) were analysed for endo-inulinase from *A. niger* from various sources, including the commercial *A. niger* endo-inulinase from company Megazyme (Megazyme, 2020; Nguyen et al., 2011). Table 1 shows the conditions for measuring insulin activity, such as pH, buffer type and time measurement.

Table 1. Conditions for measuring endo-inulinase *Aspergillus niger* activity

Source of endo-inulinase	pH, buffer	t [min]	Ref.
A. niger 12 (Japan)*	5.0 acetate	30	Zaita et al. (2000)
Sigma–Aldrich (USA)	5.4 sodium acetate	30	Karimi et al. (2014)
Megazyme International Ireland (Ireland)	4.5 sodium acetate	10	Megazyme, 2020
Megazyme International Ireland (Ireland)	5.5 sodium acetate	30	Nguyen et al. (2011)

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Strains of *Aspergillus niger* in Sigma–Aldrich and Megazyme International Ireland come from their laboratory collections of each company.

Measurements of inulinase activity were determined using dinitrosalicylic acid (Karimi et al., 2014; Zaita et al., 2000) at 575 nm and the Nelson-Somogyi method at 620 nm, (Megazyme, 2020; Nguyen et al., 2011). The initial inulin concentration measured was in the range of 0.2% to 6%.

Karimi et al. (2014) and Zaita et al. (2000) studied endo-inulinase activity during the hydrolysis of inulin buffered with various buffers and at various pH values for 30 minutes. Megazyme International Ireland (Ireland) (Megazyme, 2020) *A. niger* endo-inulinase activity was determined at a pH of 4.5 and a reaction time of 10 min. The optimal activity of Megazyme International Ireland (Ireland) endo-inulinase was also showed at pH 5.5. Nguyen et al. (2011) studied Megazyme endo-inulinase activity at a higher pH with 30 minutes of hydrolysis.

3. RESULTS AND DISCUSSION

3.1. Effect of temperature on endo-inulinases from Aspergillus niger activity

Based on experimental data on the change in the activity of exo-inulinase from *A. niger* (Zaita et al., 2000; Karimi et al., 2014; Megazyme, 2020; Nguyen et al., 2011) values of deactivation energy (E_d), β parameter and optimal temperature (T_{opt}) were determined from Eq. (1) using non-linear regression from SigmaPlot 12.3 software. Figures 1–4 show experimental data on endo-inulinase activity, along with activity curves plotted based on Eq. (1) for the values of the specified parameters E_d, T_{opt}, β listed in Table 2.

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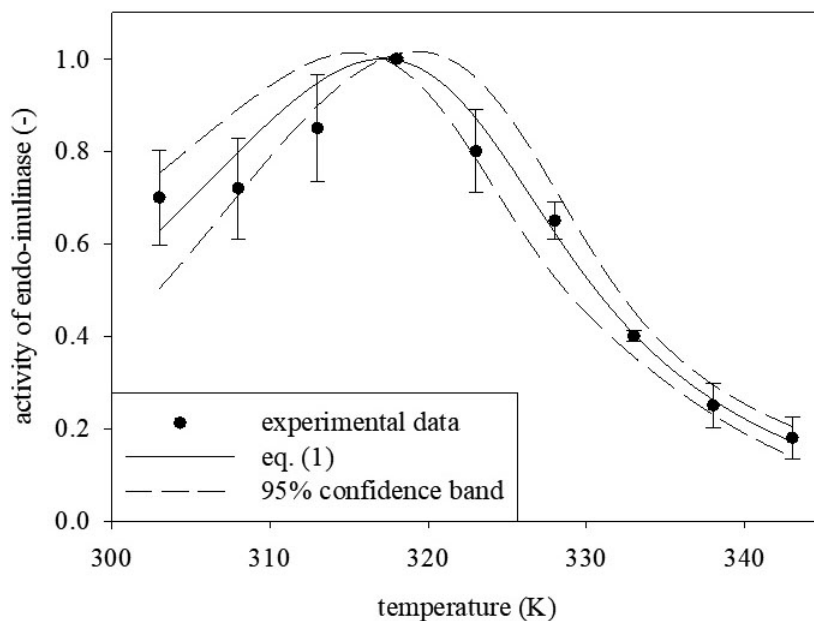*Justyna Miłek, Chem. Process Eng., 2020, 41 (3), 229–236*

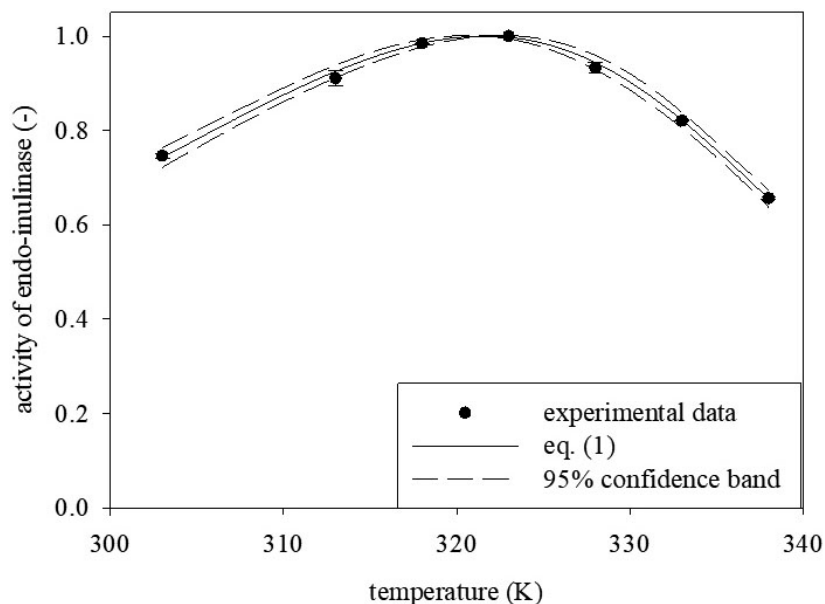Fig. 1. The activity of endo-inulinase *Aspergillus niger* by measurements Zaita et al. (2000)

Fig. 2. The activity of endo-inulinase *Aspergillus niger* by measurements Karimi et al. (2014)

Knowing the values of activation energy for the deactivation reaction E_d and the parameter β, the activation energy value was calculated from Eq. (2). The obtained results for endo-inulinase activity are shown and presented according to the increasing T_{opt} value in Table 2.

Table 3 presents statistical data obtained while determining the kinetic parameters of endo-inulinases from *Aspergillus niger*. High regression coefficients R^2 (above 0.98), standard errors of *SSE* estimation (below 0.0910) were obtained; while statistical variability of E_d and T_{opt} parameters in most analysed cases was $p < 0.0001$.

F-Fisher test values from 163.92 to 706.57 with a low probability value P equal to 0.0001 confirmed that it was appropriate to apply Eq. (1) when determining kinetics parameters. Also, Figs. 1–4 present standard deviation errors for experimental points, while the 95% confidence limits were marked for the obtained curves.
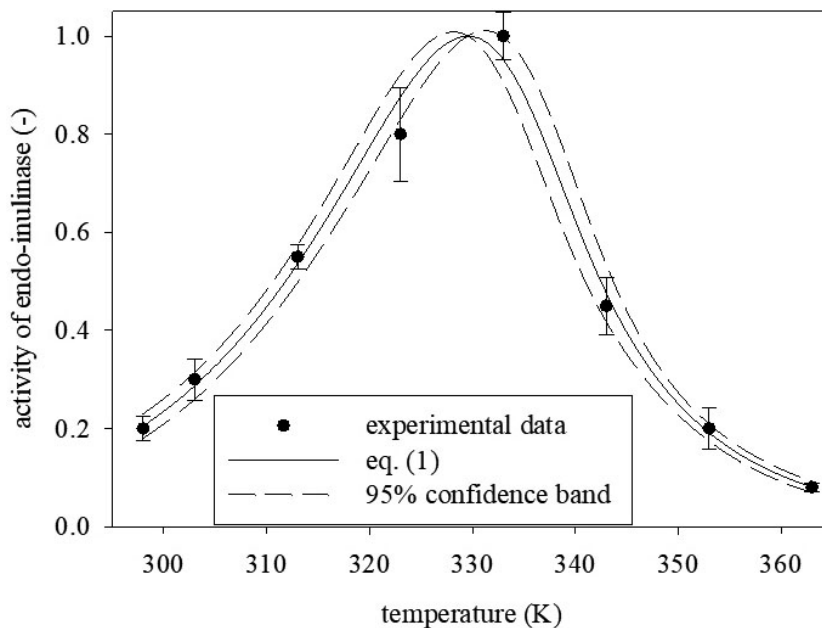
Fig. 3. The activity of commercial endo-inulinase *Aspergillus niger* (2020)
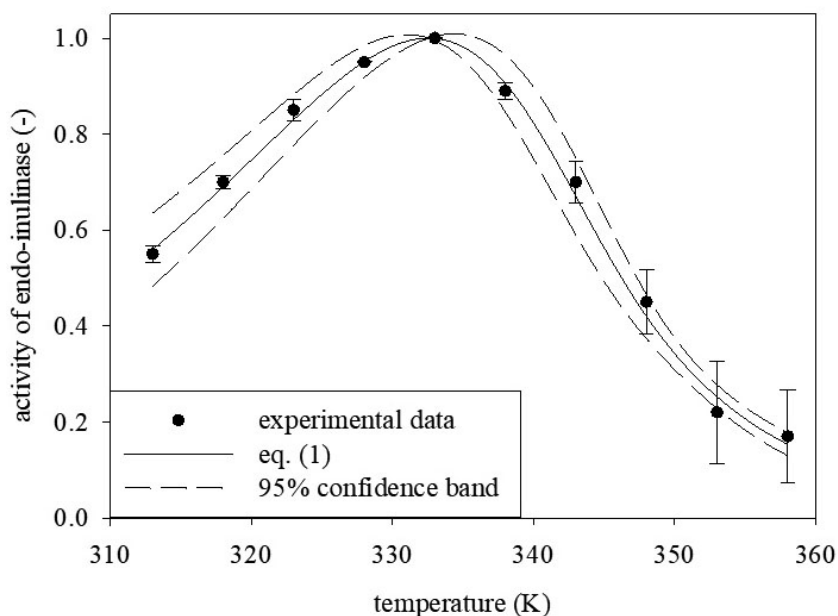

Fig. 4. The activity of commercial endo-inulinase *Aspergillus niger* by measurements Nguyen et al. (2011)

Table 2. Value of kinetic parameters estimated for endo-inulinase *Aspergillus niger*

Fig.	T, min	E_d, kJ/mol	T_{opt}, K	β	E_a, [kJ/mol]	E_d/E_a
1	30	127.78 ± 5.58	317.12 ± 0.83	0.83 ± 0.15	45.60 ± 8.87	2.80
2	30	88.42 ± 5.03	321.62 ± 0.36	0.59 ± 0.09	23.53 ± 3.20	3.46
3	10	142.87 ± 2.75	329.54 ± 0.62	0.82 ± 0.05	50.66 ± 3.61	2.82
4	30	142.09 ± 16.02	332.55 ± 0.72	0.59 ± 0.08	37.82 ± 9.28	3.77

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Table 3. Statistical data obtained by determining the kinetic parameters of endo-inulinases from *Aspergillus niger*

Fig.	R^2	SSE	p			F	P
			E_d, kJ/mol	T_{opt}, [K]	β		
1	0.9820	0.0915	< 0.0001	< 0.0001	0.0015	163.92	< 0.0001
2	0.9972	0.0105	< 0.0001	< 0.0001	0.0024	706.57	< 0.0001
3	0.9943	0.0631	< 0.0001	< 0.0001	< 0.0001	434.64	< 0.0001
4	0.9889	0.0745	< 0.0001	< 0.0001	0.0002	312.10	< 0.0001

3.2. Optimum temperature

The optimum temperature T_{opt} for endo-inulinase from *A. niger* has been determined and its value was in the range from 313 K to 328 K (Ohta et al., 2004) when the activity measurements were performed during hydrolysis of inulin aqueous solution. The estimated average values of the optimal temperatures for the experimental data (Karimi et al., 2014; Megazyme, 2020; Nguyen et al., 2011; Zaita et al., 2000) were found in the range from 317.12 ± 0.83 K to 332.55 ± 0.72 K. For the commercial *A. niger* endo-inulinase from Megazyme (Megazyme, 2020; Nguyen et al., 2011), it was found that they were 329.54 ± 0.62 K and 332.55 ± 0.72 K, respectively, and these values were about 10 K higher than those for endo-inulinase *A. niger* obtained from *A. niger* 12 strain and from Sigma–Aldrich. The values T_{opt} found in this work are well within the range of values reported in the literature (Ohta et al., 2004).

3.3. Activation energy E_a

The calculated energy values for activation of E_a are from 23.53 ± 3.20 kJ/mol to 50.66 ± 3.61 kJ/mol for endo-inulinase *A. niger*. Karimi et al. (2014) determined the activation energy E_a for *A. niger* endo-inulinase equal to 12.97 kJ/mol and this value is 45% lower than the calculated value from Eq. (2) and shown in Table 2.

Skowronek and Fiedurek (2006) determined the activation energy for *A. niger* 20 OSM (strain from Collection of Industrial Microbiology, Maria Curie-Skłodowska University, Lublin) endo-inulinase as equal to 58.33 kJ/mol. Literature activation energy data for the commercial liquid mixture exo- and endo-inulinase *A. niger* (Fructozyme L^{TM}, Novozymes, A/S, Denmark) determined by Ricca et al. (2009) was 78.57 kJ/mol. The values E_a are well within the range of values reported in the literature (Karimi et al. 2014; Ricca et al., 2009; Skowronek and Fiedurek, 2006).

3.4. The activation energy for enzyme deactivation E_a

The values of activation energies of the deactivation reaction (E_d) for endo-inulinases *A. niger* were obtained in the range from 88.42 ± 5.03 kJ/mol to 142.87 ± 2.75 kJ/mol (Table 2). The activation energy of the deactivation reaction (E_d) of the commercial exo- and endo-inulinase of *Aspergillus niger* for marked k_d Ricca et al. (2009) calculated as equal to 313.47 kJ/mol. The values E_d are well within the range of values reported in the literature.Knowing the E_d/E_a relationship for a given *A. niger* endo-inulinase one can choose the enzyme with the highest thermal stability. The endo-inulinase *Aspergillus niger* from Megazyme International Ireland (Ireland), is more thermally stable than the endo-inulinase *Aspergillus niger* from Sigma–Aldrich. The lower the E_d/E_a value of a given *A. niger* endo-inulinase, the more stable the enzyme is.

4. CONCLUSIONS

The method of determining the following thermodynamic parameters: the activation energies (E_a) and the deactivation energies (E_d) as well as the optimal temperatures T_{opt} of inulin hydrolysis reaction by endo-inulinase *Aspergillus niger* based on curves of changes in endo-inulinase *A. niger* depending on the temperature of hydrolysis was applied.

For the optimum temperatures (T_{opt}), the difference between the obtained values is about 15 K. The differences in the calculated values of the activation energy of the reaction E_a are about 27 kJ/mol. The differences in the calculated values of energy E_d are about 55 kJ/mol for endo-inulinase *A. niger* of different origin.

The commercial endo-inulinases *A. niger* were characterised by higher values of optimal temperature T_{opt} as well as higher values of energy E_d than non-commercial endo-inulinases *A. niger*. The E_a, E_d, T_{opt} values determined from activity vs. temperature curves were depending on the origin of inulinases and conditions (reaction time, pH) of activity measurement.

SYMBOLS

a	dimensionless enzyme activity, –
$a_{\exp}$	endo-inulinase from *Aspergillus niger* activity determined experimentally, –
$a_{cal}\left(E_d, \beta, T, T_{opt}\right)$	endo-inulinase from *Aspergillus niger* activity calculated from Eq. (1), –
E_a	activation energy, J/mol
E_d	activation energy of the deactivation process, J/mol
F	Fisher test values
P	probability value
R	gas constant, 8.314 (J/(mol K)
R^2	regression coefficients,
SSE	the sum of squared of errors
T	temperature, K
T_{opt}	optimum temperature, K

Greek symbols

β	dimensionless parameter, –

REFERENCES

Chi Z. M., Zhang T., Cao T.S., Liu X. Y., Cui W., Zhao C. H., 2011. Biotechnological potential of inulin for bioprocesses. *Bioresour. Technol.*, 102, 4295–4303. DOI: 10.1016/j.biortech.2010.12.086.

Chi Z., Chi Z., Zhang T., Liu G., Yue L., 2009. Inulinase-expressing microorganisms and applications of inulinases. *Appl. Microbiol. Biotechnol.*, 82, 211–220. DOI: 10.1007/s00253-008-1827-1.

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Justyna Miłek, Chem. Process Eng., 2020, 41 (3), 229–236

Karimi M., Chaudhury I., Jianjun C., Safari M., Sadeghi R., Habibi-Rezaei M., Kokini J., 2014. Immobilization of endo-inulinase on non-porous aminofunctionalized silica nanoparticles. *J. Molecular Catalysis B: Enzymatic.*, 104, 48–55. DOI: 10.1016/j.molcatb.2014.01.025.

Megazyme, 2000. Endo inulinase *Aspergillus niger*. (Lot 121002b). Available at: https://www.megazyme.com/documents/Booklet/E-ENDOIAN_DATA.pdf.

Miłek J., 2018. Estimation of the kinetic parameters for H_2O_2 enzymatic decomposition and for catalase deactivation. *Braz. J. Chem. Eng.*, 35, 995–1004. DOI: 10.1590/0104-6632.20180353s20160617.

Miłek J., 2020a. Calculation of temperature optimum as well as activation and deactivation energy for the olive oil hydrolysis with porcine pancreas lipase. *Przem. Chem.*, 99, 585–587. DOI: 10.15199/62.2020.4.14.

Miłek J., 2020b. Determination the optimum temperature and activation energies for the hydrolysis of starch by α-amylase *Bacillus licheniformis*. *Przem. Chem.*, 99, 880–881. DOI: 10.15199/62.2020.6.9.

Nguyen Q. D., Rezessy-Szabo J. M., Czukor B., Hoschke A., 2011. Continuous production of oligofructose syrup from Jerusalem artichoke juice by immobilized endo-inulinase. *Process Biochem.*, 46, 298–303. DOI: 10.1016/j.procbio.2010.08.028.

Ohta K., Akimoto H., Moriyama S., 2004. Fungal inulinases: Enzymology, molecular biology and biotechnology. *J. Appl. Glycosci.*, 51, 247–25. DOI: 10.5458/JAG.51.247.

Ricca E., Calabrò V., Curcio S., Iorio G., 2009. Optimization of inulin hydrolysis by inulinase accounting for enzyme time- and temperature-dependent deactivation. *Biochem. Eng. J.*, 48, 81–86. DOI: 10.1016/j.bej.2009.08.009.

Singh P., Gill P.K., 2006. Production of inulinases: recent advances. *Food Technol. Biotechnol.*, 44,151–162.

Singh R. S., Chauhan K., 2018. Production, purification, characterization and applications of fungal inulinases. *Current Biotechnol.*, 7, 242–260. DOI: 10.2174/2211550105666160512142330.

Skowronek M., Fiedurek J., 2006. Purification and properties of extracellular endoinulinase from *Aspergillus niger* 20 OSM. *Food Technol. Biotechnol.*, 44, 53–58.

Wojcik M., Miłek J., 2016. A new method to determine optimum temperature and activation energies for enzymatic reactions. *Bioprocess Biosyst. Eng.*, 39, 1319–1323. DOI: 10.1007/s00449-016-1596-7.

Xu Y., Zheng Z., Xu Q., Yong Q., Ouyang J., 2016. Efficient conversion of inulin to inulooligosaccharides through endoinulinase from *Aspergillus niger*. *J. Agric. Food Chem.*, 64, 2612–2618. DOI: 10.1021/acs.jafc.5b05908.

Zaita N., Fukushige T., Tokuda M., Ohta K., Nakamura T., 2000. Preparation and enzymatic properties of onto various polysaccharide supports. *Food Sci. Technol. Res.*, 6, 34–39.