Mathematical modelling of glucoamylase catalyzed saccharification process

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Glucoamylase obtained from Novozyme was used as a catalyst in the reaction of maltose hydrolysis. Several other disaccharides and oligosaccharides were tested as substrates for this enzyme as well. Commercially known as Dextrozyme, this enzyme has pullulanase (along with glucoamylase) in its contents. Pullulanase prevents the reverse reaction according to several authors. Optimal pH and temperature were found for the enzyme activity: pH 5.5 and 40 °C. Under these conditions maltose hydrolysis were carried out in different reactor types. Enzyme kinetics were determined, and mathematical models for each reactor type were developed. Maltose hydrolysis was carried out in the batch, repetitive batch, fed-batch and continuously operated enzyme membrane reactor. Dextrozyme showed good operational stability in all experiments. Enzyme deactivation was not detected during its prolonged use. This was especially showed in the continuously operated enzyme membrane reactor where Dextrozyme was active for more than 35 days with maltose conversion nearly 100 % during the whole time.

Introduction

The starch processing industry is entirely dependent upon the use of enzymes for large scale production [Crabb & Shetty, 1999]. Among these enzymes amylases and glucoamylases are the most important. They catalyze the hydrolysis of various polysaccharides to lower sugars or glucose. These hydrolyzates are used in the fermentative production of alcohols, amino acids, biopolymers, carboxylic acids and enzymes [Koch & Röper, 1988]. Therefore, they represent a starting material for chemical and biochemical syntheses.

Glucoamylases are important biocatalysts with industrial application in the production of crystalline glucose or glucose syrup. They are used both in soluble and immobilized form [Norouzian et al., 2006]. They catalyze carbohydrate hydrolysis [Nikolov et al, 1989; Beschov et al, 1984] by acting on α-1,4-linkages. α-1,6-links are also hydrolyzed, but much slower [Roy et al., 2004]. Hydrolysis is a reversible reaction and glucose as a product is involved in condensation reaction in which various α-linked di- and trisaccharides are formed [Nikolov et al, 1989; Pestlin et al., 1997]. The products are oligosaccharides linked by the resistant α-1,6-linkages [Crabb & Shetty, 1999]. The reverse reaction is especially noticed in concentrated D-glucose solutions [Pazur & Okada, 1967]. However, if pullulanase is added to the reaction solution, the reverse reaction of glucose condensation can be avoided. Pullulanase is a debranching enzyme which hydrolyzes α-1,6-links in the chain and is generally used along with glucoamylase to obtain glucose from gelatinized starch [Roy et al., 2004]. This mixture of enzymes is present in Dextrozyme, a commercial enzyme preparation used in this work.

Maltose is one of the by-products of α-amylase catalyzed starch hydrolysis. That makes it an interesting substrate for glucoamylase, if simultaneous liquefaction and saccharification are carried out. The aim of these processes is maximal glucose concentration, and glucoamylase is therefore necessary for the hydrolysis of lower sugars (oligosaccharides – including maltose) to glucose.
Optimization of the initial reaction conditions of the experiment is necessary to obtain optimal results. Besides pH and temperature optimization to obtain maximal enzyme activity, it is also necessary to optimize enzyme concentration, as well as to determine the enzyme kinetics. With determined enzyme kinetics, it is possible to develop a mathematical model of the process. Mathematical models are very useful for the design and optimization of biochemical processes [Bryjak et al., 2004]. They lead to their better understanding, and the ability to predict various situations without the necessity to carry out additional experiments. When one substrate is considered as a single-reacting component in the sugar hydrolysis, simple kinetic expressions like Michaelis-Menten kinetics are often used to describe the enzyme kinetics [Cepeda et al., 2001]. The initial reaction rate method is often used to determine enzyme kinetics. This is a method which involves the measurement of the concentration vs time curves at the initial period of the reaction. From the linear period of this dependence (<10% substrate conversion) initial reaction rate is calculated.

In this work, Dextrozyme, a commercial enzyme preparation that has pullulanase along with glucoamylase in its composition, was used in hydrolysis of various sugars. Detailed measurements were carried out with maltose as a substrate. Operational stability and enzyme activity were examined in various reactor experiments.

**Experimental part**

*Enzyme activity*

Glucoamylase activity was measured on maltose as a substrate according to the initial reaction rate method. 5 ml reactor was used for that purpose. For every point on the diagram (initial reaction rate vs maltose concentration) a batch experiment for a certain maltose concentration was carried out. Low enzyme concentration was used to achieve slow reaction. Samples at the beginning of the reaction were taken and analyzed for glucose (under 10% conversion). 1 Unit of glucoamylase activity was defined as the amount of enzyme liberating 1 µmol of glucose per one minute. Glucose concentration was measured by HPLC with RI detector.

*Determination of maltose and glucose concentration by HPLC*

Maltose and glucose concentrations were followed by HPLC (Shimadzu, Japan) with a C\textsubscript{18} column (Carbohydrate Ca\textsuperscript{2+}, 300 x 6.5 mm, CS-Chromatographie service GmbH) and RI detector. The mobile phase was redistilled water at the flow 0.5 cm\textsuperscript{3} min. The analysis was performed at 80 °C. Standard solutions were prepared by dissolving appropriate masses of glucose and maltose in redistilled water. The retention times of maltose and glucose were 7.5 and 10.0 min respectively.

*Reactor experiments*

The experiments of maltose hydrolysis were carried out in four reactor types: batch, repetitive batch, fed-batch and the continuously operated enzyme membrane reactor. A detailed description of each experiment is presented below. All experiments were carried out at 40 °C and 0.1 mol dm\textsuperscript{-3} phosphate buffer pH 5.5. The reaction was started by adding the enzyme to the reactor vessel.

Batch and the repetitive batch reactor experiments were carried out in a 25 cm\textsuperscript{3} glass vessel. It was placed on the magnetic stirrer and thermostated to ensure the homogeneity and constant temperature of the reaction solution. 10-100 µl samples were taken from the reactor and analyzed for glucose and maltose. After all substrate was hydrolyzed in the repetitive batch experiments, a new amount was added in the reactor. The product was not charged out.
of the reactor. The new amount of substrate was dissolved in a small amount of buffer which could compensate the volume loss in the first cycle (due to sampling).

The fed-batch experiment was started as a batch with the initial volume of 25 cm$^3$. The total reactor volume was 400 cm$^3$. The initial concentration of maltose was 25 g dm$^{-3}$. After initial concentration of maltose was spent, an addition of new maltose solution was started. Its concentration was 100 g cm$^{-3}$ and the flow was set to 3.4 ml h$^{-1}$. Piston pump was used to ensure the constant flow of the reaction solution. The volume of the reaction solution slowly started to increase up to 300 cm$^3$, and then the experiment was stopped. Samples were taken and analyzed during the experiment.

Continuously operated enzyme membrane reactor (EMR) [Giorno & Drioli, 2000; Vasić-Rački et al., 2003; Vasić-Rački et al., 2003a] (which operates as continuous stirred tank reactor) experiments were carried out in 10 cm$^3$ reactor volume. A polymer membrane (cut off 20 kDa) was used to retain the enzymes in the reactor. The molecular weight of glucoamylases from various fungal sources is usually in the range of 48 to 90 kDa, except for glucoamylase from *Aspergillus niger* which has a molecular weight of 125 kDa [Suresh et al., 1999]. The reaction was started by adding the enzyme through the injection septum. An alternating piston pump was used for constant delivery of substrate solution and to achieve the desired residence time in the enzyme membrane reactor. The flow was regularly checked during the experiment. The reaction mixture was stirred on a magnetic stirrer. The enzyme membrane reactor was thermostated at 40 °C. The reaction solution that was pumped in the reactor was kept in a separate bottle on a magnetic stirrer. Samples from the reactor outlet were taken and analyzed regularly during the experiment.

Data handling

Parameters of the mathematical model were estimated by non-linear regression analysis using Simplex and Least Squares method implemented in SCIENTIST software [SCIENTIST handbook]. They were estimated by fitting the model to the experimental data. The calculated data were compared with the experimental data, recalculated in the optimization routine and fitted again until a minimal error between experimental and integrated values was achieved. The residual was defined as the sum of the squares of the differences between the experimental and calculated data. The "Episode" algorithm implemented in the SCIENTIST software was used for simulations.

Results and discussion

Enzyme kinetics

Glucoamylase activity was measured at different pH's and temperatures to determine the optimal conditions for its activity. The highest activity was obtained at 40 °C and pH 5.5 (0.1 mol dm$^{-3}$ phosphate buffer). All experiments were carried out at these conditions. The kinetics of glucoamylase was measured according to the initial reaction rate method. Enzyme activity was examined with five different substrates: maltose, starch, amylose, amylopectin and dextrin. Kinetic parameters were estimated from the experimental data: initial reaction rate vs concentration of different substrates. The obtained results are presented in Table 1. They show that the enzyme has the highest activity towards maltose, and has the highest affinity towards amylopectin as a substrate.
Table 1. Kinetic parameters estimated from the dependencies of initial reaction rate on the concentration of substrate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_m$ [g dm$^{-3}$ min$^{-1}$]</th>
<th>$K_m$ [g dm$^{-3}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>amylase</td>
<td>4278.66 ± 2.93</td>
<td>16.275 ± 1.68</td>
</tr>
<tr>
<td>amyllopectin</td>
<td>1249.56 ± 17.30</td>
<td>0.0096 ± 0.0031</td>
</tr>
<tr>
<td>dextrin</td>
<td>7039.68 ± 218.84</td>
<td>1.43 ± 0.16</td>
</tr>
<tr>
<td>maltose</td>
<td>9398.50 ± 425.86</td>
<td>1.24 ± 0.34</td>
</tr>
<tr>
<td>starch</td>
<td>767.75 ± 41.27</td>
<td>0.212 ± 0.062</td>
</tr>
</tbody>
</table>

Considering these results, and substrate solubility in buffer, maltose was chosen as a substrate for further characterization of Dextrozyme. The effect of glucose (reaction product) on the initial reaction rate of maltose hydrolysis was studied as well. It was found that glucose does not inhibit the enzyme.

**Mathematical modeling**

Maltose hydrolysis kinetics was described by pure Michaelis-Menten kinetics (Eq 1). The initial reaction rate experiments showed that there is no product inhibition.

$$r = \frac{V_m \cdot c_{\text{maltose}}}{K_m + c_{\text{maltose}}}$$  \hspace{1cm} (1)

Mass balances for maltose and glucose in the batch (and repetitive batch) reactor are presented by the equations 2 and 3. Equations 4 and 5 represent the mass balances of maltose and glucose in the fed batch reactor, whereas equation 6 represents the mass balance of the enzyme in the fed-batch reactor. Namely, since maltose solution is being pumped to the reactor during the experiment, the volume is changed (increased) and the enzyme concentration is lowered - diluted. Mass balances for maltose and glucose in the continuously operated enzyme membrane reactor are presented by the equation 7 and 8.

$$\frac{dc_{\text{maltose}}}{dt} = -r$$ \hspace{1cm} (2)

$$\frac{dc_{\text{glucose}}}{dt} = r$$ \hspace{1cm} (3)

$$\frac{dc_{\text{maltose}}}{dt} = \frac{1}{V} \left( -c_{\text{maltose}} \cdot \frac{dV}{dt} + c_{\text{maltose},0} \cdot q \right) - r$$ \hspace{1cm} (4)

$$\frac{dc_{\text{glucose}}}{dt} = \frac{1}{V} \left( -c_{\text{glucose}} \cdot \frac{dV}{dt} \right) + r$$ \hspace{1cm} (5)

$$\frac{dc_{\text{enzyme}}}{dt} = \frac{1}{V} \left( -c_{\text{enzyme}} \cdot \frac{dV}{dt} \right)$$ \hspace{1cm} (6)

$$\frac{dc_{\text{maltose}}}{dt} = \frac{c_{\text{maltose},0} - c_{\text{maltose}}}{\tau} - r$$ \hspace{1cm} (7)

$$\frac{dc_{\text{glucose}}}{dt} = \frac{c_{\text{glucose},0} - c_{\text{glucose}}}{\tau} + r$$ \hspace{1cm} (8)
Reactor experiments

Maltose hydrolysis was carried out in four reactors: batch, repetitive batch, fed-batch, and continuously operated enzyme membrane reactor to examine the enzyme’s operational stability and activity. The results are presented in Figures 1-4. The purpose of these experiments was also to validate the mathematical model developed for each reactor type.

Figure 1. Maltose hydrolysis catalyzed by Dextrozyme in the batch reactor ($c_{\text{maltose,0}} = 27.29 \text{ g dm}^{-3}$, 40 °C, 0.1 mol dm$^{-3}$ phosphate buffer pH 5.5, 5216.4 U of Dextrozyme were added in the reactor, $V_{\text{reactor}} = 25 \text{ cm}^3$).

Figure 1 represents the results of the batch reactor experiment. The mathematical model used to simulate the results (kinetic equation: 1, mass balance equation: 2 and 3) fits well the data. 100 % conversion of maltose to glucose was achieved. The next step was the repetitive batch experiment presented in Figure 2. In the repetitive use of enzyme in these experiments, the enzyme deactivation was tested. The products and enzyme were not charged out of the reactor, and after all substrate (maltose) was hydrolyzed, an additional amount was added in the reactor as described in the experimental part. 100 % maltose conversion was achieved in this reactor as well. There was also no activity loss in the second and third batch. The mathematical model (kinetic equation: 1, mass balance equation: 2 and 3) simulated well the experimental data.

Figure 2. Maltose hydrolysis catalyzed by Dextrozyme in the repetitive batch experiment ($c_{\text{maltose,0}} = 27.29 \text{ g dm}^{-3}$, $c_{\text{maltose,1}} = 20.60 \text{ g dm}^{-3}$ and $c_{\text{maltose,2}} = 20.40 \text{ g dm}^{-3}$, 40 °C, 0.1 mol dm$^{-3}$ phosphate buffer pH 5.5, 5216.4 U of Dextrozyme were added in the reactor, $V_{\text{reactor}} = 0.025 \text{ dm}^3$).
Maltose hydrolysis was also carried out in the fed-batch reactor. The results are presented in Figure 3. The experiment was started as a batch in 25 cm³ volume. After initial concentration of maltose was spent, maltose solution was pumped in the reactor. The developed mathematical model (kinetic equation: 1, mass balance equation: 4, 5 and 6) described the data well. There was no enzyme deactivation in this reactor type as well. Maltose concentration was very low – 1-2 mmol dm⁻³ during the whole experiment, which proves high efficiency of the enzyme. End concentration of glucose was cca 510 mmol dm⁻³.

The fourth experiment was carried out in the continuously operated enzyme membrane reactor, and the results are presented in Figure 4. This experiment lasted for 35 days, and it was ended due to the pump failure. However, it can be seen, that Dextrozyme is very stable and active through a very long time, which makes it an ideal candidate for the continuous use. The reactor was opened at the end of this experiment, and the membrane was removed. From the way it looked, it was evident that the enzyme immobilized itself to the membrane, which probably contributed to its long-term activity. Practically 100% conversion of maltose was achieved during the whole time of the experiment. The developed mathematical model (kinetic equation: 1, mass balance equation: 7 and 8) describes the data well.
Table 2. Comparison of the volumetric productivity in different reactors.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Volumetric productivity</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>7.28 \cdot 10^{-4}</td>
<td>mmol/U</td>
</tr>
<tr>
<td>Repetitive batch</td>
<td>1.95 \cdot 10^{-3}</td>
<td>mmol/U</td>
</tr>
<tr>
<td>Fed-batch</td>
<td>1.92 \cdot 10^{-2}</td>
<td>mmol/U</td>
</tr>
<tr>
<td>Continuously operated EMR</td>
<td>3.07 \cdot 10^{-4}</td>
<td>mmol/U</td>
</tr>
</tbody>
</table>

Dextrozyme is an excellent enzyme with high activity and affinity towards maltose as a substrate. Since it shows no deactivation during the continuous or repetitive use, it is ideal for industry. Especially for the continuous processes which enable the constant product quality. Repetitive batch experiment can also be a good option, since the same amount of enzyme is being reused, which makes the process cheaper.

Different reactors were compared according to volumetric productivity as well. It was calculated according to equation 9. The results were presented in Table 2.

\[ Q_v = \frac{\text{mmol of product}}{\text{Units of enzyme}} = \frac{\text{mmol}}{U} \]  

(9)

The best volumetric productivity was achieved in the fed-batch reactor, and the lowest in the continuously operated enzyme membrane reactor.

Conclusion

Dextrozyme shows activity to different sugars: maltose, starch, amylose, amylopectin and dextrin. It has the highest activity towards maltose as a substrate (the lowest estimated Michaelis constant value). The reactor experiments showed that Dextrozyme has high operational stability at all reactor conditions when maltose was used as a substrate. Maltose conversion was 100% in all experiments. There was no sign of the reverse condensation reaction which shows that pullulanase present in the system was active and efficient in same time. The best reactor type, according to the volumetric productivity, was fed-batch reactor. The lowest volumetric productivity was achieved in the continuously operated enzyme membrane reactor.

Acknowledgements

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List of symbols

- \( c \) concentration, g dm\(^{-3} \)
- \( \text{EMR} \) enzyme membrane reactor
- \( K_m \) Michaelis constant, g dm\(^{-3} \)
- \( q \) flow rate, dm\(^3\) min\(^{-1} \)
- \( Q_v \) volumetric productivity, mmol U\(^{-1} \)
- \( r \) reaction rate or enzyme activity, g dm\(^{-3} \) min\(^{-1} \)
- \( t \) time, min, h, or d
- \( V \) reactor volume, dm\(^3\)
- \( V_m \) maximal reaction rate, g dm\(^{-3} \) min\(^{-1} \)
Greek symbols

References