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Effect of the Degree of Polymerization of Inulin on the Rate of Hydrolysis Using Immobilized Inulinase

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Summary

The present paper addresses two crucial features in the industrial development of fructose production by enzymatic hydrolysis of inulin: the use of immobilized biocatalyst in the hydrolysis of crude extracts of chicory roots and the evaluation of the effect of degree of polymerization of inulin on the overall reaction rate. The immobilized biocatalyst consisted of inulinase covalently bound to Sepabeads® supports. It was demonstrated that its catalytic activity towards crude inulin extract (real substrate) was much higher than that exhibited towards pure inulin (synthetic solution). Experiments revealed that, in applications of practical interest with real substrate, the activity of immobilized enzyme was as high as 63 % of that of free enzyme in homogeneous solution. This certainly was a driving force to potential industrial application of this immobilized enzyme preparation. Therefore, the effect of pure and crude substrates on the kinetics of the reaction catalysed by the immobilized enzyme was investigated. The kinetic analysis revealed a Michaelis-Menten dependence of the reaction rate on substrate concentration for both pure (high molecular mass) and crude (low molecular mass) inulin. Interesting results were derived from the comparison of K_m and v_{max} values in the two cases. In particular, it was found that increasing degree of polymerization of the substrate caused v_{max} decrease and K_m increase. After evaluation of mass transport effects, this was mainly associated with a different substrate/enzyme affinity when exploiting inulin characterized by different (low or high) degree of polymerization.

Key words: immobilized enzyme, kinetic analysis, degree of polymerization, inulinase, fructose syrup

Introduction

Inulin hydrolysis for fructose production is a competitive process with respect to classical sucrose inversion followed by glucose isomerisation. Glucose isomerisation is performed enzymatically, with glucose isomerase converting the glucose obtained from starch through a series of enzymatic reactions of liquefaction and saccharification; it is a reversible reaction and high fructose concentrations requested by the food industry may only be reached after costly chromatographic separations (*1*). Sucrose inversion can be run either enzymatically or through acidic catalysis; the latter is preferred because the product has better flavour quality than the one obtained by enzymatic catalysis (*2*). Inverted syrups contain 50 % fructose and 50 % glucose.

The development of enzymatic hydrolysis of inulin, which leads to more than 90 % fructose in one reaction stage, requires the comprehension of reaction characteristics and the implementation of new immobilized biocatalysts. Although the reaction mechanism is a multistep catalysis with different enzymes acting on substrates with

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different characteristics, it has been proved that, without appreciable errors, the reaction can be described by Michaelis-Menten kinetics (*3*) and no substrate or product inhibition exists (*4*). The maximum activity of inulinases from different microbial sources is observed at a temperature of 60 °C (*5–7*), but a thermal optimization is advisable so as to gain the activity of enzyme without compromising its stability (*8*). Based on this, inulinase-catalysed inulin hydrolysis is feasible, with no particular hurdles for the process implementation. As a matter of fact, many applications of immobilized inulinases have been proposed in the literature in order to provide an even more efficient process. Inulinase was successfully immobilized on Amino-Cellulofine (*9*); more recently, an enzymatic membrane reactor has been proposed (*10*). A packed bed reactor with immobilized inulinases was tested, as well (*6*). However, in these works, reaction efficiency was tested on pure (reagent grade) inulin, while in the development of a new immobilized biocatalyst it is crucial to test catalytic efficiency with respect to crude materials, such as those that would be likely used in real processes. In this direction, Wenling *et al.* (*11*) proposed the immobilization of inulinase on macroporous beads and tested the catalyst on inulin extracts from Jerusalem artichoke dry powder.

In the present paper, different inulin extracts were obtained as a result of different extraction conditions applied to different chicory root samples. Then, the activity of inulinase, immobilized on Sepabeads® (*12*), towards different extracts was tested in order to evaluate its performance as a potential industrial preparation and to put in evidence relevant aspects of hydrolysis of crude inulin extracts. In particular, the effect of the degree of polymerization (DP) on the observed reaction rate in terms of mass transport and enzyme-substrate affinity was investigated on an experimental and a theoretical basis. This could be useful for activity tests of future immobilized enzymes and for fructose production process optimization.

Theory

The degree of polymerization of inulin may affect the reaction rates of immobilized enzymes because of both mass transport resistance, for the estimation of which it is necessary to account for the dependence of diffusion coefficient on inulin molecular mass, and kinetic performance, related to enzyme-substrate affinity. From a theoretical point of view, the kinetic effect can be evaluated only after isolating mass transport contribution from the observed reaction rate. Mass transport effects can be independently evaluated on the basis of fundamental calculations. Since the enzyme, in this case, is immobilized only on the external surface of spherical particles, all mass transport effects could be measured by a mass transport coefficient k_c, defined as the proportionality factor between inulin mass flux and a driving force expressed as inulin concentration difference. Such coefficient depends on the operating conditions according to a general correlation relating Sherwood number:

$$\mathrm{Sh} = \frac{k_c D}{D_{AB}} \qquad /1/$$

to Reynolds number:

$$\mathrm{Re} = \frac{\rho v D}{\mu} \qquad /2/$$

and Schmidt number:

$$\mathrm{Sc} = \frac{\mu}{\rho D_{AB}} \qquad /3/$$

where k_c is mass transport coefficient, D is diameter of immobilized biocatalyst, D_{AB} is the diffusion coefficient of inulin in water, μ is solution viscosity and ρ is solution density.

In well mixed systems, Sherwood number can be expressed as a function of both Reynolds and Schmidt numbers according to a power law relationship:

$$\mathrm{Sh} = a \cdot \mathrm{Re}^b \cdot \mathrm{Sc}^c \qquad /4/$$

where a, b, and c are semi-empirical coefficients.

In agitated vessels, the typical value of the exponent c is 1/3 (*13–15*). If mixing conditions and physical properties of the solution are unchanged, Sh and Sc definitions and Eq. 4 imply that:

$$\frac{k_c D}{D_{AB}} \propto \left(\frac{\mu}{\rho D_{AB}}\right)^{1/3} \qquad /5/$$

This means that mass transport coefficient k_c is proportional to $D_{AB}^{2/3}$:

$$k_c \propto D_{AB}^{2/3} \qquad /6/$$

D_{AB} is typically related to solute molecular mass (M_r) through a –0.6 power:

$$D_{AB} \propto M_r^{-0.6} \qquad /7/$$

as in the case of Wilke-Chang relationship where, indeed, –0.6 exponent refers to molar volume, rather than M_r (*15*), as well as in the case of diffusion of linear polymers (*16*).

From Eqs. 6 and 7 it can be concluded that:

$$k_c \propto M_r^{-0.4} \qquad /8/$$

Thus, the dependence of k_c on M_r and, therefore, the effect of DP on mass transport were established.

The kinetic effect of different DPs in terms of enzyme-substrate affinity, instead, was evaluated by comparison of Michaelis constant (K_m) values when substrates with different DPs were adopted.

Materials and Methods

Materials

Inulin extracts were obtained from *Cichorium intybus* L. var. Orchies according to the procedure described in the Methods section; chicory roots were kindly provided by Florimond-Desprez, Cappelle-en-Pevele, France. Reagent grade inulin used for comparison was purchased from Sigma (product code I2255, inulin from chicory, Sigma-Aldrich, Milan, Italy). The enzyme was a commercial mixture of exo- and endo-inulinases (Fructozyme L™, kindly supplied by Novozymes, Bagsværd, Denmark), with invertase activity. It was used as soluble enzyme or immobilized on solid supports. Supports for immobilization were Sepabeads® EC-HA403 (amino func-

tionalization), kindly supplied by Mitsubishi Resindion, Binasco, Italy.

Standard tests of enzymatic activity were run under the following conditions: pure inulin at initial inulin concentration, S_0, equal to 10 g/L, T=323.15 K (50 °C), pH=5.0, reaction time 30 min. Soluble and immobilized enzyme activities were 109 and 43 U per mg of protein, respectively.

Extraction of inulin from fresh chicory roots

Inulin was extracted from fresh roots of chicory during September and November harvest of 2008. Roots were washed and peeled. Afterwards, in order to obtain random samples and avoid differences that could be the result of using different sizes of roots, a number of roots was cut into pieces and placed into distilled water, where extraction took place at a temperature of 318.15 K (40 °C). After a fixed extraction time of 4 h, samples of 5 mL were collected for characterization until extraction equilibrium was reached. Different root/water mass ratios were adopted in order to obtain extracts with different characteristics, namely 50, 100 and 200 g/L (grams of roots per litre of water).

Extract characterization

Extract characterization concerns the determination of inulin concentration in the extracts and average degree of polymerization (DP) of inulin; these determinations rely on the evaluation of fructose and glucose obtained from acidic hydrolysis of inulin. Samples of 5 mL collected from the extraction step were centrifuged at 5300 rpm for 5 min. A volume of 1 mL of supernatant was immediately analysed to determine fructose and glucose concentration in the extracts as free sugars, hereafter referred to as F_f and G_f, respectively. The remaining supernatant containing inulin was hydrolysed to fructose and glucose by means of complete acidic hydrolysis; the conditions of hydrolysis were: pH=1.5 (by the addition of HCl), temperature 353.15 K (80 °C), time of hydrolysis 2 h. After complete acidic hydrolysis, samples were immediately analysed to evaluate total fructose and glucose concentrations (F_t and G_t). Concentrations of fructose and glucose derived from inulin (F_i and G_i) were obtained as differences:

$$F_i=(F_t-F_f) \qquad /9/$$

and

$$G_i=(G_t-G_f) \qquad /10/$$

DP of inulin was evaluated as F_i/G_i and inulin concentration as follows:

$$S=0.9\cdot F_i+G_i \qquad /11/$$

where concentrations are expressed on a mass basis, but the proposed relationship accounts for molar stoichiometry as explained by Ricca *et al.* (*3*). Extractions were considered at their equilibrium when inulin concentration *vs.* time of extraction reached a plateau.

Enzyme immobilization

The immobilization procedure relies on covalent binding of inulinases on amino supports. It was described in detail in a previous paper (*12*).

Reaction tests

Tests were carried out in a batch stirred system (Applikon, Delft, The Netherlands) consisting of a 1.5-litre glass vessel, a six-blade impeller driven by an electric motor (Stirrer Motor Assembly P100, Applikon) controlled by a stirring controller (P100, ADI 1032, Applikon), pH and temperature sensors, and a jacket for temperature control by means of a Bio Controller (ADI 1030, Applikon). All tests were run at 40 °C, mixing rate of 300 rpm, substrate initial concentration between 3 and 40 g/L, reaction volume 200 mL. Some changes in the procedures were necessary when using pure inulin or crude extracts as substrates and soluble or immobilized enzyme as biocatalyst. They were mainly related to the way of loading enzymes and substrate, and to reaction quenching. Pure inulin was a powder and it had to be dissolved in the reaction solution before adding the catalyst, while crude inulin extracts, after a filtration step, were directly utilized as such. Immobilized enzymes were weighed and an amount of 0.4 g of wet mass was used, while soluble enzymes were collected by means of a micropipette and loaded with 22.5 μL of Fructozyme; this quantity was chosen in order to have the same amount of protein loaded as in the case of the immobilized enzyme tests, namely 2.84 mg/L. The optimal pH for the enzyme activity ranged between 4.5 and 5.5 (*17*); for this reason, reactions with pure inulin were run in acetate buffer at pH=5, as described in the literature (*3*), while the extracts were treated at pH values between 5 and 5.5. In the case of soluble enzyme, the reaction was quenched by submerging the samples into boiling water as described in the literature (*18*), while the immobilized biocatalyst was separated from the reaction mixture by means of a 90-μm filter and the samples were immediately analyzed.

Reaction conversion degree was evaluated as follows:

$$X=\frac{S_0-S}{S_0} \qquad /12/$$

where X is inulin conversion rate, S_0 is initial concentration of inulin and it is known at the beginning of every reaction run, while S represents inulin concentration at any time during the reaction progress, evaluated from the measured fructose and glucose concentrations, according to the following relationship (*3*):

$$S=0.9\cdot F+G \qquad /13/$$

Kinetic analysis

Kinetic parameters of pure inulin and crude extract hydrolysis catalyzed by immobilized inulinases were evaluated according to the procedure described in a previous work (*3*). Initial velocity rates were measured at T=323.15 K (50 °C), pH=5.0, reaction time 30 min, inulin DP=6.4 and initial substrate concentrations of 3, 5, 7, 20 and 40 g/L. Michaelis-Menten parameters were determined through Hanes-Woolf linearization.

Sugar analysis

Sugar analysis was performed using high-performance liquid chromatography (HPLC). The mobile phase was H_3PO_4 aqueous solution (volume ratio of 0.1 %) and it was pumped at a flow rate of 0.5 mL/min. The column was a Supelcogel C610-H 300×7.8 mm (Supelco, Bellefonte, PA, USA). Analyses were run at room temperature. Under these conditions, fructose and glucose had retention times of 14.5 and 13 min, respectively. The detection relied on refractive index (RI 930 Jasco Europe, Milan, Italy). Quantification was based on a previously elaborated calibration curve.

Statistical analysis

Each experiment was repeated twice; however, only the average value of each of the two measurements was reported hereafter, since the calculated standard deviations never exceeded 5 %. The statistical analysis was performed using STATGRAPHICS v. 7.0 software (Statistical Graphics, Englewood Cliffs, NJ, USA) and the level of statistical significance was $p<0.05$. The effectiveness of the proposed mathematical model was evaluated by checking the coefficient of determination, R^2, and the percentage error between the predicted and the experimental values.

Results

Inulin extraction conditions

Three extraction runs were carried out using different root/solvent ratios and chicory from different harvest times in order to obtain low concentration of the extract. The characteristics of extracts are reported in Table 1.

Table 1. Inulin concentration in the extracts and average degree of polymerization of inulin

Extraction test	$\frac{m(\text{root})}{V(\text{solvent})}$/(g/L)	S/(g/L)	DP	M_r
E1	50[a]	9.1	10	1640
E2	100[b]	15	4.8	800
E3	200[b]	17.9	6.4	1050

[a]September harvest, [b]November harvest (2008); S=inulin concentration, DP=degree of polymerization; M_r is the average molecular mass of the extracted inulin molecule (M_r=162(DP–1)+180)

An obvious result was that an increase of inulin concentration was obtained by augmenting the solid/solvent ratio. As far as the DP is concerned, it was proved that late harvesting (November) was characterized by a decrease of DP (*19*); this justifies the high DP value in extraction test E1. The reason for the slight difference between E2 and E3 must be sought in the different storage times of chicory roots for the preparations (7 days and none, respectively), which is also known to influence the characteristics of inulin contained in the roots (*20*). Aiming at determining the effect of DP on enzymatic hydrolysis without the influence of substrate concentration, extracts reported in Table 1 were subsequently diluted in order to obtain inulin syrups with different DP but identical substrate concentration (9 g/L). The major characteristics of the extracts exploited to perform the reaction tests (Table 1 summarizes the experimental conditions in which the extracts were obtained) are reported in Table 2. It is worthwhile noticing that dilution did not alter the DP values.

Table 2. Extracts used for enzymatic reaction tests

Extract	S_0/(g/L)	DP
E1	9	10
E2	9	4.8
E3	9	6.4

S_0=initial inulin concentration, DP=degree of polymerization

A subsequent extraction run was carried out in order to obtain very concentrated syrup to be then used in successive diluted forms as a substrate for kinetic analysis of enzyme reaction. The extraction was run with September roots and solid/solvent ratio of 1000 g/L, resulting in 71 g/L of inulin syrup with DP=7. From this extract, syrups for kinetic analysis at initial inulin concentrations of 3, 5, 7, 10, 20 and 40 g/L were obtained by dilution with distilled water.

As a standard, the performance of soluble enzyme on both pure and crude inulin was tested. Extract E3 was chosen as crude substrate; it had DP=6.4, while the DP of pure (reagent grade) inulin was found equal to 28, after complete acidic hydrolysis. Fig. 1 shows the activity of the enzyme in its native form on both crude extract and pure inulin.

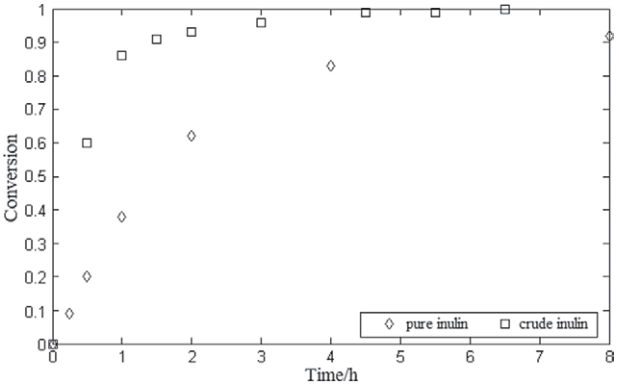

Fig. 1. Soluble enzyme activity on crude extract, as compared to pure inulin (initial inulin concentration S_0=9 g/L, degree of polymerization DP=6.4 and 28, respectively, and enzyme loading E_0=2.84 mg of protein per L)

The extremely fast reaction rate of crude extract hydrolysis is obvious; its final conversion was reached after about 4 h, and after the same time pure inulin was only converted to an extent of 80 %. When reaction rates were evaluated at the beginning of the reaction, *i.e.* at the same substrate concentration, the activity of soluble enzyme was found to be three times higher on crude than on pure inulin, with 60 and 20 % conversion, respectively, after 30 min. The faster hydrolysis of crude inulin with respect to that of reagent grade inulin indi-

cated, on the one hand, that no enzyme inhibitors were present in the extracts and, on the other hand, that there was a performance improvement when using raw extracts as substrates. This was due to the difference in DP, with small molecules having higher affinity for the enzyme. Moreover, the effect of mass transport resistances, possibly affected by inulin molecular size, could be excluded due to homogeneous catalysis provided by soluble enzymes in their native state. This topic will be addressed in the next section on kinetic analysis of the performance of immobilized catalyst.

From standard activity data on pure inulin, the ratio between soluble and immobilized standard activities (Materials section) was equal to 39 %. A test of hydrolysis of chicory extracts catalyzed by the immobilized enzyme was run to establish if the new immobilized enzyme was actually able to catalyze the reaction on crude extracts and to evaluate possible differences with respect to the soluble enzyme. The E3 extract (DP=6.4) was chosen as substrate; experimental results, as compared to those relative to the soluble enzyme, are reported in Fig. 2.

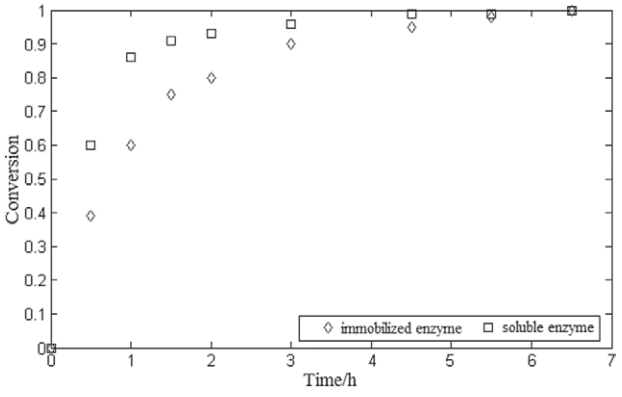

Fig. 2. Performance of immobilized *vs.* soluble enzyme on the same protein loading basis (initial inulin concentration S_0=9 g/L, degree of polymerization DP=6.4 and 28, respectively, and enzyme loading E_0=2.84 mg of protein per L)

First of all, it is of relevance that immobilized enzyme could bring crude substrate to complete conversion. Moreover, when its activity was compared to that of soluble enzyme, the latter still remained more active. It is worthwhile observing that, in the case of pure inulin, the activity ratio between immobilized and soluble enzyme was equal to 39 %, whereas in the case of crude extract hydrolysis it was only 63 %. The immobilized catalyst had a great potential in crude inulin hydrolysis; moreover, the difference in the activities of the soluble and the immobilized enzyme was not due to an intrinsic activity loss of the immobilized catalyst, but, to a certain extent, to the effect of mass transport resistances in respect to the high molecular mass of pure inulin.

With this promise of an active biocatalyst (Fig. 2) and an effect of different substrates on inulinase activity (Fig. 1), the performance of the immobilized catalyst was tested on different extracts, characterized by the properties reported in Table 2. Experimental results of the reaction tests are reported in Fig. 3.

Results shown in Fig. 3 were of crucial importance since they allowed assessing that the differences already

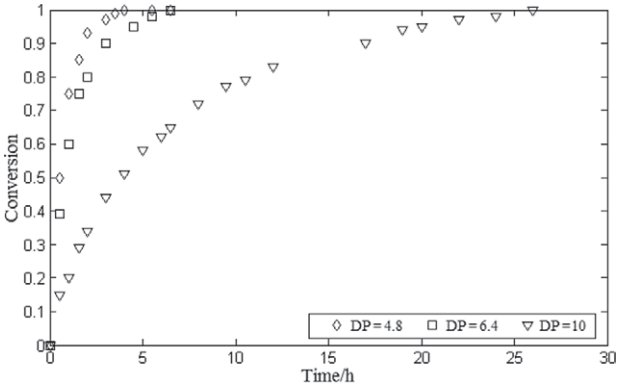

Fig. 3. Comparison of the effects of different degrees of polymerization (DP) on immobilized enzyme-catalyzed hydrolysis (initial inulin concentration S_0=9 g/L, and enzyme loading E_0= 2.84 mg of protein per L)

observed in Fig. 1 did not depend on the use of crude or reagent grade inulin, but rather on the faster catalysis achievable with lower molecular mass of substrates. In fact, the three series of data in Fig. 3 were all relative to crude inulin from chicory root extracts; the only difference between them was the molecular mass of the substrate. For instance, the reaction of the substrate with DP=4.8 was completed after 4 h, while in the case of DP=6.4, 6 h were necessary; after the same time, substrate with DP=10 reached only 60 % conversion. The effect of DP values, ranging between 4.8 and 28, on the reaction rate (initial activities after 30 min) is reported in Fig. 4.

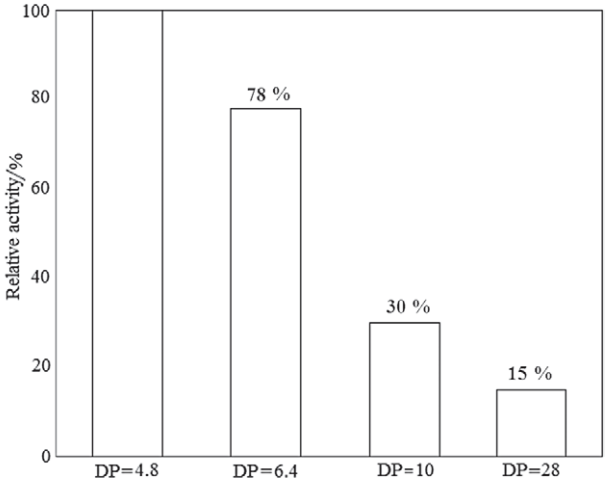

Fig. 4. Effect of the degree of polymerization of inulin (DP) on the reaction rate evaluated after 30 min (initial inulin concentration S_0=9 g/L, and enzyme loading E_0=2.84 mg of protein per L)

With reaction rates decreasing to 30 % when passing from DP=4.8 to 10, the degree of polymerization must be definitely considered as a process variable in enzymatic hydrolysis of inulin. In a process design perspective, though, the kinetics of crude chicory extracts must be known. In this context, using data from pure inulin hydrolysis could lead to mistaken quantification of the appropriate enzyme loading. In order to obtain a tool

for quantitative comparison of pure and crude inulin kinetics, a kinetic study for these two cases was undertaken and reported in the next section.

Kinetic analysis

As far as the kinetics of the hydrolysis of crude inulin extracts catalyzed by immobilized enzymes was concerned, duplicate reaction runs were carried out at different substrate initial concentrations; the coefficients of variation of duplicate data (not shown) never exceeded 5 %. From these data, initial slopes were evaluated in order to obtain reaction rates at any substrate concentration; initial velocity experiments were always stopped before 10 % conversion and linear interpolation of initial concentration data gave statistically satisfactory fitting. Once reaction rates *vs.* substrate concentrations data were available, they were correlated through Michaelis-Menten kinetics. The applicability of Michaelis-Menten model to this system and the values of kinetic parameters were evaluated through different linear manipulations of Michaelis-Menten equation. Hanes-Woolf plot offered best data fitting and it was chosen for the estimation of parameters (Fig. 5).

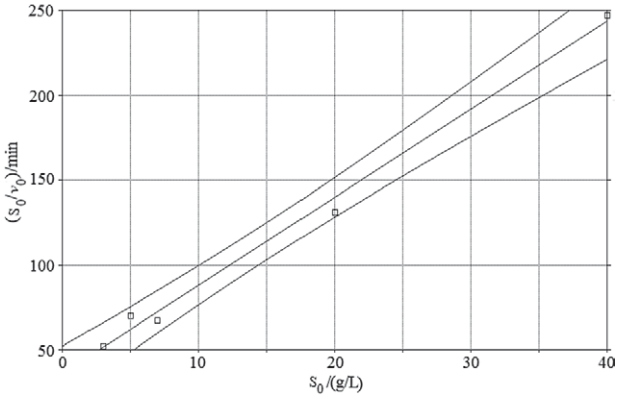

Fig. 5. Kinetic fitting and parameter determination for crude inulin hydrolysis (degree of polymerization DP=6.4). Hanes--Woolf plot (K_m/v_{max}=36.18 min; $1/v_{max}$=5.19 (min·L/g)). Lines around the fitting curve represent 95 % confidence interval. S_0=initial inulin concentration, v_0=initial reaction rate

The fitting within 95 % confidence interval proved that Michaelis-Menten model was appropriate for the reaction under study and kinetic parameters could be estimated with satisfactory accuracy. From intercept and slope of the straight line in Fig. 5, the following values of kinetic parameters were obtained: v_{max}=0.156 mM/min= 0.19 (g/L)/min and K_m=5.75 mM =7.0 g/L.

If the rate equation is substituted into a substrate mass balance equation for a batch reactor, the following integrated solution, expressed as a relationship between reaction time and current substrate concentration, can be used for validation over a wide range of reaction conversions:

$$t = \frac{K_m}{v_{max}} \ln\left(\frac{S_0}{S}\right) + \frac{(S_0 - S)}{v_{max}} \qquad /14/$$

Validation was shown in Fig. 6.

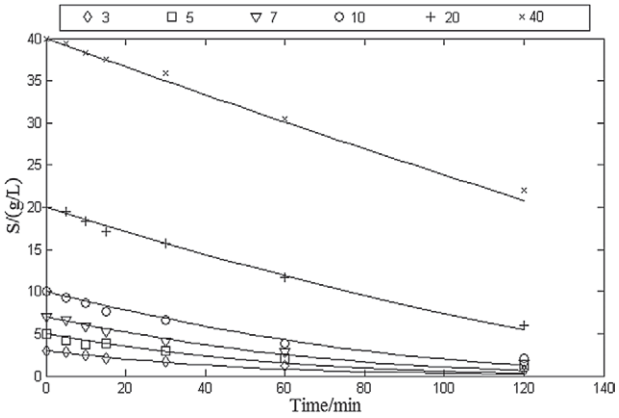

Fig. 6. Kinetic model validation for crude inulin hydrolysis. Continuous lines represent model predictions. S=inulin concentration

The comparison shown in Fig. 6 was referred to conversion values, which were often above 50 % (much higher than those at 10 % used for the kinetic model implementation). The good agreement between experimental and predicted data validated the model, with the deviation being often below 5 %, especially at high substrate concentrations, which is definitely more interesting from a process development perspective.

A kinetic analysis identical to that performed on crude inulin at DP=6.4 was performed with pure inulin at DP=28, finding slower kinetics as already observed in Figs. 1 and 3. The rate equation was confirmed to follow the Michaelis-Menten model and the reliability of fitting and parameter estimation were very similar to that shown for crude inulin. The results in terms of kinetic parameters are reported in Table 3 as compared to the results found for crude inulin.

Table 3. Comparison of Michaelis-Menten kinetic parameters at different degrees of polymerization of inulin

Inulin	K_m		v_{max}	
Pure DP=28	10.4 mM	49 g/L	0.042 mM/min	0.2 (g/L)/min
Crude DP=6.4	5.7 mM	7.0 g/L	0.16 mM/min	0.2 (g/L)/min

K_m=Michaelis constant, v_{max}=maximum reaction rate, DP=degree of polymerization

Analysis of mass transport effects

As demonstrated in the theoretical section, the effect of DP on the observed reaction rates in terms of mass transport could be evaluated through calculation of mass transport coefficients. According to Eq. 8, the ratio of the mass transport coefficients, k_c, for pure inulin (M_r=4700) and crude inulin (M_r=1300) is:

$$\frac{k_{c,pure}}{k_{c,crude}} = \left(\frac{4700}{1300}\right)^{-0.4} = 0.6 \qquad /15/$$

Eq. 15 states the importance that the effect of DP on mass flux, of which k_c is a measure, be taken into ac-

count, since k_c can almost double when passing from big to small molecules. However, in the present case, it was crucial to estimate the effect of mass transport on the observed process rate, as it was determined by both mass transport and intrinsic kinetics. This could be done by comparison of experimental data of soluble enzyme (no mass transport resistances) and immobilized enzyme. It can be seen in Fig. 1 that the ratio of (intrinsic) reaction rate, v_{intr}, of soluble enzyme to crude and pure inulin was equal to 3; this was due only to the effect of different DP.

$$\frac{v_{intr,\,crude}}{v_{intr,\,pure}} = 3 \qquad /16/$$

It can be seen from the data on immobilized enzyme shown in Table 3 that the reaction rate ratio with crude and pure inulin under the same conditions as those reported in Fig. 1 was equal to 3.4, and this was to be ascribed to both kinetics and transport.

$$\frac{v_{imm,\,crude}}{v_{imm,\,pure}} = 3.4 \qquad /17/$$

By comparison of the ratios for soluble and immobilized enzymes (Eqs. 16 and 17), mass transport affected the observed reaction rate by 13 %:

$$\frac{\left(\frac{v_{imm,\,crude}}{v_{imm,\,pure}}\right) - \left(\frac{v_{intr,\,crude}}{v_{intr,\,pure}}\right)}{\left(\frac{v_{intr,\,crude}}{v_{intr,\,pure}}\right)} \cdot 100 = \frac{3.4-3}{3} \cdot 100 = 13\ \% \qquad /18/$$

The remaining difference (87 %, the major part) between crude and pure inulin hydrolysis performance by immobilized biocatalyst was solely due to kinetic effects.

Discussion

From the data in Table 3, all the difference between the kinetic behaviour of crude (low DP) and pure (high DP) inulin could be interpreted according to the different values of K_m and v_{max}. This opened up a discussion on the phenomenological interpretation of the difference of reaction rates between low and high DP of inulin substrates. In Michaelis-Menten model, at a fixed enzyme loading, v_{max} was proportional to the kinetic constant of the reaction. From this point of view, the molar changes of v_{max} should be interpreted as a faster release of the product once the enzyme had come in contact with the substrate. Instead, when v_{max} was calculated in terms of mass changes, it was constant, meaning that the mass rate of product release was only dependent on the enzyme-substrate (ES) complex concentration and not on the type of substrate (small or big inulin molecule). Moreover, at low E/S ratios, as in this work, ES concentration depended on the enzyme concentration only, and not on the substrate molar concentration. On the other hand, K_m can be considered as an indirect measure of the free energy change due to substrate binding to the enzyme (*21*) or, in other words, an inverse measure of the affinity between the enzyme and substrate: the higher K_m value, the lower the affinity (higher free energy change). Therefore, back to the results in Table 3, faster kinetics of crude inulin substrate could be explained as a higher affinity of inulinases to small molecules than to high molecular mass molecules, with K_m becoming seven times bigger when expressed as mass concentration and only twice when expressed as molar concentration, when the DP decreased by a factor of four.

This certainly clarified the role of the degree of polymerization of inulin on the rate of reaction and revealed the potential of a classical tool, such as kinetic analysis, in giving a direction for the phenomenological explanation of the evidence of practical interest.

Conclusions

In the present paper, the efficiency of an immobilized catalyst in the hydrolysis of raw inulin extracts for fructose production was proved. The immobilized enzyme exhibited lower activity with respect to that of native enzyme, which is expected in enzyme immobilization. However, in the present work, it was demonstrated that this was true only when reagent grade inulin with high DP was adopted as a substrate; in that case, it was thought that molecular size of the substrate represented a limiting factor for efficient interaction between the immobilized enzyme and the substrate itself. This hypothesis was supported by the fact that the divergence between soluble and immobilized biocatalyst was significantly reduced when low molecular mass substrates were processed, as it is the case for crude inulin. On the basis of that, the exploited immobilized enzyme proved to have a great potential in industrial application of inulin hydrolysis.

In addition to these practical findings, a relevant contribution to the scientific understanding of this reaction was ascertained: the importance of the degree of polymerization of inulin in the reaction performance. This was proved experimentally, quantified and explained on the basis of a kinetic analysis. In particular, the effect of inulin molecular mass was related to the dependence of enzyme-substrate affinity here expressed in terms of Michaelis constant and, to a lesser extent, to mass transport resistances. It was evidenced that different DPs of typical inulin extracts determined relevant changes of reaction performance in terms of conversion; these could, in turn, lead to an erroneous estimation of the reaction time or of the enzyme loading necessary for a reaction to get a given conversion in a given reaction time. Due to the generally high impact of enzyme costs on the overall costs of an enzymatic process, it is our belief that these effects should always be considered during calculations and design of bioprocesses of natural polymers.

Nomenclature

a, b, c	semi-empirical coefficients
D	immobilized biocatalyst particle diameter
D_{AB}	inulin diffusivity in water
DP	degree of polymerization
E	enzyme loading
ES	enzyme-substrate complex
F	fructose concentration
G	glucose concentration
k_c	mass transport coefficient

K_m	kinetic parameter, Michaelis constant
M_r	molecular mass
Re	Reynolds number
S	substrate (inulin) concentration
Sc	Schmidt number
Sh	Sherwood number
T	temperature
v	reaction rate
v_{max}	kinetic parameter, maximum velocity
X	inulin conversion rate

Greek symbols

ρ	solution density
μ	solution viscosity

Subscripts

crude	raw inulin extracts from chicory roots
f	free sugar in the extract
i	sugar from inulin hydrolysis
imm	immobilized enzyme
intr	intrinsic
pure	reagent grade inulin
t	total sugars after extract hydrolysis (t=f+i)
0	initial (time of reaction=0)

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