

# Kinetics of glucose isomerization to fructose by immobilized glucose isomerase in the presence of substrate protection

A. Converti, M. Del Borghi

**Abstract** The activity of immobilized glucose isomerase of *Streptomyces murinus* has been tested batchwise under different conditions in order to gather the related kinetic parameters necessary to optimize an immobilized enzyme column for the continuous production of high fructose corn syrup (HFCS). To this purpose, the Briggs-Haldane model incorporating an apparent first-order inactivation constant has been used with success. A comparison of the equilibrium constants and of the maximum theoretical conversion yields calculated at different temperatures with those estimated for the native enzyme demonstrates that the immobilization favours the transformation of glucose to fructose only at  $T > 70^\circ\text{C}$ , as a possible consequence of a combined effect of catalysis and equilibrium thermodynamics enhancement. Enzyme inactivation has also been tested at different temperatures and sugar concentrations to evaluate the related kinetic parameters under different conditions of substrate protection.

## List of symbols

$e$	inactivated form of the free enzyme
$E$	active enzyme concentration (mol/l)
EF	intermediate complex of the reverse reaction
EG	intermediate complex of the forward reaction
$f$	fractional conversion of glucose to fructose (-)
$F$	fructose concentration (mol/l)
$G$	glucose or sugar concentrations (mol/l)
GI	glucose isomerase
$h$	Plank's constant (J s)
HFCS	high-fructose corn syrup
$k$	Boltzmann's constant (J/K)
$K$	equilibrium constant (-)
$k_{+1}$	rate constant of EG formation from G ( $\text{l min}^{-1} \text{mol}^{-1}$ )
$k_{-1}$	rate constant of EF consumption to form G ( $\text{min}^{-1}$ )
$k_{+2}$	rate constant of EG consumption to form F ( $\text{min}^{-1}$ )
$k_{-2}$	rate constant of EF formation from F ( $\text{l min}^{-1} \text{mol}^{-1}$ )
$k_d$	decay constant ( $\text{h}^{-1}$ )

$k_m$	kinetic parameter defined in Eq. (4) (mol/l)
$k_{mf}$	Michaelis constant of the forward reaction (mol/l)
$k_{mr}$	Michaelis constant of the reverse reaction (mol/l)
$n$	factor to express the ratio of X to free enzyme inactivation rate (-)
$P$	integral productivity at time $t$ (mol/l)
$P_{1/2}$	integral productivity at enzyme half-life (mol/l)
$P_\infty$	integral productivity at infinite time (mol/l)
$R$	ideal gas constant ( $\text{J mol}^{-1} \text{K}^{-1}$ )
$t$	time (h, min or s)
$T$	temperature (K or $^\circ\text{C}$ )
$V$	specific reaction rate ( $\text{mol min}^{-1} \text{g}_{\text{cat}}^{-1}$ )
$V_m$	kinetic parameter defined in Eq. (3) ( $\text{mol min}^{-1} \text{g}_{\text{cat}}^{-1}$ )
$V_{mf}$	maximum velocity of the forward reaction ( $\text{mol min}^{-1} \text{l}^{-1}$ )
$V_{mr}$	maximum velocity of the reverse reaction ( $\text{mol min}^{-1} \text{l}^{-1}$ )
$x$	inactivated form of the complexed enzyme
$X$	generic intermediate complex

## Greek letters

$\theta$	half-life of the enzyme (h)
$\sigma$	protection factor (-)
$\psi$	activity coefficient (-)

## Subscripts

cat	catalyst
max	maximum value
$o$	starting value
$t$	total value

## Superscripts

*	value at the thermodynamic equilibrium
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## 1

### Introduction

High-fructose corn syrup (HFCS), which is increasingly used as an alternative sweetener, can be produced by the industrial enzymic conversion of glucose to fructose by immobilized glucose isomerase (GI). Due to the problems related to restriction of the production in European Union (EU), it is profitable to push the enzymic conversion of glucose to fructose as much as possible in order to satisfy the demand of products always more and more sweet, up to the limits imposed by the equilibrium thermodynamics. Both fructose and HFCS are produced in EU by continuous isomerization of concentrated syr-

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A. Converti, M. Del Borghi  
Institute of Chemical and Process Engineering "G.B. Bonino",  
Faculty of Engineering, University of Genoa, Via Opera Pia,  
15, I-16145 Genoa, Italy

Correspondence to: A. Converti

ups coming from starch hydrolysis, that can be performed in columns containing immobilized GI fed at residence times consistent with fructose levels in the product no less than 42% [1, 2].

However, the performance of these reactors is largely influenced by both diffusion resistance and enzyme inactivation. In fact, although diffusion resistance seems to enhance the apparent stability of the immobilized enzyme [3-6], it obviously affects productivity mainly at low residence times. Also enzyme inactivation, which is influenced by several factors, among which pH, ionic strength, temperature, and poisonous substances [7-10], progressively reduces productivity. On the other hand, glucose isomerase is protected by substrate against inactivation, thanks to the stabilization of the tertiary structure exerted in the complex by the link between active site and glucose [11-13].

Several authors demonstrated that a statistical approach considering enzyme microheterogeneity is in general the best way to study immobilized enzyme inactivation [14-16]. In fact, this property, that is the result of minor differences between individual protein molecules, becomes of great significance mainly in immobilized enzymes or in systems undergoing to denaturation [17], and may cause the inactivation reaction to appear to be of higher order as well as of more complex nature. Nevertheless, the conventional, reversible Briggs-Haldane kinetic model, which considers the molar amount of enzyme negligible with respect to the total amount of product and reactant, described sufficiently well the decrease of the thermal decay rate constant of the complexed immobilized glucose isomerase with respect to the free enzyme [12]. Chen and Wu calculated a decay constant for this complex that is half that of the uncomplexed form [12], while Houg et al. [18] and Illanes et al. [19] subsequently used these values for modelling immobilized glucose isomerase reactors.

From these considerations it is evident that a deep knowledge of immobilized glucose isomerase kinetics both in the presence or not of substrate protection is of great importance to select the optimum conditions to push the equilibrium towards fructose formation. However, although kinetics of native enzyme catalysis and inactivation have already been studied in detail [20, 21], only few efforts were addressed in the past to immobilized industrial enzyme catalysis under actual conditions [22].

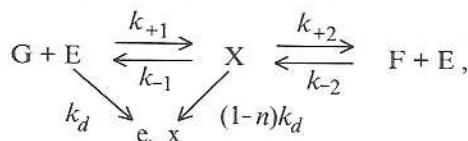
The first part of this work deals with the evaluation of the main kinetic parameters of glucose isomerization to fructose by immobilized GI, at different temperatures and starting sugar levels, while the final section is devoted to the inactivation process both in the presence or in the absence of substrate as well as to the comparison of the integral productivity under different operating conditions.

## 2 Theoreticals

### 2.1 Equilibrium parameters

As well known, glucose isomerase activity can be described by the reversible Briggs-Haldane model, supposing that

the inactivation of the enzyme, both in complexed or free forms, follows first order kinetics:



where E is the active free enzyme, G the glucose, F the fructose, X the intermediate complex between the enzyme and glucose or fructose, e and x the inactivated forms of the free and the complexed enzyme, respectively. Constants  $k_{+1}$ ,  $k_{-1}$ ,  $k_{+2}$  and  $k_{-2}$  are the rate constants of the elementary reactions,  $k_d$  and  $(1-n)k_d$  the decay constant of the active free enzyme and that of the enzyme complexed with the substrate. Chen and Wu demonstrated that  $n$  is 0.5 for the enzyme under consideration [12], which means that the link with substrate exerts an enzyme stabilization (protection) against inactivation.

Applying the pseudo-steady-state hypothesis for X, the Briggs-Haldane approach leads to the Michaelis-Menten-type equation [20]:

$$V = \frac{V_m(G - G^*)}{k_m + (G - G^*)}, \quad (2)$$

where:

$$V_m = [1 + (1/K)] \frac{k_{mr} V_{mf}}{k_{mr} - k_{mf}}, \quad (3)$$

$$k_m = \frac{k_{mf} k_{mr}}{k_{mr} - k_{mf}} \left[ 1 + \left( \frac{1}{k_{mf}} + \frac{K}{k_{mr}} \right) \frac{G_0}{1 + K} \right], \quad (4)$$

$$k_{mf} = (k_{-1} + k_{+2})/k_{+1}, \quad (5)$$

$$k_{mr} = (k_{-1} + k_{+2})/k_{-2}, \quad (6)$$

$$V_{mf} = k_{+2} E_t, \quad (7)$$

$$V_{mr} = k_{-1} E_t, \quad (8)$$

$$K = F^*/G^* = \frac{k_{mr} V_{mf}}{k_{mf} V_{mr}}, \quad (9)$$

$G$ ,  $G^*$ ,  $G_0$  being actual, equilibrium, and starting glucose concentrations, respectively,  $F^*$  fructose concentration at the equilibrium, and  $E_t$  total concentration of active enzyme. The Michaelis constants and the maximum velocities of the forward and reverse reactions ( $k_{mf}$ ,  $V_{mf}$  and  $k_{mr}$ ,  $V_{mr}$ , respectively) can be estimated by plotting, according to Lineweaver-Burk, the starting experimental data of batch runs carried out using only glucose or fructose as substrate. The rate constants  $k_{+1}$ ,  $k_{+2}$ ,  $k_{-1}$ , and  $k_{-2}$ , as well as the equilibrium constant,  $K$ , can then be calculated through Eqs. (5-9).

Defining the fractional conversion of glucose to fructose,  $f$ , as:

$$f = F/G_0, \quad (10)$$

the maximum fraction of glucose which can be present as fructose at the end of a batch conversion process,  $f_{\max}$ , is:

$$f_{\max} = K/(1 + K). \quad (11)$$

## 2.2

### Enzyme inactivation

It is well accepted by now that the so-called kinetic approach [20], rather than the thermodynamic approach [23], is the best choice to describe glucose isomerase inactivation. In particular, first-order kinetics have successfully been proposed for thermal inactivation of both free and immobilized forms of this enzyme, supposing that this non-reversible process is very slow if compared with the equilibrium (kinetic control) [12]. According to these considerations and defining the activity coefficient  $\psi$  as the ratio of the total active enzyme concentration at any time to the starting concentration ( $\psi = E_t/E_{t_0}$ ), one can write, in the absence of any substrate protection ( $n = 0$ ):

$$d\psi/dt = -k_d\psi \quad (12)$$

or, in the presence of substrate protection ( $n = 0.5$ ):

$$d\psi/dt = -k_d\psi(1 - \sigma) \quad (13)$$

The so-called protection factor,  $\sigma$ , which gives a quantitative measure of the extent of substrate protection, can then easily be calculated from the equation:

$$\sigma = 1 - (\theta'/\theta) \quad (14)$$

where  $\theta' = \ln 2/k_d$  is the half-life of the unprotected enzyme and  $\theta = \ln 2/k_d(1 - \sigma)$  that of the protected enzyme.

The apparent first-order inactivation constant,  $k_d(1 - \sigma)$ , can easily be estimated from the slopes of the straight lines obtained plotting the experimental data of  $\ln \psi$  versus time, according to the integrated form of Eq. (13).

## 2.3

### Integral productivity

If thermal inactivation is taken into consideration, the specific activity of the enzyme becomes a function of the time. So, the total productivity up to a given time,  $P$ , can be obtained, either in the presence ( $0 < \sigma < 1$ ) or in the absence of substrate protection ( $\sigma = 0$ ), by integrating the product of the starting forward reaction rate to the activity coefficient:

$$P = \int V_{mfo} \exp[-k_d(1 - \sigma)t] dt \\ = [V_{mfo}/k_d(1 - \sigma)][1 - \exp(-k_d t)] \quad (15)$$

Two useful parameters, that, contrary to integral productivity, are independent of time, are the values such a productivity achieves at infinite time:

$$P_\infty = V_{mfo}/k_d(1 - \sigma) \quad (16)$$

and at the activity half-life:

$$P_{1/2} = V_{mfo}/2k_d(1 - \sigma) \quad (17)$$

## 3

### Materials and methods

#### 3.1

##### Materials

The commercial Sweetzyme T,<sup>®</sup> supplied by Novo Nordisk, is an active immobilized glucose isomerase (EC

**Table 1.** Characteristics of the commercial immobilized glucose isomerase used for kinetic studies

Enzyme classification	EC 5.3.1.5 D-xylose ketolisomerase
Microorganism	Streptomyces murinus
Dry specific activity	350 IGIU/g
Fixed-bed density	0.33 g/l
Consistency	granules
Particle size	0.3–1.0 mm

5.3.1.5 D-xylose ketolisomerase) from a selected strain of *Streptomyces murinus*, whose main characteristics are summarized in Table 1. Glucose and fructose for microbiology were used for broths preparation while those used as standards were of reagent grade. All chemicals, including salts and buffers, were from Carlo Erba, Milan, Italy.

#### 3.2

##### Analytical methods

Fructose and glucose were determined by high performance liquid chromatography, as previously described [24].

#### 3.3

##### Operating conditions and analysis

After preliminary rehydration of the enzyme in distilled water for 24 h, activity tests were carried out at 60 °C in Erlenmeyer flasks by adding 0.6 g of the enzyme to 50 ml of a 0.05 M Tris-buffer solution containing 1.00 g/l  $MgSO_4 \cdot 7 H_2O$ , 1.00 g/l  $Na_2SO_3$  and 450.0 g/l glucose at pH 7.5.

Kinetic parameters were estimated through batch runs carried out at pH 7.0 at different temperatures under conditions of negligible thermal inactivation, that is using the same high enzyme concentration as that of the activity tests. The Michaelis constants and the maximum velocities of both forward and reverse reactions were determined using starting broths containing either 2 mol/l glucose or fructose. The equilibrium constant was then estimated by Eq. (9).

The first-order inactivation constants either in the presence or in the absence of substrate protection have been estimated through batch tests carried out at pH 7.0 in a 1.0 litre-chemostat Gallenkamp FBL-195 stirred at 150 rpm and containing 50 g of fresh immobilized enzyme.

## 4

### Results and discussion

#### 4.1

##### Kinetic parameters of enzyme activity

Figures 1 and 2 show the influence of temperature on the maximum velocities of both forward and reverse reactions and on the Michaelis constants, calculated by Lineweaver-Burk plots of the experimental data of either glucose or fructose isomerizations. The related kinetic constants, all increasing with temperature, can only partially be compared in Fig. 3 because the units employed for bimolecular events ( $k_{+1}$  and  $k_{-2}$ ) differ from those used for

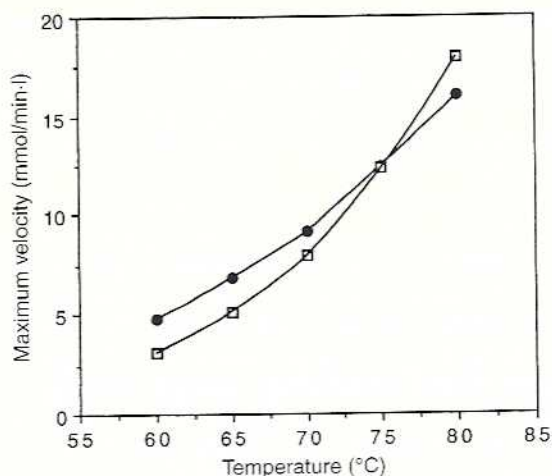


Fig. 1. Temperature dependence of the maximum velocities of both forward (●) and reverse (□) reactions of isomerization by immobilized glucose isomerase

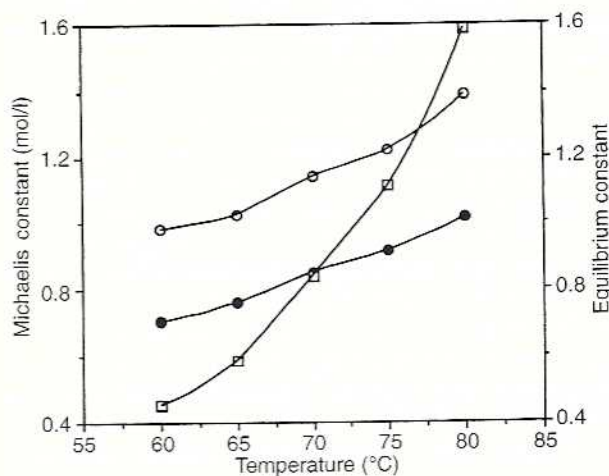


Fig. 2. Temperature dependence of the equilibrium constant (○) and the Michaelis constants of both forward (●) and reverse (□) reactions of isomerization by immobilized glucose isomerase

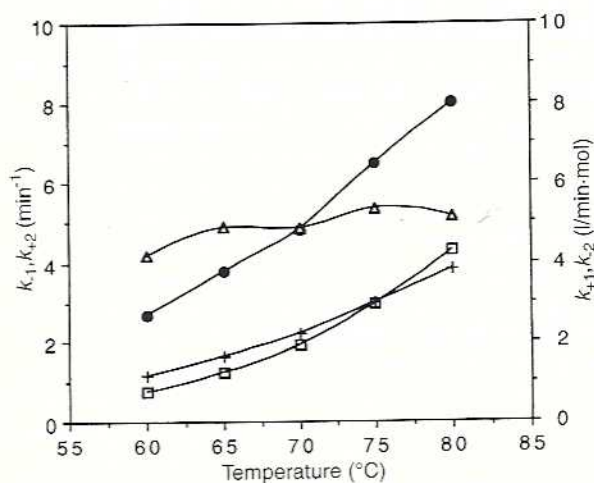


Fig. 3. Behaviours of the rate constants of the elementary reactions, according to the Briggs-Haldane approach. (□)  $k_{-1}$ ; (●)  $k_{+1}$ ; (Δ)  $k_{-2}$ ; (+)  $k_{+2}$

monomolecular ones ( $k_{+2}$  and  $k_{-1}$ ). Some interesting considerations can anyhow be made. First of all, the comparable values of  $k_{+2}$  and  $k_{-1}$  confirm, at the same time, the impossibility of handling the reversible glucose isomerase kinetics with the traditional Michaelis-Menten model and the validity of the Briggs-Haldane approach. As shown in the same figure,  $k_{+2}$  grows less rapidly with increasing temperature than  $k_{-1}$ , which means that, in theory, the reverse conversion of fructose to glucose should be favoured by heating. This behaviour, however, is not confirmed by the continuous increase of the equilibrium constant observed within the tested range of temperature (Fig. 2), which is a consequence of the simultaneous much faster growth of  $k_{+1}$  than  $k_{-2}$ .

Comparison of the values of the equilibrium constant with those reported in the literature for the native enzyme [20] shows that immobilization favours the forward reaction only at  $T > 70$  °C. This result, which is rather surprising considering that not even the presence of a catalyst should be able, in general, to appreciably modify the position of chemical equilibria, suggests that immobilization could influence the equilibrium thermodynamics.

A final attempt has been made to study the effect of substrate concentration on the kinetic parameters of immobilized glucose isomerase activity at 75 °C. The results of Table 2 show that  $V_m$  is not appreciably influenced by starting substrate concentration, while  $k_m$  increases almost linearly within the tested range of  $G_0$ , thus suggesting the existence of a competitive-type saturation phenomenon exerted by substrate.

Table 3 shows the temperature dependence of the maximum theoretical fraction,  $f_{max}$ , of total starting glucose which can be present as fructose at the end of batch isomerization either by native or immobilized glucose isomerase. As can be seen, the product (fructose) fraction will hardly be able to exceed 50% of the total sugar using the native enzyme. As suggested by Roels [20], it is worthwhile to emphasize that this result is independent of the way of carrying out the process, that is subject to the constraint dictated by thermodynamics. One can then deduce that also the higher  $f_{max}$  values observed at  $T > 70$  °C using the immobilized biocatalyst have to be ascribed to the previously supposed enhancement of process thermodynamics.

Table 2. Influence of the starting substrate concentration on the main kinetic parameters of immobilized glucose isomerase activity at 75 °C

$G_0$ (mol/l)	0.5	1.0	2.0	3.0
$V_m$ ( $\mu$ -mol min <sup>-1</sup> g <sub>cat</sub> <sup>-1</sup> )	10551	11149	10759	10713
$k_m$ (mol/l)	6.92	10.52	15.53	21.55

Table 3. Temperature dependence of the maximum theoretical fraction of  $G_0$  which can be present as fructose at the equilibrium ( $f_{max}$ )

$T$ (°C)	50	55	60	65	70	75	80
Native	0.495	0.504	0.510	0.520	0.526	-	-
Immobilized	-	-	0.495	0.507	0.533	0.550	0.582

## 4.2

### Kinetic parameters of enzyme inactivation

Thermal inactivation of the immobilized glucose isomerase in the absence of substrate protection has been studied following the residual activity of the enzyme at time intervals quite longer than those selected for enzyme activity tests. To avoid any possible influence either of the reaction environment or of the substrate protection phenomenon on the kinetic parameters, batch isomerization tests have been carried out in a buffer solution at pH 7.0, that is in the absence of either glucose or fructose, within the temperature range 60–80 °C. The results listed in Table 4 show the progressive increase of the first-order inactivation constants of both native enzyme and immobilized system with increasing temperature, that is the result of a growing significance of the denaturation phenomenon. In addition, the values of  $k_d$  for the native enzyme are substantially higher than those calculated for the immobilized system, which confirms a possible stabilizing role of the immobilizing material against enzyme inactivation [25].

The main kinetic results of batch isomerization tests carried out at different total sugar equilibrium concentrations are summarized in Table 5. Once again, the apparent first-order inactivation constant remarkably increases, at a given value of  $G_o$ , with increasing temperature. But the most interesting result is that  $k_d(1 - \sigma)$  progressively decreases with increasing  $G_o$  at a given temperature, which put in evidence the occurrence of an additional phenomenon of substrate protection against glucose isomerase inactivation, besides the previous effect due to immobilization. This result is confirmed in Fig. 4 where the growth of enzyme half-life with either increasing sugar concentration or decreasing temperature is clearly shown.

## 4.3

### Integral productivity

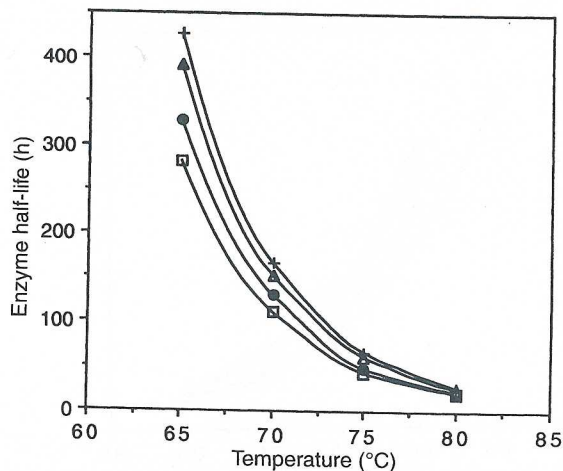
Taking into account thermal inactivation, the specific activity referred to a given amount of immobilized enzyme becomes a decreasing function of time. Defining the integral productivity as the cumulative enzyme activity and

**Table 4.** Temperature dependence of the first-order inactivation constants,  $k_d$  ( $\text{h}^{-1}$ ), of both native and immobilized glucose isomerase in the absence of substrate protection

$T$ (°C)	60	65	70	75	80
Native	0.0194	0.0682	0.2306	0.7534	2.380
Immobilized	$9.59 \cdot 10^{-4}$	0.0025	0.0062	0.0161	0.0343

**Table 5.** Dependence of the first-order inactivation constant,  $k_d(1 - \sigma) \cdot 10^3$  ( $\text{h}^{-1}$ ), on the temperature, under different conditions of substrate protection

$T$ (°C)	65	70	75	80
$G_o$ (mol/l)				
0.0	2.47	6.22	16.09	34.28
0.5	2.10	5.30	14.52	31.40
1.0	1.77	4.54	11.32	27.48
2.0	1.62	4.16	10.38	25.24

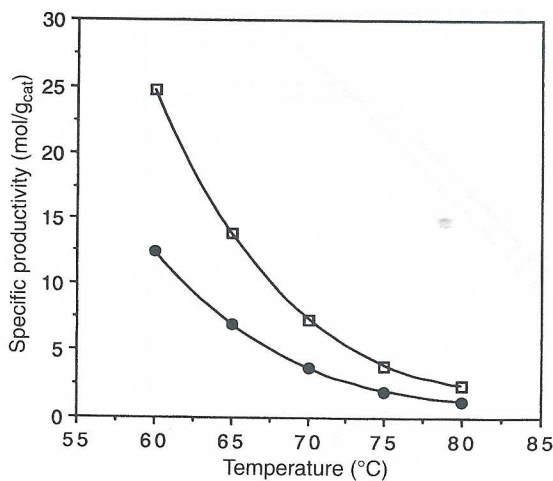


**Fig. 4.** Dependence of the immobilized GI half-life on both temperature and sugar concentration.  $G_o$  (mol/l): (□) 0.0; (●) 0.5; (▲) 1.0; (+) 2.0

calling  $V_{mfo}$  the maximum starting velocity of the forward reaction, which is proportional to the active enzyme concentration, integral productivity up to a given time,  $P(t)$ , up to infinite time,  $P_\infty$ , and up to enzyme half-life,  $P_{1/2}$ , can be estimated through Eqs. (15–17), respectively.

Substituting in these equations the values of  $V_{mfo}$  and  $k_d$  for glucose isomerization by immobilized glucose isomerase, the behaviours of Fig. 5 would be obtained for both  $P_\infty$  and  $P_{1/2}$  in the hypothetical situation of absent substrate, while Fig. 6 put in evidence the marked influence of substrate protection on  $P_\infty$ , at a given temperature. These results show on the whole that both productivities strongly fall at higher temperature, due to the quicker enzyme inactivation, but this effect seems to be only partially counterbalanced by the increasing substrate protection at higher equilibrium sugar levels.

Selection of the optimal operating conditions for immobilized glucose isomerase reactors requires, however, to get information on the way productivity achieves its maximum value either in the presence or in the absence of substrate protection. To this purpose, integral specific productivity at



**Fig. 5.** Temperature dependence of the integral productivities of 1.0 g of immobilized glucose isomerase at enzyme half-life (●) and at infinite time (□), in the absence of substrate protection

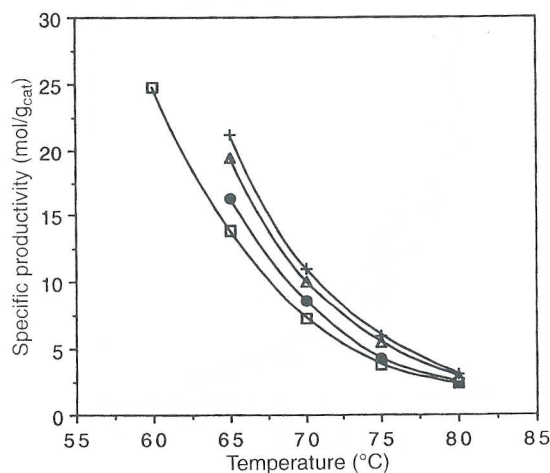


Fig. 6. Substrate protection effect on the integral productivity of 1.0 g of immobilized glucose isomerase at infinite time.  $G_0$  (mol/l): (□) 0.0; (●) 0.5; (Δ) 1.0; (+) 2.0

60 and 80 °C has been plotted versus time in Fig. 7, using different sugar levels. Although the use of logarithmic scale tends to reshuffle the effect of substrate protection, temperature appears to be the most important parameter controlling the productivity, which has to be considered to optimize the yield of continuous isomerization.

Increasing sugar concentration shows, on the other hand, remarkable improvement of  $P_\infty$  but no appreciable influence on the integral productivity at a given intermediate time. Since for practical purposes continuous operation is carried out under conditions ensuring productivities close to  $P_\infty$  (high residence times), substrate protection does not appear to be actually exploitable to improve productivity.

## 5

### Conclusion

The main kinetic parameters of immobilized glucose isomerase activity and inactivation have been estimated in

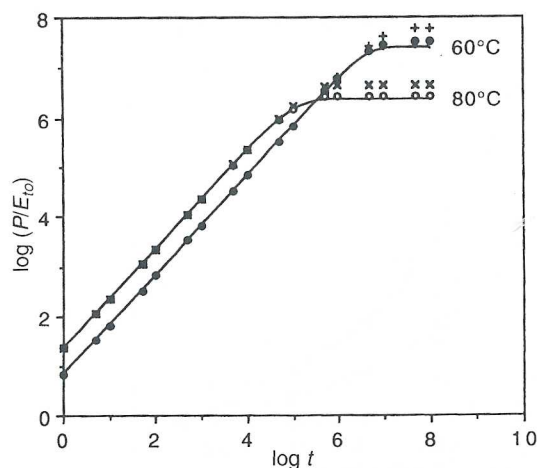


Fig. 7. Integral productivity of 1.0 g of immobilized glucose isomerase vs time.  $T = 60$  °C:  $G_0$  (mol/l): (—) 0.0; (●) 0.5; (+) 2.0.  $T = 80$  °C:  $G_0$  (mol/l): (—) 0.0; (○) 0.5; (×) 2.0. Units:  $t$  (s);  $P/E_{t_0}$   $\mu\text{-mol/g}_{\text{cat}}$

this study, either in the presence or in the absence of substrate protection, in order to provide suggestions for continuous HFCS production from starch hydrolysate with immobilized enzyme columns. Although there is evidence that immobilization of the enzyme is able to push the equilibrium towards the formation of fructose at  $T > 70$  °C, it seems that substrate protection cannot be usefully exploited to overcome thermal inactivation.

Also the enzyme productivity strongly falls at high temperature due to the quick enzyme inactivation, but this effect is only partially counterbalanced by the increasing substrate protection at higher sugar levels. This means that temperature control is the only viable way to limit thermal inactivation.

The next effort in this field will be addressed to the study of the effects of immobilization on glucose isomerase thermodynamic quantities, either in the presence or in the absence of substrate protection.

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