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RESEARCH ARTICLE

Mixture design of starchy substrates hydrolysis by an immobilized glucoamylase from *Aspergillus brasiliensis*

Paula Zaghetto Almeida[a], Josana Maria Messias[b], Marita Gimenez Pereira[c], Vanessa Elisa Pinheiro[a], Lummy Maria Oliveira Monteiro[a], Paulo Ricardo Heinen[a], George Cunha Cardoso[d], João Atílio Jorge[c] and Maria de Lourdes Teixeira de Moraes Polizeli[c]

[a]Departamento de Bioquímica e Imunologia, Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto, Brazil; [b]Departamento de Química, Universidade de São Paulo, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Ribeirão Preto, Brazil; [c]Departamento de Biologia, Universidade de São Paulo, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Ribeirão Preto, Brazil; [d]Departamento de Física, Universidade de São Paulo, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Ribeirão Preto, Brazil

ABSTRACT

Starch has great importance in human diet, since it is a heteropolymer of plants, mainly found in roots, as potato, cassava and arrowroots. This carbohydrate is composed by a highly-branched chain: amylopectin; and a linear chain: amylose. The proportion between the chains varies according to the botanical source. Starch hydrolysis is catalyzed by enzymes of the amilolytic system, named amylases. Among the various enzymes of this system, the glucoamylases (EC 3.2.1.3 glucan 1,4-alpha-glucosidases) are the majority because they hydrolyze the glycosidic linkages at the end of starch chains releasing glucose monomers. In this work, a glucoamylase secreted in the culture medium, by the ascomycete *Aspergillus brasiliensis*, was immobilized in Dietilaminoetil Sepharose-Polyethylene Glycol (DEAE-PEG), since immobilized biocatalysts are more stable in long periods of hydrolysis, and can be recovered from the final product and reused for several cycles. Glucoamylase immobilization has shown great thermal stability improvement over the soluble enzyme, reaching 66% more activity after 6 h at 60 °C, and 68% of the activity after 10 hydrolysis cycles. A simplex centroid experimental mixture design was applied as a tool to characterize the affinity of the immobilized enzyme for different starchy substrates. In assays containing several proportions of amylose, amylopectin and starch, the glucoamylase from *A. brasiliensis* mainly hydrolyzed the amylopectin chains, showing to have preference by branched substrates.

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Introduction

Starch is the main carbohydrate reserve of plants and it is the second more abundant in nature. It is an important part of human feeding and can be hydrolyzed generating maltose, glucose and oligosaccharide syrups (Vieille and Zeikus 2001).

Starch is composed by two glucose polymers, amylose and amylopectin. The first one is almost linear, with relative low molecular weight, and it is constituted by long chains of D-glucose with α-1,4 linkage. Amylopectin has many short branches, with α-1,6 linkage at the ramification points, and high molecular weight (Vilaplana and Gilbert 2010).

The application of starch in industry varies primary by its rheological and gelatinization properties (Ai and Jane 2015). Amylopectin is responsible for the swelling power and viscosity of gelatinized starch, while amylose interlaces with amylopectin limiting the granules swelling (Zeng et al. 1997). Starch can be classified according to its amylose content as waxy (0–8%), normal (20–30%) or high-amylose (>40%) (Schwartz and Whistler 2009). The composition of starch, and its crystallization level, varies with botanic source. In maize, the amylose content is in the range of 23–28%, in cassava 18–24%, in wheat 25–26%, in rice 4–21% and in potato 17–27% (Waterschoot et al. 2015).

Conventionally, the first industrial step in the degradation of starch to obtain high glucose syrups is the liquefaction. This energy consuming step is carried at 90–100 °C, in the presence of highly thermostable α-amylase. After that, in the saccharification phase, the syrup is cooled and glucoamylases are added. This hydrolysis occurs at less costly temperatures, around

CONTACT Maria de Lourdes Teixeira de Moraes Polizeli polizeli@ffclrp.usp.br Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Bandeirantes, 3900 Avenue, 14040-901 Ribeirão Preto, SP, Brazil

60 °C, resulting in high glucose concentrations (James and Lee 1997; Slivinski et al. 2011).

Glucoamylases (E.C. 3.2.1.3 glucan 1,4-alfa-glucosidase) are exo-amylases that release β-D-glucose monomers at the non-reducing starch ending. They hydrolyze α-1,4 linkage and, in a slower rhythm, the α-1,6 linkage of ramification points (Hiromi et al. 1966). Biotechnological industries demand enzymes with greater efficiencies, lower costs and longer shelf lives. Immobilized biocatalysts have prolonged activity, stability and simpler downstream purification steps aiming to generate the final product. In addition, they work in a wide range of pH and temperatures, have a great turnover and can be reused for several hydrolysis cycles (Datta et al. 2013; DiCosimo et al. 2013). While immobilized biocatalysts still represent a small portion of enzyme market, they are of interest due to the characteristics mentioned above (DiCosimo et al. 2013).

Design of experiment (DOE) is a powerful tool that results in more economical and profitable bioprocess. It is possible to optimize the process with a reduced number of experiments, by analyzing the interaction among the involved variables and the value of the experimental variation (Castro and Sato 2013; Arias et al. 2016; Zuorro et al. 2016; Kim et al. 2017). Mixture design is a DOE approach developed for optimizing mixtures, in which the final product depends on the relative proportion of its constituents (Anderson and Whitcomb 1998). In enzyme field, it is usually applied to obtain more efficient cocktails or optimized culture media (Rispoli and Shah 2008; Suwannarangsee et al. 2012).

In this work, the same methodology was applied to characterize the enzyme action upon a heteropolymeric subtract. The mixture design was planned with several proportions of amylose, amylopectin and starch in order to determine which of the starchy chains are more hydrolyzed by the immobilized glucoamylase from *Aspergillus brasiliensis*. These studies contribute to a better understanding in enzyme action and may reflect in a more precise and focused application in food industry.

Methods

Microorganism and amylolytic production

The fungi *A. brasiliensis* was isolated from Brazilian Atlantic Forest in Peruíbe – SP. It is part of the filamentous fungi culture collection from Laboratory of Microbiology and Cell Biology – FFCLRP, where it is catalogued as CFF124 (Almeida 2015). The experimental procedures are summarized in Figure 1. This strain was cultivated in SR (Segato Rizzatti) medium (Rizzatti et al. 2001), during 5 days, at 30 °C, with 1% maltose as carbon source. The culture was filtrated, dialyzed in distilled water, clarified with activated charcoal (5 mg per mL of crude extract) and buffered with 10 mM Tris-HCl buffer, pH 7.0.

Enzyme immobilization

Enzyme immobilization was carried out with 1 g of Dietilaminoetil-Sepharose (DEAE-Sepharose) equilibrated with 10 mM Tris-HCl buffer, pH 7.0. The mixture (100 mL clarified enzymatic extract + DEAE-Sepharose) was incubated under agitation in cold room at 4 °C, for 1 h. After this process, the mixture of enzyme plus resin (derivative) was filtered, washed with buffer and added to a solution of 30% polyethylene glycol 4000

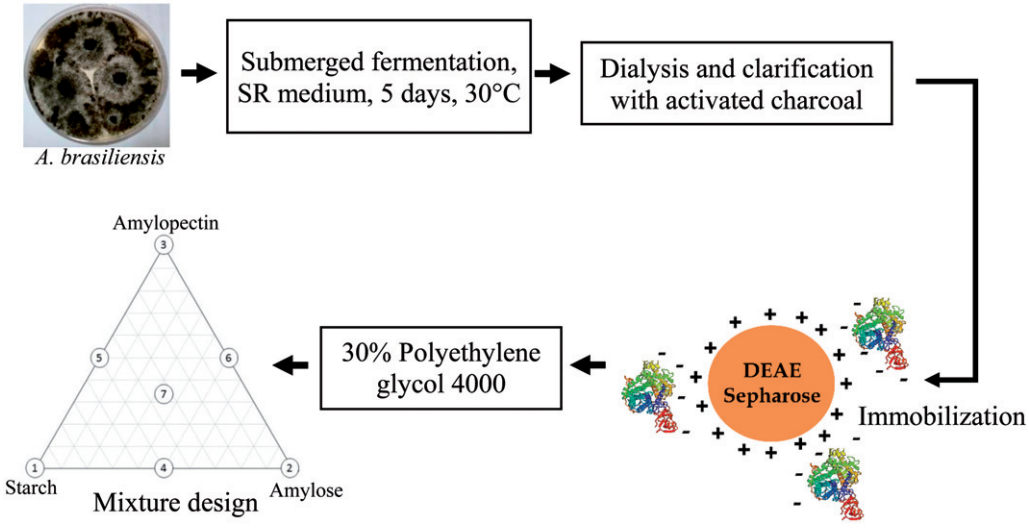

Figure 1. Experimental steps to glucoamylase production, immobilization and characterization by mixture design.

(PEG 4000), overnight, at 4 °C. After that, the derivative was filtered again, washed with buffer and used for enzymatic assays. The protein content was quantified by Bradford (1976).

Enzymatic assay

Amylolytic activity was measured with 3,5-dinitrosalicylic acid (DNS) (Miller 1959). The assay was composed of 50 μL crude extract and 50 μL 1% soluble starch in sodium citrate buffer 50 mM, pH 4.5. The mixture was incubated at 70 °C, for 5 min, interrupted by the addition of 100 μL DNS reagent, and boiled for 5 min. After cooling, 1 mL of distilled water was added in the assay tube and the reaction was read at 540 nm in SpectraMax Plus 384 Microplate Reade. The blank of the enzymatic assay was composed by the enzyme inactivated by DNS prior the addition of substrate. One unit of enzyme activity was defined as that catalyzing the conversion of 1 μmol of glucose, per minute, in the assay conditions.

Thermal denaturation

The thermal denaturation assay occurred with the crude extract and the derivative in the absence of substrate or buffer. The free enzyme as well as the one immobilized in DEAE-PEG was incubated at 50 °C, up to 6 h. The enzymatic assay proceeded as described above. The activity of the first assay (time zero) was considered 100%.

Reuse of the derivative

In order to evaluate the possibility of biocatalyst reuse, the DEAE-PEG derivative was assayed for ten hydrolysis cycles. Each cycle was performed at 50 °C in sodium citrate buffer 50 mM, pH 4.5 and 1% soluble starch for 5 min. After each cycle the derivative was separated from the products by centrifugation 4000*g* for 5 min and washed three times in 1 mL of distilled water. The activity was measured by the formation of reducing sugars after hydrolysis, as described above.

Mixture design

The reducing sugar yield (μmol/mL), originated from starch substrate hydrolysis by *A. brasiliensis* glucoamylase was determined through a statistical mixture design matrix. The total proportion sum for each assay was one. The representation of a three-component simplex centroid mixture system is made by triangular diagrams which contains: three experiments with pure components (diagram vertex), three binary mixture of components (midpoint of the edges) and one ternary mixture (diagram central point), performed in triplicate to calculate the model error (Calado and Montgomery 2003).

In Table 1 are summarized the factors and their minimum and maximum proportions 1. Each assay consisted of different concentrations and mixtures of substrate: 5 mL of 0.167%, 0.25% or 0.5% substrate (starch, amylose or amylopectin from potato, Sigma, St. Louis, MO, USA) in 50 mM sodium acetate buffer,

Table 1. Matrix to construct the mixture design.

Point	Concentration (%) and proportion (0–1) of substrates		
	Starch	Amylose	Amylopectin
1	0.5 (1)	0 (0)	0 (0)
2	0 (0)	0.5 (1)	0 (0)
3	0 (0)	0 (0)	0.5 (1)
4	0.25 (0.5)	0.25 (0.5)	0 (0)
5	0.25 (0.5)	0 (0)	0.25 (0.5)
6	0 (0)	0.25 (0.5)	0.25 (0.5)
7 – central	0.167 (0.33)	0.167 (0.33)	0.167 (0.33)
8 – central	0.167 (0.33)	0.167 (0.33)	0.167 (0.33)
9 – central	0.167 (0.33)	0.167 (0.33)	0.167 (0.33)

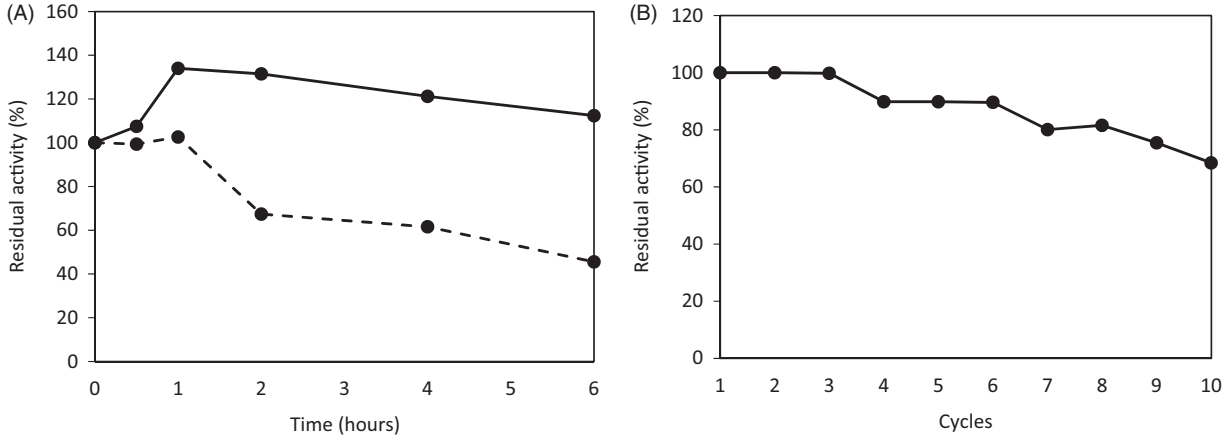

Figure 2. Immobilized enzyme: (A) thermal denaturation of immobilized (continuous line) and free enzyme (dashed line); (B) Activity of immobilized enzyme after 10 hydrolysis cycles.

pH 4.5, with 15 mM sodium azide to prevent bacterial growth. The test was performed with 0.5 enzymatic unit of derivative. The experiments occurred at 50 °C in dry bath under continuous stirring at 500 rpm up to 4 h. Samples were collected after 0.5, 1, 2, 3 and 4 h and boiled to stop the enzymatic reaction. The amount of reducing sugars was determined by DNS method (Miller 1959).

The results of the experimental mixture design were analyzed and interpreted using the software Statistica 12 (StatSoft Inc., Palo Alto, CA, USA). Regression coefficients (R^2) were used to express quality of the fit of the models, and its statistical significance was determined by the F test. Analysis of variance was performed at the significance levels of 5% or 10%.

Results and discussion

The DEAE-PEG support immobilized 83% (3.6 mg) of proteins from de extract. This biocatalyst had a greater stability at 50 °C when compared to the free enzyme (Figure 2(a)). After 6 h of incubation, 112% of the initial activity was detected in the derivative, while the activity of the free enzyme declined to 45%. The DEAE-PEG derivative remained efficient after 10 hydrolysis cycles (Figure 2(b)). The activity was unaltered after the three first cycles, and, after the tenth cycle 68% of the initial activity was still detected.

Table 2. Equations produced by the mixture desing model.

Time of hydrolysis (h)	Equations
0.5	$y = 0.43A + 0.5B + 0.7C + 0.32AB - 1.59ABC$ [1]
1	$y = 0.6A + 0.47B + 0.71C + 0.572AB - 1.31ABC$ [2]
2	$y = 0.83A + 0.43B + 0.89C + 0.4AB + 0.50BC - 1.65ABC$ [2]
3	$y = 0.66A + 0.55B + 0.81C + 0.25AB$ [1]
4	$y = 0.84A + 0.65B + 0.96C - 3.23ABC$ [1]

Legend: y = expected value μmol/mL (reducing sugar); A = proportion of starch; B = proportion of amylose; C = proportion of amylopectin. The equations are significant at [1] 90% and [2] 95% confidence level.

Torres et al. (2004) immobilized a commercial *Aspergillus niger* glucoamylase in DEAE-agarose, 75% of the protein was successfully bound to the resin, but, the thermostability had a small decrease, with a half-life of less than 20 min at 55 °C. Tomar and Prabhu (1985) used a DEAE-cellulose activated with cyanuric chloride to immobilize a partially purified glucoamylase of *A. niger* and also obtained a slightly less thermostable derivative when compared to the free enzyme at several temperatures.

The addition of polyethylene glycol promotes steric stabilization, thereby preventing proteins and other biomolecules from approaching the surface, avoiding the adsorbed protein on the support to aggregate (Osmond et al. 1975).

Regarding the mixture design, the equations constructed from the model coefficients are shown in Table 2. The ANOVA values and the models used are listed in Table 3. In the graphical representations of response surface (Figure 3) it is possible to visualize the hydrolysis of substrates at each time of the

Table 3. Analysis or variance for mixture design of derivative DEAE-PEG.

		ANOVA						
						F-test		
Time of hydrolysis (h)	Model	Source	SS	DF	MS	*F* calculated	Critical value	r^2
0.5	Special cubic	Model	0.045582	4	0.011395	17.639	4.11	0.946
		Total error	0.002584	4	0.000646			
		Lack of fit	0.001896	2	0.000948	2.757	9.00	
		Pure error	0.000688	2	0.000344		$\alpha = 0.1$	
		Total	0.048166					
1	Special cubic	Model	0.041805	4	0.010451	76.579	6.39	0.987
		Total error	0.000546	4	0.000136			
		Lack of fit	0.000239	2	0.000120	0.780	19.00	
		Pure error	0.000307	2	0.000153		$\alpha = 0.05$	
		Total	0.042351					
2	Special cubic	Model	0.142043	5	0.028409	72.650	9.01	0.992
		Total error	0.001173	3	0.000391			
		Lack of fit	0.000592	1	0.000592	2.040	18.51	
		Pure error	0.000581	2	0.000290		$\alpha = 0.05$	
		Total	0.143217					
3	Quadratic	Model	0.051999	5	0.010400	21.004	5.31	0.972
		Total error	0.001485	3	0.000495			
		Lack of fit	0.000784	1	0.000784	2.236	8.53	
		Pure error	0.000701	2	0.000351		$\alpha = 0.1$	
		Total	0.053484					
4	Special cubic	Model	0.088616	3	0.029539	20.421	3.62	0.925
		Total error	0.007232	5	0.001446			
		Lack of fit	0.004616	3	0.001539	1.177	9.16	
		Pure error	0.002616	2	0.001308		$\alpha = 0.1$	
		Total	0.095848					

enzymatic assay. The following results were obtained: (i) at 0.5 h, most of the amylopectin was hydrolyzed; (ii) at 1 and 2 h starch and amylopectin were hydrolyzed; (iii) at 3 and 4 h of incubation, amylopectin is still the most hydrolyzed substrate by the immobilized glucoamylase, and amylose remains poorly hydrolyzed. In order to validate the model, it was performed a triplicate with points of 1 and 3 h at concentrations of 25% starch and 75% amylose (A) and 25% starch and 75% amylopectin (B) as shown in Table 4.

The value predicted for 1 h in A was 0.69 ± 0.11 μmol/mL and the observed was 0.700 μmol/mL; in B the predicted value was 0.62 ± 0.11 μmol/mL and the observed was 0.512 μmol/mL. At the point of

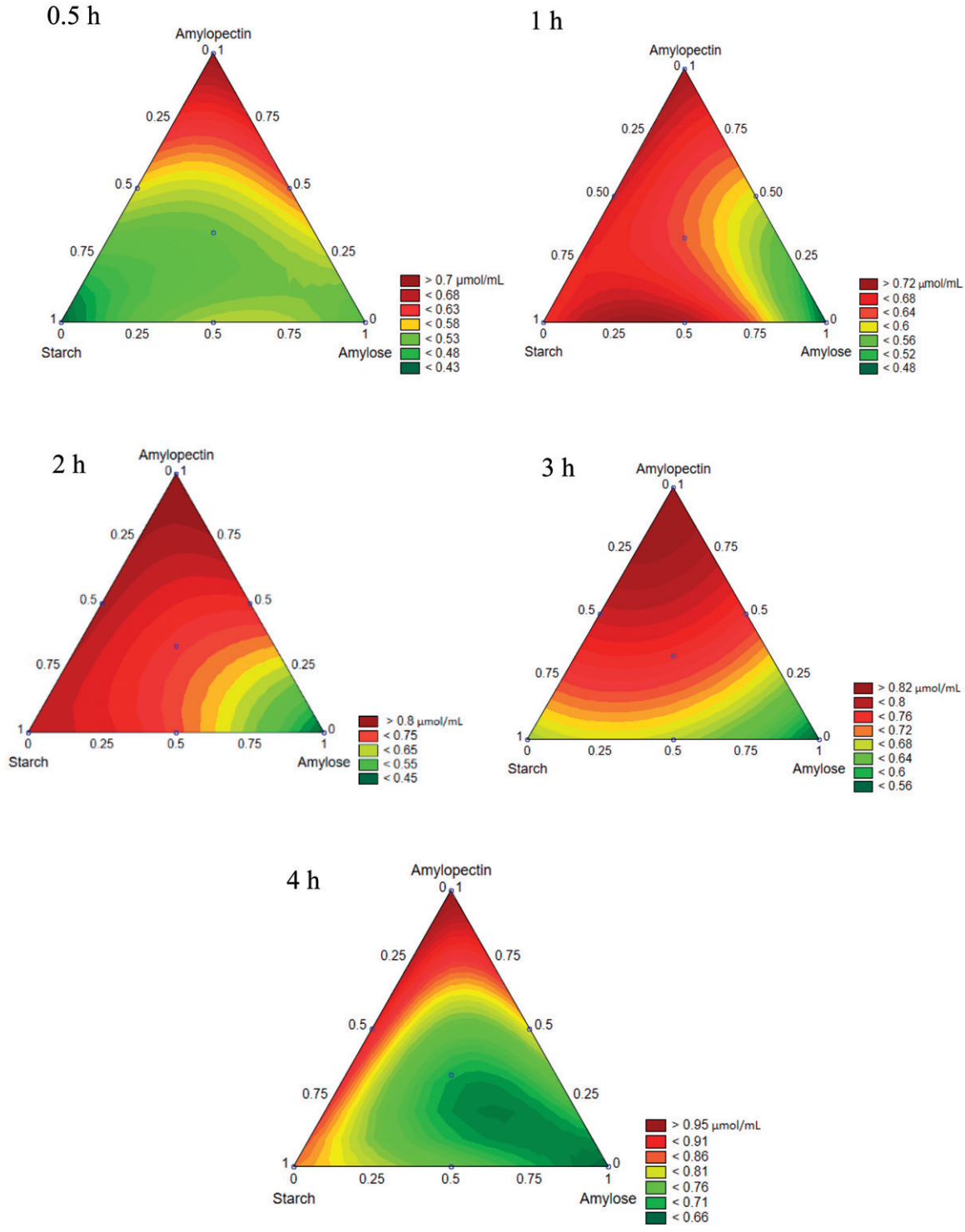

Figure 3. Biochemical behaviour of *Aspergillus brasiliensis* glucoamylase during starch, amylose and amylopectin hydrolysis in several times. The response surface shows the detected amount of reducing sugar (μmol/mL).

Table 4. Matrix of validation experiments.

Validation points (1 and 3 h)	Concentration (%) and proportion (0–1) of substrates		
	Starch	Amylose	Amylopectin
A	0.125 (0.25)	0.375 (0.75)	0 (0)
B	0.125 (0.25)	0 (0)	0.375 (0.75)

3 h, for A the predicted value was 0.814 ± 0.12 μmol/mL and the observed value was 0.883 μmol/mL. In B, the predicted value was 0.63 ± 0.06 μmol/mL and the observed value was 0.596 μmol/mL.

Starch is constituted by approximately 15–20% of amylose and 75–80% of amylopectin. Amylose is formed by long chains with about 1000 units of glucose, while amylopectin is made of short chains, with 10–60 units of glucose (Soccol et al. 2006). *A. brasiliensis* glucomylase showed preference to shorter amylaceous substrates. This characteristic may have been evolutionary selected due to the abundance of amylopectin in the starch composition. Amirul et al. (1996) reported that *A. niger* glucoamylase I shows a smaller Michaelis–Menten kinetics (km) for amylopectin (2.1 mg/mL) as substrate than to amylose (2.6 mg/mL), which suggests more affinity to the first substrate. *Paecilomyces variotti* glucoamylase shows a greater km for amylose (2.5 mg/mL) than amylopectin (2 mg/mL) (Michelin et al. 2008). On the other hand, *Aspergillus fumigatus* and *Aspergillus awamori* glucoamylases had more activity in amylose than amylopectin, with a difference around 10% (Yamasaki et al. 1977; Silva and Peralta 1998).

Conclusion

The immobilization of the glucoamylase resulted in a more stable and reusable biocatalyst. Amylopectin, the most abundant starch chain, was preferentially hydrolyzed by the glucoamylase from *A. brasiliensis* immobilized in DEAE-PEG. At 1 and 2 h, the heteropolymer starch showed high levels of hydrolyzation and minor levels at the other tested periods. The mixture planning proved to be a valid tool to enzyme characterization upon an heterogeneous substrate and efficiently showed the action of the immobilized glucoamylase from *A. brasiliensis* in the diverse starch chains through the time, which may reflect a more specific and precise application in food industry.

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Disclosure statement

The authors declare no conflict of interests.

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References

Ai Y, Jane JL. 2015. Gelatinization and rheological properties of starch. Starch-Stärke 67:213–224.

Almeida PZ. 2015. Diversidade do potencial amilolítico em fungos filamentosos: purificação e caracterização de uma glucoamilase de Aspergillus brasiliensis [dissertation]. Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto.

Amirul AA, Khoo SL, Nazalan MN, Razip MS, Azizan MN. 1996. Purification and properties of two forms of glucoamylase from *Aspergillus niger*. Folia Microbiol 41:165–174.

Anderson MJ, Whitcomb PJ. 1998. Find the most favorable formulations. Chem Eng Prog 94:63–67.

Arias MJ, Modesto LFA, Polikarpov I, Pereira N. 2016. Design of an enzyme cocktail consisting of different fungal platforms for efficient hydrolysis of sugarcane bagasse: optimization and synergism studies. Biotechnol Prog 32(5): 1222–1229.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Calado V, Montgomery DC. 2003. Planejamento de Experimentos usando o Statistica. Rio de Janeiro: Ed E-papers.

Castro RJS, Sato HH. 2013. Synergistic effects of agroindustrial wastes on simultaneous production of protease and α-amylase under solid state fermentation using a simplex centroid mixture design. Ind Crops Prod 49:813–821.

Datta S, Christena LR, Rajaram YRS. 2013. Enzyme immobilization: an overview on techniques and support materials. Biotech 3:1–9.

DiCosimo R, Mcauliffe J, Poulose J, Bohlmann G. 2013. Industrial use of immobilized enzymes. Chem Soc Rev 42:6437–6474.

Hiromi K, Hamazu Z, Takarashi K, Ono S. 1966. Kinect studies on glucoamylase II: competition between two types of substrates having α-1.4 and α-1.6 glucosidic linkage. J Biochem 59:411–418.

James JA, Lee BH. 1997. Glucoamylases: microbial sources, industrial applications and molecular biology: a review. J Food Biochem 21:1–52.

Kim IJ, Lee HJ, Kim KH. 2017. Pure enzyme cocktails tailored for the saccharification of sugarcane bagasse

pretreated by using different methods. Process Biochem 57:167–174.

Michelin M, Ruller R, Ward RJ, Moraes LAB, Jorge JA, Terenzi HF, Polizeli MLTM. 2008. Purification and biochemical characterization of a thermostable extracellular glucoamylase produced by the thermotolerant fungus *Paecilomyces variotii*. J Ind Microbiol Biotechnol 35:17–25.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31:426–428.

Osmond DWJ, Vincent B, Waite FA. 1975. Steric stabilization: a reappraisal of current theory. Colloid Polymer Sci 253:676–682.

Rispoli F, Shah V. 2008. Optimization of the media ingredients for cutinase production from *Colleotrichum lindemuthianum* using mixture design experiments. Biotechnol Prog 24:648–654.

Rizzatti ACS, Jorge JA, Terenzi HF, Rechia CGV, Polizeli MLTM. 2001. Purification and properties of a thermostable extracellular β-D-xylosidase produced by thermotolerant *Aspergillus phoenicis*. J Ind Microbiol Biotechnol 26: 156–160.

Schwartz D, Whistler RL. 2009. History and future of starch. In: BeMiller JN, Whistler RL, editors. Starch: chemistry and technology. 3rd ed. Burlington, MA: Academic Press, Elsevier.

Silva WB, Peralta RM. 1998. Purification and characterization of a thermostable glucoamylase from *Aspergillus fumigatus*. Can J Microbiol 44:493–497.

Slivinski CT, Machado AVL, Iulek J, Ayub RA, Almeida MMD. 2011. Biochemical characterization of a glucoamylase from *Aspergillus niger* produced by solid-state fermentation. Braz Arch Biol Technol 54:559–568.

Soccol CR, Rojan PJ, Patel AK, Woiciechowski AL, Vandenberghe LPS, Pandey A. 2006. Glucoamylase. In: Pandey A, Webb C, Socool CR, Larroche C, editors. Enzyme technology. New York: Springer. p. 221–238.

Suwannarangsee S, Bunterngsook B, Arnthong J, Paemanee A, Thamchaipenet A, Eurwilaichitr L, Laosiripojana N, Champreda V. 2012. Optimisation of synergistic biomass-degrading enzyme systems for efficient rice straw hydrolysis using an experimental mixture design. Bioresour Technol 119:252–261.

Tomar M, Prabhu KA. 1985. Immobilization of glucoamylase on DEAE-cellulose activated with chloride compounds. Enzyme Microb Technol 7:557–560.

Torres R, Pessela BC, Mateo C, Ortiz C, Fuentes M, Guisa JM, Fernandez-Lafuente R. 2004. Reversible immobilization of glucoamylase by ionic adsorption on sephabeads coated with polyethyleneimine. Biotechnol Prog 20:1297–1300.

Vieille C, Zeikus GJ. 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 65:1–43.

Vilaplana F, Gilbert RG. 2010. Characterization of branched polysaccharides using multiple-detection size separation techniques. J Sep Sci 33:3537–3554.

Waterschoot J, Gomand SV, Fierens E, Delcour JA. 2015. Production, structure, physicochemical and functional properties of maize, cassava, wheat, potato and rice starches. Starch 67:14–29.

Yamasaki Y, Suzuki Y, Ozawa O. 1977. Three forms of α-glucosidase and a glucoamylase from *Aspergillus awamori*. Agric Biol Chem 41:2149–2161.

Zeng M, Morris CF, Batey IL, Wrigley CW. 1997. Sources of variation for starch gelatinization, pasting, and gelation properties in wheat. Cereal Chem 74:63–71.

Zuorro A, Miglietta S, Familiari G, Lavecchia R. 2016. Enhanced lipid recovery from *Nannochloropsis* microalgae by treatment with optimized cell wall degrading enzyme mixtures. Bioresour Technol 212:35–41.