Design of operational temperature for immobilized glucose isomerise using an accelerated inactivation method

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Abstract: Thermal inactivation of immobilized glucose isomerase in a concentrated glucose solution was investigated in the batch mode and temperature range of 83—95 °C, which is substantially higher than the temperature used in the industrial production of high-fructose corn syrup. Simultaneous evaluation of all inactivation data showed that first-order kinetics with the Arrhenius temperature dependence of the rate constant provided a good approximation of the biocatalyst stability under the investigated conditions. The model parameters were then used to predict the operational temperature for this biocatalyst in the production of high-fructose corn syrup based on the set operational life-time of the biocatalyst. The simulation predicted a window of operational temperature of 60—65 °C, which corresponds very well with the industrial applications of this biocatalyst. This observation demonstrates that the multi-temperature method of enzyme inactivation can provide a good estimate of biocatalyst process stability and is thus a useful tool in the development of biocatalytic processes.

Keywords: high-fructose corn syrup, glucose isomerase, process stability, inactivation kinetics

Introduction

Reversible isomerization of D-glucose to D-fructose catalyzed by D-xylose ketol-isomerase (EC 5.3.1.5; called also glucose isomerase or xylose isomerase) is a reaction with an enormous importance in starch industry. Production of high-fructose corn syrup (HFCS) is one of the most important industrial enzymatic processes. The world annual production of HFCS is currently more than 10 million tons (Anonymous, 2018) and is by far the largest among the processes employing immobilized enzymes (DiCosimo et al., 2013).

The success story of immobilized glucose isomerase is based on several factors (Bhosale et al., 1996). The principle ones are that glucose isomerase is an expensive enzyme and HCFS is a cheap bulk sweetener. Immobilization of glucose isomerase and the use of continuous packed bed reactors were thus necessary to implement the HCFS production process in a large scale thus reducing the contribution of biocatalyst cost to the overall production costs to an acceptable amount.

Industrial glucose isomerization is carried out using concentrated glucose solutions and elevated temperatures. High saccharide concentrations have two major benefits: they significantly stabilize the biocatalyst against thermal denaturation, and they reduce downstream processing costs by reducing the need to evaporate large water volumes. Moreover, elevated temperature reduces the risk of microbial contamination and the viscosity of saccharide solutions improving their processing.

A disadvantage of higher temperatures is that the biocatalyst becomes susceptible to enzyme denaturation. In case of glucose isomerase, this phenomenon has been the subject of numerous studies (DiCosimo et al., 2013). Operational temperature of the glucose isomerization reactor has to be chosen as a trade-off between the mentioned positive effects of elevated temperature and good operational stability of the biocatalyst. Starch industry currently uses the operational temperature range of 50—65 °C, when temperatures between 55 and 60 °C are the most common choice (DiCosimo et al., 2013). The value of operational temperature had to be originally achieved by the trial-and-error method but it is nowadays confirmed by four decades of industrial practice.

The operational temperature is definitely the optimal temperature for this industrial process. The concept of optimal temperature is often presented in biochemical and biotechnological literature. It is basically a temperature at which an apparent or true maximum activity, i.e. initial reaction rate is achieved in the activity assay (Daniel et al., 2001). Such temperature is however in the region of fast enzyme inactivation and it is often too high even for processes employing soluble enzymes as the enzyme can completely lose its activity in the relatively short time of a few hours. Such fast inactivation significantly increases the amount of enzyme spent.

An interesting determination method for glucose isomerization operational temperature was presented by Gibbs et al. (2005). The method is based on a sophisticated design of experiments and modeling. Inactivation experiments were carried out in a continuous stirred tank reactor which temperature was gradually increased. In order to collect enough data to validate the complex model of concomitant reaction and inactivation, several experiments were carried out at different linear temperature gradients (called ramps by the authors). The model obtained was then used to predict the total turnover number per biocatalyst mass at isothermal conditions. The maximum value of the total turnover number determines the optimal temperature. They found that this single criterion preferred the lowest temperature and very short space time. When the space time was fixed to a reference value, the dependence of the total turnover number on temperature was very flat with a plateau in the region of 40—60 °C.

The total turnover number predicted at 55 °C was however about 50 times higher than those achieved in industrial conditions. Possible causes of the discrepancy can lie in the stability of both carrier and enzyme. Thermal unfolding does not have to be the single factor responsible for glucose isomerase activity loss in industrial conditions. Other phenomena may include thiol oxidation of cysteine residues or "Maillard-like" reactions between the enzyme and saccharides (Volkin and Klibanov, 1989; Lim and Saville, 2007).

The objective of this work was to show that a much simpler method can provide a good first estimate of the operational temperature of the immobilized enzyme reactor. Experimental part of the method is based on a series of batch inactivation experiments of immobilized glucose isomerase carried out at several temperatures at which the inactivation rate was relatively fast. The resulting time-activity data were processed using the so-called multi-temperature evaluation method (Vrábel et al., 1997; Polakovič and Bryjak, 2002). This method has been successfully applied to the description of a complex inactivation mechanism of a hexameric enzyme (Illeová et al., 2003) or different immobilized preparations (Vrábel et al., 1997). The resulting model of immobilized glucose isomerase inactivation can then be used to predict the extrapolated biocatalyst lifetime at moderate temperatures. The operational temperature window can then be estimated from the required biocatalyst lifetime.

Materials and Methods

Biocatalyst

Immobilized glucose isomerase Sweetzyme from *Streptomyces murinus* (Novozymes, Bagsværd, Denmark), a glutaraldehyde cross-linked enzyme, was used throughout this work. The biocatalyst had a form of dry brown cylindrical granules with the particle size in the range of 0.5—2.0 mm and the specific activity of 350 U g^{-1}.

Substrate solution

Glucose, the substrate for glucose isomerase, was used in the single concentration of 2.5 mol dm^{-3}. Glucose was dissolved in a Tris buffer with pH 7.0 containing 1.0 g dm^{-3} of $MgSO_4 \cdot 7\ H_2O$ and 1.0 g dm^{-3} of Na_2SO_3. All chemicals were of analytical grade and obtained from local vendors.

Biocatalyst activity assay

Glucose isomerase activity of immobilized biocatalyst samples was determined using the initial rate method. A stirred reactor filled with 12.5 ml of the substrate solution was preheated to 60 °C and a precisely weighed amount (approximately 0.3 g) of wet immobilized glucose isomerase was added. The reaction was carried out for 40 minutes at 60 °C. A 300 µl sample was withdrawn every 10 minutes and cooled in ice water to stop the enzymatic reaction. The sample was diluted and the concentration of liberated fructose was determined using a HPLC column Watrex 250 × 8 mm Polymer IEC Pb form (Watrex International, Clearwater, U.S.A.) at the temperature of 80 °C with re-distilled water as a mobile phase with the flow rate of 0.8 ml min^{-1}.

Inactivation experiments

Particles of the immobilized enzyme were rehydrated in the 0.05 M Tris buffer solution for two hours, rinsed with the buffer solution and placed onto a sintered glass filter. The buffer solution excess was removed by a vacuum pump. Dry residue mass of the wet biocatalyst was determined at 60 °C using the moisture analyzer Kern MLS (Frommern, Germany). Inactivation batch experiments were carried out in sealed test tubes placed in a water bath, 0.5 g of the wet biocatalyst was added to 2 ml of the substrate solution preheated to the inactivation temperature. Different time exposures at the inactivation temperature were used. At the chosen time, a test tube was taken out of the bath and rapidly cooled in ice water. The particles were separated by filtration and the activity assay was carried out as described above.

Mathematical modeling

One-step irreversible inactivation mechanism,

$$N \xrightarrow{k_d} I \qquad (1)$$

was applied to derive a model for the description of immobilized glucose isomerase inactivation. In Eq. (1), N and I are the active and inactive form of the enzyme, respectively, and k_d is the first-order inactivation rate constant.

Gibbs et al. (2005) declared that diffusional limitations are not significant for commercial glucose isomerase preparations. This assumption was accepted and the batch inactivation process was then described by the following equation:

$$\frac{\mathrm{d}a}{\mathrm{d}t} = -k_d a \tag{2}$$

where a is the relative biocatalyst activity in time t. The initial activity was thus set to the value of 1. Temperature dependence of the rate constant was expressed in the form of a rearranged Arrhenius equation:

$$k_d = k_{d0}\exp\left[\frac{E}{\mathrm{R}T_0}\left(1-\frac{T_0}{T}\right)\right] \tag{3}$$

where E is the activation energy of the inactivation reaction, R the universal gas constant, and k_{d0} the value of the rate constant at the reference temperature T_0. The so-called multi-temperature evaluation (Vrábel et al., 1997; Polakovič and Bryjak, 2002), where inactivation data for all temperatures are fitted simultaneously using non-linear regression, was applied. The mathematical modeling software Athena Visual Workbench (Stewart & Associates, Madison, WI, USA) was used for parameter estimation.

Using the estimated parameters E and k_{d0}, the values of k_d were calculated from Eq. (3) for a temperature range broader than the one used in the inactivation experiments. Subsequently, Eq. (2) was integrated to provide the biocatalyst half-life $t_{1/2}$ (Eq. (4)) at different temperatures. The half-life is a common measure of biocatalyst process stability.

$$t_{1/2} = \frac{\ln(2)}{k_d} \tag{4}$$

Results and discussion

Batch inactivation experiments were carried out in the temperature range of 83—95 °C which ensured accelerated inactivation compared to the HCFS process conditions. Fig. 1 shows the measured activity courses at the four temperatures applied. Experimental half-lives were from 10 minutes at 95 °C to 10 hours at 83 °C. In order to identify the adequate mechanism of GI inactivation, all experimental data were evaluated simultaneously (Vrábel et al., 1997; Polakovič and Bryjak, 2002) as described above. The estimated parameter values and their 95 % confidence intervals were as follows:

$$k_{\mathrm{d0}} = (7.46 \pm 0.48) \times 10^{-3}\ \mathrm{min}^{-1}$$

$$E = 304 \pm 15\ \mathrm{kJ\ mol}^{-1}$$

The reference temperature T_0 was set to 89 °C, which was in the middle of the experimental temperature range. Standard error of predicted activity values was 3.5 %.

Fig. 1 also shows the modeled activity courses which confirm that a good fit of the experimental

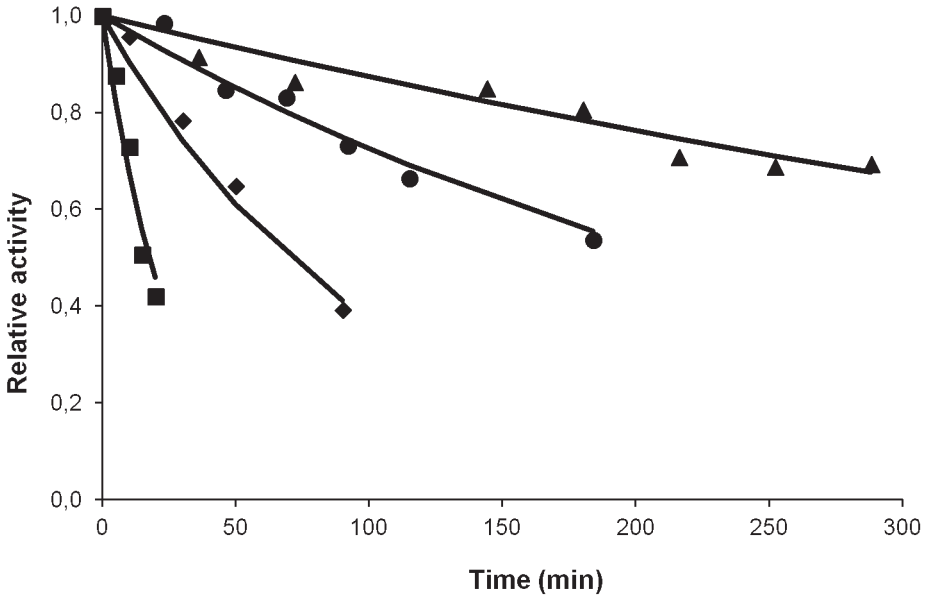

Fig. 1. Thermal inactivation of immobilized glucose isomerase in 2.5 mol dm^{-3} glucose solution: 83 °C (▲), 86 °C (●), 90 °C (♦), 95 °C (■).
Lines represent fitted data obtained by the simultaneous evaluation method.

data in the whole temperature range was achieved for the model assuming simple first-order kinetics of inactivation and negligible mass-transfer limitations. First-order inactivation kinetics for immobilized glucose isomerase from *Streptomyces* species has been reported by most authors (Volkin and Klibanov, 1989; Converti and Del Borghi, 1997; Bandlish et al., 2002; Lim and Saville, 2007). Only Gibbs et al. (2005) verified the Lumry-Eyring model as a more convenient. A principal difference of their study compared to the previous ones is that they applied a very broad temperature range of 40—90 °C in their investigation. The first reversible step of the Lumry-Eyring mechanism was however exhibited only at the lower temperatures studied whereas the inactivation was completely governed by the first-order kinetics at higher temperatures.

The results presented in Fig. 1 demonstrate a considerable influence of temperature on the inactivation rate. A slow decline of the residual activity was observed at 83 °C when the extrapolated half-life is about 10 h. On the other hand, an increase of temperature by 12 °C to 95 °C resulted in a high loss of stability and the half-life was reached already in 18 minutes. This sharp increase of the inactivation rate with temperature is reflected in the high value of activation energy, of about 300 kJ mol^{-1}.

Other authors reported lower values of activation energy. Converti and Del Borghi (1997) reported a value of 180 kJ mol^{-1} for Sweetzyme and Gibbs et al. (2005) only 100 and 120 kJ mol^{-1} for Gensweet from Genencor, respectively. The inactivation temperatures considered in other studies were mostly lower than those used in our work. Only Borgi et al. (2004) presented the half-life of a *Streptomyces* sp. glucose isomerase at 90 °C which was essentially the same as the value of 60 min observed in this work.

As it has been mentioned above, the main objective of this work was to demonstrate the suitability of the accelerated inactivation experiments to provide an estimate of the operational temperature. Using the estimated values of the rate constant and activation energy, half-life of the immobilized biocatalyst was calculated for a chosen temperature using Eqs. (3) and (4). Figure 2 presents the relationship between the half-life and operational temperature in the interval from 60 °C to 95 °C.

It is evident from this plot that a region of long-term stability of the immobilized biocatalyst starts at temperatures below 65 °C. The predicted half-life at this temperature is about 80 days and it increases to 390 days at 60 °C. It means that that the indicated operational temperature window of 60—65 °C is by about 20 °C lower than the lower boundary of the temperature interval used in the inactivation experiments. The predicted half-life values can be compared with the results of inactivation experiments carried out for some commercial immobilized glucose isomerases at temperatures of around 60 °C.

Very diverse half-lives were observed or predicted by different authors. Gensweet, Lim et al. (2007) observed the values of about 30 h at 60 °C, whereas Gibbs et al. (2005) predicted the value of 6 months at 55 °C using their method of accelerated inactivation. Bandlish et al. (2002) also determined very low half-life of about 10 h at 60 °C for Sweetzyme while Converti and Del Borghi (1998) declared the half-life of about 300—400 h at 65 °C for the same enzyme depending on the glucose concentration.

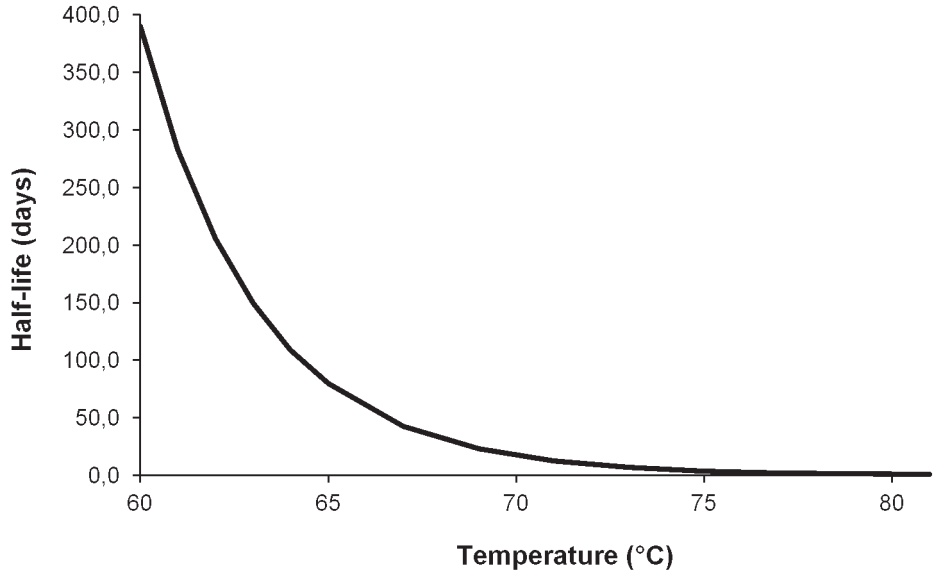

Fig. 2. Predicted half-life of immobilized glucose isomerase vs. operational temperature.

Our highly extrapolated values of half-life were naturally much higher than the above mentioned ones and they can be expected to be overestimated. The upper boundary of the predicted operational temperature of 65 °C was a logical choice to verify our prediction. A packed-bed reactor experiment was designed for that purpose. The biocatalyst bed had the length of 3 cm and the diameter of 1 cm and was fed with 2.5 mol dm^{-3} glucose solution of the same composition as in the inactivation experiments. The substrate flowrate was kept constant at 0.3 ml min^{-1} for 14 days. The initial glucose conversion was 40 %, similarly to industrial conditions, and it decreased to 10 % after 14 days (Fig. 3). The conversion and therewith also the volumetric productivity of the reactor decreased to the half of the initial value after 400 hours.

The experimental biocatalyst half-life was thus only about 10 % of the predicted value. It was however identical with the value found by Converti and Del Borghi 20 years ago (1998). Sweetzyme has been on the market since 1975 (Zittan et al., 1975) and its activity has increased significantly over the decades but its thermal stability has evidently remained about the same *(DiCosimo et al., 2013).* If we assume a 10-fold difference between the simulated and true half-life at lower operational temperatures, the simulated half-lives at 60 °C and 55 °C will be reduced to 40 days and 220 days, respectively. Since the operational lifetime of isomerization biocatalysts is typically about three half-lives (Gibbs et al., 2005), the temperature of 55 °C with the assumed operational lifetime of 660 days seems to be a safer choice. The confirmation of a selected temperature, however, requires further long-term experiments.

Conclusions

The objective of this work was to examine the potential of accelerated inactivation experiments to predict the operational temperature for an immobilized biocatalyst. A commercial preparation of immobilized glucose isomerase used in the production of high-fructose corn syrup was chosen as a representative biocatalyst. A suitable range of temperatures to carry out batch inactivation experiments was found to be 83—95 °C. It was verified that the rate of inactivation is governed by the first-order kinetics in the whole temperature range. The multi-temperature inactivation data evaluation method provided a pre-exponential rate constant and activation energy that were used for the estimation of operational temperature.

Since the activation energy was quite high, the simulated half-lives extrapolated the operational temperature to the operational window by about 25 °C lower than the mean temperature used in the inactivation experiments. A long-term stability experiment in a packed bed reactor proved that the extrapolated biocatalyst lifetime was significantly overestimated. Nonetheless, the estimated operational temperature window is a good starting point for biocatalyst stability examination. The presented procedure speeds up the development of processes employing immobilized biocatalysts.

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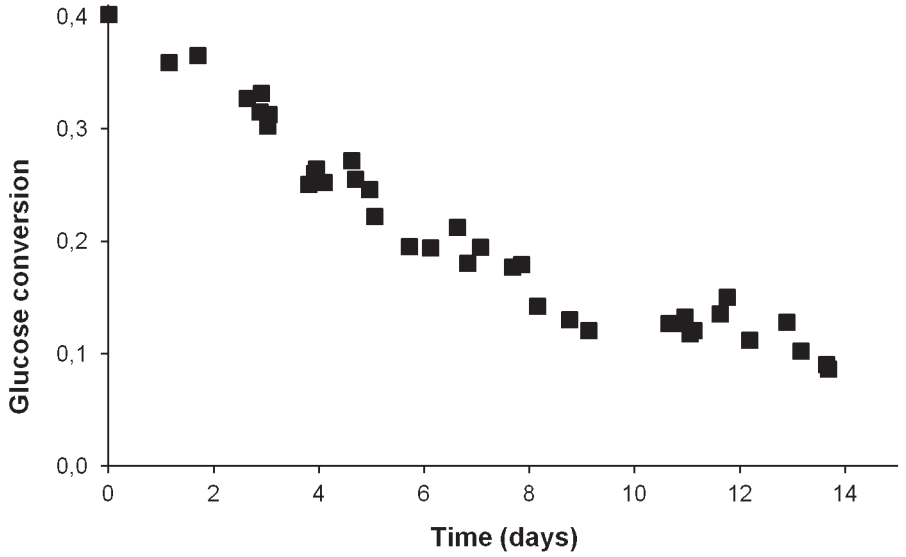

Fig. 3. Operational stability of glucose isomerase in a packed-bed reactor at 65 °C. Outlet glucose conversion was measured at the constant flowrate of 2.5 mol dm^{-3} glucose feed solution.

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