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Studies on the Adsorption of Inulinase from *Kluyveromyces marxianus* ATCC 16045 onto an Ion Exchange Resin

Estudos de Adsorção de Inulinase a partir de *Kluyveromyces marxianus* ATCC 16045 em Resina de Troca Iônica

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SUMMARY

The adsorption of an extracellular inulinase directly onto an ion exchange resin from a clarified crude broth was investigated in this work. The enzyme inulinase was obtained from *Kluyveromyces marxianus* ATCC 16045 by fermentation in shaken flasks in a medium containing peptone, sucrose, yeast extract and K_2HPO_4 at pH 3.5, 30°C and 150 rpm for 48 hours. The final enzymatic activity was about 72 U mL^{-1}. The crude broth filtrate was used for kinetic studies and for the adsorption isotherm of the extracellular inulinase from *Kluyveromyces bulgaricus* ATCC 16045 onto an ion exchange resin. The trials were carried out at pH 3.5 and 25°C in stirred tank reactors containing STREAMLINE™ SP, developed for expanded bed adsorption. It was observed that the adsorption was well described by the Langmuir isotherm, and the values determined for Q_m and K_d were 1,254 U mL^{-1} and 0.325 UmL^{-1}, respectively. The kinetic parameters k_1 (6.52 . 10^{-3} mL U^{-1} min) and k_2 (2.09. 10^{-3} min^{-1}) as well as the average values of 1.71 . 10^{-2} cm s^{-1} for the film coefficient (K_s) and 6.96 . 10^{-7} cm^2 s^{-1} for the effective diffusion coefficient (D_{ef}), were determined using the experimental results and mathematical modelling.

RESUMO

A adsorção de uma inulinase extracelular em resina de troca iônica diretamente a partir de um caldo filtrado foi investigada neste trabalho. A enzima inulinase foi obtida a partir de fermentação por *Kluyveromyces marxianus* ATCC 16045 em frascos erlenmeyers com meio contendo peptona, sacarose, extrato de levedura e K_2HPO_4, a pH 3,5, 30°C, 150 rpm durante 48 horas. A atividade enzimática final foi ao redor de 72 U mL^{-1}. O caldo filtrado foi utilizado para os estudos cinéticos e de obtenção da isoterma de adsorção da inulinase extracelular de *Kluyveromyces bulgaricus* ATCC 16045 usando resina de troca iônica. Os ensaios foram realizados a pH 3,5 e 25°C em reatores agitados encamisados contendo resina STREAMLINE™ SP, material desenvolvido para utilização em colunas de leito expandido. Observou-se que a adsorção pode ser bem descrita pela isoterma de Langmuir, sendo determinado o Q_m e o K_d, 1254 U mL^{-1} e 0,325 U mL^{-1}, respectivamente. Os parâmetros cinéticos k_1 (6,52 . 10^{-3} mL U^{-1} min) e k_2 (2,09. 10^{-3} min^{-1}) bem como os valores médios 1,71 . 10^{-2} cm s^{-1} para o coeficiente de transferência de massa na película (K_s) e 6,96 . 10^{-7} cm^2 s^{-1} para o coeficiente de difusão efetiva (D_{ef}) foram determinados utilizando os resultados experimentais e modelagem matemática.

PALAVRAS-CHAVE
KEY WORDS

Inulinase; Streamline; Purification; Kluyveromyces; Ion Exchange

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1. INTRODUCTION

Sucrose has been widely used as a sweetener in the food industry for a long time. However, because of its medicinal and nutrition properties, D-fructose has gained popularity in the food and beverage industries. This sugar has emerged as an alternative to sucrose, which is known to cause problems related to corpulence, cariogenicity, atherosclerosis and diabetes (GUPTA *et al.*, 1994).

Inulin is the storage carbohydrate in roots and tubers of plants such as the Jerusalem artichoke, chicory and dahlia. It represents a good source of high fructose, reduced calorie sweeteners and consists of linear β-2,1-linked poly-fructose chains displaying a terminal glucose unit (VANDAMME & DERYCKE, 1983). The conventional production of fructose from starch requires at least three enzymatic steps, yielding only 45% of fructose (ZITTAN, 1981). In contrast, the formation of fructose from the complete hydrolysis of inulin is a single-step inulinase reaction and yields up to 95% of fructose. Inulin also has an industrial potential as a source of functional sweeteners like other oligosaccharides, provided that an endoinulinase acts on the inulin. YUN *et al.* (1997) reported high yields of inulo-oligosaccharide production using purified endoinulinase. Biotransformations can compete with optimised chemical productions if improved techniques involving the choice of convenient methods of downstream processing, are employed (KAMIMURA *et al.*, 2001).

Ion exchange chromatography, one of the methods applied in bioproduct recovery, is suitable for inulinase purification. BORNNERJEA *et al.* (1986) analysed 100 papers on protein purification and found that ion exchange chromatography was the most commonly used method, occurring in 75 percent of purification schemes. The reasons for the success of ion exchange chromatography are its widespread applicability, high resolving power, high binding capacity and relative cheapness (CHANG & CHASE, 1996). STREAMLINE™ adsorbents, which are modified sepharose matrices, were developed specifically for use in expanded bed adsorption. The choice of STREAMLINE™ was because it was designed with a well defined density distribution and allows for operation at flow rates eightfold higher than those used with conventional sepharose (CHANG *et al.*, 1993), besides feed-stocks containing particulates can be processed directly using fluidised or expanded bed adsorption procedures, which are able to operate efficiently in the presence of particulates (CHANG & CHASE, 1996), such as the broth from inulinase production by *Kluyveromyces*.

There is considerable industrial interest in both the scale-up and optimisation of chromatographic unit operations used in the purification of bioproducts. All the information required for characterization can be used for prediction purposes. Whilst there are many published studies on the adsorption of single proteins to different adsorbents, few studies have been reported using realistic systems involving more than one adsorbing protein, such as crude broths. The main objective of this work was to investigate the adsorption of crude inulinase produced by *Kluyveromyces marxianus* ATCC 16045 onto the STREAMLINE™ cation exchange resin. The adsorption kinetics and equilibrium isotherms were carried out in order to determine the model parameters.

2. MATERIAL AND METHODS

2.1 Fermentation

Kluyveromyces marxianus ATCC 16045 was employed for inulinase production. The microorganism was grown in Malt Extract Yeast (MY) broth. The inoculum cultures were grown on a medium containing 20 g L^{-1} sucrose (20 g L^{-1} sucrose, 5 g L^{-1} yeast extract, 5 g L^{-1} K_2HPO_4, 1.5 g L^{-1} NH_4Cl, 1.15 g L^{-1} KCl and 0.65 g L^{-1} $MgSO_4.7H_2O$) at pH 6.5. Inulinase was produced in a 500 mL flask with 100 mL of culture medium (14 g L^{-1} sucrose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract and 1 g L^{-1} K_2HPO_4) at pH 3.5. The fermentation was carried out with 10% inoculum at 30°C in a rotary shaker at 150 rpm for 48 hours (KALIL *et al.*, 2001).

2.2 Inulinase Assay

The activity was assayed as follows: 1 mL enzyme solution was mixed with 9 mL of 2% (w/v) sucrose or inulin in 0.1 M acetate buffer pH 4.5. The mixture was maintained at 50°C and the rate of appearance of reducing sugar was determined by the DNS method (MILLER, 1959) using glucose as the standard from sucrose and fructose as the standard from inulin. One unit of inulinase activity was defined as the amount of enzyme that hydrolysed 1 μmol of sucrose per min or the amount of enzyme catalysing the liberation of 1 μmol of fructose equivalent from inulin per min. For inulinase the ratio of the activity on inulin versus sucrose (I/S) ratio is higher than 10^{-2} while for invertase it is lower than 10^{-4} (ETTALIBI & BARATTI, 1987). KALIL *et al.* (2001) determined a ratio of 7 . 10^{-2} for this enzyme, characterized as inulinase.

2.3 Protein Determination

The protein concentrations in the crude fermentation broth filtrates were determined according to LOWRY *et al.* (1951). Bovine serum albumin was used as the standard protein.

2.4 Ion Exchange Resin

The cation exchange resin STREAMLINETM SP from Amersham-Pharmacia was used.

2.5 Adsorption Kinetics

The kinetic experiments were performed in a stirred reactor at 25°C, using the filtered fermentation broth. Trials were carried out with 5 mL of wet resin (1:4 w/v) in 50 mL of inulinase solution in 20 mM acetate buffer at pH 3.5. The samples were quantified according to enzymatic activity in order to determine the adsorption kinetic profiles.

2.6 Adsorption Isotherms

Experimental data were obtained from batch adsorption using 1 mL of resin suspension (1:4) in 10 mL of different concentrations of crude inulinase in 20 mM acetate buffer at pH 3.5. After two hours of incubation in the shaker at 25°C and 175 rpm, the amount of adsorbed inulinase was determined from the remaining activity in solution.

For the adsorption of a protein onto an ion exchange adsorbent, the adoption of a favourable reversible isotherm of the Langmuir type has been widely used (CHANG & CHASE, 1996; SILVA *et al.*, 1999; PESSOA Jr. *et al.*, 1996).

From the Langmuir isotherm, the following expression relates the amount of adsorbate bound to the adsorbent Q^*, to the concentration $C^*$of enzymatic activity in solution:

$$Q^* = \frac{Q_m.C^*}{K_d + C^*} \quad (1)$$

where C and Q are the enzymatic activity in the liquid and solid, respectively, Q_m represents the maximum binding capacity of the resin and K_d is the equilibrium constant. The symbol * represents the concentrations at equilibrium.

2.7 Mathematical Modelling for Adsorption and Desorption in Stirred Tank Reactors

If a volume of adsorbent, V_s, is immersed in a volume V_l of liquid with inulinase showing an initial enzymatic activity C_0, in a perfectly agitated tank, the solute diffuses into the resin particles and is adsorbed up to a certain equilibrium concentration. To formulate the differential equations it is assumed that:

a) the resin particles are spherical;

b) diffusion of inulinase inside the solid particles obeys Fick's law;

c) the diffusion process is only in the direction of r;

d) isothermal conditions.

Considering these assumptions, the adsorption process in a constant volume stirred tank can be described by equation (2)

$$\frac{d\,C_b}{d\,t} = -\frac{3}{R}\,\frac{V_s}{V_l}\,K_s\,(C_b - C_i)\big|_{r=R} \quad (2)$$

where C_b is the enzymatic activity in the liquid phase, C_i is the enzymatic activity in the liquid phase in the interior of the particle pores, K_s is the film coefficient, t and r are the temporal (time) and spatial (radial) variables, respectively, and R is the particle radius.

The initial condition for Equation (2) is:

$$t = 0,\ C_b = C_0 \quad (3)$$

the material balance in the solid particle with adsorption onto the available surface inside the particle is:

$$\varepsilon_p \frac{\partial\,C_i}{\partial\,t} = D_{ef}\varepsilon_p\left(\frac{\partial^2 C_i}{\partial\,r^2} + \frac{2}{r}\frac{\partial\,C_i}{\partial\,r}\right) - (1-\varepsilon_p)\frac{\partial\,Q_i}{\partial\,t} \quad (4)$$

where ε_p is the particle porosity, D_{ef} is the effective diffusion coefficient and Q_i is the enzymatic activity in the solid phase.

If it is considered that no diffusion occurs on the external particle surface (diffusion only in the r direction), the material balance for a given adsorbed quantity is actually the balance between accumulation and consumption, as in equation (5).

$$\frac{\partial\,Q_i}{\partial\,t} = k_1 C_i\,(Q_m - Q_i) - k_2.Q_i \quad (5)$$

where k_1 and k_2 are the adsorption and desorption kinetic constants, respectively.

The initial and boundary conditions associated with the diffusion process inside the solid particles are:

$$t = 0,\ C_i = Q_i = 0 \quad (6)$$

$$r = R,\ \frac{\partial\,C_i}{\partial\,r} = \frac{K_s}{\varepsilon_p\,D_{ef}}\,(C_b - C_i) \quad (7)$$

$$r = 0,\ \frac{\partial\,C_i}{\partial\,r} = 0 \quad (8)$$

The diffusion equation inside the particles was discretised and solved using the method of orthogonal collocation (VILLADSEN & MICHELSEN, 1978), where the boundary condition referring to the film resistance was used as the collocation point. To integrate the two differential equations, the DASSL code was used (PETZOLD, 1989). In order to optimise the effective diffusion coefficient (D_{ef}), the film coefficient (K_s) and the kinetic constant (k_1), the NEDLER & MEAD (1965) method was used simultaneously with the numerical solution of the differential equations. These optimum values where obtained for a minimum value of the sum of the square error difference, the latter involving the simulated concentration values and the experimental ones:

$$\Phi = \sum_1^N (C_b - C_{exp})^2 \quad (9)$$

When the values for Φ_i in the simplex vortexes and their mean values $\overline{\Phi}_i$ satisfied inequality (10), the optimisation was considered complete.

$$\left(\frac{\sum_1^m (\Phi_i - \overline{\Phi}_i)^2}{m+1}\right)^{1/2} < e \quad (10)$$

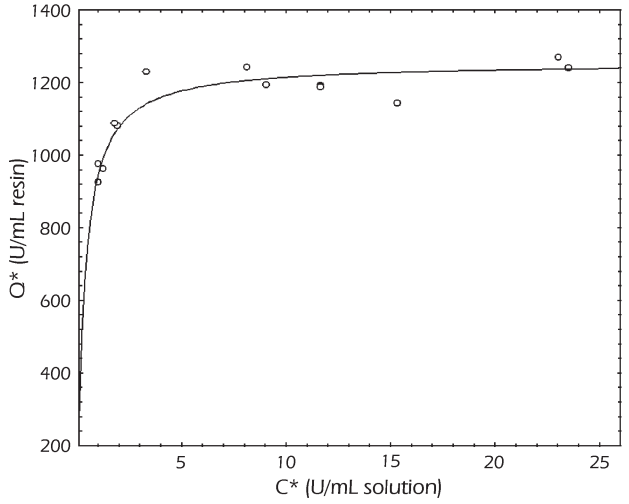
where *m* is the number of parameters to be optimised and *e* is the convergence parameter taken as 10^{-5}.

3. RESULTS AND DISCUSSION

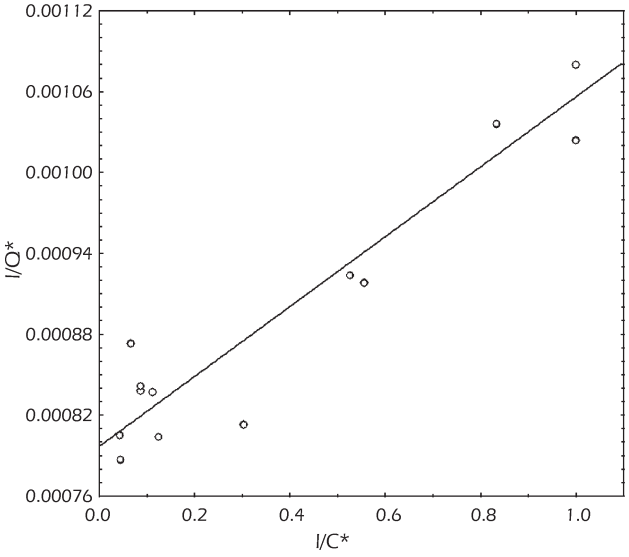
3.2 Adsorption Isotherm

The results for free enzymatic activity versus the amount adsorbed at equilibrium are shown in Figure 1 (a). A Langmuir-type curve (Equation 2), fitted to these experimental data, was represented by a solid line. Figure 1 (b) shows the linear fitting

of the Langmuir equation used to obtain the values for Q_m and K_d, 1,254 U mL^{-1} and 0.325 U mL^{-1}, respectively. It can be seen that the isotherm for inulinase adsorption can be described by the Langmuir equation with good agreement. PESSOA Jr. *et al.* (1996) obtained the adsorption isotherm for inulinase from *Candida kefyr* on STREAMLINE^{TM} SP at pH 4.0 with Q_m of 5.3 . 10^{-6} U g^{-1} and a K_d of 14.0 . 10^{-8} U mL^{-1}.



(a)



(b)

FIGURE 1. Adsorption isotherm for the inulinase from *Kluyveromyces marxianus* ATCC 16045 on STREAMLINE ^{TM} SP resin in 20 mM acetate buffer at pH 3.5 and 25°C. (a) The solid line represents the fitted Langmuir equation. (b) Linear fitting for the determination of Q_m and K_d (R= 0.96).

BARBOZA *et al.* (2002) studying clavulanic acid adsorption on an anion exchange resin observed that equilibrium was also obtained for a Langmuir isotherm with Q_m 2.28 . 10^{-2} g g^{-1}, K_d 3.94 . 10^{-2} g L^{-1}, k_1 1.1 L g^{-1} min^{-1} and k_2 0.0043 min^{-1}.

3.3 Adsorption Kinetics

In Figure 2, the dynamic fitting of the experimental data to obtain the parameters presented in Table 1 can be seen. This table shows the parameters for each kinetic adsorption assay with different initial concentration, where Q_m and ε_p were1,254 U mL^{-1} and 0.51, respectively, for all assays.

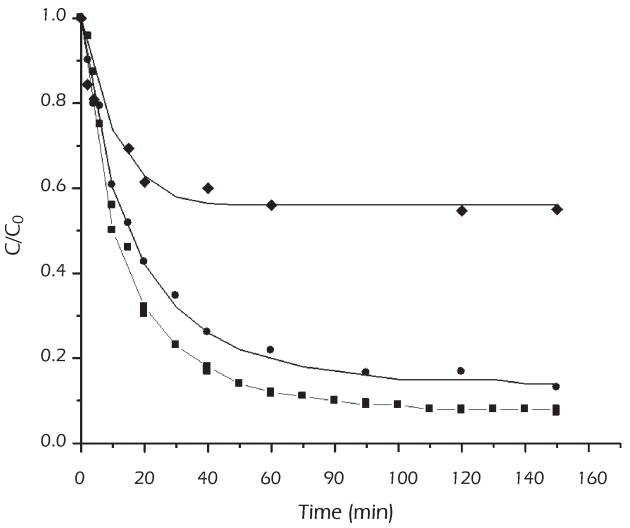


FIGURE 2. Dynamic fitting of inulinase adsorption on ion exchange resin. Solid lines represent the predicted curves and the symbols, the experimental data. (▪) C_0 = 19.95 U.mL^{-1}; (•) C_0 = 28.57 U.mL^{-1} ; (♦) C_0 = 51.28 U.mL^{-1}.

TABLE 1. Fitted values for D_{ef}, K_s, k_1, k_2, Bi and K_d.

Parameters	C_0		
	19.95 U mL^{-1}	28.57 U mL^{-1}	51.28 U mL^{-1}
D_{ef} (cm^2 s^{-1})	1.105 x 10^{-6}	6.58 x 10^{-7}	3.25 x 10^{-7}
K_s (cm s^{-1})	5.03 x 10^{-2}	5.03 x 10^{-4}	5.03 x 10^{-4}
k_1 (mL U^{-1} min^{-1})	6.52 x 10^{-3}	6.52 x 10^{-3}	6.52 x 10^{-3}
k_2 (min^{-1})*	2.09 x 10^{-3}	2.09 x 10^{-3}	2.09 x 10^{-3}
Bi**	455.2	7.6	15.5
K_d (U mL^{-1})	0.32	0.32	0.32

*k_2 was obtained from $K_d = k_2/k_1$
**Biot from the expression $Bi = K_s.R/D_{ef}$ (R= 100μm).

It was observed that k_1 and k_2 were essentially the same for all runs. The same behaviour was obtained when KAMIMURA *et al.* (2001) studied lipase adsorption onto a biospecific resin where k_1 and k_2 were 6 . 10^{-2} mL U^{-1} min^{-1} and 0.14, respectively for all the kinetic adsorption assays. It can be seen in Table 1 that D_{ef} changes according to the broth concentration. SILVA *et al.* (1999) found that D_{ef} was dependent on the initial BSA concentration for adsorption on ACCELL PLUS QMA^{TM}, with this parameter decreasing as the initial BSA concentration increased. GARKE *et al.* (1999) studying lysozyme and γ-globulin for a two-component system, presented two

trends: first, D_{ef} was concentration dependent for both proteins, decreasing with increasing concentration; second, the effective pore diffusivity of lysozyme in the binary system decreased with the mass fraction of the competing γ-globulin.

4. CONCLUSIONS

The experimental method of determining isotherms and kinetic curves was shown to be adequate in obtaining fundamental information about inulinase adsorption onto the STREAMLINE^{TM} SP resin. Adsorption modelling showed that the process could be mathematically described by the Langmuir equation. The resin showed a Q_m of 1,254 U mL^{-1} and K_d of 0.325 U mL^{-1}. The dynamic fitting allowed one to determine the kinetic parameters k_1 (6.52 . 10^{-3} mL U^{-1} min) and k_2 (2.09 . 10^{-3} min^{-1}) as well as the average values of 1.71 . 10^{-2} cm s^{-1} for the film coefficient (K_s) and 6.96 . 10^{-7} cm^2 s^{-1} for the effective diffusion coefficient (D_{ef}).

Notation

C_b	enzymatic activity in the liquid phase, U mL^{-1}
C_{exp}	experimental enzymatic activity in the liquid, U mL^{-1}
C_i	enzymatic activity of liquid inside the particle, U mL^{-1}
C_0	initial or inlet enzymatic activity in the liquid phase, U mL^{-1}
C^*	equilibrium enzymatic activity in the liquid, U mL^{-1}
D_{ef}	effective diffusion coefficient, cm^2 s^{-1}
e	Convergence parameter
K_d	dissociation constant, U mL^{-1}
K_s	film coefficient, cm s^{-1}
k_1	adsorption kinetic constant, mL U^{-1} min^{-1}
k_2	desorption kinetic constant, min^{-1}
m	number of parameters to be optimised
N	number of collocation points
Q_i	enzymatic activity in the solid phase, U mL^{-1}
Q_m	Langmuir isotherm constant, U mL^{-1}
Q^*	equilibrium enzymatic activity in solid phase, U mL^{-1}
r	radial co-ordinate, m
R	particle radius, m
t	time, s
V_l	volume of liquid, mL
V_s	volume of adsorbent, mL
Greek letters	
ε_p	particle porosity
Φ	sum of the square error, (U mL^{-1})^2

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