

ADSORPTION OF THE INULINASE FROM *Kluyveromyces marxianus* NRRL Y-7571 ON STREAMLINE[®] DEAE RESIN

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(Received: October 20, 2004 ; Accepted: May 25, 2005)

Abstract - The enzyme inulinase is used to produce oligosaccharides and fructose, with up to 95% fructose in a single stage of inulina hydrolysis. With in the aim to purify the enzyme, studies on the conditions of enzyme adsorption in an expanded-bed column were conducted using phosphate and tris-HCl buffers. The inulinase used in this work was obtained from *Kluyveromyces marxianus* NRRL Y-7571 by fermentation in an industrial medium. Using the anionic resin Streamline DEAE, the adsorption equilibrium time was determined. It was observed that the adsorption isotherm follows the Langmuir model; the parameters for the maximum amount of adsorbed inulinase (Q_m) and the dissociation constant (k_d) were determined. With 0.05 M sodium phosphate buffer at pH 6.0, the parameter values 1428 UI/mL and 2 UI/mL with a correlation coefficient of 0.96 were obtained. For 0.02 M tris-HCl buffer at pH 7.5, the same parameters were 5000 UI/mL and 0.05 UI/mL with a correlation coefficient of 0.99. The best purification conditions for the fixed bed were shown to be a 0.4 M phosphate buffer with NaCl as eluter, a purification factor of 11.4, and a recovery yield of up to 79%.

Keywords: Enzyme; Adsorption; Oligosaccharides and fructose.

INTRODUCTION

Inulinase has been very well studied due to its recognized potential for the production of syrups with a high fructose content (Ettalibi & Baratti, 1987) and for the synthesis of oligosaccharides (Kim et al., 1997), considered prebiotic compounds. Due to some advantages of fermentation processes, such as short processing time, the possibility of conduction at any time of year, and the low cost of substrate, many enzymes are produced by this means, supplanting production based on animal and vegetable sources.

Studying inulinase production by *K. marxianus var. bulgarius* ATCC 16045, Treichel (2001) reduced production costs to 1/50 using industrial

medium (corn steep liquor and molasses), keeping the same enzyme activity of 120 UI/mL as that optimized by Kalil (2000) with a defined medium. Makino et al. (2002) carried out a maximization study of inulinase production by *Kluyveromyces marxianus* NRRL Y 7571, using the industrial medium. In this study a production as high as 700 UI/mL was obtained. However, to make it commercially viable, development of an appropriate process for its recovery and purification is required.

Traditionally, the adsorption of proteins from fermented medium is carried out in fixed beds, where the raw medium requires a pretreatment to remove solids and cells. In the case of expanded beds, the resultant crude medium can be used directly with the solid material passing through the

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stream bed, resulting in a single process with fewer steps, smaller losses of the desired product and cheaper operational costs. Kalil (2004) studied the adsorption of inulinase on the cationic resin Streamline® SP and verified that the adsorption behavior follows the Langmuir model. Skidmore and Chase (1989) obtained experimental results on systems in which the proteins are adsorbed on an ionic exchanger with an isotherm that can be described by the Langmuir equation.

The main goal of this work is to maximize Q_m and k_d in the adsorption of inulinase produced by *Kluyveromyces marxianus* NRRL Y-7571, using industrial medium. The adsorption study for the Streamline®DEAE resin was carried out through the construction of isotherms. An appropriate saline concentration for the elution of the enzyme in the ionic exchange chromatographic column was also determined.

MATERIALS AND METHODS

▪ Microorganism and Inoculum

Kluyveromyces marxianus NRRL Y-7571 was preserved in malt-agar slants. The microorganism was activated in tubes with 10 mL volume of medium, incubated for 24 hours at 30°C. Each tube was transferred to the inoculum medium composed of 20 g/L of sucrose, 5 g/L of yeast extract, 5 g/L of K_2HPO_4 , 1.5 g/L of NH_4Cl , 1.15 g/L of KCl , and 0.65 g/L of $MgSO_4 \cdot 7H_2O$.

▪ Fermentation

Fermentations were carried out in 500 mL flasks, inoculated with 10% inoculum and incubated at 30°C and 150 rpm in a shaker (Pycrotherm - New Brunswick Scientific, N.J.). The fermentation medium was composed of 90 g/L of molasses, 45 g/L of corn steep liquor, and 4 g/L of yeast extract at pH 5.5 (Makino et al. 2002).

▪ Adsorption Test at Different pH Values

0.05 M phosphate buffer solutions were prepared at different pH values, ranging from 5.2 to 8.0. For the 0.02 M tris-HCl buffer, the pH ranged from 6.0 to 8.0. Nine mL of solution buffer, 1 mL of resin Streamline®DEAE, and 1 mL of enzyme solution were added to each flask and stirred during 10 minutes before determination of the final activity.

▪ Determination of the Adsorption Equilibrium Time

Forty-five mL of enzyme solution diluted in 0.05 M phosphate buffer at pH 6.0 and 5 mL of resin solution in phosphate buffer (1:3) were introduced into a double-wall reactor kept at 25°C. Two different initial concentrations of the enzyme were used: 111 UI/mL (A) and 50 UI/mL (B). The C/C_0 (final-to-initial activity) ratio as a function of time was determined for each assay.

The same procedure was carried out for 0.02 M tris-HCl buffer solutions at pH 7.5 with the initial activities of 45 UI/mL (A), 108 UI/mL (B), and 200 UI/mL (C).

▪ Adsorption Isotherms

To determine the isotherm model and its parameters that best fit the process, solutions with different enzyme concentrations were prepared. One mL of 1:3 diluted buffered resin solution and 9 mL of the enzyme solution were added to 50 mL flasks and shaken at 25°C during 2 hours, before activities in the liquid and solid phases were measured.

▪ Determination of Enzymatic Activity

Enzyme activity was determined by measuring the initial production speed of free sugars under controlled conditions, as described by Santos (1998). The unit of inulinase activity per milliliter is defined as the capacity of the enzyme to hydrolyze 1 $\mu\text{mol/mL}$ of sucrose (or inulin) per minute. The DNS method was used to measure reducing sugar was carried out by (Miller, 1959), with the modifications suggested by Costa (1986).

RESULTS AND DISCUSSIONS

Adsorption Test for Different pH Values

▪ 0.05 M Phosphate Buffer

The adsorption assay was carried out at a pH ranging from 5.2 to 8.0 with an initial activity of 57 UI/mL. The amount of enzyme adsorbed by the resin (C) was calculated according to the difference between initial activity (C_0) and final activity in solution. The phosphate buffer was selected for this study. It must be mentioned that Santos (1998) studied the adsorption of inulinase on Q-Sepharose resin, using a fixed-bed column with this buffer and obtained better purification results than obtained with other buffers.

▪ 0.05M Tris-HCl Buffer

pH values of 6.0 6.5 7.0 7.5, and 8.0 were used. For all the conditions tested, as well as in the preceding case, there was no final enzyme activity indicating that the entire amount of enzyme in the medium was adsorbed on the resin. Thus a pH of 7.5 was selected for the subsequent tests with tris-HCl buffer.

▪ Adsorption Kinetic

It was verified that 50 minutes was enough to achieve adsorption equilibrium, independently of the buffer type, pH, and initial concentration of the

enzyme, as depicted in Figures 1 and 2. Thus, 120 minutes was defined as the time required to obtain the adsorption isotherms.

It was observed that, for an initial enzyme activity of about 50 UI/mL, approximately 70% in the phosphate buffer and 100% in the tris-HCl buffer were adsorbed. For an initial enzyme activity of about 100 UI/mL, adsorption was only 30% in the phosphate buffer and 70% in tris-HCl buffer. With an initial enzyme activity of 200 UI/mL and tris-HCl buffer solution, only 50% adsorption was obtained. The best adsorption capacity of the enzyme inulinase by the Streamline®DEAE resin was with tris-HCl buffer.

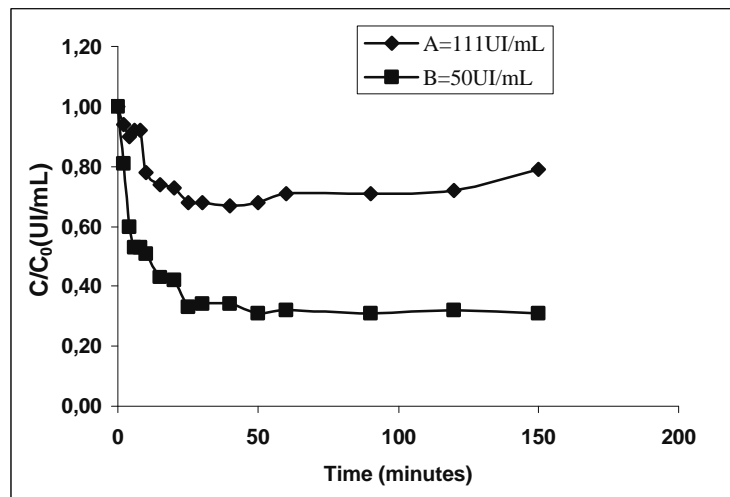


Figure 1: Inulinase adsorption for the system with Streamline®DEAE resin, clean medium diluted in phosphate buffer at pH 6.0, and 25°C as a function of time.

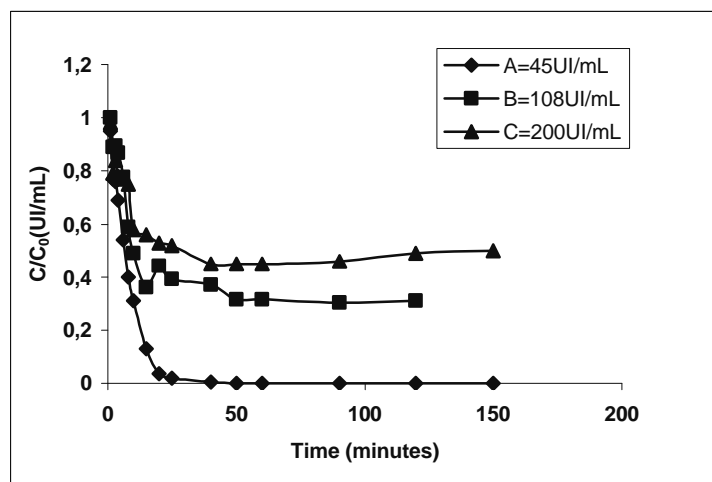


Figure 2: Inulinase adsorption for the system with Streamline®DEAE resin, clean medium diluted in Tris-HCl drain plug at pH 7.5 and 25°C as a function of time.

▪ Adsorption Isotherm

The adsorption results were plotted as Q^* versus C^* (the Langmuir model, equation 1) to determine k_d , the dissociation constant, and Q_m , the maximum adsorption capacity, and as $1/Q^*$ versus $1/C^*$ the linear form, equation 2.

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

$$\frac{1}{Q^*} = \frac{1}{Q_m} + \left(\frac{k_d}{Q_m} \right) \left(\frac{1}{C^*} \right) \quad (2)$$

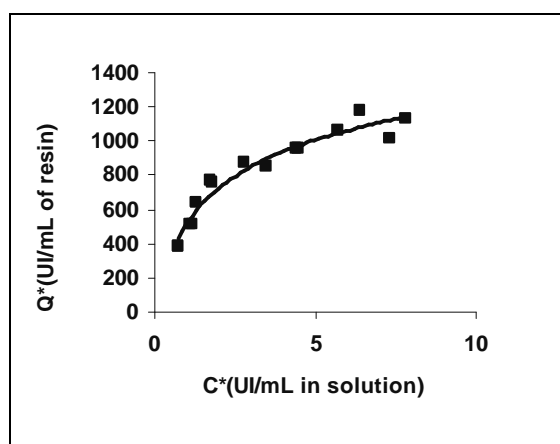
▪ Using 0.05 M Phosphate Buffer, pH 6.0

The adsorption isotherm points were the values of initial activity (C_0), activity in the equilibrium

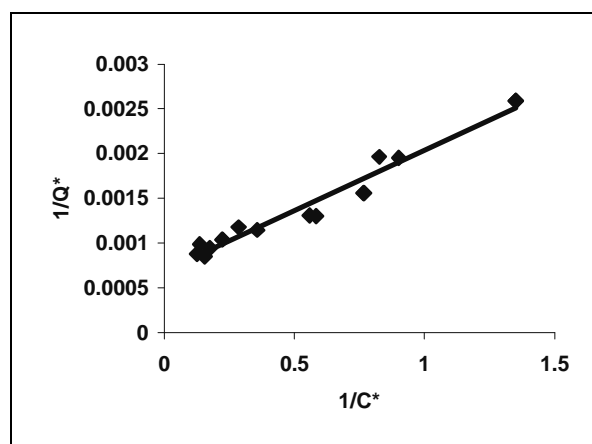
solution (C^*), and adsorbed activity by milliliter of resin (Q^*) (Figure 3). The values for the initial activity varied from 50 to 14 UI/mL. From the graph $1/Q^*$ versus $1/C^*$ in Figure 3 b, the values of 1428 UI/mL for Q_m and 2 UI/mL for k_d with a correlation coefficient of 0.96 were determined.

▪ Using 0.05M Tris-HCl Buffer, pH 7.5

Following the same procedure as in the previous item, tris-HCl buffer was tested with the initial activity in the range of 186 to 108 UI/mL. The plot $1/Q^*$ versus $1/C^*$ was constructed as shown in Figure 4 b. The values of 5000 UI/mL for Q_m and 0.05 UI/mL for k_d with a correlation coefficient of 0.99 were obtained.

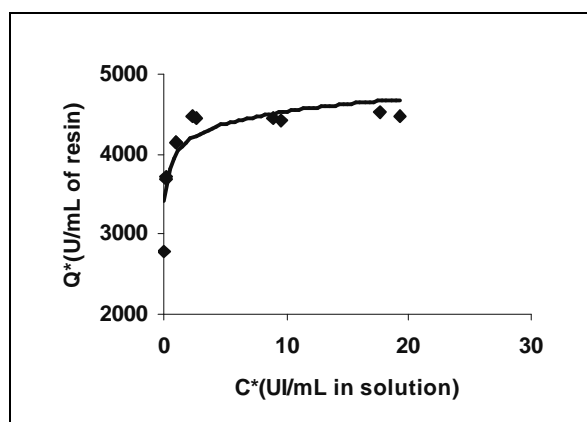


(a)

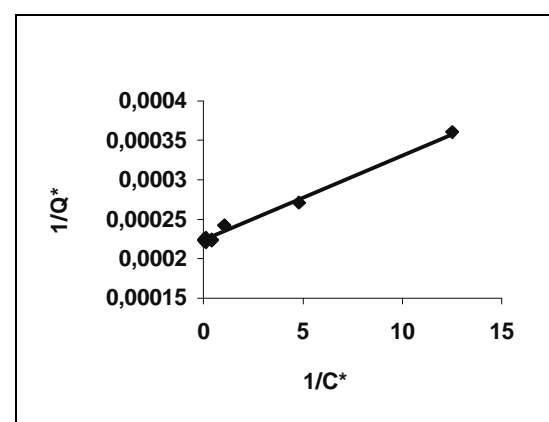


(b)

Figure 3: (a) Plot of experimental data on inulinase adsorption and (b) Linearization of Langmuir model to determine Q_m and k_d .



(a)



(b)

Figure 4: (a) Plot of experimental data on inulinase adsorption and (b) Linearization of Langmuir model to determine Q_m and k_d .

▪ Purification in the Fixed Bed Column

Based on knowledge of the adsorption capacity of the resin, purification studies on adsorption and elution of the enzyme for each type of buffer were carried out in the fixed bed using FPLC equipment (Fast Protein Liquid Chromatography from Pharmacia).

▪ Phosphate Buffer

The phosphate buffer was tested in a 20 mL column bed, feeding 10 mL of centrifuged medium with an initial activity of 510 UI/mL. The enzyme was eluted with 0.4 M NaCl, as can be observed in the chromatogram in Figure 5. The fraction that had the highest level of enzyme activity was fraction 23 which had a purification factor of 11.4 and 79% recovered enzyme (Table 1).

▪ Tris-HCl Buffer

Purification using 0.02 M tris-HCl buffer at pH 7.5 was also tested. Figure 6 contains the chromatogram, where the elution of the enzyme with 0.3 M NaCl can be observed. The better recovery of the enzyme was 19% in fraction 20 with a purification factor of 2.64

The better performance of the fixed-bed column with phosphate buffer was verified, even though the tris-HCl buffer had shown better adsorption capacity in previous results. The reason may be that denaturation of the enzyme was higher when the tris-HCl buffer was used. The Donnan effect increases the pH by approximately 1 unit for an anionic resin, which means that the real pH was near 8.5, a value that may be harmful to the enzyme (Scopes, 1988)

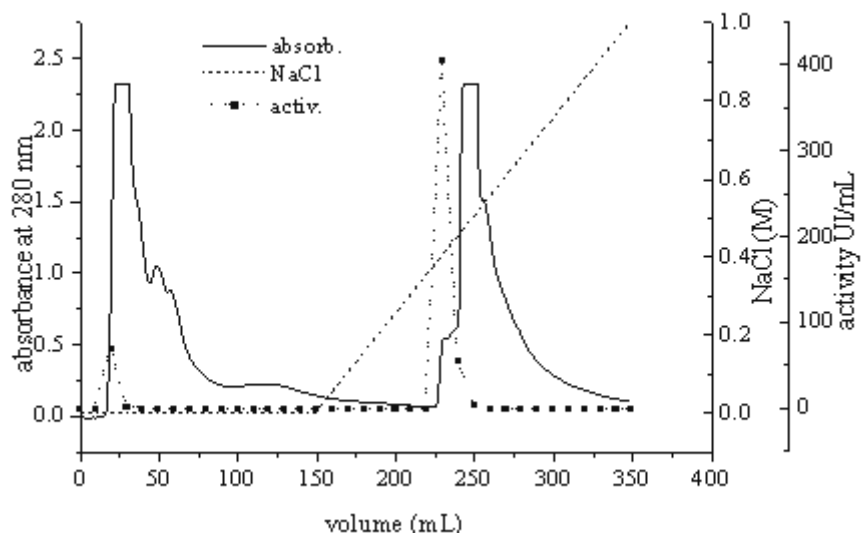


Figure 5: Chromatogram for inulinase, using phosphate buffer in a fixed bed.

Table 1: Purification in a fixed-bed column with phosphate buffer.

Fraction	Activity UI/mL	Total activity UI	Total protein mg	Specific activity UI/mg	Purification factor	Recovery (%)
medium	510.20	5102	100	50.97	1	
Fraction 23	405.60	4056	7	579.43	11.37	79

Table 2: Purification in a fixed-bed column with tris-HCl.

Fraction	Activity UI/mL	Total activity UI	Total protein mg	Specific activity UI/mg	Purification factor	Recovery (%)
medium	570	5700	107.06	53.24	1	
Fraction 20	108.22	1082	7.69	140.73	2.64	19

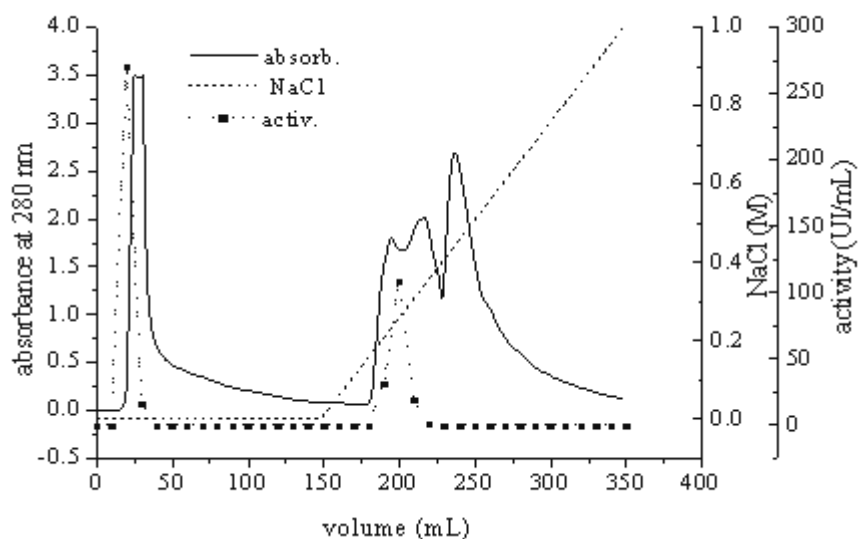


Figure 6: Chromatogram for inulinase, using tris-HCl buffer in a fixed bed.

CONCLUSIONS

The adsorption study on the anionic resin Streamline®DEAE and isotherm constructions allow determination of the maximum adsorption capacity, Q_m , which was about 1428 UI/mL, and the dissociation constant, k_d , which was about 2 UI/mL with a correlation coefficient of 0.96, using 0.05 M sodium phosphate buffer at pH 6.0. For 0.02 M tris-HCl buffer at pH 7.5 the same parameters were 5000 UI/mL for Q_m and 0.05 UI/mL for k_d . These results demonstrate that the tris-HCl buffer provided an adsorption capacity that was about 3.5 times higher than that provided by the phosphate buffer. The adsorption capacities with the two types of buffers were better than those obtained by Kalil (2000), who used cationic resin Streamline®SP with a maximum adsorption capacity of 1254 UI/mL. Purifications carried out in the fixed bed for the purpose of determining the elution conditions with 0.4 M NaCl, showed a 79% recovery of the enzyme added to the 0.02 M sodium phosphate buffer at pH 6.0 with a purification factor of 11.4. In the case of the tris-HCl buffer eluted with 0.3 M NaCl recovery was 19% with a purification factor of 2.6.

ACKNOWLEDGEMENT

The authors would like to acknowledge for the financial support received from CNPq for this work.

NOMENCLATURE

Q_m	maximum adsorption capacity	UI/mL of resin
k_d	dissociation constant	UI/mL
Q^*	adsorbed activity	UI/mL of resin
C	final activity	UI/mL
C^*	activity in the equilibrium solution	UI/mL
C_0	initial activity	UI/mL

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