Determinition 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 $23.53 \pm 3.20$ kJ/mol to $50.66 \pm 3.61$ kJ/mol, the deactivation energies $E_d$ were in the range from $88.42 \pm 5.03$ kJ/mol to $142.87 \pm 2.75$ kJ/mol and the optimum temperatures $T_{opt}$ were obtained in the range from $317.12 \pm 0.83$ K to $332.55 \pm 0.72$ for endo-inulinase A. niger.

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

1. INTRODUCTION

Inulin consists of linear chains of $\beta$-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
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:

$$a = \frac{\exp \left( \frac{(T_{opt} - T)}{RT_{opt}} \cdot \frac{E_d}{(\exp \beta - 1)} \right) \left( 1 - \exp \left( -\beta \exp \left( \frac{E_d}{RT_{opt}} \right) \right) \right)}{1 - \exp(-\beta)}$$  \hspace{1cm} (1)

The activation energy $E_a$ is determined by the equation

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

Dimensionless parameter $\beta$ determines the relationship

$$\beta = k_{d0} t_a \exp \left( \frac{E_d}{RT_{opt}} \right)$$  \hspace{1cm} (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 Milek (2016). Based on Eqs. (1) and (2), the kinetic parameters $E_d$, $\beta$ 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)

\[
SSE(E_d, \beta, T_{opt}) = \sum_{i=1}^{n} \frac{a_{exp}}{a_{exp}} - a_{cal}(E_d, \beta, T; T_{opt})^2
\]

(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

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

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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), \( \beta \) 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}, \( \beta \) listed in Table 2.
Knowing the values of activation energy for the deactivation reaction $E_d$ and the parameter $\beta$, 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-inulinsases 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.
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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*

<table>
<thead>
<tr>
<th>Fig.</th>
<th>$T$, min</th>
<th>$E_d$, [kJ/mol]</th>
<th>$T_{opt}$, K</th>
<th>$\beta$</th>
<th>$E_a$, [kJ/mol]</th>
<th>$E_d/E_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>127.78 ± 5.58</td>
<td>317.12 ± 0.83</td>
<td>0.83 ± 0.15</td>
<td>45.60 ± 8.87</td>
<td>2.80</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>88.42 ± 5.03</td>
<td>321.62 ± 0.36</td>
<td>0.59 ± 0.09</td>
<td>23.53 ± 3.20</td>
<td>3.46</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>142.87 ± 2.75</td>
<td>329.54 ± 0.62</td>
<td>0.82 ± 0.05</td>
<td>50.66 ± 3.61</td>
<td>2.82</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>142.09 ± 16.02</td>
<td>332.55 ± 0.72</td>
<td>0.59 ± 0.08</td>
<td>37.82 ± 9.28</td>
<td>3.77</td>
</tr>
</tbody>
</table>
Table 3. Statistical data obtained by determining the kinetic parameters of endo-inulinases from *Aspergillus niger*

<table>
<thead>
<tr>
<th>Fig.</th>
<th>$R^2$</th>
<th>SSE</th>
<th>$E_d$, kJ/mol</th>
<th>$T_{opt}$, [K]</th>
<th>$\beta$</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9820</td>
<td>0.0915</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0015</td>
<td>163.92</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>2</td>
<td>0.9972</td>
<td>0.0105</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0024</td>
<td>706.57</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>3</td>
<td>0.9943</td>
<td>0.0631</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0001</td>
<td>434.64</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>4</td>
<td>0.9889</td>
<td>0.0745</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0002</td>
<td>312.10</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

### 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 ± 0.83 K to 332 ± 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™, 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 $E_d$

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

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

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REFERENCES


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