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Artigo

Evaluation of Sweet Potato Cultivars to the Formation of Sugars with Potential for the Production of Ethanol

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Otimização do Processo de Hidrólise de Batata-doce a partir de Biocatalisadores Comerciais Visando Produção de Álcool

Resumo: Atualmente, a produção de etanol a partir de fontes renováveis como o amido e resíduos lignocelulósicos tem sido extensamente investigado. Visando a maior viabilidade da produção de álcool a partir de amiláceas, objetivou-se otimizar a hidrólise de batata-doce utilizando biocatalisadores comerciais. A otimização foi conduzida a partir de 1 g do clone UGA 5 em pH 5,6, 60 °C e tempo de 120 minutos. A evolução do processo enzimático foi avaliada em função de diferentes combinações de concentrações entre as enzimas comerciais α-amilase de *Bacillus* sp. e amiloglucosidase de *A. niger*. Para cada ensaio foram determinadas as concentrações de açúcares redutores e glicose. Visando o ótimo operacional foi calculada uma análise de variância (ANOVA) com delineamento inteiramente casualizado em um planejamento fatorial 2x5x5. Conforme o resultado de interação, apresentado pela ANOVA verificou-se que existe sinergismo significativo entre as enzimas na geração de açúcares redutores. A combinação A3 revelou elevada taxa de conversão (0,96 ± 0,02 g) a qual foi selecionada para os demais clones. Em condições otimizadas as melhores taxas de coversão corresponderam ao clone UGA56 onde obteve-se 0,97 ± 0,03 g de açúcares redutores e 0,89 ± 0,02 g de glicose. No entanto, este clone foi que apresentou menor produtividade (t ha^{-1}), indicando que este parâmetro não é eficiente para discriminar o rendimento visando a produção de álcool.

Palavras-chave: Biomassa; amido; sacarificação; ANOVA.

Abstract

Currently, the etanol production from renewable sources, such as starch or lignocellulosic materials has been extensively investigated. Aiming at creating viability to the production of alcohol from starch raw material, this study was an attempt to optimize the sweet potato hydrolysis employing commercial biocatalysts. The hydrolytic procedure was developed from 1 g to access Unicentro / Guarapuava / Agronomy 5 (UGA 5) at pH 5.6, 60 °C for 120 minutes. The enzymatic process evolution was evaluated as a function of different concentration combinations between the commercial enzymes α-amylase from Bacillus sp. and amyloglucosidase from *Aspergillus niger*. In each test, the concentration of reducing sugars and glycose were determined. Aiming at the optimal operational, analysis of variance (ANOVA) was performed with a completely randomized design in a 2x5x5 full factorial. According to the interaction result presented by the ANOVA, it was seen that there is significant synergy between the enzymes in the generation of reducing sugars. The combination A3 revealed high conversion rate (0.96 ± 0.02 g) which was selected for the other accesses. The best conversion rates corresponded to the access UGA 56, in which 0.97 ± 0.03 g reducing sugars and 0.89 ± 0.02 g glycose were obtained. Despite this lowest productivity (t ha^{-1}), this access indicating that this parameter is not enough to discriminate yield aiming at alcohol production.

Keywords: Biomass; starch; saccharification; reducing sugars; ANOVA.

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Evaluation of Sweet Potato Cultivars to the Formation of Sugars with Potential for the Production of Ethanol

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1. Introduction

Oil (a non-renewable natural resource) is still the world's main energy matrix. The pollution (CO_2 emission responsible for the greenhouse effect intensification) generated by its extraction, transportation, processing and when its byproducts are burnt, is considered a great disadvantage of using this kind of energy. In such context, new sources have been searched, mainly renewable ones.[1]

Regarding environmental issues, the alcohol – produced from sugars, starch or lignocellulosic biomass (second-generation) – has been recognized worldwide as a promising source, since it presents potential to partially substitute gasoline.[2]

Although nowadays most of the ethanol produced in the world is from sugar cane and sweet corn, projections indicate the need for alternative raw materials (starchy, agriculture and forestry residues) to meet this biofuel demand.[3] Sweet potato (*Ipomoea batatas)* appears as an interesting alternative. Some characteristics of using this starchy based material are: short development cycle (4-5 months), rusticity in the field, adaptation to the weather conditions, high yield per ton of raw material and use of the byproduct resulting from its fermentation in animal food, since the sweet potato concentrates around 23 % protein in its composition.[4]

However, one of the main technological constraints hampering the use of sweet potato as a viable and safe substrate to produce alcohol is its hydrolysis process (saccharification), since the highest carbohydrate concentration is in starchy form, therefore, a pre-treatment is necessary to convert the starch into fermentable sugars.[5]

Starch saccharification can occur through acid or enzymatic processes. The former presents as its main advantage the short conversion time, however, it has disadvantages as the need for the reactional medium neutralization, equipment corrosion and the generation of non-fermentative sugars.[6-7] The starch enzymatic saccharification process usually occurs in conversion reactors with the use of enzymes (biocatalysts) which might be of vegetable or microbial origin.[8]

Amylases are classified according to their action mechanism on the starch or regarding the kind of linkage they hydrolyze. Regarding the action mechanisms, there are two categories: endoamylases and exoamylases.[9] The endoamylases hydrolyze starch randomly inside its molecules, forming linear branches of oligosaccharides of different lengths and in this way they break the glycosidic linkages α-1,4 present inside (endo) the amylase or amylopectin chains. While the exoamylases act externally on the substrate linkages from the non-reducing ends, producing low molecular weight products.[10]

The α-amylase hydrolyzes the polysaccharide linkages which have three or more D-glycose units in α-1,4 union. The attack (non-selective) occurs on the several points of the chain simultaneously, forming initially oligosaccharides from five to seven glycose units, presenting the configuration α in the carbon C, in the reducing glycose unit produced.[11] Most of the α-amylases are able to contour the α-1,6 linkages found in the branching points without breaking them.

The amyloglucosidases or glucoamylases break the amylose and amylopectin α-1,4 linkages, forming β-D-glycose as a product. Some amyloglucosidases are able to hydrolyze type α-1,6 glycosidic linkages, however, more slowly than the α-1,4 linkage hydrolysis. Besides the starch amylose and amylopectin fractions, other molecules such as maltase, dextrin and glycogen are hydrolyzed by this biocatalyst, which might also act upon α-1,3 linkages.[11]

It seems important to highlight that the amyloglucosidase plays the main role *in natura* starch hydrolysis and, when associated to α-amylase, reveals great synergic effect.[12] Enzyme mixtures containing α-amylases and amyloglucosidase are more effective regarding starch hydrolysis.[13]

Although the enzymatic hydrolysis has been consolidated as the most effective procedure to convert starch into fermentable sugars, its economic viability is strongly hampered by the cost of the enzymes employed in the biomass hydrolysis process.[14] In addition, the use of these biocatalysts during the process requires high temperatures, which make the process even less viable.

The hydrolysis of starchy raw materials, including sweet potato, to provide fermentable sugars and their bioconversion into lactic acid and bioethanol were recently reported[15-16]. However, there is still the need for establishing a better defined biological approach to convert sweet potato starch, evaluating the enzymes and the most suitable processing conditions to produce fermentable sugars.[4]

Therefore, the objective of this study was to evaluate the sweet potato access hydrolysis best conditions (enzyme concentration) using commercial biocatalysts.

2. Material and Methods

2.1. Raw material selection

Sweet potato Accesses coming from the Germplasm Banks of the State University of Centro Oeste (UNICENTRO) and the Federal University of Tocantins (UFT) (UGA5 and UGA7) were selected for the hydrolysis process due to their productivity (all roots harvested in t ha^{-1}) and their average mass (ratio between the root total mass (g) and the number of roots with high nutrient concentration), see Table 1.

Table 1. Productivity and average mass of different sweet potato accesses

Access	ROOTS	
	PRODUCTIVITY (t ha^{-1})	AVERAGE MASS (g)
UGA5	62.01	360
UGA7	56.70	440
UGA64	50.88	330
UGA56	16.68	140
UGA49	41.50	690

*UGA – Unicentro/Guarapuava/Agronomy

All the roots in the access were washed, processed, dried and ground (Figure 1). A digital oven with continuous air flow was used for drying, at 60 °C constant temperature for 24 hours. Finally, the processed and dried tuberous roots were milled in a Wiley type mill, passed through 1.0 mm (20 mesh).

(a) (b) (c)

Figure 1. (a) *in natura* sweet potato; (b) after grated and dried, (c) after the grinding process

2.2. Optimization of the UGA5 access hydrolysis process using commercial enzymes

The UGA5 access was previously selected for the hydrolysis process optimization, since its productivity levels (t ha^{-1}) were higher than the remaining clones (Table 1). Initially, the enzymatic process evolution was evaluated as a function of different combinations between the commercial enzymes α-amylase from *Bacillus* sp. (Sigma-Aldrich) and amyloglucosidase from *A. niger* (Sigma-Aldrich). The hydrolysis temperature and the medium pH were 60 °C and 5.6, respectively, for a period of 2 hours. According to the enzyme manufacturer's instructions, the pH range for □-amylase and amyloglucosidase activities are 5.0-7.5 and 4.5-6.0, respectively. For this reason, an intermediate pH value of 5.6 was adopted.

In all tests, a 1 g sample was incubated in McIlvaine buffer, with the addition of different volumes of enzymes, so that the total reactional volume 20 mL was reached. Table 2 presents the enzyme combinations for each gram of substrate to be hydrolyzed as well as their respective volumes.

Table 2. Combinations of enzymes used in the UGA5 hydrolysis process

α -Amylase	Ug^{-1} sample	Volume (µL)	Amyloglucosidase	Ug^{-1} sample	Volume (µL)
A	0.5	13	**1**	250	19
B	1.0	26	**2**	500	38
C	1.5	39	**3**	750	57
D	3.0	78	**4**	1500	114
E	6.0	156	**5**	2000	150

All the enzyme combinations were evaluated, that is, A1 to A5. The same sequences were evaluated in relation to B, C, D and E. All the tests were carried out in triplicate.

2.3. Determination of reducing sugars

The reducing sugars dosage was carried out through the DNS (3,5-dinitrosalicylic acid) method, proposed by Miller.[17] The reactional solution had 250 µL sample and 250 µL DNS reagent. After homogenization for 5 minutes in bathwater, the solution was cooled in water and diluted with 2.5 mL distilled water. The spectrophotometer reading was carried out at 540 nm. The absorbance value conversion into mg of reducing sugars was carried out through the glycose calibration curve with a 99,9 % correlation coefficient reliability.

2.4. Glycose determination

The amount of glycose generated in the sweet potato hydrolysis process was determined through the glycose oxidase method, employing the oxidase enzymatic kit (Laborlab). The reaction solution had 20 µL of the hydrolyzed conveniently diluted in 2.0 mL of the work reactive. After incubation of the reaction mixture at 37 °C for 10 minutes, the absorbance reading was carried out in spectrophotometer at 505 nm.

2.5. Data analysis and sampling design

In order to describe the system under study and consequently explore the best experimental conditions aiming at the optimal operational, the analysis of variance (ANOVA)

with a completely randomized design in full factorial 2x5x5 was developed. The differences between averages were ensured by the Fisher LSD ($P < 0.05$) test.

After the establishment of the best conditions for UGA5 clone hydrolysis process, the same procedure was carried out for the remaining clones. In order to determine the clone with the highest potential aiming at ethanol production, a one-way ANOVA (cultivar) was calculated, the dependent variables were reducing sugars and glycose. Next, the Fisher LSD ($P < 0.05$) test was used to ensure the differences between averages.

The residue normality and variance homogeneity were verified through the Shapiro-Wilks and Bartlett tests.[18] The analyses were carried out aided by the program SAS/STAT 9.13.

3. Results and Discussion

The enzymatic hydrolysis of UGA5 clone was evaluated as a function of different combinations between the commercial enzymes α-amylase and amyloglucosidase. The assays were performed at 60 °C and pH 5.6. Although not evaluated in the current study, the relevance of reactional pH in sweet potato hydrolysis seems to be significant, since changes of pH may result in the loss of enzyme activity or dissociation between substrate and catalytic group of enzyme active center, leading to the reduced speed of enzymatic reaction.[20]According to the interaction result, - there is significant synergism between the enzymes under study in the production of reducing sugars, since the F test showed significant differences among averages (($F(4,16) = 6,17$; $p<.001$).

After the seventy-five tests had been completed (combining α-amylases A to E with amyloglucosidase 1 to 5, all in triplicate), it was seen, as shown in Table 3, that the combinations E4 (1,0 ± 0,05 g), D5 (1,0 ± 0,08 g), D3 (1,0 ± 0,08 g), C5 (0,98 ± 1,1 g) and A3 (0,95 ± 0,02 g) were the best generators of reducing sugars, while the worst combinations were C1 (0,58), B1 (0,59) and D2 (0,64). From the results, it is possible to infer that the effect of amyloglucosidase dosage was more critical than that of -amylase. In this case, high amyloglucosidase concentration was required for achieve efficient hydrolysis. This fact can occur since this enzyme hydrolyzes not only α-l,4 glucosidic linkages, but also α-l,6 glucosidic linkages, providing the ability to completely digest starch into glucose. [19]

Due to the enzymes high cost and the small variation of reducing sugars obtained at the end of the process, the combination A3 was selected (0.5 U g^{-1} amyloglucosidase and 750 U g^{-1} α-amylase), because of the smallest volumes of enzymes used, which, consequently, can imply in significant cost reduction in larger scale applications.

The combined use of enzymes allowed high conversion rates (0,96 ± 0,02 g) of the starchy material into fermentable sugars to be obtained. This was due to the α-amylase dextrinizant activity, which breaks the starch into smaller molecules (dextrins, while the amyloglucosidase hydrolyzes the dextrin molecules and starch through the non-reducing ends, producing glycose units. It seems relevant to emphasize that this enzyme is the only one able to hydrolyze simultaneously the starch molecule linkages α-1,4 and α-1,6 in glycose.[21]

Table 3. Average of reducing sugars generated through different combinations of α-amylases and Amyloglucosidase

α-amylases	Amyloglucosidase	Reducing sugars (g)	Group
C	1	0.58	f
B	1	0.59	f
D	2	0.64	f
B	2	0.66	ef
B	4	0.67	ef
D	1	0.67	ef
E	1	0.68	def
A	1	0.70	def
B	3	0.71	def
C	2	0.72	def
A	2	0.78	cde
C	3	0.80	cde
C	4	0.81	bcd
E	2	0.86	abc
E	5	0.86	abc
D	4	0.87	abc
A	4	0.89	abc
E	3	0.90	abc
A	5	0.90	abc
B	5	0.93	abc
A	3	0.95	a
C	5	0.98	a
D	3	1.00	a
D	5	1.00	a
E	4	1.00	a

Averages followed by the same letter did not differ statistically in the Tukey test at 5 % significance

The best enzyme concentrations established by Yingling[22] to promote sweet potato hydrolysis were 214 U g^{-1} for α -amylase and 398 U g^{-1} for amyloglucosidase, obtaining starch conversion rates slightly lower than the ones found in this study, equivalent to 92.5 %. Similar results were found by Shanavas [23] with manioc flour. When using α-amylase and amyloglucosidase in the hydrolysis of sweet potato starch, Souza,[24] found out that the enzyme concentrations corresponded to 12.71 U g^{-1} (Termamyl) and 101.1 U g^{-1} (AMG-300L). This suggests that the differences observed in relation to the

combination of enzymes established in this study are probably due to the different nature of the accesses under analysis.

3.1. UGA7; UGA64; UGA56 and UGA49 access hydrolysis process

Based on best enzymes concentrations for UGA5 access hydrolysis, which corresponded to the combination A3, allied to the reaction time of 120 minutes, the hydrolysis of the remaining clones was carried out.

Figure 2 shows that the highest levels, both of reducing sugars and glycose were produced from the clone UGA56, in which 0.97 ± 0.03 g reducing sugars and 0.89 ± 0.02 g glycose were obtained from 1.0 g starch. However, this clone revealed the lowest productivity indices (t ha^{-1}) (Table 1). Therefore, these clones productivity was not an efficient parameter to discriminate yield aiming at alcohol production. Good levels regarding reducing sugars were also reached with the accesses UGA5 and UGA64, corresponding to 0.95 ± 0.02 g and 0.94 ± 0.02 g, respectively, which were statistically different. However, UGA 49 was the one with the lowest reducing sugar and glycose averages.

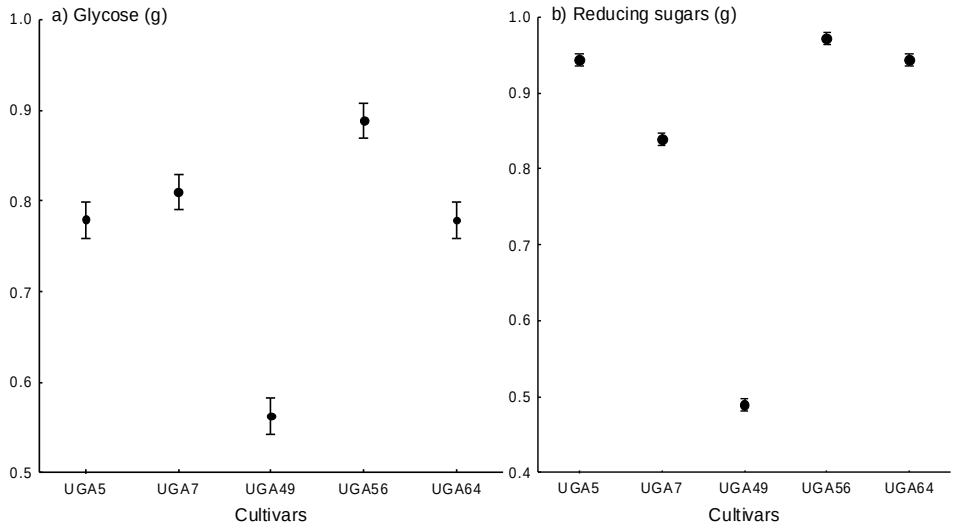

Figure 2. Hydrolysis of UGA7; UGA64; UGA56 and UGA49 access using commercial enzymes

Kansou[25] explained that the amount of sugars generated in the hydrolytic process is directly proportional to the starch percentage present in the sweet potato. These authors also pointed out that the starch percentage depends on the amylacea cultivation time. The longer the cultivation time is, the higher the levels of starch generated by the plant are. Therefore, UGA56 clone is expected to have had initially higher starch content than the remaining accesses.

From the quantification of the glycose present in 1.0 g hydrolyzed sweet potato, it was possible to calculate theoretically the amount of alcohol generated through the Gay-Lussac equation, which can be used as a reference for efficacy calculations regarding the alcohol to be produced.

$$C_6H_{12}O_{6(s)} \rightarrow 2C_2H_5OH_{(l)} + 2CO_{2(g)}$$

Therefore, from 0.89 g glycose, it would be possible to obtain stoichiometrically 0.45 g ethanol or 0.57 mL ethanol. However, despite the working conditions rigor, it is important to emphasize that a decrease in the amount of alcohol obtained at the end of the process was observed, due to possible experimental errors, both in the hydrolytic process and in fermentation, as well as part of the sugars (around 5 %) being destined to the cell growth and the formation of fermentation byproducts, such as glycerol, succinic acid, etc.

The alcohol production estimates for the remaining accesses is shown in Table 4.

Table 4. Estimates of alcohol production by the remaining accesses via Gay-Lussac equation

Access	Glycose (g)	Alcohol (mL)
UGA5	0.78±0.01	0.51±0.01
UGA7	0.80±0.02	0.52±0.02
UGA49	0.59±0.02	0.38±0.02
UGA56	0.89±0.02	0.57±0,.02
UGA64	0.77±0.02	0.50±0.02

Although access UGA5 presented the highest productivity index in the field, other accesses with lower indices were shown suitable for the production of alcohol regarding fermentable sugars. Therefore, it was seen that the productivity index alone is not an indication of excellence regarding alcohol generation from sweet potato.

4. Final Considerations

In the present study, we established a combination of α-amylases and amyloglucosidases that presented great efficacy in converting sweet potato starch into fermentable sugars. Data indicated the effect of amyloglucosidase dosage on the hydrolysis of sweet potato starch was more critical than that of α-amylase.

It seems relevant to highlight that clones with low productivity indices (t ha^{-1}), such as UGA56, revealed excellent results regarding alcohol generation. Finally, it is emphasized that important aspects need to be evaluated, such as pH, temperature and the reaction kinetics, in addition to studies of the optimal fermentation conditions for this kind of raw material.

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