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Purification by expanded bed adsorption and characterization of an α-amylases FORILASE NTL® from *A. niger*

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Abstract

In this work the purification and biochemistry characterization of α-amylases from *Aspergillus niger* (FORILASE NTL®) were studied. The effects of expansion degree of resin bed on enzyme purification by expanded bed adsorption (EBA) have also been studied. Residence time distributions (RTD) studies were done to achieve the optimal conditions of the amylases recovery on ion-exchange resin, and glucose solution was used as a new tracer. Results showed that height equivalent of the theoretical plates (HETP), axial dispersion and the Prandt number increased with bed height, bed voidage and linear velocity. The adsorption capacity of α-amylases, on the resin, increased with bed height and the best condition was at four-expansion degree. α-Amylase characterization showed that this enzyme has high affinity with soluble starch, good hydrolysis potential and molecular weight of 116 kDa.

Keywords: *Aspergillus niger*; α-Amylase; Expanded bed adsorption; Expansion degree; Purification; Biochemistry characterization

1. Introduction

Downstream process is defined as a sequence of procedures that when followed, results in a purified product. Aqueous two-phase systems extraction, membrane separation and fluidized bed sorption are three recent downstream processes. Expanded bed adsorption (EBA) was developed from protein chromatography. It permits crude feeding into the chromatographic column without an initial treatment to eliminate the biological material suspension, and as the bed expands, it increases adsorbent surface contact, making interaction with the targeted molecule more effective [1–3]. Several researches on expanded bed adsorption behaviors are made to achieve a better understanding of the effects of adsorbent type and size [4,5], bed height, linear velocity [6], fluidization and elution solutions effects on residence time distribution (RTD) [3,7] for application in the recovery of important biomolecules.

In biomolecules purification by EBA, we must be attempted for the sedimentation and particle fluidization to obtain optimal conditions of system operation [6]. Fernandez-Lahore et al. [3] examined the suitability of ion-selective electrodes (ISE) for the determination of RTD in turbid, cell-containing fluids. The enhanced feedstock compatibility of IES is better than that of other tracer sensing devices and allows a better study of bed system hydrodynamics under relevant operating conditions. Within the linear range of the corresponding ISE-tracer pair, both the rate and the pH are normally measured during EBA of proteins. According to the authors, the data provided a powerful tool for predicting the overall process of adsorption with a defined feedstock type and composition.

The (EC 3.2.1.1; α-1,4 glucan 4-glucanhydroxilase) α-amylase is a hydrolytic enzyme used more in several starch hydrolyses processing feeding industry, principally in the beer industry. They were sourced by microorganisms (*Bacillus sub-*

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tilis, *B. coagulans*, *B. licheniformis* and *B. stearothermophilus*) and veg plants, such as the cereal malt (barley, rice and rye) [8–11]. According to Sigma [12], this purified enzyme have an aggregated price of U$/g 1500.

In the present work, an α-amylase from culture medium contained *Aspergillum niger* debris cell, and BSA protein (FORILASE NTL®) was purified by expanded bed adsorption on the Amberlite IRA 410 ion-exchange resin, and subsequently its enzyme was biochemitrically characterized. The effect of expansion degree of resin bed on enzyme purification was studied. A residence time distribution (RTD) study was made.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Amberlite IRA 410 ion-exchange resin from VETEC (São Paulo, Brazil) was used. α-Amylase from *Aspergillus* sp. of the type FORILASE NTL® was provided by COGNIS LTDA (Brazil). Bovine serum albumin (BSA) PA reagent was provided by QUIMEX (Brazil). Alcohol, cupric sulfate, di-nitric salicylic acid (DNS), sodium chloride and potassium sodium tartrate PA reagents and 80% phosphoric acid were provided by VETEC (Brazil). Coomasie brilliant blue G, potassium and di-sodium hydrogen phosphate, and sodium hydroxide PA reagents were provided by MERCK (Germany).

2.1.2. EBA column

Fig. 1 shows a scheme of the EBA column used in the present work. The glass column is 1 cm × 30 cm with an adjustable piston and feed flow inlet at the bottom of the column and a product flow outlet at the top. Sixty mesh plates at the feed inlet and at the product outlet were used to avoid loss of adsorbents particles. A ruler was placed at the side of the column for measurement of bed height.

2.1.3. Reservoir solution of enzymes/BSA

Reservoir solution was made with 50 ml of FORILASE NTL® amylase solution and 50 ml of BSA at 250 μg/ml of concentration. All solutions were obtained in 0.07 M phosphate buffer at pH 6. FORILASE NTL is a commercial product used in textile processes; this product contain cell debris of *Aspergillus niger* and residue of this culture medium.

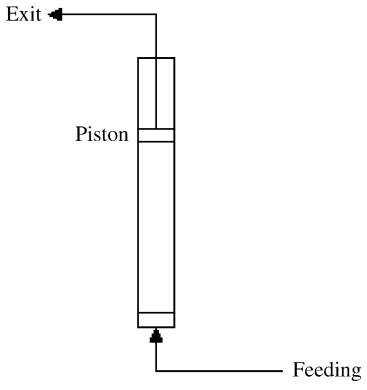

Fig. 1. Scheme of the EBA column.

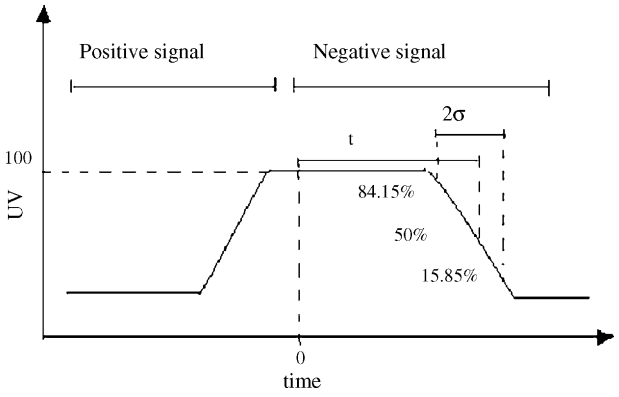

Fig. 2. Scheme for determination of RTD [1].

2.2. Methods

2.2.1. Determination of bed voidage (ε)

Bed voidage was obtained by substitution of data on specific mass (ρ_P) and mass (m_P) of the adsorbent particles, area of the cross section of the column (A_T) and bed height (H), in the following equation [3,4,7]:

$$\varepsilon = 1 - \frac{V_P}{V_L} = 1 - \frac{V_P}{A_T H} = 1 - \frac{m_P}{\rho_P A_T H} \quad (1)$$

where V_P is the particle volume.

2.2.2. Study of residence time distribution (RTD)

Phosphate buffer at 0.07 M concentration and at pH 6.0 was used as a fluidizer; the particle bed was fluidized until bed height of the study was achieved (approximately two, three and four times the initial bed height). All experiments were carried out at home conditions of temperature and pressure (22 °C and 1 atm), and the internal temperature of column was not controlled. Glucose solution was used as a tracer in substitution to acetone. Five milliliters of the tracer (glucose solution) was injected at the bottom of the column (below the particle bed). At the column outlet samples were collected from time to time. Glucose concentration was measured in all samples by Milles method [13]. To 1 mL of samples containing glucose, 1 mL of water distiller and 3 mL DNS reagent is added, and this mixture is carried out at the boiling point for 5 min. After the mixture is cooled, the absorbance analysis made at 540 nm [13].

The RTD curves were obtained by the pulse method. In Fig. 2 it is shown how DRT was determined experimentally. The mean residence time (t) and the standard deviation (σ) are substituted in Eq. (2), to obtain the height equivalent of the theoretical plates (HETP).

$$\mathrm{HETP} = \frac{t^2}{\sigma^2} \times H \quad (2)$$

Axial dispersion (D_{axial}) was calculated with Eq. (3), as follows:

$$\text{HETP} = \frac{UH}{2\varepsilon D_{axl}} \quad (3)$$

The Peclet number (*Pe*) was obtained with Eq. (4) [3,4,7]:

$$Pe = \frac{HU}{D_{axial}} \quad (4)$$

2.2.3. Enzymes assays

α-Amylase activity was measured according to Milles laboratory method [13]. Twenty millilitres of 2% soluble starch at pH 4.8 (0.1 M acetate buffer) were hydrolyzed by 500 μL of enzyme sample in 10 min at 30 ± 2 °C [8–11]. Total protein concentration was determined according to the dye binding method of Bradford [14] with BSA as protein standard. One unit of enzyme activity was defined as μmol of glucose released per min under the assay conditions. The specific activity was calculated as activity for mg protein.

2.2.4. Amylases purification

Expanded bed adsorption was carried out at 22 °C, 1 atm and pH 6. Adsorbent bed was pre-equilibrated to the height of working, with 0.07 M phosphate buffer at pH 6. A 4.2 cm bed height was expanded at 8.5, 11.6 and 15.4 cm; these bed heights are corresponded to approximately 2, 3 and 4 expansion degrees, respectively. Five milliliters of enzyme solution (crude) in phosphate buffer at pH 6 was loaded in column bottom for promoting the adsorption of enzymes on Amberlite IRA 410 ion-exchange resin. The elution was profiled with 0.25 M NaCl at 14 mL/min in descendent flux. The activity and the protein content were measured time to time during the adsorption, washing and elution periods [7,16,17].

2.2.5. Enzyme characterization

The optimum pH of the enzyme was determined by measuring its activity per 10 min; the pH varied in the rage of 3.0–10.0 at 30 ± 2 °C, using 5 mL of distilled water and 20 mL of a 2% (w/v) soluble starch solution in the following buffers: 0.1 M acetate (pH 3.0–5.0), 0.1 M phosphate (pH 5.5–7.5) and 0.1 M ammonium (pH 8.0–10). The optimum temperature was obtained by measuring its activity per 10 min, among 10 at 95 ± 2 °C, using some soluble starch solution in 0.1 M acetate buffer at pH 4.8 [9–11,15,18]. The thermal stability of enzyme was observed at 30, 60 and 80 °C per 70 min of reaction time. Activation energy of the soluble starch hydrolysis by α-amylase of type FORILASE NTL® was calculated by Arrhenius law [8]. Initial rates of starch hydrolysis were determined at various substrate concentrations (0.01–20 mg/mL). The kinetic constants K_m and V_{max} were estimated by Lineweaver-Burk method [9–11,15,18]. SDS-PAGE was performed on mini-PROTEAN II cell (Bio-Rad, USA) with 12% acrylamide gel, using buffer protein molecular weight marker [9–11,18].

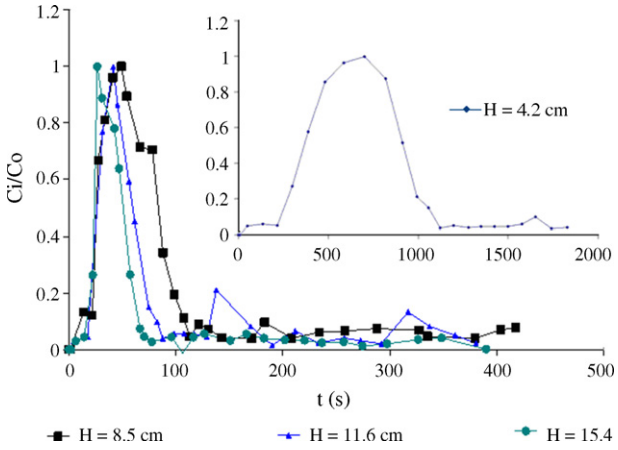

Fig. 3. Experimental RTD curves for 0.07 M phosphate buffer at pH 6, 22 °C and 1 atm.

3. Results and discussion

3.1. Residence time distribution study

Fig. 3 shows RTD curves for glucose tracer as it passed into the column bed. They have peaks that show the meanwhile glucose variation their passage into column bed; it is a characteristic of the RTD curves, illustrating that the glucose solution may be used as tracer and the RTD parameter can be determined [1,3,4]. Analyzing the DRT obtained after perfect ion-tracer pulse (axial dispersion, plug-flow exchange of mass with stagnant zones) gave a quantitative description of the underlying hydrodynamic situation during EBA processing [3].

Table 1 shows the RTD results after substitution of the data into Eqs. (1)–(4), respectively, according to the methodology used by Fernádez-Lahore et al. [3], Santos [7] and Yamamoto et al. [4]. In the table we can see that the liquid axial dispersion increased with height and bed voidage and linear velocity. There was a 10-fold increase when the initial bed height doubled and a 30-fold increase when the initial bed height quadrupled. This facilitates the flow of biological material into the particle bed and increases the contact between biological material and adsorbent particles, so that it is possible to feed the crude material into the fermented tank directly, thereby avoiding fouling and reducing the costs of pretreatment and prepurification, which are the main chromatographic problems [1,3]. The Peclet number (*Pe*) is the parameter that measures the mass transfer flow into the system. It increased with bed height, doubled with the maximum bed

Table 1
Experimental parameters obtained by RTD curves at pH 6, 22 °C and 1 atm

Bed	H (cm)	ε	U (m/s)	HETP	D_{axial} (cm²/s)	*Pe*
Fluidized	15.4	0.848	0.0058	32.96	2.477	3.628
	11.6	0.797	0.0045	24.82	1.536	3.411
	8.5	0.724	0.0030	18.19	0.835	3.097
Fixed	4.2	0.440	0.0004	2.14	0.0887	1.885

While H is the bed height, ε the bed voidage, U the linear velociy, HETP the height equivalent of the theoretical plates, D_{axial} the axial dispersion and the *Pe* Peclet number.

Table 2
Influence of expanded bed height on the recovery of α-amylase from *Aspergillus niger* (FORILASE NTL®) by expanded bed adsorption using Amberlite IRA 410 ion-exchange resin, at pH 6, 22 °C and 1 atm

Expanded bed height	Samples	Activity (U/mL)	Protein (μg/mL)	SA (U/μg)	AR (%)	PF
	Crude	45367.5	2242.80	20.2190	100.000	1.00000
8 cm	Recovered	527.211	16.1120	32.7217	1.16209	1.61836
12 cm	Recovered	1804.54	19.4543	92.7579	3.97761	4.58765
16 cm	Recovered	2135.90	12.4891	171.020	4.70799	8.45839

While AR is the activity recovery, SA the specific activity and PF the purification factor.

height, and facilitates mass transfer in the system unlike what occurs in fixed bed systems. HETP changed with all the parameters studied.

3.2. Expanded bed adsorption of α-amylases

Table 2 shows the results of α-amylase recovery by expanded bed adsorption on Amberlite IRA 410 ion exchange. The recovery yield increased to expansion degree, showing that the expansion bed carries out the best biomolecules recovery. It occurs because of more particles–biomolecules interaction in expanded bed adsorption than in the fixed bed by increase of the bed voidage influence. Its effect was also seen by Chang et al. [16] and Santos [7]; however, Kalil [17] did not find any evidence of this phenomenon in her work.

When the bed height was doubled, tripled or quadrupled (2, 3 and 4 expansion degree, respectively), there was an increase of two, three and four times in the recovery yield. This showed that fungal α-amylase purification is proportional to the expansion degree in the conditions studied in this work. It is concordant to the RTD study because to the increase of bed height facilitates the contact between enzymes and Amberlite IRA 410 ion-exchange resin.

The recovery yield obtained in this work was likely to facilitate the α-amylase recovery by chromatography, and they are more than acetone precipitation, ultrafiltration and dialysis and aqueous two-phase system purifications [9,19–21]. Majority of these authors used DEAE-sephacel and DEAE-cellulose resin, which increased the cost price of α-amylases purification. By the use of Amberlite IRA 410 ion-exchange resin and the chromatography on expanded bed condition, it was possible to obtain pure α-amylases from crude source with low cost price [1,3].

3.3. Biochemistry characterization

Fig. 4 shows that α-amylase kept 80% of activity in a pH range between 6.5 and 7.5; it is analogous to the α-amylase from *Heterorhabdits bacteriophora* [11]. However, FORILASE NTL® α-amylase shows optimum condition at pH 7.0 (it is the maximum activity) by Fig. 4. This pH optimum is analogous to the α-amylase from *Lipomyces herperus*, *L. lineolaris*, *A. suum*, and *Hyalomma dromedarii* [11]. Literatures cite that pH optimum of the greater number of α-amylase is between pH 4.5 and 6.5 [8,11,15,22]; however, there is a basic pH optimum of α-amylases as from *Spodoptera frugiperda*, *S. littoralis* (pH 9.6) and *T. gondii* (pH 8.4) [11].

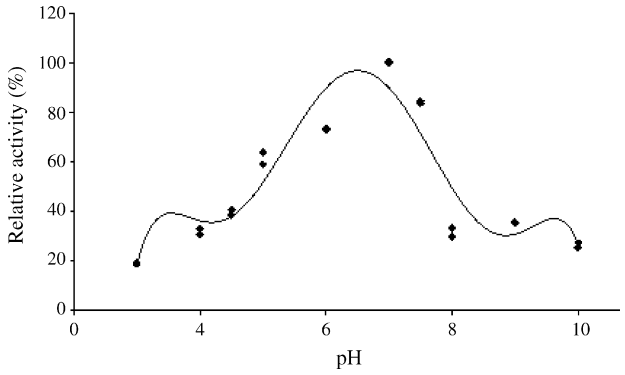

Fig. 4. pH effect on the activity of the FORILASE NTL® α-amylase at 30 °C.

Fig. 5 shows that 80% of activity of FORILASE NTL® α-amylase were kept in the temperatures between 60 and 95 °C, but the optimum temperature was about 80 °C. From Fig. 6 is noted that about 70% of activity were kept per 70 min at 80 °C.

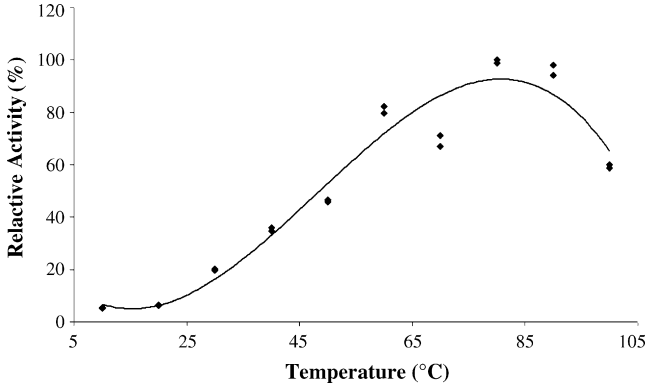

Fig. 5. Temperature effect on the activity of the FORILASE NTL® α-amylase at pH 4.8.

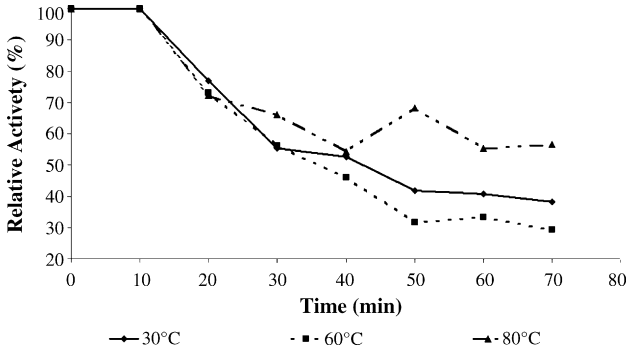

Fig. 6. Thermal stability study of the FORILASE NTL® α-amylase at pH 4.8.

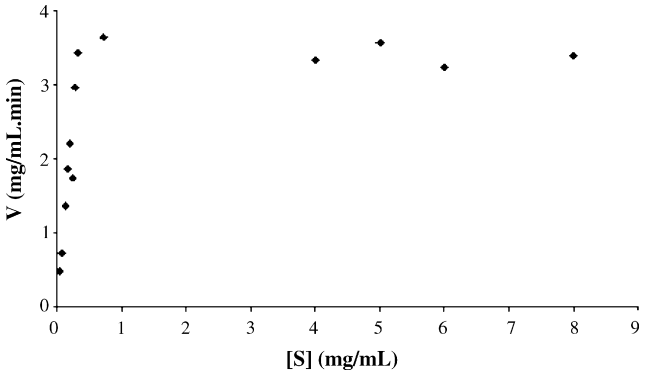

Fig. 7. Starch hydrolyses kinetic by the FORILASE NTL® α-amylase at pH 4.8 and 30 ± 2 °C.

This thermal stability is a good factor to justify the employment of this enzyme in several industrial process. Literature reports that the optimum temperature of α-amylases activity is at 40 °C for α-amylases from *H. bacteriophora*, *A. suum*, or *S. litorallis*, of 50 °C for α-amylases from *C. flavus* or *S. alluvius* ATCC 26074, or from *L. kononenkoae* or *C. antarctica* CBS 667 [15], of 65 °C for α-amylases from *T. gondii* [11], of 55 °C for α-amylases from *L. manihotivorans* [9] and of 70 °C for α-amylases *T. lanuginosus* [23]. In the literature it has been cited that between 70 and 75 °C is the optimum temperature of the greater number of α-amylases [8,11,15,22].

Fig. 7 shows that to obtain K_m and V_{max} constants by Lineweaver-burk method, their values were 7.4×10^{-3} mg/mL and 1.36×10^2 mol/ml min, respectively. The K_m value of FORILASE NTL® α-amylase was less than the α-amylase from species of granivorous coleopterans (0.061–0.42 mg/mL) and lepidopterans (0.037–0.38 mg/mL), from *A. suum* (0.483.33 mg/mL), from *H. dromedarii* (10 mg/ml), from *H. bacteriophora* (6.5 mg/mL) and from *L. manihotivorans* (3.44 mg/mL) [9]. The V_{max} value was more than their cited amylases [9]. This shows that FORILASE NTL® amylase have high affinity to the soluble starch.

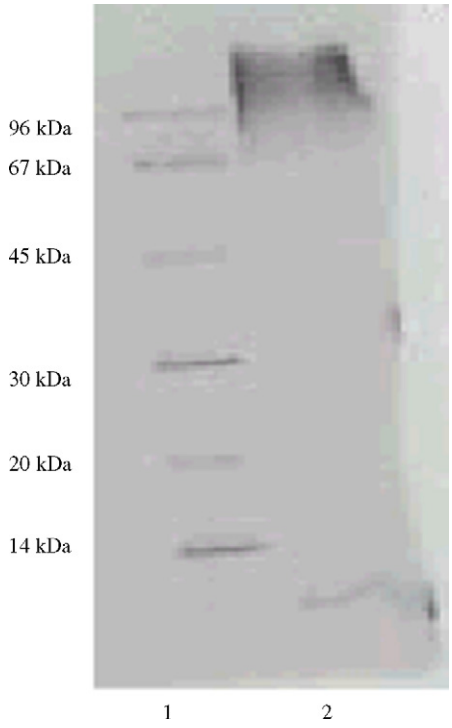

Fig. 8. SDS-PAGE analysis after α-amylase recovery by EBA process. While 1 is the marker, 2 is the α-amylase samples.

After EBC process, the eluted samples were analyzed by SDS-PAGE for the determination of enzyme molecular weight and purification degree (Fig. 7). The analysis of this figure shows that eluted material is pure (no BSA) and molecular weight of FORILASE NTL® α-amylase is 116 kDa, approximately. According to the literature, the molecular weight is dependent on α-amylase source, as from α-amylase from *Bacillus clausii* [18] and *H. bacteriophora* (47 kDa), *A. suum* (83 kDa), *H. dromedarii* (106 kDa) [11], from *B. stearothermophilus* (59 kDa) [10] and *L. manihotivoras* (135 kDa) [9] (Fig. 8).

4. Conclusions

The RTD study showed that HEPT, axial dispersion and the Prandt number increased with bed height, bed voidage and linear velocity. Phosphate buffer was promoted a good affinity between amylase and resin, and that at 16 cm of bed height it is the best system for amylase recovery by the expanded bed chromatography in Amberlite IRA 410 ion-exchange resin.

The recovery yield increased to augment the bed height, showing that the expansion bed carries out the best biomolecules recovery; it occurs due to more particles–biomolecules interaction in expanded bed than in fixed bed by increase to bed voidage influence.

Fungal amylase characterization showed that the K_m and V_{max} constants were 7.4×10^{-3} mg/mL and 1.36×10^2 mol/ml min, respectively. This introduces that enzyme has high affinity with soluble starch and good hydrolysis potential and has molecular weight is 116 kDa.

This work showed that the employment of Amberlite IRA 410 ion exchange in the expanded bed adsorption is the best system for recovery of α-amylases from *Aspergillus niger* (FORILASE NTL®) and it is more economically viability than the other purification methods.

5. Nomenclature

AR	Activity recovery (%, U/mL/U/mL)
A_T	Area of cross section (m^2)
Calc	Calculated data
D_{axial}	Axial dispersion (cm^2/s)
Exp	Experimental data
g	Gravity acceleration (m/s^2)
H	Bed height (cm)
HETP	Height equivalent of the theoretical plate (cm)
K_m	Michaelis-Menten constant (mg/mL)
m_p	Particle mass (kg)
n	Expansion of coefficient (dimensionless)
Pe	Pecletl number (dimensionless)
PF	Purification factor (U/μg/U/μg)

R_p	Particle Reynolds number (dimensionless)
R_t	Terminal Reynolds (dimensionless)
RTD	Residence time distribution
[S]	Substrate concentration (mg/mL)
SA	Specific activity (U/μg)
t	Time (s)
U	Linear velocity (m/s)
U_T	End velocity (m/s)
V	Velocity of starch hydrolysis (mol/mL min)
V_{max}	Maximum velocity of starch hydrolysis (mol/mL min)
V_L	Liquid volume (m^3)
V_P	Particle volume (m^3)

Greek symbols

ε	Voidage
μ	Dynamic viscosity (kg/s m)
ρ_L	Specific liquid mass (kg/m^3)
ρ_p	Specific particle mass (kg/m^3)
σ	Standard deviation (dimensionless)

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