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Design and characterisation of an enzyme system for inulin hydrolysis

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Abstract

The optimal conditions for inulin hydrolysis using a commercial inulinase preparation, either free or immobilised in activated Amberlite were established by factorial design and surface response methodology. The immobilised biocatalyst displayed highest activity at pH 5.5 and 50 °C, whereas the optimum pH for the free form was slightly more acidic (4.5), and the optimum temperature was a little higher (55 °C). The model system estimated optimal pH and temperature values of 5.4 and 52 °C for the immobilised system and 4.9 and 56 °C for the free system. Michaelis–Menten type kinetics adequately described both free and immobilised bioconversion systems, which were evaluated under the respective optimal pH and temperature conditions. The use of a non-linear regression method for the determination of the kinetic parameters provided a best fit to the experimental data, as compared to a conventional Lineweaver–Burk linearisation. The K_m for inulin of the free biocatalyst was 153 g l^{-1} at 55 °C and pH 4.5, whereas the apparent K_m for inulin of the immobilised biocatalyst was 108 g l^{-1} at pH 5.5 and 50 °C. The reutilisation of the immobilised biocatalyst throughout consecutive batches was evaluated. A significant decrease of enzyme activity was observed in the first two batches, after which the system exhibited significant stability. The low cost of the support, the stability of the immobilised biocatalyst towards pH and temperature and its high affinity for the substrate suggests its potential for inulin hydrolysis.

Keywords: Biotransformation; Enzyme immobilisation; Inulin; Inulinase

1. Introduction

Inulin belongs to a class of carbohydrates known as fructans, in which most of the glycosidic bonds are made of fructosyl-fructose bonds, and that usually have a terminal glucose unit. Inulin is a linear β-(2 → 1)-linked fructose polymer that occurs as a reserve carbohydrate in garlic, asparagus root, Jerusalem artichoke, dahlia tubers or chicory root. The percent inulin content, on fresh weight basis, from these sources ranges from 10% to 20% (Kaur & Gupta, 2002). This polymer is a recognised source for the production of either ultra-high fructose syrups, with a D-fructose content over 75% (Vandamme & Derycke, 1983), alternatively to the multi-enzyme hydrolysis of starch or the less favoured inverted sugar production with invertase, or for the production of oligofructose syrups. Fructose is a GRAS sweetener, sweeter than sucrose (up to 1.5 times), with lower cost, and functional properties that enhance flavour, colour, and product stability, and is thus widely used in many foods and beverages instead of sucrose (Hanover & White, 1993). Furthermore, fructose metabolism bypasses the known metabolic pathway of glucose and therefore does not require insulin (Millo & Werman, 2000).

Oligofructose is a prebiotic, and its positive effect on human health has been widely acknowledged (Kaur & Gupta, 2002; Roberfroid, 2002). Inulin hydrolysis to

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fructose is carried out by an exoinulinase (EC 3.2.1.26; β-D-fructofuranosidase) acting either alone or synergistically with an endoinulinase (EC 3.2.1.7; β-fructan-fructanohydrolase) (Yun et al., 2000). The latter enzyme is responsible for the partial hydrolysis of the polymer to fructooligosaccharide (Cho, Sinha, Park, & Yun, 2001a, 2001b; Park, Kim, Kim, & Yun, 1998; Park, Bae, You, Kim, & Yun, 1999; Yun, Kim, Kim, & Song, 1997a; Yun, Kim, Uhm, & Song, 1997b; Yun et al., 2000).

Chemical hydrolysis of inulin can be carried out by treatment with organic or mineral acids (Beglov & Golubev, 2004; Kim & Hamdy, 1986) or through heterogeneous catalysis using solid acidic catalysts, such as acid-cation exchange resins (Matsumoto and Yamazaki, 1986), zeolites (Abasaeed & Lee, 1995, 1996; Jacobs and Hinnekens, 1990) or oxidised activated carbon (Heinen, Peters, & van Bekkum, 2001). However, the chemical approach is currently associated with some drawbacks, namely formation of unwanted by-products and coloured and colour forming compounds, which lower product yield and require a more demanding downstream processing (Matsumoto and Yamazaki, 1986). These shortcomings can be overcome if the more specific enzymatic route is used. This approach is particularly effective if an immobilised biocatalyst is used, since it allows either biocatalyst reuse or continuous mode of operation and prevents contamination of the processed product. Such systems have been evaluated by several authors, by using either immobilised inulinase (Ettalibi & Baratti, 1992; Gupta, Kaur, Kaur, & Singh, 1992; Kim, Byun, & Uhm, 1982; Nakamura, Ogata, Shitara, Nakamura, & Ohta, 1995; Peters & Kerkhoofs, 1983; Wenling, Huiying, & Shiyuan, 1999; Yun et al., 1997a; Yun et al., 2000), or whole cells with inulinase activity (Barranco-Florido, García-Garibay, Gómez-Ruiz, & Azaola, 2001; Yun, Song, Choi, Choi, & Song, 1999). The use of a cheap and simple, yet effective, immobilisation method can provide a key asset, if an experimental set-up amenable for scale-up is envisaged (Katchalski-Katzir & Kraemer, 2000; Schmid et al., 2001). Careful characterisation of the experimental system is required if an effective system is to be developed. In the present work, a systematic evaluation of key operational parameters for inulin hydrolysis, based on the use of a simple immobilised biocatalyst, is carried out and matched to the free system. Thus, the optimum pH and temperature conditions are established, using a factorial design and response surface methodology. The kinetic parameters were computed by both conventional linearisation of the Michaelis–Menten kinetics and non-linear optimisation. This work can provide a preliminary basis for the production of fructose syrups with a commercial inulinase preparation (Fructozyme L) immobilised onto Amberlite, a cheap immobilisation support (Obón, Castellar, Iborra, & Manjón, 2000).

2. Materials and methods

2.1. Biocatalyst

Fructozyme L, a commercial preparation of inulinases from *Aspergillus niger*, was provided by Novozymes.

2.2. Enzyme immobilisation

Fructozyme L was immobilised onto Amberlite IRC 50 (Rohm and Haas) based on the process described by Obón et al. (2000). Briefly, 30 g of Amberlite were successively washed with 200 ml distilled water and with 100 mM acetate buffer, pH 4.5, and then filtered through qualitative filter paper. 75 ml of a 100 g l^{-1} polyethylenimine solution were then added to the support and the suspension incubated at room temperature under gentle magnetic stirring for 2 h. The modified support was again recovered by filtration and incubated at room temperature under gentle magnetic stirring for 2 h in the presence of 150 ml of a 10% aqueous solution of glutaraldehyde. The activated support was then incubated at 4 °C under gentle magnetic stirring for 2 h in the presence of 30 ml of a solution of Fructozyme L. This solution was prepared by diluting, 10-fold, the commercial preparation with 100 mM acetate buffer, pH 4.5. The support was again recovered by filtration, thoroughly washed with 100 mM acetate buffer, pH 4.5, filtered and stored at 4 °C until use.

2.3. Inulin hydrolysis

All bioconversion trials were performed in duplicate, at least, in a magnetically stirred jacketed glass vessel, with 25 ml volume. Reactions were started by adding 10 μl of a 10-fold diluted solution of Fructozyme L, whether pH and temperature or substrate concentration effects on biocatalytic activity were being evaluated, to 4 ml of an inulin solution in 100 mM acetate buffer, or 120 mg of the immobilised biocatalyst, so as to give similar enzyme concentrations. Occasionally the inulin solution was incubated with no enzyme added. Samples (100 μl) were taken periodically, in one-minute intervals up to 5 min, when the free biocatalyst was used, or up to 20 minutes in 4 minintervals, when the immobilised biocatalyst was evaluated, and immediately assayed for quantification of reducing sugars. Bioconversion runs were performed in a temperature range of 40–70 °C, in a pH range of 4.0–6.5, in an inulin concentration range of 1.0–200 g l^{-1} and with a stirring speed of 600 rpm. This allowed external mass transfer resistances to be ruled out, as determined in preliminary trials. Occasionally the supernatant was assayed for free protein.

2.4. Estimation of the kinetic parameters

The reaction was assumed to exhibit Michaelis–Menten kinetics. The kinetic parameters were first estimated by linear regression using the Lineweaver–Burk plot, which results from the linearisation of the Michaelis–Menten equation:

$$V = \frac{V_{\max} S}{S + K_m} \rightarrow \frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \cdot \frac{1}{S}. \quad (1)$$

The kinetic parameters were also computed by non-linear optimisation, by minimising the following error function:

$$\phi = \sum_i e_i \cdot w_i \quad (2)$$

in which e_i is the relative error at each of the i data points (as opposed to the absolute error used in standard linear regression fitting) and w_i is a weight calculated as the inverse of the standard relative error associated with the experimental determination, so that the most trusted determinations have a higher weight than those with a higher associated error.

2.5. Biocatalyst reuse

Four hundred milligrammes of the immobilised biocatalyst was added to 10 ml of a 5 g l^{-1} inulin solution in pH 5.5 acetate buffer at 50 °C. Samples (100 μl) were taken periodically up to 20 minutes at 4-min intervals and immediately assayed for quantification of reducing sugars. Occasionally the supernatant was assayed for free protein. The biocatalyst was recovered by filtration in qualitative filter paper, thoroughly washed with pH 5.5 acetate buffer, and again incubated in 10 ml of a fresh 5 g l^{-1} inulin solution in pH 5.5 acetate buffer at 50 °C for a new bioconversion run. Two independent runs were performed in each batch.

2.6. Analytical methods

Quantification of reducing sugars was performed by the DNS method (Miller, 1959). Protein was quantified by the Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951).

Any contribution of thermal hydrolysis was ruled out as no reducing sugars were observed after incubating the inulin mixture at 50 °C.

3. Results and discussion

3.1. Activity optima

The influence of temperature and pH on the biocatalyst activity of the free and immobilised enzyme was investigated. In order to detect the operational conditions that optimise the biocatalyst activity, the results were fitted to a polynomial model:

$$Y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} a_{ij} x_i x_j, \quad (1)$$

where Y is the response variable (biocatalyst activity) and x_i is the ith independent variable (X_1 = pH or $X_2 = T$) all scaled to the same range (between 0 and 1), a_0 the constant of the model, a_i the first order model coefficients, and a_{ij} the second order coefficients. When $i \neq j$, then a_{ij} is the coefficient for the interaction between factors i and j. The experimental data were first fitted to the model by multiple regression. The statistical significance of each of the terms was evaluated by analysis of variances (ANOVA). The following relationships were obtained:

Free enzyme :

$$Y = (0.24 \pm 0.10) + (0.97 \pm 0.34) \cdot X_1 + (1.7 \pm 0.3) \cdot X_2 - (1.3 \pm 0.3) \cdot X_1^2 - (1.6 \pm 0.3) \cdot X_2^2, \quad r^2 = 0.80. \quad (2)$$

Immobilised enzyme :

$$Y = (0.28 \pm 0.14) + (1.0 \pm 0.4) \cdot X_1 + (1.4 \pm 0.4) \cdot X_2 - (1.4 \pm 0.4) \cdot X_1^2 + +(0.59 \pm 0.33) \cdot X_1 \cdot X_2 - (1.7 \pm 0.4) \cdot X_2^2, \quad r^2 = 0.81. \quad (3)$$

Both free and immobilised enzymes show activity optima within the tested ranges. Table 1 compares the predicted maximum activity conditions and values with those obtained experimentally.

A shift in the optimum pH from 4.5 to 5.5 could be observed as a result of immobilisation. Both optima are in agreement with the optimum pH of 5 reported for inulinases from *Aspergillus ficuum* (Ettalibi & Baratti, 1992). The activity maximum of the immobilised enzyme form was shifted from 55 to 50 °C as compared to the free form. A broader-shaped curve was observed for the temperature–pH–activity profile of the immobilised enzyme preparation, as compared to the free form

Table 1
Optimal pH and temperature for the hydrolysis of inulin promoted by free and immobilised inulinase, according to experimental data and surface response methodology

Biocatalyst form	Experimental	Surface response
Free	pH 4.5, 55 °C $V = 0.048$ g l^{-1} min^{-1}	pH 4.9, 56 °C $V = 0.043 \pm 0.008$ g l^{-1} min^{-1}
Immobilised	pH 5.5, 50 °C $V = 0.012$ g l^{-1} min^{-1}	pH 5.4, 52 °C $V = 0.011 \pm 0.002$ g l^{-1} min^{-1}

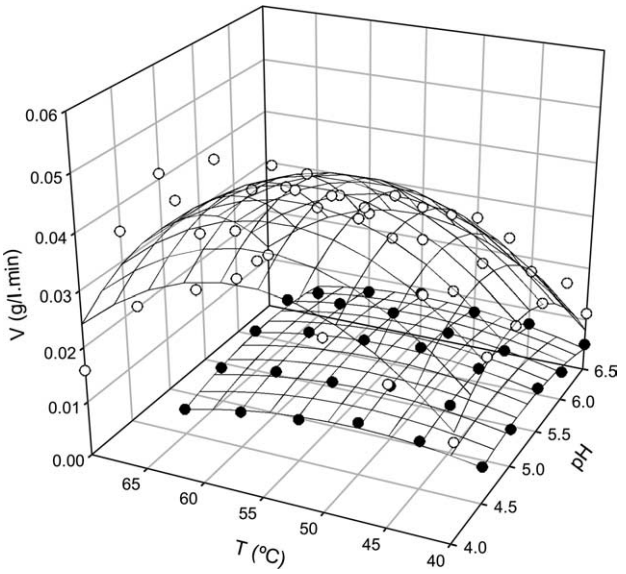

Fig. 1. Combined effect of the temperature and pH of the bioconversion medium in the biocatalytic activity of free (open dots) and Amberlite-immobilised (closed dots) inulinase. A 5 g l^{-1} inulin solution was used.

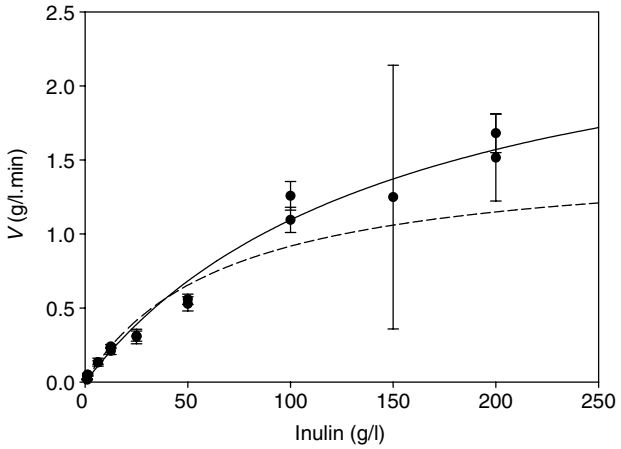

Fig. 2. Influence of the substrate concentration on the activity of free inulinase. Bioconversion runs were carried out at pH 4.5 and 55 °C. Model predictions based on kinetic parameters estimated by Lineweaver–Burk linearisation (dashed line) and by non-linear regression (full line) are displayed.

(Fig. 1). The immobilised biocatalyst thus presents a high relative activity over a wider range of temperature and pH, as compared to data reported for inulinase immobilised on porous glass (Ettalibi & Baratti, 1992) and on amino-cellulose (Nakamura et al., 1995).

3.2. Kinetic parameters

The kinetic parameters were first determined for the free enzyme, at pH 4.5 and 55 °C. Fig. 2 compares the curves obtained when using the Michaelis–Menten parameters estimated through the Lineweaver–Burk linearisation and the non-linear regression method. The Figure clearly shows that Lineweaver–Burk linearisation may lead to poorly accurate assessments. Since the method inverts both V and S, it becomes particularly sensitive to the data points at low substrate concentration, i.e., low V, due to two factors: (i) the higher value of the error of $1/V$ contributes to the regression error and (ii) the higher (S, V) values are clustered into a small region of ($1/S$, $1/V$) values, while the lower (S, V) values are spanned into a wide region of ($1/S$, $1/V$) values. This explains why the parameters estimated according to the Lineweaver–Burk plot lead to a curve that well fits the data at low inulin concentrations, but highly underestimates the reaction rate at higher inulin concentrations. Further comments on the limitation of this, as well as other linear regression methods, in the estimation of enzyme kinetic parameters can also be found in the literature (Cornish-Bowden & Eisenthal, 1974; Learmonth, 1995; Sagnella, 1985; Selwyn, 1995). The parameters estimated through the non-linear optimisation procedure yield a curve that well describes the experimental data over the full range of inulin concentration. The estimated parameters were $V_{max} = 2.77$ g l^{-1} min^{-1} and $K_m = 153$ g l^{-1}. These results show that most trials were carried out at concentrations below K_m, i.e. at conditions for which the reaction rate is less than half the maximum theoretical reaction rate. However, it is not feasible to work at higher inulin concentrations due to the inulin solubility limit, roughly 0.15 weight fraction at 55 °C (Bot, Erle, U, Vreeker, & Agterof, 2004), and since reaction systems involving polysaccharide solutions become increasingly viscous with a concomitant concentration increase (Azis, Chin, Deacon, Harding, & Pavlov, 1999). The system viscosity further limits the operation of the immobilised enzyme reactor since it leads to poor mass transfer conditions and to high-pressure drops, when a packed bed reactor is used. Thus the maximum inulin concentration used when performing the kinetic characterisation of the system was 100 g l^{-1}. Only the region of low substrate concentrations of the Michaelis–Menten kinetic curve was therefore obtained (Fig. 3). The estimated parameters for the immobilised form were $V_{max} = 0.542$ g l^{-1} min^{-1} and $K_m = 108$ g l^{-1} at pH 5.5 and 50 °C. Although the derived parameters allow a very accurate description of the enzyme kinetics within the studied concentration range, they are likely to have significant associated errors. This is most likely to lead to ill-advised extrapolations beyond the concentration range tested.

The observed K_m of the immobilised inulinase was not below the K_m of the free enzyme, suggesting a high affinity of the immobilised biocatalyst for inulin, contrary to data reported for the immobilisation of inulinase on DEAE–cellulose and on supports isolated from soybean and eggs (Gupta et al., 1992).

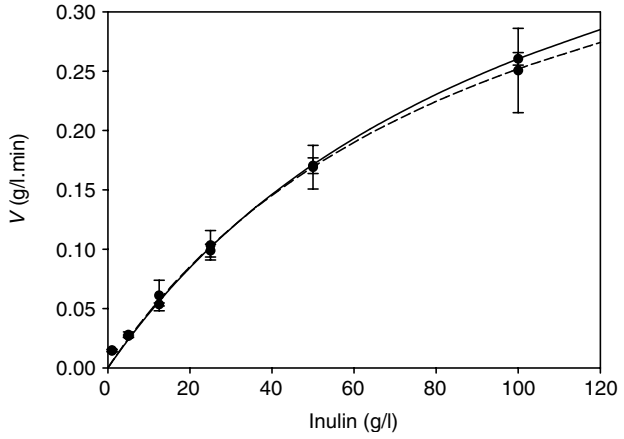

Fig. 3. Influence of the substrate concentration on the activity of immobilised inulinase. Bioconversion runs were carried out at pH 5.5 and 50 °C. Model predictions based on kinetic parameters estimated by Lineweaver–Burk linearisation (dashed line) and by non-linear regression (full line) are displayed.

3.3. Biocatalyst reuse

The ease of separation and the increased stability are among the advantages of using an immobilised biocatalyst. The reutilisation of the immobilised enzyme in two reactors throughout seven consecutive batches was evaluated (Fig. 4). A significant decrease of enzyme activity was observed in the first two batches, after which the system exhibited a remarkable stability. The decay could be due the loss of loosely bound enzyme during the first batches, mainly during washing procedures, since no significant protein levels were detected in the bioconversion medium once the reaction was stopped (data not shown).

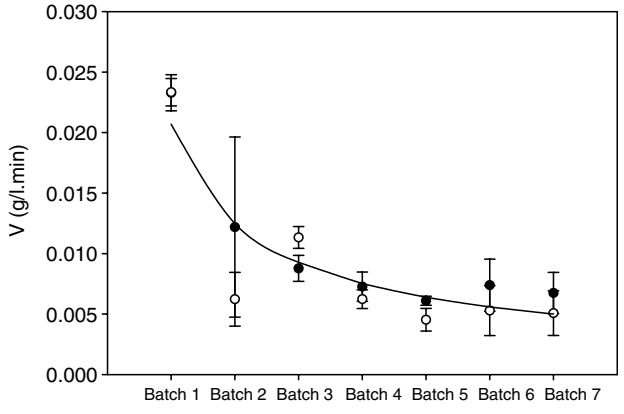

Fig. 4. Repeated, batch hydrolysis of inulin with immobilised biocatalyst. Trials were performed at 50 °C using a 5 g l^{-1} inulin solution. Open and closed symbols correspond to the activity data of independent runs.

4. Conclusions

The use of a factorial design and surface response methodology were used to access the optimum pH and temperature condition to maximise hydrolytic activity of inulin by free and immobilised inulinase. The model system estimated optimal pH and temperature values of 5.4 and 52 °C for the immobilised system and 4.9 and 56 °C for the free system, which are quite close to the experimental data gathered of pH 5.5 and 50 °C for the immobilised form and pH 4.5 and 55 °C for the free biocatalyst. Michaelis–Menten-type kinetics provided the best fit for the experimental data, where the use of a non-linear regression method proved more accurate in the estimation of the kinetic parameters than did Lineweaver–Burk linearisation. The immobilised biocatalyst was effectively reused in seven consecutive batches, although significant activity decay was observed in the first two runs.

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