

# Production of glucose and fructose syrups from cassava (*Manihot esculenta* Crantz) starch using enzymes produced by microorganisms isolated from Brazilian Cerrado soil

*Produção de xaropes de glicose e de frutose a partir de amido de mandioca (Manihot esculenta Crantz) usando enzimas produzidas por microorganismos isolados do solo de Cerrado Brasileiro*

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## Abstract

The high demands for sugars and the development of enzymatic technology have increased the production of sweeteners, especially for glucose and fructose syrups. This work describe a technology for glucose and fructose syrups from Brazilian cassava starch using enzymes produced by soil microorganisms isolated from the Brazilian Cerrado soil. Firstly, *Aspergillus niger* and *Streptomyces* sp. were isolated from the soil and used as glucoamylase (GA) and glucose isomerase (GI) producer sources. After characterization, GA and GI exhibited optimum pH 4.5 and 8.0, respectively. GA showed maximum activity at 60 °C and GI at 85 °C. GA and GI retained 65 and 80%, respectively, of initial activity after 180 minutes of incubation at 60 °C. The kinetic parameters  $K_m$  and  $V_{max}$  were 0.476 (mg.mL<sup>-1</sup>) and 8.58 (μmol/minute) for GA and 0.082 (M) and 48.20 (μmol/minute) for GI. The maximum glucose syrups production occurred after 24 hours of reaction with a 98% yield. The production of fructose syrups with 42% (w/v) was reached after 96 hours of reaction.

**Keywords:** glucoamylase; glucose isomerase; glucose-fructose syrups; cassava.

## Resumo

A alta demanda por açúcares e o desenvolvimento da tecnologia enzimática têm aumentado a produção de adoçantes, principalmente xaropes de glicose e frutose. Este trabalho descreve uma tecnologia para a produção de xaropes de glicose e frutose a partir de amido de mandioca usando enzimas produzidas por microorganismos isolados do solo do Cerrado Brasileiro. Primeiramente, *Aspergillus niger* e *Streptomyces* sp. foram isolados a partir do solo e usados como fontes produtoras de glicoamilase (GA) e glicose isomerase (GI). Após caracterização GA e GI exibiram pH ótimo de 4,5 e 8,0, respectivamente. GA apresentou uma atividade máxima a 60 °C enquanto que GI a 80 °C. GA e GI mantiveram 65 e 80%, respectivamente, da atividade inicial, após 180 minutos de incubação a 60 °C. Os parâmetros cinéticos  $K_m$  e  $V_{max}$  foram 0,476 (mg mL<sup>-1</sup>) e 8,58 (μmol min<sup>-1</sup>) para GA e 0,082 (M) e 48,20 (μmol min<sup>-1</sup>) para GI. A máxima produção de xarope de glicose ocorreu após 24 horas de reação com um rendimento de 98%. A produção de xarope de frutose com 42% (m/v) foi alcançada após 96 horas de reação.

**Palavras-chave:** glicoamilase; glicose isomerase; xarope de frutose; mandioca.

## 1 Introduction

Corn starch is the major industrial raw material for glucose and fructose syrup production in the US and in many other parts of the world (CABELLO, 1999). However, good results have been obtained for starch processing in Europe, South America and Asia when alternative starch sources are used (SCHENCK; HEBEDA, 1992). The industrial processing of starch to sugars can be carried out either by acid or enzymatic hydrolysis. However, the use of enzymes is preferred to acid, once it produces high yields of desired products and less formation of undesired products such as toxic compounds (SANJUST et al., 2004). In recent years, biological approaches using enzymes in starch processing have been extensively studied to obtain high-

purity sugars from starch. These processes became economically attractive for biotechnological industries (VORAGEN, 1998; PARK et al., 2005). Furthermore, with the current worldwide increased sugar demand, a new research era in enzymatic technology has concentrated on decreasing the high costs of syrup production (SANJUST et al., 2004; VORAGEN, 1998).

The glucose and fructose syrup are commonly produced by processing corn starch (VORAGEN, 1998). First, corn starch is treated with alpha-amylase to produce oligosaccharides. Next, an enzyme called glucoamylase (GA) breaks down the sugar chains even further to yield the simple sugar glucose

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(PONTOH; LOW, 1995). Finally glucose-isomerase (GI) converts glucose to a mixture of about 26 to 59% of fructose, depending on the enzyme source and/or production conditions (BANDLISH et al., 2002). These syrups are largely used in pharmaceutical applications as well as in food industries (VORAGEN, 1998). Although, such processes are consolidated, a large number of researches are conducting studies in order to decrease the production costs and find a new and economically attractive starch source for this process (SANJUST et al., 2004; VORAGEN, 1998; BANDLISH et al., 2002; SAUER et al., 2000; VAN DER VEEN; VAN DER GOOT; BOOM, 2005).

Cassava (*Manihot esculenta*), also called manioc, is a perennial plant widely grown in many tropical countries, including Brazil. Since cassava roots contain high starch content and low quantity of impurities such as protein and lipid, they are acknowledged as an excellent source of pure suitable starch for a wide range of applications (TONUKARI, 2004; SANGUANPONG et al., 2003).

This work describes a technology for glucose and fructose syrup production using Brazilian cassava starch and enzymes produced by soil microorganisms isolated from the Brazilian Cerrado soil.

## 2 Materials and methods

### 2.1 Organisms, cultures and enzymes production

*Aspergillus niger* was isolated from Cerrado soil samples and was identified according to Gilman (1971). Briefly: 1 g of soil samples taken from the top 20 cm of the soil were dissolved in 10 mL of distilled water and serial dilutions were transferred to Petri plates containing Sabouraud agar (glucose 4%, peptone 1%,  $\text{CuSO}_4$  0.001% and agar 1.5%) and 500  $\text{mg}\cdot\text{L}^{-1}$  of penicillin. After inoculating the soil samples, the culture plates were incubated for 7 days at 30 °C. After growth, the fungal species were further isolated by dilution plate and single spore culture method and then subjected to taxonomic studies.

*Streptomyces* sp. strain was also isolated from the Cerrado soil sample according to Sanchez and Quinto (1975). First, the soil sample was dried at room temperature for about a week. After that, 1 g of this sample was mixed with 10 mL of sterile water and 100  $\mu\text{L}$  of this solution was spread out on Petri plates containing isolation medium (casein 0.04%, starch 0.1%,  $\text{KNO}_3$  0.05%,  $\text{KH}_2\text{PO}_4$  0.02%,  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  0.01%,  $\text{CaCO}_3$  0.01% and agar 1.5%) supplemented with 500  $\text{mg}\cdot\text{L}^{-1}$  of penicillin. The culture plates were incubated for 7 days at 30 °C. After growth, the species were subjected to taxonomic studies also according to Gilman (1971). Finally, the *Streptomyces* sp. strain was maintained in Yamanaka medium (xylose 1%, glucose 1%, peptone 1%, sodium acetate 1%,  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  0.02%,  $\text{MnSO}_4\cdot 6\text{H}_2\text{O}$  0.03%,  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$  0.024% and agar 2%).

For GA production, the spores ( $10^7\cdot\text{mL}^{-1}$ ) were transferred to 500 mL of liquid medium consisting of yeast extract 1%,  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  1%,  $\text{FeSO}_4$  0.01%,  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$  0.01%,  $\text{KH}_2\text{PO}_4$  0.02%,  $(\text{NH}_2)_2\text{SO}_4$  0.125% and starch 1%. After incubation under constant shaking (150 rpm) for 48 hours at 32 °C, the culture

supernatant was collected by filtration through Whatman n° 1 paper and kept at -20 °C until use.

For GI production, 8 discs of 5 mm in diameter from *Streptomyces* sp. culture plates were transferred to 500 mL of Yamanaka liquid medium. After incubation under constant shaking (150 rpm) for 96 hours at 35 °C, the mycelium were harvested by filtration through filter paper Whatman n° 1, washed with 250 mL of NaCl 0.9%, frozen in liquid nitrogen and stored at -80 °C until use.

### 2.2 Enzyme assays

The GA activity was determined as previously described (SILVA; ASQUIERI; FERNANDES, 2005) by monitoring the starch hydrolysis. 60  $\mu\text{L}$  of enzyme solution was mixed with 40  $\mu\text{L}$  of 100 mM sodium acetate buffer pH 4.2 and 100  $\mu\text{L}$  of starch solution 0.5% (w/v). The mixture was incubated at 60 °C for 10 minutes. The amount of glucose released was determined at 510 nm, using a glucose oxidase method (BERGMEYER; BERNT, 1974). One unit of GA corresponds to the amount of enzyme that released 1.0  $\mu\text{mol}$  of glucose per minute under the assay conditions.

As GI is an intracellular enzyme, all the procedures were carried out using the mycelium directly, according to Mirta et al. (1983). First, the enzyme was activated incubating 30 mg of the previously obtained mycelium resuspended in 5.0 mL of distilled water at 50 °C for 10 minutes. Next, the sample was centrifuged at 6,000 rpm for 10 minutes at 4 °C. The supernatant was discarded and the cells were resuspended in 2.0 mL of Tris HCl 50 mM buffer pH 8.0 containing  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  50 mM,  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$  0.25 mM and 250  $\mu\text{L}$  of glucose solution 0.1  $\text{mg}\cdot\text{mL}^{-1}$ . For determining the GI activity, the system was incubated at 70 °C for 30 minutes. The reaction was stopped with the addition of 2.5 mL of  $\text{HClO}_4$  0.5 M. The fructose amount was determined using the cysteine-carbazol method proposed by Dishe and Borenfreud (1951). One unit of GI corresponds to the amount of enzyme that converts 1.0  $\mu\text{mol}$  of glucose per minute under the assay conditions.

### 2.3 Determination of enzyme characteristics

All enzymes characteristics, both GA and GI, were tested under previously described optimal testing conditions. The pH effect on the enzymes activity was determined by varying the pH of the reaction mixtures using 100 mM of sodium citrate (pH 2.0-4.0), 100 mM of sodium acetate (pH 4.2-5.5), 100 mM of sodium phosphate (pH 6.0-8.0) and Tris HCl 50 mM (pH 8.5-9.5) buffers supplemented with  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  50 mM and  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$  0.25 mM when the GI was analyzed. The optimum temperature for the enzyme activities was from 0 to 100 °C for both enzymes and an additional 110 °C point for the GI analysis. The thermal stability of GA enzymes was determined by incubating an enzyme solution for GA and mycelium suspension for GI at 50 and 60 °C and 60 and 70 °C for 180 minutes for GA and GI, respectively. At every 20 minutes, an appropriate aliquot of samples was taken out and used to analyze the enzymes as described above. Kinetic parameters ( $K_M$  and  $V_{max}$ ) were determined at best conditions for

the enzymatic analysis by measuring the initial reaction yields with soluble starch at various concentrations (1-25 mg.mL<sup>-1</sup>) for GA and for glucose solution at various concentrations (1-100 µg.mL<sup>-1</sup>) for GI. The constant values were calculated using the software Microcal-Origin 5.0.

#### 2.4 Production of glucose syrup

For the glucose syrup production, a suspension of 35% (w/v) of previously extracted cassava starch was prepared. The suspension pH was adjusted using 0.2 M pH 6.0 phosphate buffer. This suspension was maintained under heating, increasing the temperature in 1 °C/minute, until the starch was completely solubilized. After, it was cooled down, the solution was incubated in water bath at 50 °C with 10 mL of commercial α-amylase Sigma\* (1 mg.mL<sup>-1</sup>) for 2 hours for total liquefaction. For scarification, this resulting maltodextrin syrup had its pH adjusted to 4.2 with 0.2 M of acetate buffer. Then, 100 mL of previously produced and characterized glucoamylase solution was added and incubated at 60 °C for 48 hours under constant agitation. The glucose production was monitored using the glucose oxidase method as before. After this process, the resulting syrup was purified by ion exchanged chromatography using Amberlite Ira 120 (Cationic resin) and Amberlite Ira 410 (Anionic resin) and then concentrated by evaporation under reduced pressure.

#### 2.5 Production of fructose syrup

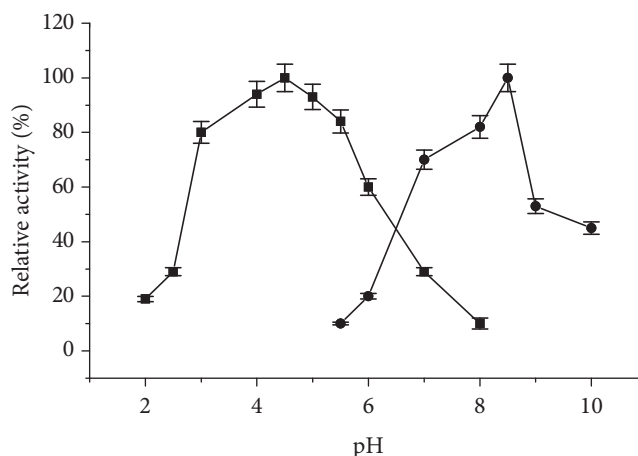
The production of fructose syrup was carried out using previously obtained glucose syrup. The solution had its pH adjusted to pH 8.0 with 0.2 M Tris-HCl buffer containing MgSO<sub>4</sub>.7H<sub>2</sub>O 50 mM and CoCl<sub>2</sub>.6H<sub>2</sub>O 0.25 mM. Differently of glucose syrup production, the isomerization was performed using *Streptomyces* mycelium instead of the enzyme solution. For that, 30 g of *Streptomyces* sp. Mycelium was fastened to dialysis bags to avoid contaminating the solution. The system was incubated under constant agitation at 70 °C for 96 hours. The fructose production was monitored using the cysteine-carbazol method proposed by Dishe and Borenfreud (1951), as before. Next, the resulting fructose syrup was purified and concentrated as glucose syrup.

### 3 Results and discussion

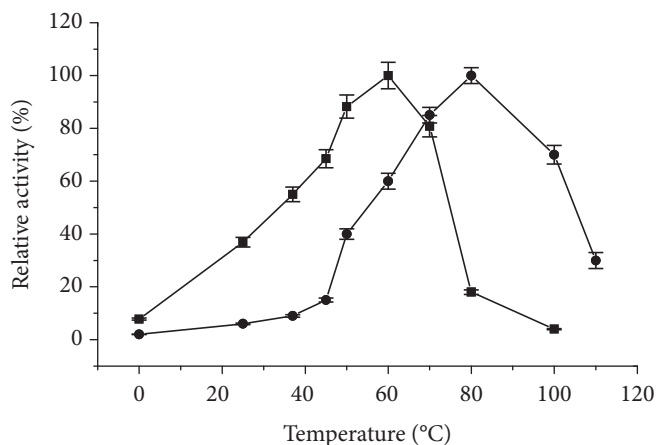
Figure 1 shows the pH effect on the enzyme activity. GA exhibits a good activity, ranging from pH 3.0 to 5.5 and its optimal pH is 4.5. GI showed maximal activity at pH 8.0. The optimum pH range of GI is generally between pH 7.0 and 9.0. However, GI exhibited activity above 70% in the pH range of 6.0 to 8.0 which is desirable for commercial applications of GI (SNEHALATA; BHOSALE; VASANTI, 1996).

Figure 2 shows the optimum temperature profiles for the enzymes. GA presented maximum activity at 60 °C. The industrial applications of glucoamylases require temperatures at around 60-70 °C (FORD, 1999). This finding is in agreement with other reported works for GA from *Aspergillus niger* (SILVA; ASQUIERI; FERNANDES, 2005). The optimum temperature for GI was 85 °C. The optimum temperature for GI ranges

from 60 to 80 °C and can increase in the presence of Co<sup>2+</sup>. The glucose isomerase from *Streptomyces* spp., *Bacillus* spp., *Actinoplanes missouriensis*, and *Thermus thermosulfurogenes* shows high optimum temperatures (70-90 °C) while that from *Lactobacillus* and *Escherichia* spp. shows low temperatures (37-50 °C) (BANDLISH et al., 2002; SNEHALATA; BHOSALE; VASATI, 1996).



**Figure 1.** Effect of pH on the activity of glucoamylase (■) and glucose isomerase (●). GA and mycelium of *Streptomyces* sp. (GI) were incubated with different buffers, 100 mM of sodium citrate (pH 2.0-4.0), 100 mM of sodium acetate (pH 4.2-5.5), 100 mM of sodium phosphate (pH 6.0-8.0) and Tris HCl of 50 mM (pH 8.5-9.5) under standard condition of activity for both enzymes. The buffers were supplemented with MgSO<sub>4</sub>.7H<sub>2</sub>O 50 mM and CoCl<sub>2</sub>.6H<sub>2</sub>O 0.25 mM for the analysis of GI. All tests were performed in triplicate and the results showed average and standard deviation.



**Figure 2.** Temperature effect on the activity of glucoamylase (■) and glucose isomerase (●). GA and mycelium of *Streptomyces* sp. (GI) were incubated at different temperatures under standard conditions and the enzymatic activity was determined. All tests were performed in triplicate and the results were average and standard deviation.

The thermal stability of enzymes was also investigated by incubating the enzymes at 50 and 60 °C and 60 and 70 °C for GA and GI, respectively, for 180 minutes prior to the analysis of the enzyme. After 1 hour of incubation at 60 °C the GA retained approximately 65% of its initial activity, whereas GI retained approximately 80% (data not shown). This finding is interesting because an energy reduction and lower times to cool the reaction mixture represents advantages for industries process applications (SANJUST et al., 2004; SNEHALATA; BHOSALE; VASATI, 1996).

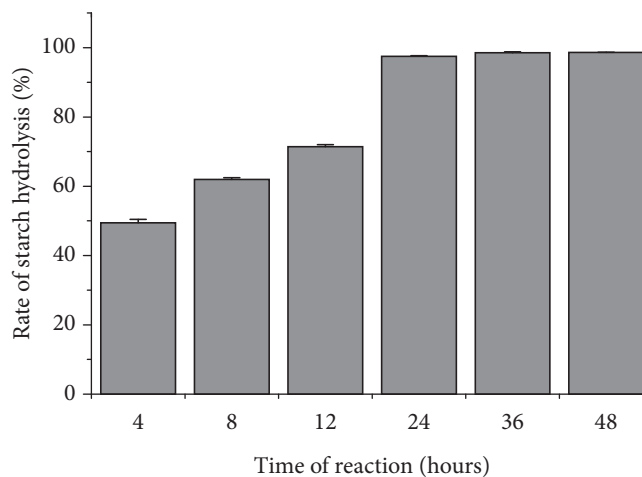
The Michaelis-Menten constant ( $K_M$ ) and the maximum reaction rate ( $V_{max}$ ) are important parameters for the characterization of any enzyme. These parameters are fundamental to choosing the enzymes for large scale industrial applications (SILVA; ASQUIERI; FERNANDES, 2005). After determining the optimum conditions for GA and GI activity, the kinetic parameters of  $k_m$  and  $V_{max}$  for both enzymes were determined. GA showed  $k_M$  and  $V_{max}$  values of  $0.476 \pm 0.021$  (mg.mL<sup>-1</sup>) and  $8.58 \pm 0.047$  (μmol/minute) respectively. The GA  $k_M$  values depend intrinsically on the substrate, if starch or maltose were used. Since starch was used, this is according to the literature (POLAKOVIC; BRYJAK, 2004). The conversion ratios of D-glucose to D-fructose catalyzed by GI from various organisms in soluble or immobilized form are in the range of 26 to 59%. The  $K_m$  value of GI for D-glucose is in the range of 0.086 to 0.920 (M) (CHEN, 1980). GI showed  $0.082 \pm 0.003$  (M) for  $k_M$  value and  $48.20 \pm 0.200$  (μmol/minute) for  $V_{max}$ . The low value found for GI  $k_M$  is explained by the fact that here the mycelium of *Streptomyces sp.* was used directly, instead of the enzyme solution and factors such as the transporter of glucose should be included in the calculation of the final  $k_M$  value, however, this is not the objective of this work. In any case, this  $k_M$  value could be used as a parameter for fructose syrup production without problems.

Figure 3 shows the conversion of cassava starch in glucose. The maximum conversion occurred after 24 hours of reaction and no significant difference was observed after that. Most works report that this conversion rate is reached only after 48 hours of reaction (PARK; PAPINI, 1970; CADMUS; HENSLEY, 1996; AKERBERG et al., 2000; TANRISEVEN; ULUDAG; DOGAN, 2002), indicating that the GA produced by *A. niger* isolated from Cerrado soil has high affinity with cassava starch. This finding is interesting for industrial applications since costs can be reduced. Higher conversion rates are not reported in the literature due to the irregular structure of starch (TANRISEVEN; ULUDAG; DOGAN, 2002).

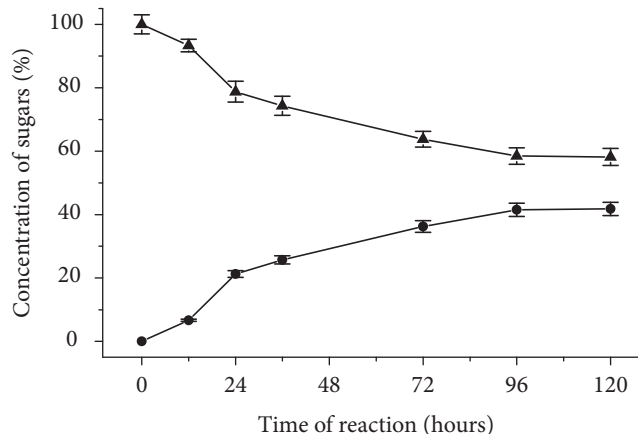
Figure 4 shows the isomerization rate of glucose during the glucose to fructose conversion. The results revealed that the maximum isomerization rate occurred after 96 hours of reaction with approximately 42% of conversion. The reaction of glucose to fructose conversion has a constant equilibrium value of 1, which means that the maximum rate of isomerization reaction that can be obtained is of about 50%. Syrups with high fructose content above 42% produced from corn, such as HFC-55 or HFC-90, can be reached either by chromatography concentration or by evaporation under reduced pressure (BLANCHARD; GEIGER, 1984). Vissuri and Klibavov (1987)

reported on the production of fructose syrup with 55% of fructose, working with the fructose solubility in ethanol and then dislocating the reaction equilibrium, resulting in increased fructose content.

The results presented in this paper indicate that the GA and GI produced by *A. niger* and *Streptomyces sp.*, respectively, are attractive candidates for industrial applications. Further experiments of co-immobilization using both enzymes or both mycelia may provide strategies to improve the characteristics of these enzymes and enable their use in large-scale glucose and fructose syrup production from cassava starch.



**Figure 3.** Time course of glucose syrup production from cassava starch. Maltodextrin solution previously obtained from cassava starch using commercial  $\alpha$ -amylase was incubated with GA from *A. niger* for 48 hours. The formation of glucose was monitored using the glucose oxidase method at different times. All tests were performed in triplicate and the results were average and standard deviation.



**Figure 4.** Time course of fructose syrup from glucose syrup of cassava starch. The glucose syrup resulted from early step was incubated with mycelium of *Streptomyces sp.* for 120 hours. The production of fructose was monitoring using the cysteine-carbazol method at different times. All tests were performed in triplicate and the results were average and standard deviation. (▲) Glucose consumption and (●) Fructose formation.

## 4 Conclusions

We conclude that the enzymes, glucoamylase, produced by *A. niger* and, glucose isomerase, produced by *Streptomyces sp.*, both isolated from Brazilian Cerrado soil showed biochemical characteristics, such as optimum pH and temperature and as well good thermal stability, that can be used in industrial process as starch processing. Furthermore, the technology used for glucose and fructose syrup production using Brazilian cassava starch and enzymes produced by soil microorganisms was successful and can be up scaled for industrial purpose.

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