Operational Stability of Glucoamylase in Continuously Operated Ultrafiltration Membrane Reactor – Experimental Methods and Mathematical Model

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doi: 10.15255/CABEQ.2014.1916

Original scientific paper
Received: January 14, 2014
Accepted: November 16, 2014

Dedicated to prof. Egon Bauman

Operational stability of glucoamylase was studied at 45, 60 and 70 °C in the reaction of maltose hydrolysis. The experiments were carried out in a continuously operated ultrafiltration membrane reactor (UFMR) at constant residence time of 176.5 minutes. The rate of enzyme operational stability decay increased with temperature. This could be quantitatively observed from the measurements of volume activity during the experiments, which were used to estimate enzyme operational stability decay rate constants. The results have shown that stationary conditions in UFMR can be maintained if sufficiently high enzyme concentration is used in the reactor, regardless of the enzyme operational stability decay that occurs. This was shown by the experiment carried out at 45 °C where it was proved that the enzyme operational decay occurs even though maltose conversion was at maximum during the entire experiment. Thus, the operational stability decay can be masked.

Key words:
biotransformation, Dextrozyme, ultrafiltration enzyme membrane reactor, enzyme activity, enzyme operational stability decay

Introduction

The rate of a reaction catalyzed by enzymes is influenced by various factors, including temperature, pH, chemical substances, etc.[1] In this work, the influence of temperature on enzyme operational stability was studied. Temperature deactivation of an enzyme is usually defined as a transformation of an active enzyme into denaturated (deactivated) form, described by an irreversible first-order reaction[2–5]. Denaturation of most proteins occurs in the temperature range of 45 – 50 °C. Due to temperature increase, the energy level of atoms in a protein molecule grows higher and denaturation, *i.e.* thermodenaturation[6,7,8], occurs when the atoms attain enough energy to break the connections inside the globular protein structure[1]. This causes the loss of the protein's biological activity, which is directly related to molecule conformation[9]. The Van't Hoff rule applies to enzyme catalyzed reactions, as well as to chemical reactions. This means that a temperature increase of 10 °C increases the reaction rate by two to three times[2,10]. Further temperature increase causes a decrease in the reaction rate[11,12] due to the limited stability of proteins[8,10]. Therefore, an increase in temperature increases the reaction rate but also the rate of deactivation, *i.e.* it decreases enzyme stability[2]. The dependence of the reaction rate on temperature shows a maximum. The temperature at which the maximum of the reaction rate is achieved is not an optimal temperature, because at that point starts the irreversible process of denaturation[10]. Enzyme deactivation is also influenced by the time of exposure to elevated temperature[10,13,14]. Therefore, the temperature maximum of an enzyme is not a constant value[10].

The reaction of maltose hydrolysis catalyzed by Dextrozyme was studied in this work. Dextrozyme is a mixture of glucoamylase and pullulanase. Glucoamylase catalyzes the maltose hydrolysis, and pullulanase prevents the reverse reaction. Hence, the enzyme of main interest in this system was glucoamylase, and its operational stability was studied at different temperatures.

Levenspiel[15] suggested two methods for the investigation of catalyst operational stability in a continuous process. The first method involves attaining constant conversion in a continuously operated reactor, which is achieved by decreasing the flow rate, *i.e.* increasing the residence time. Fast and efficient analysis is essential for rapid response to

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the conversion changes when using this method. Considering the system in question and slow sample analysis in this work, the second method was used. In this method, the flow rate of the reaction solution (*i.e.* residence time) is kept constant and the substrate conversion at the reactor outlet is followed. Thus, the conversion decrease refers to the decrease in enzyme activity and its operational stability decay[15]. The main disadvantage of this method is that the enzyme operational stability decay can go undetected due to high enzyme concentration in the reactor, which was seen in previous research of the system in question[16]. To avoid such a problem in this research, enzyme activity was followed directly in the reactor and parallel and independently of substrate conversion.

Experimental part

Chemicals

Potassium dihydrogenphosphate and sodium hydrogenphosphate were purchased from Kemika, glucose from Fluka, and maltose monohydrate from Sigma. Dextrozyme DX 1.5X was a gift from Novozymes.

Protein concentration

Protein concentration was measured to determine if enzyme was immobilized on the surface of the membrane during the experiments in the continuously operated UFMR. The membrane was removed from the reactor at the end of the experiment and put in 10 cm^3 of 0.1 mol dm^{-3} phosphate buffer pH 5.5 to dissolve the protein molecules that might be present on its surface. The concentration of the proteins dissolved in buffer was measured using Bradford method[17].

HPLC analysis

Glucose and maltose concentrations were determined using high performance liquid chromatography (HPLC) (Shimadzu, Japan) with RI detector at 80 °C. The used HPLC column was C_{18} (Carbohydrate Ca^{2+}, 300 x 6.5 mm, CS-Chromatographie Service GmbH) and the mobile phase was redistilled water at a flow rate of 0.9 cm^3 min^{-1} [16]. Retention times for maltose and glucose were 11.5 and 13.5 minutes, respectively. Samples were diluted in water and filtered (regenerated cellulose filter with 0.2 μm pores) to remove the enzyme and stop the reaction.

Glucoamylase kinetics and volume activity

The influence of maltose monohydrate concentration on the volume activity of glucoamylase in Dextrozyme was determined using the initial reaction rate method. Experiments were carried out in 5 cm^3 batch reactor at 45, 60 and 70 °C in a 0.1 mol dm^{-3} phosphate buffer pH 5.5. From the changes of product (glucose) concentration in time at the beginning of the reaction (less than 10 % substrate conversion) the linear slope ($\mathrm{d}c_G/\mathrm{d}t$) was estimated and used to calculate the enzyme activities *i.e.* initial reaction rates according to Eq. 1. In this equation, $V_{reactor}$ is reactor volume (5 cm^3), V_{enzyme} is volume of the enzyme added to the reactor, and f is dilution factor (1, 10 or 100 x) of the enzyme taken from the reactor. One unit of glucoamylase activity was defined as the amount of enzyme necessary to produce one μmol of glucose in one minute at 40 °C and in a 0.1 mol dm^{-3} phosphate buffer pH 5.5.

$$V.A. = \frac{\mathrm{d}c_G}{\mathrm{d}t}\frac{V_{reactor}}{V_{enzyme}}\cdot f \qquad (1)$$

Using these experimental data, kinetic parameters, V_m and K_m^M , were estimated. The experimental method was described in detail in previous work[16].

Measurements of enzyme activity in UFMR

Enzyme volume activity was followed during the experiments in continuously operated UFMR. These activities were measured at 40 °C and at maltose monohydrate concentration of 25 g dm^{-3} in 5 cm^3 batch reactor. Samples of enzyme (10 μL) were taken out of the reactor through the injection septum of UFMR, and then used to start the reaction in the batch reactor. Glucoamylase activity was measured at different enzyme dilutions to ensure that the activity is high enough to catalyze the reaction. Each measurement of enzyme activity was done in triplicate.

Continuously operated enzyme membrane reactor experiments

Continuous maltose hydrolysis was carried out in a 10 cm^3 enzyme ultrafiltration membrane reactor (Bioengineering, Switzerland) (Figure 1) at 45, 60 and 70 ºC. The experiments were carried out in 0.1 mol dm^{-3} phosphate buffer pH 5.5 at initial concentration of maltose monohydrate of 25 g dm^{-3}. Piston pump (RTC-Präzisionsdosierpumpe M 160, Reichelt GmbH & Co, Heidelberg, Germany) ensured the constant flow rate of the reaction solution of 3.4 cm^3 h^{-1}. The enzyme was retained in the reactor by the polyaramide membrane (UF-PA-20H, Hoechst-Celanese, Frankfurt, Germany, cut off 20 kDa). The ultrafiltration membrane consisted of a layer of polymer on a sheet of paper, and was placed on a flow splitter in the reactor, which ensured equal flow pressure on the membrane. The volume of the

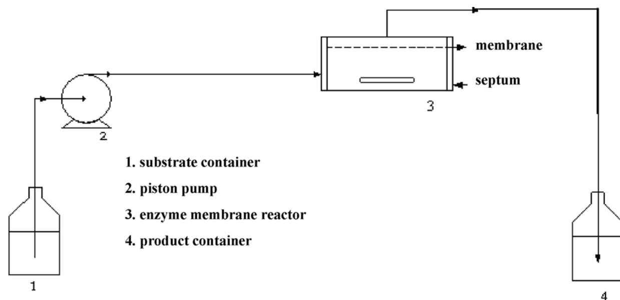


Fig. 1 – *Scheme of the experimental apparatus – continuously operated enzyme ultrafiltration membrane reactor*

membrane was negligible in comparison to the volume of the bioreactor. The reaction was started by adding the enzyme through the injection septum in the reactor. Concentrations of maltose and glucose, as well as enzyme activity, were monitored during the experiment. An experiment without the substrate at 60 °C was carried out in order to determine the influence of the substrate on enzyme stability.

Mathematical model of maltose hydrolysis in a continuously operated enzyme membrane reactor

Kinetic model of maltose hydrolysis was developed in previous research[16] using initial reaction rate method and followed Michaelis-Menten kinetics (Eq. 2).

$$r = \frac{V_m \cdot \gamma_M \cdot \varphi_{enzyme}}{K_m^M + \gamma_M} \tag{2}$$

where V_m is the maximal reaction rate of maltose hydrolysis, γ_M mass concentration of maltose, K_m^M Michaelis constant for maltose, and φ_{enzyme} volume fraction of the enzyme in the reactor.

Considering that the enzyme operational stability decay rate was investigated, and the condition of enzyme stability in the reactor was not fulfilled, mass balances for a continuously operated reactor in unsteady state were used (Eqs. 3 and 4).

$$\frac{d\gamma_M}{dt} = \frac{\gamma_{M,0} - \gamma_M}{\tau} - r \tag{3}$$

$$\frac{d\gamma_G}{dt} = \frac{\gamma_{G,0} - \gamma_G}{\tau} + 1.05 \cdot r \tag{4}$$

$$\frac{dV_m}{dt} = -k_d \cdot V_m \tag{5}$$

It was assumed that the glucoamylase operational stability decay rate can be described by the kinetics of first order (Eq. 5)[18]. In Eqs. 3 and 4, γ_G is mass concentration of glucose, τ is residence time, and k_d is operational stability decay rate constant.

Since Eqs. 3 and 4 contain mass concentration, the coefficient of 1.05 was introduced in Eq. 4 because the reaction of maltose hydrolysis from 1 g of maltose produced 1.05 g of glucose.

Data handling

Non-linear regression methods (simplex and least squares fit) implemented in SCIENTIST software[19] were used for the estimation of the enzyme operational stability decay rate constant at different temperatures. The same program was used to simulate the reaction. Kinetic parameters used in the simulation, maximal reaction rate, V_m and Michaelis constants, K_m, were estimated from the dependence of the initial reaction rate on the concentration of substrate.

Enzyme operational stability decay rate constants for the experiments at 45, 60 and 70 °C and for the experiment without the substrate at 60 °C were estimated from the volume activity *vs.* time dependence. The mathematical model presented by Eqs. 2 – 5 and the estimated operational stability decay rate constants and kinetic parameters, V_m and K_m^M, were used for the simulation of experiments in continuously operated UFMR.

The residual error was defined as the sum of 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

Kinetic parameters of maltose hydrolysis

Kinetic parameters (V_m and K_m^M) shown in Table 1 were estimated from the dependence of enzyme volume activity on the concentration of maltose. The data shows that maximal reaction rate is at 60 °C, but the affinity of enzyme towards maltose is higher at 45 °C (lower K_m value) than at 60 °C.

Table 1 – *Kinetic constants for maltose hydrolysis catalyzed by glucoamylase at different temperatures*

T [°C]	V_m [g dm^{-3} min^{-1}]	K_m^M [g dm^{-3}]
45	10164.2	1.80
60	12487.3	3.20
70	11352.7	4.25

The influence of process parameters on substrate conversion in continuous reaction system

Enzyme concentration and residence time have a significant influence on substrate conversion in a continuously operated reactor. The interaction between these parameters and their influence on maltose conversion in a continuously operated enzyme membrane reactor is presented in Figure 2. It shows that lower enzyme concentration can be compensated with longer residence time to achieve high conversion and *vice versa.*

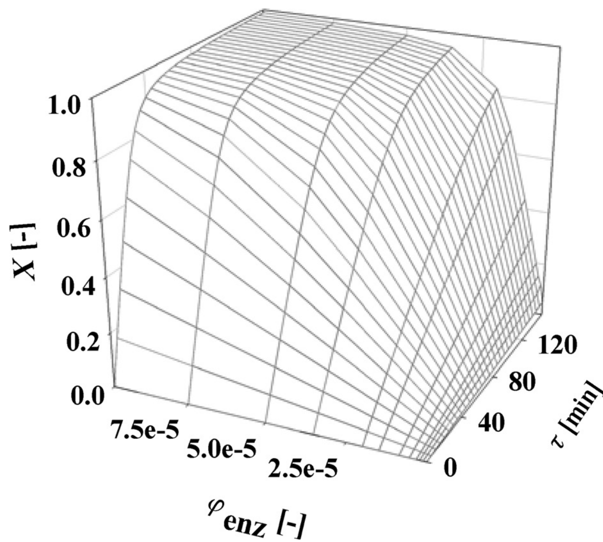


Fig. 2 – *Influence of enzyme concentration and residence time on substrate conversion in continuously operated enzyme ultrafiltration membrane reactor*

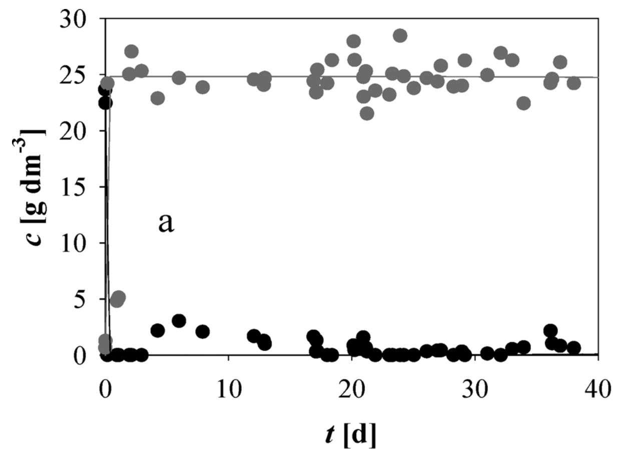
Maltose hydrolysis in a continuously operated enzyme membrane reactor at 45 °C

Maltose hydrolysis was carried out in a continuously operated UFMR at 45 °C and constant flow of the reaction mixture (3.4 mL h^{-1}). The results are presented in Figure 3a. In the previous research[16] the reaction at 40 °C was monitored over 35 days and the stationary maltose conversion was approximately 100 % during the whole experiment. The experiment at 45 °C (Figure 3a) lasted 40 days and 100 % stationary maltose conversion indicated that the enzyme was stabile, but this was not the case. The results of enzyme operational stability decay are presented in Figure 3b, and it can be seen that despite 100 % maltose conversion, the enzyme volume activity decreased quickly. The enzyme operational stability decay would be apparent from the concentration *vs.* time dependence if lower enzyme concentration was used. This can be confirmed by the simulation presented in Figure 2.

The enzyme operational stability decay rate constant shown in Table 2 was estimated from the experimental data presented in Figure 3b. Mathematical model (Eqs. 2 – 5) and the estimated enzyme operational stability decay rate constant were used to simulate the reaction in UFMR and described the data well.

In order to verify that the membrane retains the enzyme inside the reactor, enzyme activity at the reactor outlet was measured. The output solution contained no enzyme activity or proteins, and therefore, it can be concluded that the enzyme was quantitatively retained in the reactor.

After completion of the experiment, the membrane was removed from the reactor and put into a buffer (10 cm^3) to dissolve the protein molecules



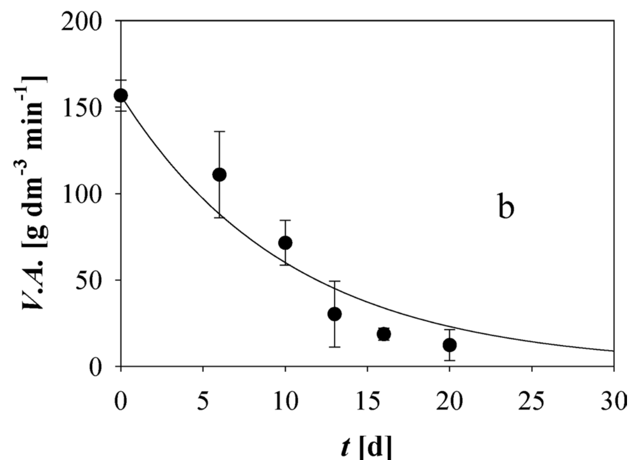


Fig. 3 – *a) Maltose hydrolysis in continuously operated enzyme ultrafiltration membrane reactor carried out in 0.1 mol dm^{-3} phosphate buffer, pH 5.5 (T = 45 °C, $V_{reactor}$ = 10 cm^3, n = 350 rpm, q_V = 3.4 mL h^{-1}, $c_{maltose\ monohydrate,0}$ = 25 g dm^{-3}, φ_{enzyme} = 0.01, $V.A._0$ = 156.8 g dm^{-3} min^{-1}, ● glucose, ● maltose, line – model),* b. *Enzyme activity during maltose hydrolysis in continuously operated enzyme ultrafiltration membrane reactor (T = 40 °C, $V_{reactor}$ = 5 cm^3, n = 350 rpm, $c_{maltose\ monohydrate,0}$ = 25 g dm^{-3}, enzyme sample from the enzyme membrane reactor was diluted 100 x for the activity measurement, ● enzyme activity, line – model)*

Table 2 – *Enzyme deactivation rate constants estimated from the experiments in continuously operated enzyme membrane reactor.*

T [°C]	k_d [d^{-1}]	$t_{1/2}$ [d]
45	0.0960 ± 0.0136	7.22
60	0.4604 ± 0.0345	1.51
60 (without substrate)	0.1609 ± 0.0197	4.31
70	0.5501 ± 0.0456	1.26

that might be present on its surface. This revealed that approximately 82 % of the proteins added to the reactor were stuck to the membrane. This could explain the enzyme operational stability decay. If enzyme accumulates on the membrane in several layers, overlaying of the enzyme active sites can occur, which then can cause enzyme operational stability decay.

Maltose hydrolysis in a continuously operated enzyme membrane reactor at 60 °C

Maltose hydrolysis in a continuously operated UFMR was also carried out at 60 °C. The results are presented in Figure 4a. Enzyme operational stability decay was monitored during the experiment, and the results are shown in Figure 4b.

This experiment showed that enzyme activity decreased faster at higher temperature, and that stationary conversion of substrate was not achieved, as may be seen in Figure 4a. At the beginning of the experiment, 100 % maltose conversion was achieved but it decreased quickly in the first hours of the experiment. Enzyme operational stability decay was complete after 2 weeks when glucose concentration dropped to zero. If enzyme operational stability decay rate in the experiment at 45 °C (Figure 3a) is compared with the experiment at 60 °C (Figure 4b), it may be seen from the estimated enzyme operational stability decay rate constants in Table 2 that the enzyme operational stability decay is faster in the experiment at 60 °C. Mathematical model (Eqs. 2 – 5) described the data well.

Maltose hydrolysis in a continuously operated enzyme membrane reactor at 70 °C

The results of maltose hydrolysis carried out in continuously operated enzyme membrane reactor at 70 °C are presented in Figure 5a. The change in enzyme volume activity during the experiment is presented in Figure 5b.

This experiment showed that enzyme operational stability decay rate is the highest at 70 °C which may be seen from the concentration *vs.* time dependence (Figure 5a) and the dependence of volume activity *vs.* time (Figure 5b). Maximum substrate conversion in the reactor was achieved after the first hour of the experiment. Afterwards, the product concentration started to decrease and dropped to zero after six days, which is twofold higher than in the experiment at 60 °C. All the experiments (at 45, 60 and 70 °C) were carried out with similar initial substrate and enzyme concentrations and it is therefore possible to compare the results. The enzyme operational stability decay rate constant at 70 °C (Table 2) was estimated from the experimental data presented in Figure 5b, and used to simulate the reaction. Other kinetic parameters used for the simulation are shown in Table 1. Mathematical model (Eqs. 2 – 5) described the data well.

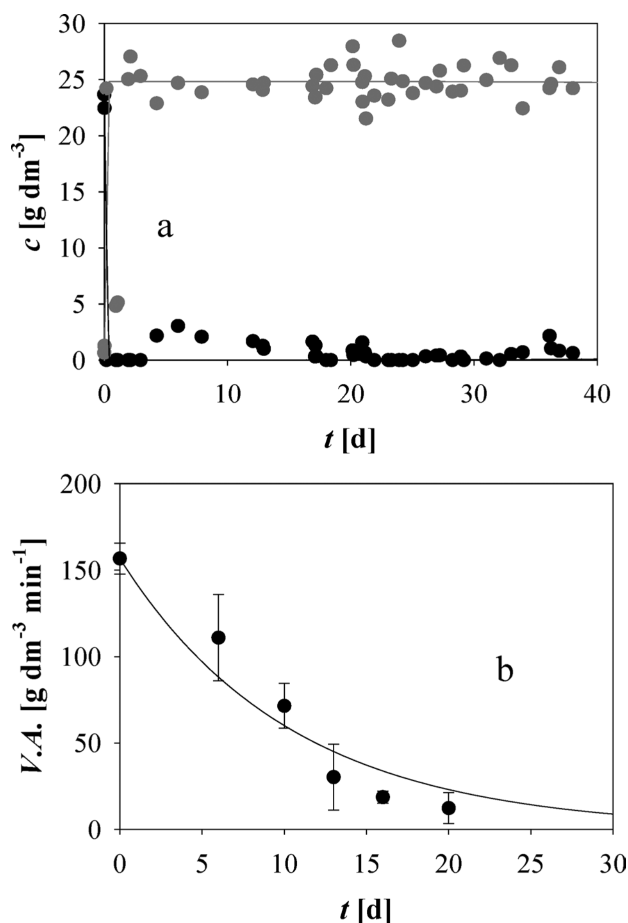


Fig. 4 – *a. Maltose hydrolysis in continuously operated enzyme ultrafiltration membrane reactor carried out in 0.1 mol dm*$^{-3}$ *phosphate buffer, pH 5.5 (*T *= 60 °C,* $V_{reactor}$ *= 10 cm*^{3}*,* n *= 350 rpm,* q_V *= 3.4 mL h*$^{-1}$*,* $c_{maltose\ monohydrate,0}$ *= 22.5 g dm*$^{-3}$*,* φ_{enzyme} *= 0.01,* $V.A._0$ *= 156.8 g dm*$^{-3}$ *min*$^{-1}$*, ● glucose, ● maltose, line – model), b. Enzyme activity during maltose hydrolysis in the continuously operated enzyme ultrafiltration membrane reactor (*T *= 40 °C,* $V_{reactor}$ *= 5 cm*^{3}*,* n *= 350 rpm,* $c_{maltose\ monohydrate,0}$ *= 25 g dm*$^{-3}$*, enzyme sample from the enzyme ultrafiltration membrane reactor was diluted 100 x for the activity measurement, ● enzyme activity, line – model)*

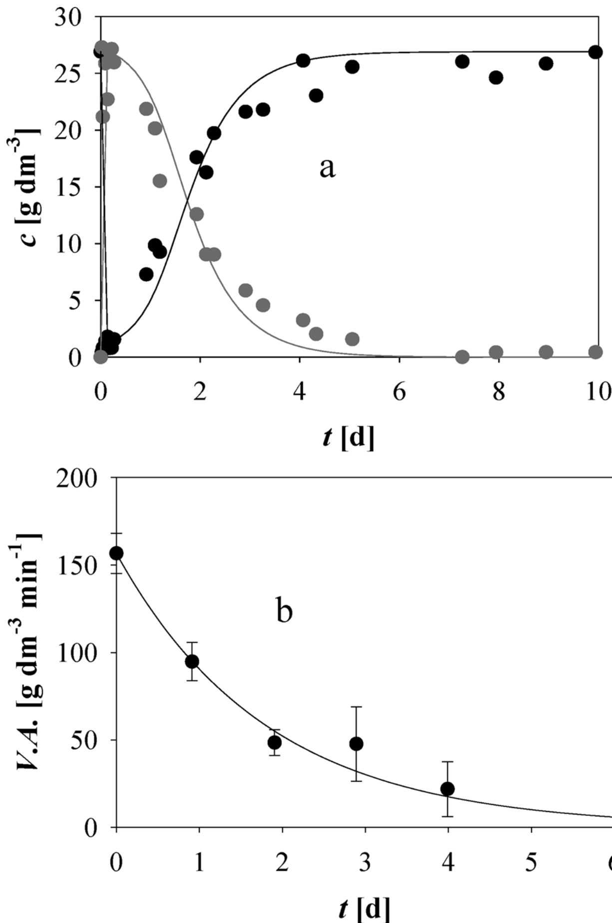


Fig. 5 – *a. Maltose hydrolysis in continuously operated enzyme ultrafiltration membrane reactor carried out in 0.1 mol dm*$^{-3}$ *phosphate buffer, pH 5.5 (T = 70 °C,* $V_{reactor}$ *= 10 cm*^{3}*, n = 350 rpm,* q_V *= 3.4 mL h*$^{-1}$*,* $c_{maltose\ monohydrate,0}$ *= 27 g dm*$^{-3}$*,* φ_{enzyme} *= 0.01,* $V.A._0$ *= 156.8 g dm*$^{-3}$ *min*$^{-1}$*, ● glucose, ● maltose, line – model), b. Enzyme activity during maltose hydrolysis in the continuously operated enzyme ultrafiltration membrane reactor (T = 40 °C,* $V_{reactor}$ *= 5 cm*^{3}*, n = 350 rpm,* $c_{maltose\ monohydrate,0}$ *= 25 g dm*$^{-3}$*, enzyme sample from the enzyme ultrafiltration membrane reactor was diluted 100 x for the activity measurement, ● enzyme activity, line – model)*

Enzyme operational stability decay in continuously operated enzyme membrane reactor without substrate at 60 °C

An experiment without the substrate at 60 °C was carried out to ascertain if the presence of substrate had any positive effect on enzyme stability, which is quite common for enzymes[21]. A phosphate buffer (0.1 mol dm^{-3}, pH 5.5) was pumped through the reactor at a constant flow of 3.4 mL h^{-1}. The enzyme was injected in the reactor and the volume activity was monitored during the experiment (Figure 6). Enzyme activity decreased even without the substrate, but it happened slower than in the experiment with the substrate at the same temperature (Figure 4). Hence, the substrate did not have a stabilizing effect on this enzyme. The enzyme operational stability decay rate constant estimated from

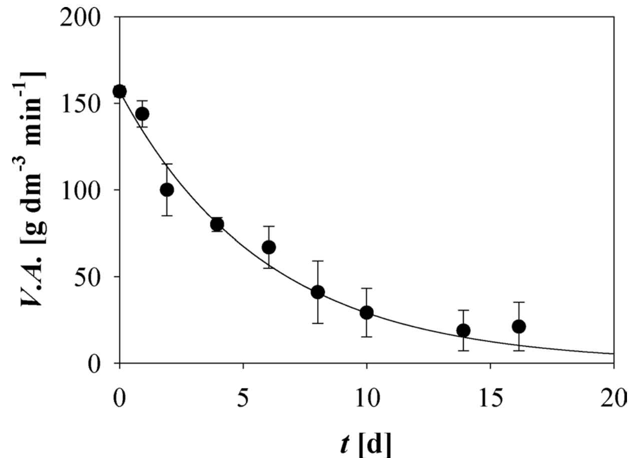


Fig. 6 – *Enzyme activity in continuously operated enzyme ultrafiltration membrane reactor during its incubation with 0.1 mol dm*$^{-3}$ *phosphate buffer, pH 5.5 – substrate was not present in the reactor (T = 40 °C,* $V_{reactor}$ *= 5 cm*^{3}*, n = 350 rpm,* $c_{maltose\ monohydrate,0}$ *= 25 g dm*$^{-3}$*, enzyme sample from the enzyme ultrafiltration membrane reactor was diluted 100 x for the activity measurement, ● enzyme activity, line – model)*

the experimental data presented in Figure 6 is shown in Table 2.

Enzyme half-life was calculated using the estimated enzyme operational stability decay rate constants at different temperatures. Since it is assumed that the enzyme operational stability decay could be described by the kinetics of the first order, enzyme half-life could be calculated according to Eq. 6.

$$t_{1/2} = \frac{\ln 0.5}{-k_d} \qquad (6)$$

Enzyme operational stability decay rate constants at different temperatures (Table 2) show that the temperature increase causes an increase in the operational stability decay rate. Therefore, enzyme half-life decreases with the increase in temperature.

The influence of the enzyme operational stability decay rate constant on maltose conversion is presented in Figure 7. SCIENTIST software and the developed mathematical model (Eqs. 2 – 5) were used for simulations. Figure 7 reveals that if enzyme operational stability decay does not occur (k_d = 0 min^{-1}), maltose conversion is high and constant. The increase in the operational stability decay rate constant causes a faster decrease in maltose conversion, and stationary conditions in the continuously operated reactor cannot be achieved.

Conclusions

The results of the concentration *vs.* time dependence in the experiment at 45 °C during 40 days could lead to the wrong conclusion that the enzyme is stabile. Enzyme activity measurement during this

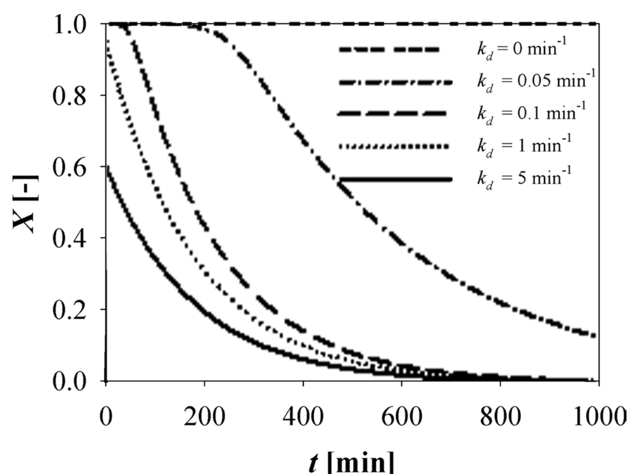


Fig. 7 – *Influence of the enzyme deactivation rate constant on substrate conversion in the continuously operated enzyme ultrafiltration membrane reactor at constant residence time of t = 3.4 cm^3 h^{-1} (the arrow presents the direction of increase of k_d)*

experiment showed that the enzyme activity decreased. Enzyme operational stability decay was easier to notice in the experiment at 60 °C while it was not possible to achieve stationary state in the reactor. In this experiment, enzyme operational stability decay was complete after approximately two weeks. In the experiment carried out at 70 °C, the enzyme operational stability decay rate was the highest; enzyme volume activity dropped to zero after six days. The temperature increase caused an increase in the enzyme operational stability decay rate and a decrease in enzyme half-life. Based on this study, it can be concluded that, with the unstable enzymes, the steady state in the continuous process can be controlled using residence time and/or the concentration of enzyme. At shorter residence time, a higher amount of enzyme is required, and at longer residence time, a lower amount of enzyme is necessary to achieve the same substrate conversion.

To retain an enzyme in the reactor, an ultrafiltration membrane reactor or immobilization of the enzyme can be used. The use of an ultrafiltration membrane reactor offers preservation of enzyme activity[22] while immobilization can cause a change in enzyme activity, specificity or selectivity[23]. Unfortunately, in the presented case, a decrease in enzyme activity was observed at higher temperatures, and to prevent the enzyme operational stability decay, the enzyme should be immobilized. Different approaches to immobilization of enzymes exist, e.g. adsorption or covalent binding to a carrier, encapsulation and entrapment or crosslinking[24]. The best immobilization method for an enzyme has to be determined experimentally since it is not possible to predict if enzyme immobilization will lead to a more stable biocatalyst[25] which is also a disadvantage. Furthermore, the cost of enzyme should be taken into consideration when deciding on the method. In this case, the enzyme was cheap and only cheap immobilization methods would be cost effective.

The developed mathematical model described the experimental data well, and is a valuable tool for studying the experimental results and drawing conclusions.

ACKNOWLEDGEMENTS

This work was supported by the Croatian Ministry of Science, Education and Sports (Contract Grant Number 125–1252086–2793).

List of symbols

G – glucose
k_d – enzyme operational stability rate constant, min^{-1}
K_m – Michaelis constant, g dm^{-3}
M – maltose
r – reaction rate, g dm^{-3} min^{-1}
t – time, min.
T – temperature, °C
V – volume, dm^3
$V.A.$ – volume activity, g dm^{-3} min^{-1}
V_m – maximal reaction rate, g dm^{-3} min^{-1}
X – conversion, – or %
g – mass concentration, mg cm^{-3}
φ – volume ratio of enzyme ($V_{enzyme}/V_{reactor}$), –

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