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# Mixture design of Starchy Substrates Hydrolysis by an Immobilized Glucoamylase of *Aspergillus brasiliensis*

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## SUMMARY

*Starch is an heteropolymer composed by a highly branched chain: the amylopectin; and a linear chain: the amylose. Glucoamylases are enzymes that hydrolyze glycosidic linkage at the end of starch chains releasing glucose. In this work it was determined by a mixture design with starch, amylose and amylopectin that the immobilized glucoamylase of Aspergillus brasiliensis mainly hydrolisys amylopectin chains.*

Keywords: Glucoamylase; *Aspergillus brasiliensis*; Starch, Amylose; Amylopectin.

## INTRODUCTION

The starch is the main reserve carbohydrate of plants and it is the second more abundant in nature (VIELLE; ZEIKUS, 2001). Starch is composed by two glucose polymers, the amylose and the amylopectin. The first one is linear and composed by long chains of D-glucose with α-1,4 linkage. The amylopectin is highly branched and it is composed by α-1,4 linkage at the linear chain and α-1,6 linkage at branching points (NELSON; COX, 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 end of starch. They hydrolyze α-1,4 linkage and in a slower step α-1,6 linkage of ramification points (HIROMI *et al*., 1966).

This work aimed to determine, through a mixture design with amylose, amylopectin and starch as substrates, which of the starchy chains are more hydrolyzed by the immobilized glucomylase of *A. brasiliensis* at several reaction times.

## MATERIAL AND METHODS

The fungi *A. brasiliensis* was cultivated in SR medium (RIZZATTI *et al*., 2001) during 5 days, at 30°C, with 1% maltose as carbon source. The culture was filtrated, dialyzed in distillated water, clarified with activated charcoal (5 mg per mL of crude extract), and buffered with 10 mM Tris-HCl pH 7.0. In order to immobilization of enzymes it was used 1g

of Dietilaminoetil-Sepharose (DEAE-Sepharose) equilibrated in the same buffer After that, the mixture was incubated under agitation with 100 mL of the enzymatic extract in cold room for 1 hour. Then, the derivative was filtered, washed with buffer, and added to a solution of 30% polietilenoglicol 4000 (PEG 4000), overnight, at 4°C. Next day the derivative was filtered again, washed with buffer, and used for enzymatic assays.

The assay consisted of different mixtures of substrate concentration (Table 1). Each tube contained 5 mL of 0, 0.167%, 0.25% or 0.5% substrate (starch, amylose or amylopectin) in 50mM sodium acetate buffer pH 4.5 with 15 mM of sodium azide to prevent bacterial growth. The test was performed with 0.5 unit of derivative with 3 repetitions of central point. The experiments occurred at 50°C in dry bath with stirring of 500 rpm during 4 hours. The samples were collected after 0.5, 2, 3 and 4 hours and boiled to stop the enzymatic reaction. The amount of reducing sugars was determined by DNS method (Miller, 1959). The data were analyzed with Statistica 12 software.

Table 1. Assays performed 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) |

## RESULTS AND DISCUSSION

The equations constructed from the model coefficients are shown in Table 2. The ANOVA values and the models used are shown in Table 3. In the graphs of response surface (Figure 1) it is possible to visualize the hydrolyze of the substrates in each time of reaction. The following results were obtained: (i) at the time of 0.5 hour the most of amylopectin was hydrolyzed; (ii) at 1 and 2 hours both polysaccharide were hydrolyzed - starch and amylopectin; (iii) at 3 and 4 hours of incubation, amylopectin is again the most hydrolyzed substrate and amylose remains it little hydrolyzed by the immobilized glucoamylase.

To validate the model, it was performed a triplicate with points of 1 and 3 hours at concentrations of 25% starch and 75% amylose (A) and another with 25% starch and 75% amylopectin (B).

The value predicted for 1 hour in A was 0.689 µmol/mL with a standard variation of 0.108 µmol/mL and the observed was 0.700 µmol/mL; to B the predicted valor was 0.625 µmol/mL and the observed valor was 0.512 µmol/mL, this value is within the standard derivation of 0.124 µmol/mL.

To the point of 3 hours in A the predicted value was 0.814 µmol/mL with a standard derivation of 0.123 µmol/mL and the observed valor was 0.883 µmol/mL. In order to B the predicted valor was 0.628 µmol/mL and the observed valor was 0.596 µmol/mL, again within the standard derivation of 0.055 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 to 60 units of glucose (PANDEY *et al.*, 2008). Thus *A. brasiliensis* glucomylase showed more affinity to amylaceous short substrates. This characteristic may have been selected due the abundance of amylopectin in the starch composition. *A. niger* Glucoamylase I has a smaller km with amylopectin (2.1 mg/mL) as substrate than to amylose (2.6 mg/mL), which suggests more affinity to the first substrate (AMIRUL *et al.*, 2005). *Paecilomyces variotti* glucoamylase has a bigger km to amylose (2.5 mg/mL) than amylopectin (2 mg/mL) (MICHELIN *et al.*, 2008). On the other hand, *A. fumigatus* and *A. awamori* glucoamylases have more activity in amylose than amylopectin, with a difference around 10% (SILVA; PERALTA, 1998; YAMASAKI *et al.*, 1977).

Table 2. Equations of mixture desing model

| Time (hours) | Equation |
|---|---|
| 0.5 | y = 0.43*A + 0.5*B +0.7*C + 0.32*AB – 1.59*ABC |
| 1 | y = 0.65*A + 0.47*B +0.71*C + 0.572*AB – 1.31*ABC |
| 2 | y = 0.83*A + 0.43*B +0.89*C + 0.4*AB + 0.50BC – 1.65*ABC |
| 3 | y = 0.66*A + 0.55*B +0.81*C + 0.25*AB |
| 4 | y = 0.84*A + 0.65*B +0.96*C– 3.23*ABC |

Legend: y = expected value µmol/mL; A = proportion of starch; B = proportion of amylose; C = proportion of amylopectin.

Table 3. Mixture design of derivative DEAE-PEG

| Time of hydrolysis (hours) | Model | ANOVA | | | | | | $r^2$ |
|---|---|---|---|---|---|---|---|---|
| | | Source | SS | DF | MS | *F*-test | | |
| | | | | | | *F calculated* | *Critical value* | |
| 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 | | | |
| | | Total | 0.048166 | | | | *α=0.1* | |
| 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 | | | |
| | | Total | 0.042351 | | | | *α=0.05* | |
| 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 | | | |
| | | Total | 0.143217 | | | | *α=0.05* | |
| 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 | | | |
| | | Total | 0.053484 | | | | *α=0.1* | |
| 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 | | | |
| | | Total | 0.095848 | | | | *α=0.1* | |

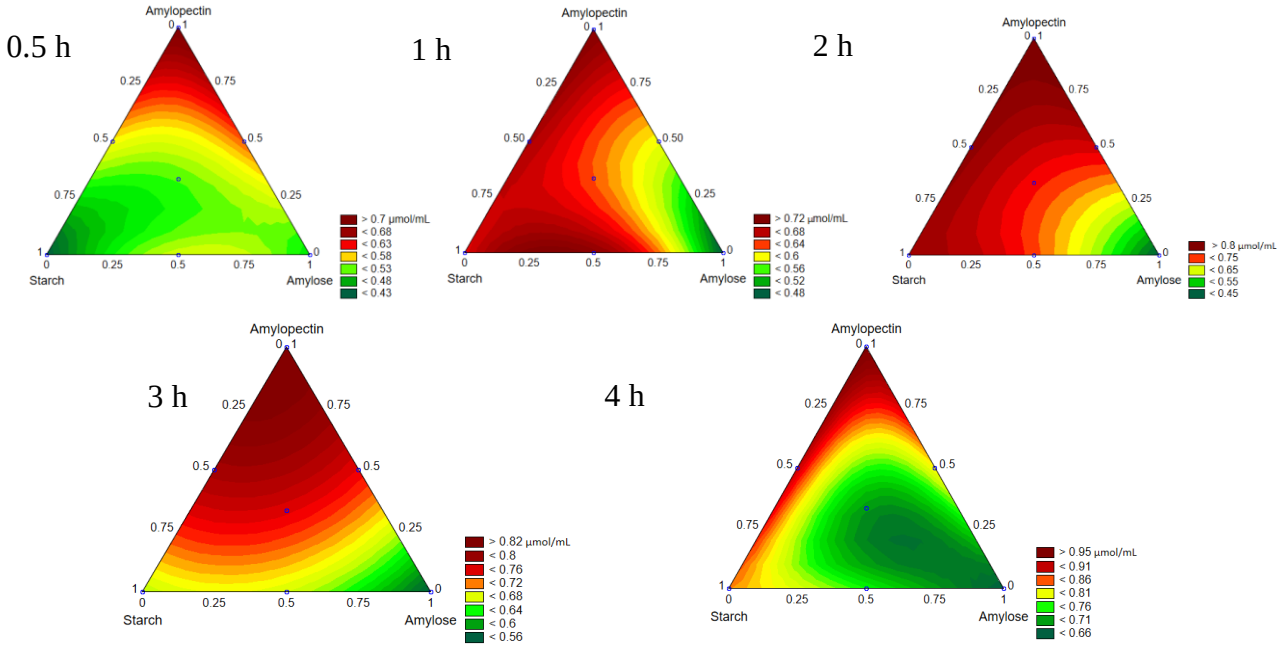


Figure 1. Hydrolysis surface response at several times of reaction

## CONCLUSION

The amylopectin, the most abundant starch chain, was preferentially hydrolysate by the DEAE-PEG immobilized glucoamylase of *Aspergillus brasiliensis*. The heteropolymer starch was hydrolyzed in high levels at the points of 1 and 2 hours and in minor levels at the other tested periods. The mixture planning showed efficiently the action of immobilized *A. brasiliensis* glucomylase in the diverse starch chains through the time.

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