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Kinetics of Sucrose Hydrolysis by Immobilized Recombinant *Pichia pastoris* Cells in a Batch reactors

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Abstract

Sucrose hydrolysis was carried out in a constant-volume batch reactor, using recombinant *Pichia pastoris* BfrA4X whole cells expressing *Thermotoga maritima* invertase, entrapped in calcium alginate beads. The kinetics of the enzymatic hydrolysis of sucrose by the biocatalyst was examined at substrate concentrations ranging between 0.03 M and 2.04 M. The reaction rate increases until 0.31 M after which the reaction velocity was constant until 1.16 M, above this concentration, the reaction rate decreases with increasing sucrose concentration. The experimental data obtained with two weight of the biocatalyst were incorporated into two kinetic models to predict the reaction time needed for sucrose hydrolysis. One model was applied for sucrose concentrations bellow 1.16 M while a second one could be used at inhibitory range between 1.46 and 2.04 M with a *k* value as function of initial sucrose concentration and biocatalyst weight.

Keywords: Kinetics; Invertase; Sucrose hydrolysis; *Pichia pastoris;* Batch reactor; Cells immobilization

Introduction

Sucrose hydrolysis catalysed by invertase (EC 3.2.1.26) produces an equimolar mixture of glucose and fructose named invert sugar, which has higher sweetener power. This mixture is largely employed as a sweetener in food and pharmaceutical industries because it has more desirable functional properties than those displayed by sucrose syrup such as low crystallization problem in food products, high osmotic pressure, high solubility, etc. Also, invert syrups have other useful applications as source of instant energy and as plasticizing agents in cosmetics, medicines and paper industry [1-3]. Food and biotechnological industries have increasingly used immobilized enzymes for various processes. The main advantages of the immobilized enzyme technology are: lower production costs and improvement in production capacity due to the ability to recover and re-use the biological catalysts that also is more stable than free enzymes [4].

Repeated batch processing is a well-known method to produce invert sugar from sucrose hydrolysis using immobilized cell-free invertases [5-8]. The constant-volume batch reactor permits an easy catalytic conversion operation, product separation and biocatalyst recovery. The batch reactor is simple, needs little supporting equipment, and is therefore ideal for small-scale experimental studies of kinetic models to describe the enzymatic hydrolysis of sucrose [9].

The aim of this work is to evaluate kinetic models to predict the reaction time needed for sucrose hydrolysis by calcium alginate immobilized recombinant *Pichia pastoris* BfrA4X, expressing a periplasmic *Thermotoga maritima* invertase, in a constant-volume batch reactor. According to the gathered experimental data, it was observed by the first time, how two kinetic models predict the reaction time at low and high sucrose concentrations respectively.

Materials and Methods

Strain and culture conditions

Pichia pastoris GS115 strain BfrA4X was obtained from the culture collection of the Center for Genetic Engineering and Biotechnology, Havana, Cuba [10].

Fed-batch fermentation was performed in a 7.5-L fermenter (INFORS) containing 3 L of growth medium [1% (w/v) cane sugar, 0.5% (w/v) yeast extract, 2.2% (w/v) $(NH_4)_2SO_4$, 1.82% (w/v) K_2HPO_4, 0.75% (w/v) $MgSO_4\ 7H_2O$, and 0.05% (w/v) $CaCl_2\ 2H_2O$, with vitamins and traces prepared as recommended by Cregg [11], and inoculated with 0.2 L of a shaking batch culture to an initial cell concentration of 3 g L^{-1} (wet biomass). The operation conditions during the batch and exponential fed batch phases were as described by [12].

The culture broth was centrifuged; 15 grams of the pellet were resuspended up to 30 mL in deionized water and yeast cells were heat-inactivated at 70°C for 30 min minutes. Heat killed yeast cells were then pelleted by centrifugation and used for whole cell immobilization or as a free cell source. The heat-killed cells in amounts of 15 g (wet weight) were resuspended in distilled water (50 mL) to achieve final biomass concentrations of 300 g L^{-1}.

Preparation of calcium alginate beads

Sodium alginate solutions were prepared by the stepwise addition of 1 g of alginate powder to 15 g of wet biomass suspended in 50 mL of deionized water and stirred thoroughly to ensure a homogenous distribution of the cells in the alginate solution, the mix was completed

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to 50 mL with deionized water before extrusion in the $CaCl_2$ solution. The alginate/cell suspension was added drop-wise through a silicone tube (using a peristaltic pump at a flow rate of 20 mL min^{-1} and a fine needle) to 500 mL $CaCl_2$ (0.55 % w/v) solution. The $CaCl_2$ solution was stirred at constant speed (100 rpm) using an impeller type marine propeller to avoid droplet aggregation. Gelation time was restricted to one hour after which the $CaCl_2$ solution was discarded. Subsequently the beads were washed three times and stored in 1.46 M sucrose solution at 4°C.

Invertase activity assay

The activity assay was performed in a batch reactor, 25 beads or 25 mg of free cells were mixed in 10 mL of sucrose solution. The assay was carried out 15 minutes at 60°C, in 50 mM sodium acetate buffer, pH 5.5 using sucrose solutions between 0.03 and 2.04 M. The mix was performed by rotating a stirring rod (4 mm Ø x 17 mm) magnetically at approximately 100 rpm. An equimolar mixture of glucose and fructose was used as standard. Sucrose hydrolysis was determined by measuring the release of reducing sugars using 3'5-dinitrosalicylic acid (DNS) as described by Miller [13].

The batch process

The immobilized cells biocatalyst was tested in a 500 mL constant-volume batch reactor. The beads of calcium alginate immobilised cells at a concentration of 100 g L^{-1} and 200 g L^{-1} were incubated 10 h into different sucrose concentrations (0.29, 0.87, 1.16, 1.46, 1.75 and 2.04 M) at 60°C with constant stirring (100 rpm). Samples were withdrawn at regular intervals to measure the reducing sugars as describe above.

Statistical analysis

Each data point represents the mean of three independent assays. Statistical significance of the multiple comparisons of slops was conducted using the t-Student test using the personal computer software program Microsoft Excel [14].

Results and Discussion

Effect of sucrose concentration on reaction rate

Data showed in figure 1 reveals that for free and calcium alginate immobilized *Pichia pastoris* BfrA4X the reaction rate increases gradually until the sucrose concentration reaches about 0.29 M (100 g L^{-1}) however, after this increment, the reaction velocity remained constant until sucrose concentration of 1.16 M (400 g L^{-1}). It was further observed that above this concentration, the reaction rate decreases with increased sucrose concentration. Free *Thermotoga maritima* invertase reaches the saturation condition around 500 g L^{-1} [15]. Differences between free and immobilized *T. maritima* invertases could be caused by the limitation in substrate diffusion into the alginate gel. *Sacharomyces cerevisiae* invertase immobilized in calcium alginate also reaches the maximum activity at similar sucrose concentration of 0.27 M [16]. Bowski [17] and Combes and Monsan [18], also found a shift in peak activity at sucrose concentrations of 0.29 M however, they observed a fast decrease in the free invertase activity, but in our experiments, a plateau between 0.31 and 1.16 M was obtained. Several reasons could explain the reaction rate decrease with an increment of sucrose concentration, the inhibitory effect of substrate, the reduction in the free water, and the oligosaccharides synthesis [19].

Biochemical characterization of the sucrose hydrolysis reaction in the biocatalyst

The rates of batch sucrose hydrolysis reaction by the immobilized *Pichia pastoris* BfrA4X were determined at 60°C and pH 5.5, with two biocatalyst weight of 100 and 200 g L^{-1} respectively, in a stirred sucrose solution at various concentrations, between 0.29 M and 2.04 M. The correlation between the hydrolysis percentage and the reaction time is shown in Figure 2. When the reaction time was extended to 10 h, the complete sucrose hydrolysis was obtained until the sucrose concentration reached 1.16 M and 1.46 M for biocatalyst weight of 100 and 200 g L^{-1}, respectively.

To correlate the experimental data for the sucrose-invertase reaction with a kinetic model that predicts the reaction time needed for sucrose hydrolysis, we assayed the kinetic equation for constant-volume batch reactor, according to Levenspiel [9].

Equation 1 is the expression for constant-volume batch system.

$$\frac{t}{S_{AO}} = \frac{V}{W}\int_0^{S_A} \frac{dS_A}{-r_A} \quad (1)$$

According to Michaelis-Menten the rate expression can be written as

$$r_A = \frac{V_{\max} S_A}{K_M + S_A} \quad (2)$$

From Equation 1 *t* can be expressed as

$$t = \frac{V}{W} S_{AO} \int_0^{S_A} \frac{dS_A}{-r_A} = -\frac{V}{W} \int_{S_{A0}}^{S_A} \frac{dS_A}{-r_A} \quad (3)$$

When Equation 2 and 3 are combined and integrated, we obtain

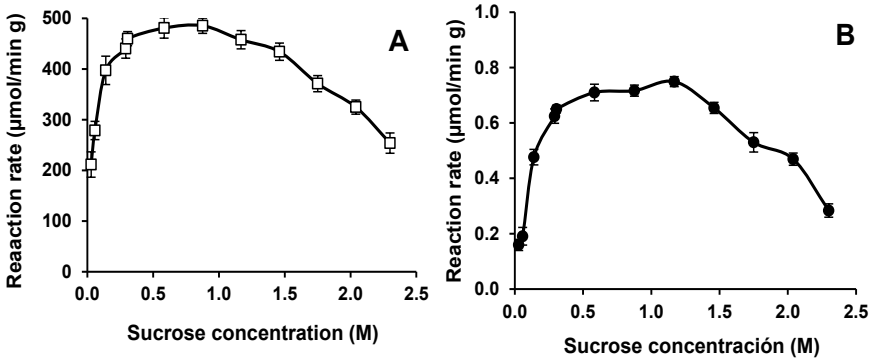

Figure 1: Michaelis-Menten plots of the (A) free and (B) immobilized *Pichia pastoris* BfrA4X expressing the *Thermotoga maritima* invertase (60ºC, 15 min, pH 5.5).

$$t = \frac{V}{W}\left(\frac{K_M}{V_{max}} \times \mathbf{h}\ \frac{S_{A0}}{S_A} + \frac{S_{A0} - S_A}{V_{max}}\right) \quad (4)$$

Where t is reaction time (min), V is the reaction volume, W biocatalyst weight (g), S_A sucrose concentration (mmol L^{-1}), r_A is the reaction rate (mmol min^{-1} g^{-1}), S_{A0} is the initial sucrose concentration (mmol L^{-1}), K_M is the Michaelis constant (mmol L^{-1}), V_{max} is the maximum rate (mmol min^{-1} g^{-1}).

The constants K_M 141 mM and V_{max} 48 μmol min^{-1} g^{-1} previously determined [12], were substituted in Equation 4 at different initial sucrose concentrations. Experimental data for S_A (Figure 2) were used to determine the theoretical time for sucrose hydrolysis.

Table 1 shows the values of theoretical times calculated according to Equation 4 and compared with those obtained practically, showing differences with the experimental time for both biocatalyst weights. The calculated predicted time differed from the experimental time in less than 1 h when experiments were conducted with sucrose concentrations bellow 1.16 M and in more than 1 h when substrate concentration above 1.16 M was used. The statistical analysis showed significant differences in the slopes of the plot of experimental and theoretical time versus the S_A at high sucrose concentration.

Although the Equation 4 permits a close fit to the experimental data for low sucrose concentration, another kinetic model must be applied for the observed deviation at high sucrose concentration.

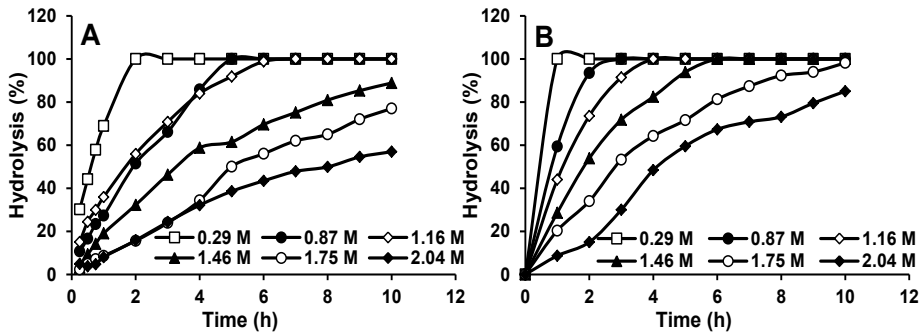

Figure 2: Sucrose hydrolysis in a constant-volume batch reactor by immobilized *Pichia pastoris* BfrA4X expressing the *Thermotoga maritima* invertase. (A) 100 g L^{-1} (B) 200 g L^{-1}.

100 gL^{-1}	0.29 M[nd]		0.87 M[nd]		1.16 M[nd]		1.46 M*		1.75 M*		2.04 M*	
t_e	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$
0.25	0.48	+0.23	0.36	+0.11	0.69	+0.44	0.18	-0.07	0.15	-0.10	0.41	0.16
0.50	0.73	+0.23	0.57	+0.07	1.12	+0.62	0.52	+0.02	0.32	-0.18	0.33	-0.16
0.75	1.00	+0.25	0.82	+0.07	1.38	+0.63	0.79	+0.04	0.46	-0.29	0.41	-0.34
1	1.26	+0.26	0.96	-0.04	1.66	+0.66	1.07	+0.07	0.57	-0.43	0.65	-0.34
2			1.90	-0.10	2.65	+0.65	1.83	-0.17	1.03	-0.97	1.24	-0.75
3			2.52	-0.48	3.45	+0.45	2.65	-0.35	1.59	-1.41	1.90	-1.09
4			3.54	-0.46	4.28	+0.28	3.41	-0.59	2.30	-1.70	2.50	-1.49
5					4.92	-0.08	3.58	-1.42	2.84	-2.16	3.00	-1.99
6					6.16	+0.16	4.11	-1.89	3.24	-2.76	3.38	-2.61
7							4.49	-2.51	3.69	-3.31	3.73	-3.26
8							4.91	-3.09	3.94	-4.06	3.90	-4.10
9							5.26	-3.74	4.24	-4.76	4.27	-4.72
10							5.58	-4.42	4.34	-5.66	4.47	-5.52

200 g L^{-1}	0.29 M[nd]		0.87 M[nd]		1.16 M[nd]		1.46 M*		1.75 M*		2.04 M*	
t_e	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$	t_t	$t_t - t_e$
1			1.09	+0.09	1.03	+0.03	0.81	-0.19	0.67	-0.33	0.35	-0.64
2			1.99	-0.01	1.81	-0.19	1.55	-0.45	1.26	-0.74	0.88	-1.11
3					2.44	-0.56	2.13	-0.87	1.80	-1.20	1.46	-1.53
4							2.51	-1.49	2.20	-1.80	2.25	-1.74
5							3.06	-1.94	2.48	-2.52	2.34	-2.66
6									2.88	-3.12	2.66	-3.33
7									3.16	-3.84	2.81	-4.18
8									3.43	-4.57	2.92	-5.08
9									3.54	-5.46	3.20	-5.79
10									3.97	-6.03	3.20	-6.79

t_e experimental time (h), t_t theoretical time (h), the space in blank means that at this time the hydrolysis was 100%; * means significant differences in the slopes of the plot of t_e and the t_t versus the S_A at different sucrose concentration. (Student t test, $p \leq 0.05$) [nd] means not significant differences in the slopes of the plot of t_e and the t_t versus the S_A at different sucrose concentration. (Student t test, $p \leq 0.05$)

Table 1: Theoretical time calculated according Eq. 4 at different sucrose concentrations for 100 and 200 g L^{-1} of biocatalyst.

To predict the reaction time needed to sucrose hydrolysis at sucrose concentrations above 1.16 M we tested the first-order rate equation for irreversible monomolecular type reactions [9], as follow.

$$-r_A = \frac{dS_A}{dt} = kS_A \quad (5)$$

Separating and integrating we obtain

$$-\int_{C_{Ao}}^{C_A} \frac{dS_A}{S_A} = k\int_o^t dt \quad \text{which is} \quad -\ln\frac{S_A}{S_{Ao}} = kt \quad (6)$$

The fractional conversion X_A of a given reactant is defined as the fraction of reactant converted into product $X_A = \frac{N_A - N_A}{N_A}$, Equation 6 can be derived using conversions and we obtain $S_A = \frac{N_{Ao}}{V} = \frac{N_{Ao}(1-X_A)}{V} = S_{Ao}(1-X_A)$ and $-dS_A = S_{Ao}dX_A$

Hence Equation 5 becomes $\frac{dX_A}{dt} = k(1-X_A)$

Integrating we obtain

$$\int_o^{X_A} \frac{dX_A}{1-X_A} = k\int_o^t dt \quad \text{or} \quad -\ln(1-X_A) = kt \quad (7)$$

A result equivalent to Equation 6.

Where t is the time (h); N_{Ao} is the initial sucrose (mol), r_A reaction rate (mol $min^{-1}g^{-1}$), S_{A0} is the initial sucrose concentration (mol L^{-1}), k is the reaction kinetic coefficient (h^{-1}).

A plot of -ln(1- X_A) versus t gives a straight line through the origin of slop k. The figure 3 shows the plot of the experimental data obtained for the biocatalyst weight (W) 100 g L^{-1} for initial sucrose concentrations S_{Ao}, between 1.46 (500 g L^{-1}) and 2.04 M (700 g L^{-1}).

From the k values obtained to 1 g of biocatalyst, the plot of k/W versus S_{Ao} yields a straight line which slope gives the expression $k = (-0.0432 g^{-1}h^{-1}M^{-1}S_{Ao} + 0.1054)W$ (Figure 4)

The k value was substituted in Equation 7. The experimental data for sucrose concentrations between 1.46 and 2.04 M showed in figure 2 were transformed as fractional conversion X_A and were used to determine the theoretical time for sucrose hydrolysis as function of S_{Ao} and W. Table 2 shows the theoretical times calculated according to Equation 7 and the difference with the experimental time for both biocatalyst weights. The predicted time differed from the experimental time in less than 1 h for the three sucrose concentrations. The statistical analysis showed not significant differences in the slopes of experimental and theoretical time plots versus the X_A at high sucrose concentration.

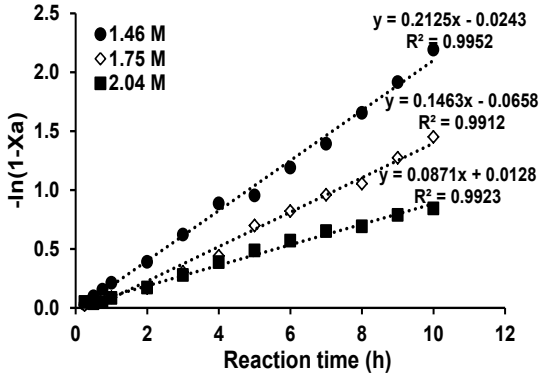

Figure 3: Application of the first order reaction at sucrose concentrations between 1.46 and 2.04 M

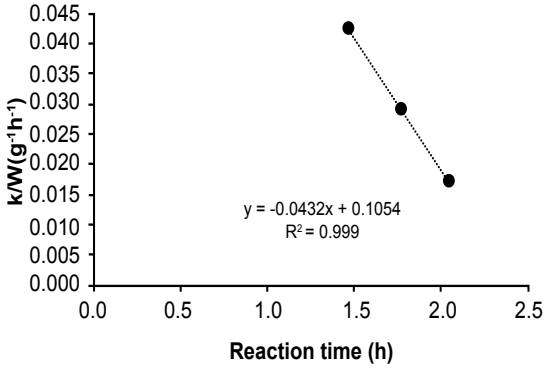

Figure 4: Dependence of the reaction kinetic coefficient k with initial sucrose concentration and biocatalyst weight.

100 g L^{-1}	1.46 Mnd		1.75 Mnd		2.04 Mnd	
t_e	t_t	t_t - t_e	t_t	t_t - t_e	t_t	t_t - t_e
1	1.00	-0.00	0.60	-0.40	0.97	-0.03
2	1.84	-0.16	1.14	-0.86	2.00	+0.00
3	2.93	-0.07	2.05	-0.95	3.24	+0.24
4	4.19	+0.19	2.96	-1.04	4.51	+0.51
5	4.51	-0.49	4.70	-0.30	5.67	+0.67
6	5.62	-0.38	5.52	-0.48	6.63	+0.63
7	6.58	-0.42	6.46	-0.54	7.20	+0.20
8	7.82	-0.18	7.09	-0.91	8.04	+0.04
9	9.06	+0.06	8.56	-0.44	9.17	+0.17
10	10.36	+0.36	9.76	-0.24	9.81	-0.18

200 g L^{-1}	1.46 Mnd		1.75 Mnd		2.04 Mnd	
t_e	t_t	t_t - t_e	t_t	t_t - t_e	t_t	t_t - t_e
1	0.80	-0.20	0.76	-0.24	0.53	-0.47
2	1.83	-0.27	1.59	-0.41	1.50	-0.50
3	3.09	+0.01	2.56	-0.44	2.74	-0.26
4	4.11	+0.11	3.46	-0.54	4.97	+0.97
5	6.61	+1.61	4.23	-0.77	5.26	+0.26
6			5.64	-0.36	6.51	+0.51
7			6.96	-0.04	7.17	+0.17
8			8.62	+0.62	7.65	-0.35
9			9.45	+0.45	9.22	+0.22
10					9.18	-0.82

t_e experimental time (h), t_t theoretical time (h), the space in blank means that at this time the hydrolysis was 100%; * means significant differences in the slopes of the plot of t_e and the t_t versus the X_A at different sucrose concentration. (Student t test, $p \leq 0.05$) nd means not significant differences in the slopes of the plot of t_e and the t_t versus the X_A at different sucrose concentration. (Student t test, $p \leq 0.05$)

Table 2: Theoretical time calculated according Equation 7 at different sucrose concentrations for 100 and 200 g L^{-1} of biocatalyst.

Conclusion

According to the described results the enzymatic hydrolysis of sucrose by the immobilized *Pichia pastoris* GS115 strain BfrA4X can be satisfactorily described by two kinetic models. The kinetic equation for constant-volume batch reactor (Equation 4) can be applied essentially to the region of low substrate concentration (bellow 1.16 M) while the first-order rate equation for irreversible monomolecular type reactions (Equation 7) with the k value from the region of high substrate concentration between 1.46 and 2.04 M. The k value as function of initial substrate concentration and the biocatalyst weight

involve different factors that can affect the reaction rate at high sucrose concentration, such as diffusion limitations and substrate inhibition. The knowledge of these kinetic models can be used for the successful and efficient production of invert syrup in a wide range of sucrose solution.

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