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Enzymatic Hydrolysis of Cassava Starch Using Barley Malt Amylases

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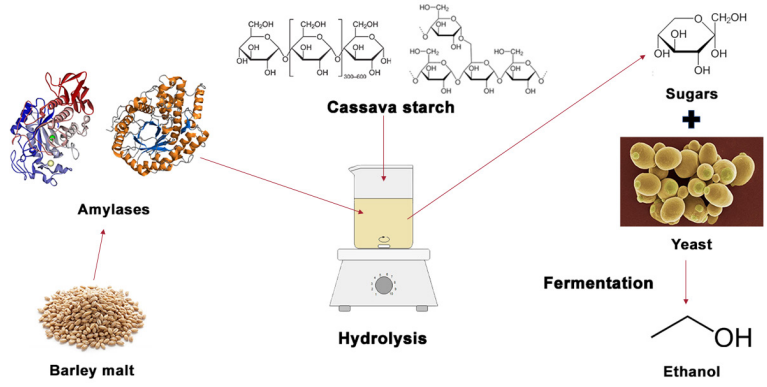
Barley malt was used as a source of amylases for the hydrolysis of cassava starch to produce reducing sugars for the alcoholic fermentation. Two routes of hydrolysis were evaluated in this work. One using milled barley malt and the other using the enzyme extract of this grain. The first one evaluated three concentrations of milled barley malt: 5, 10 and 15% (w/w) and there was no significant difference between the values of reducing sugars obtained as a function of the three concentrations. Three concentrations were also tested for barley malt extract: 0.5, 1.0 and 1.5 mL of extract. The higher content of reducing sugars was found for the 0.5 mL concentration of extract. The barley malt extract was more efficient in the enzymatic hydrolysis of cassava starch due to a better contact of the enzymes with substrate. The alcoholic fermentation of the wort obtained with 0.5 mL yielded an ethanol content of 7.74 ± 3.19 g/L with an efficiency of 88.6%.

Graphical abstract



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1. Introduction

Bioethanol is a biofuel that is becoming increasingly important within the energy matrix due to the need to exchange fossil fuels for renewable energy sources, supplying energy demand, reducing the emission of pollutants into the atmosphere and enabling a more sustainable future. Obtained through the alcoholic fermentation of sugars made by microorganisms as *Saccharomyces cerevisiae* and *Escherichia coli*, it is possible to produce it in several regions worldwide from different raw materials [1–4].

The raw materials used can be divided into two groups: those that have a high concentration of fermentable sugars and those that are composed mostly of non-fermentable sugars. Within the latter group, cassava (*Manihot esculenta*) is a crop of interest for the production of bioethanol due to its low cost and availability, easily produced in tropical and sub-tropical regions [5, 6]. The main carbohydrate present in the latter is starch, a polysaccharide composed of two polymers: amylose and amylopectin. These sugars are not directly

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fermentable by the microorganisms used in alcoholic fermentation, and therefore it is necessary to be hydrolyzed in order to produce smaller and fermentable sugars. Hydrolysis can be carried out by acid or enzymatic catalysis. Among these, enzymatic hydrolysis is more advantageous due to the selectivity of the catalyst; the purity of the products generated and avoids problems such as acid neutralization and corrosion of metallic materials used in the process [7, 8].

The usual process of enzymatic hydrolysis reported in literature uses α-amylase for the liquefaction and amyloglucosidase for the saccharification, both enzymes used are commercial or from fungal origin. [8–10]. However, these enzymes have a high price and obtaining them by fungal cultures requires a series of precautions that make the hydrolysis process costly [7, 9]. Therefore, a cheaper source of enzymes could reduce the cost and optimize the processes of starch hydrolysis and fermentation of the sugars generated, expanding alcohol production.

Barley malt is a cereal modified through the malting process, being one of the main ingredients used in brewing. The malting process modifies the physical structure of the barley grain and promotes the synthesis and activation of several enzymes such as amylases, xylanases and proteases. There is a wide range of amylases present in malt, but the main ones found in this cereal are α-amylase, β-amylase, α-glucosidase and limit dextrinase. These enzymes are able to act on the liquefaction and saccharification of starch to produce fermentable sugars, which makes it possible to replace conventional enzymes (α-amylase and amyloglucosidase) with barley malt during the enzymatic hydrolysis of starch [11, 12].

There is a wide range of studies in the literature related to barley malt. However, the vast majority are focused on its application in brewing's mashing, where the malt starch is hydrolyzed by the malt's own amylases. However, there are few reports of the use of malt as a source of amylases for the hydrolysis of starches from other sources to obtain bioethanol. Farias and coworkers [13] evaluated the hydrolysis of maize starch with malted maize enzymes and fermented this hydrolysate to obtain 20 to 60 g/L of ethanol. Santana [14] evaluated the hydrolysis of cassava starch using barley, corn, wheat and rye malts obtaining reducing sugar levels in the range of 80 to 100 g/L and ethanol levels close to 45 g/L. Park and coworkers [15] evaluated the use of organic malt extract to hydrolyze starches from different sources, obtaining levels of reducing sugars of up to 150 g/L. However, the latter evaluated this process for use in organic processed food. Therefore, further investigations are needed to evaluate the use of barley malt as a source of amylases to hydrolyze starch and obtain bioethanol.

In this work, the milled barley malt and the enzymatic extract of the malt were evaluated as a source of amylolytic enzymes in the hydrolysis of cassava starch in order to generate fermentable sugars for the production of bioethanol.

2. Results and Discussion

The Figure 1 shows the images obtained from scanning electron microscopy of cassava starch. The large cassava granules are spherical in shape, whereas small granules are irregular and polygonal in shape. Furthermore, the surface is smooth with no cracks or holes. Wang et al. [16] suggests that the presence of holes or cracks in the surface may increase the granule's susceptibility to enzymatic attack. Therefore, it is possible to suggest that cassava starch has its susceptibility to enzymatic attack reduced due to the absence of these holes and cracks on its surface. α-Amylase plays an important role in the initial stage of hydrolysis. This enzyme is the only barley malt present capable of cleavage any α (1-4) linkage in the amylaceous chain. As reported by Muralikrishna [17], at the beginning of hydrolysis, α-amylase hydrolyzes portions of the starch chain present on the surface of the granule creating holes and tunnels. These holes and tunnels allow other enzymes to enter the starch granule and consequently the hydrolysis of more internal glycosidic bonds.

Figure 2 shows the diameter distribution of the cassava starch granules. Within the analyzed area, the distribution found was normal, the average diameter was 13.70 ± 2.594 μm, in a range of 3 to 22 μm, range within the reported diameter size (3 - 32 μm) [18].

The enzymatic hydrolysis assays of cassava starch and the values of reducing sugars (RS) measured by the DNS method [19] are shown in Table 1. The analysis of variance (ANOVA) showed that there is no statistically significant difference between the three values of reducing sugars found for the different concentrations of malt used. The variation in the concentration of barley malt was not significant in the reducing sugars obtained, which is confirmed by the Table 1 and Figure 3.

Table 1. Amount of reducing sugars obtained for each malt concentration. The mean values with the same letters are not statistically different by the Tukey test at a 5% significance ($p < 0.05$).

Malt concentration (% w/w)	Reducing sugars (g/L)
5	2.52 ± 0.261 [a]
10	3.09 ± 0.891 [a]
15	3.01 ± 1.22 [a]

The Table 2 shows the values of reducing sugars found for the enzymatic hydrolysis using barley malt extract. The ANOVA showed that the means can be divided in two groups (a and b). The mean values with the same letters do not differ statistically by the Tukey test at a 5% significance ($p < 0.05$). Thus, it is possible to see that the concentrations of 0.5 mL and 1.0 mL of malt extract yielded a higher level of reducing sugars. The Figure 4 confirms that the lower concentration of enzymatic extract yields higher levels of RS.

Table 2. Amount of reducing sugars obtained for each malt extract concentration.

Malt extract concentration (mL)	Reducing sugars (g/L)
0.5	8.56 ± 2.01 [a]
1.0	4.50 ± 0.721 [a,b]
1.5	4.35 ± 0.318 [b]

The barley malt extract was more effective than the milled barley malt in hydrolysis of cassava starch. The Figure 5 can confirm this hypothesis, where is possible to see that the amount of RS obtained was higher in the assay using 0.5 mL of malt extract. A probable reason for the better performance of the malt enzymatic extract is the higher contact between the amylases and the substrate, and possibly the distribution of the enzymes in the extract is more homogeneous than in the milled malt. Ruiz [6] and collaborators used commercial enzymes α-amylase from *Aspergillus kawachi* and glucoamylase from *Aspergillus niger* to hydrolyze cassava starch and the reducing sugar content reported was lower than those shown in this work.

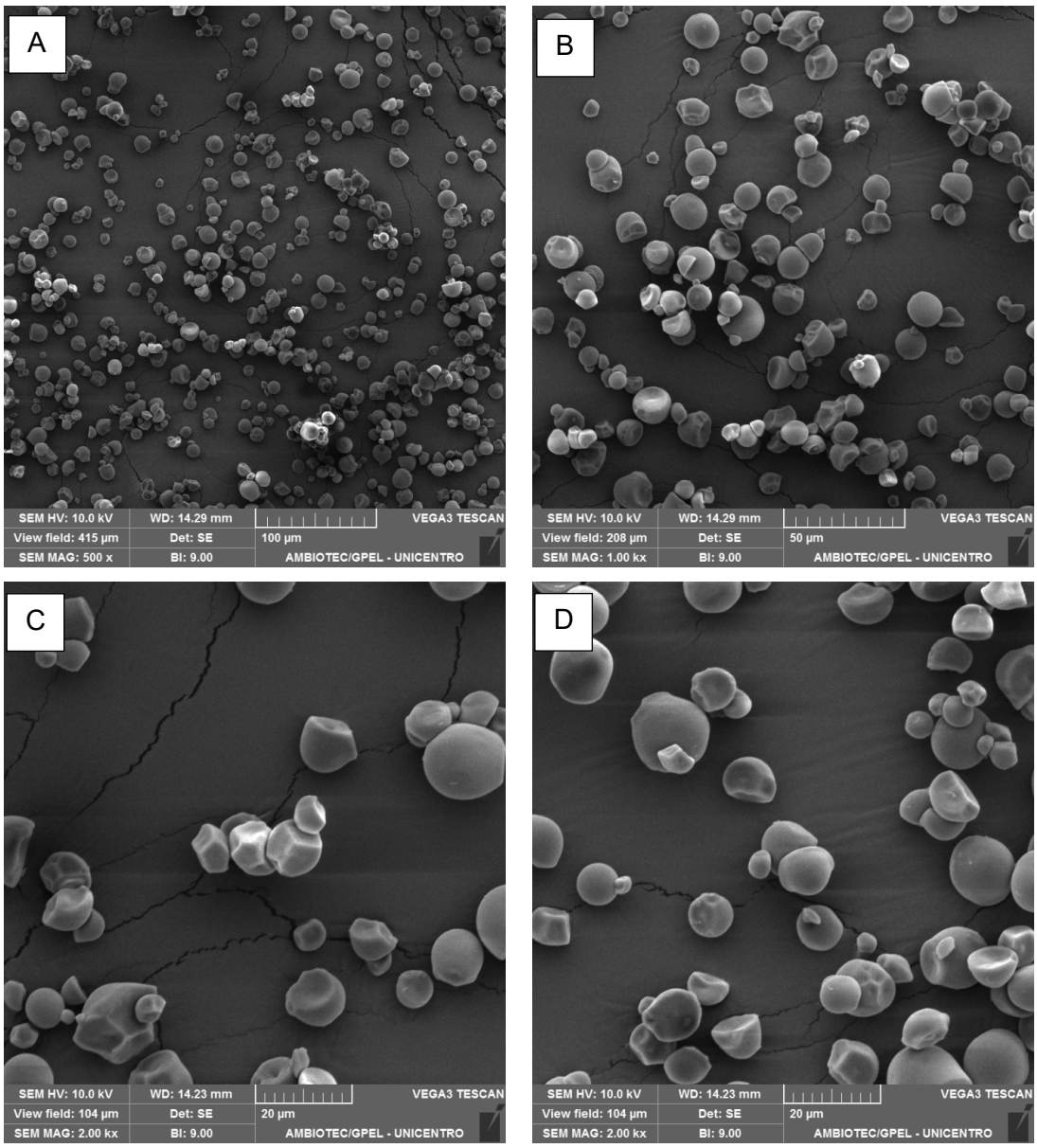

Fig. 1. SEM images for the cassava starch with (A) 500, (B) 1000 and (C,D) 2000 x magnification.

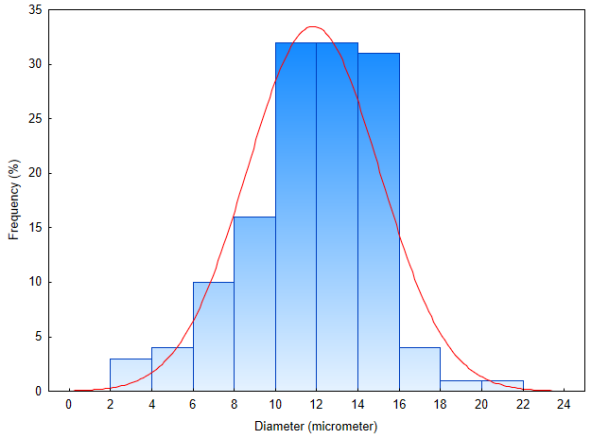

Fig. 2. Histogram of the diameter distribution of the cassava starch granules.

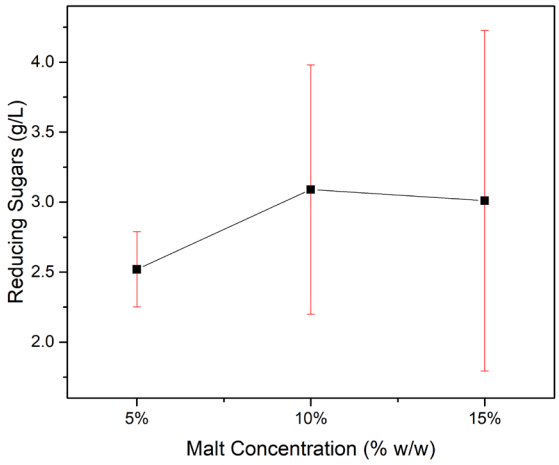

Fig. 3. Mean values of reducing sugars as a function of the barley malt concentration.

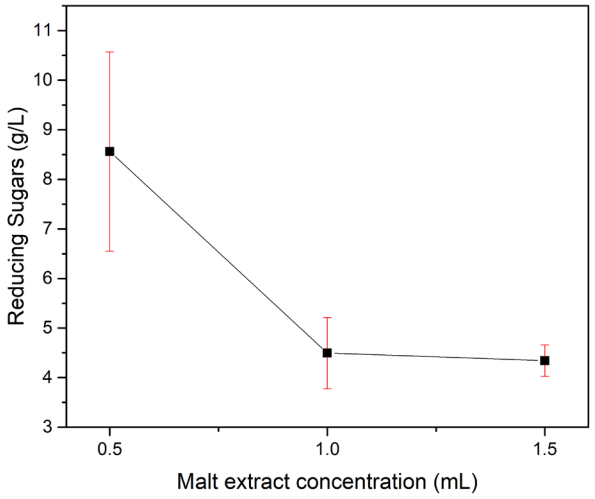

Fig. 4. RS yielded as a function of the enzymatic extract concentration.

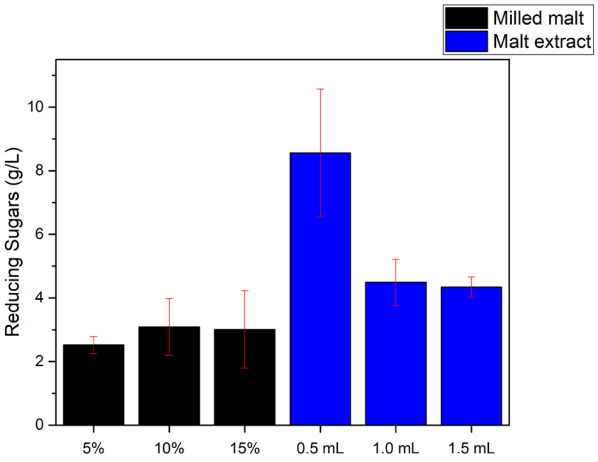

Fig. 5. Reducing Sugars yielded in the test with milled malt compared to the reducing sugars obtained with malt extract.

The hydrolysis temperature and pH used is within the optimum range of the enzyme β-amylase, which cleaves α (1-4) glycosidic bond of starch at the non-reducing ends producing maltose and some dextrins. However, there is also a catalytic action from α-amylase, but it was less effective because it was outside the temperature range for the optimal activity of this enzyme [20]. Sammartino [21] states that the two enzymes act complementarily during the hydrolysis of starch, since α-amylase is able to attack α (1-4) glycosidic bond of starch, this enzyme is essential to provide a greater number of non-reducing chains making the activity of β-amylase more effective. A balance between the catalytic activity of α-amylase and β-amylase is essential to increase the generation of reducing sugars during the hydrolysis of starch using barley malt as a source of amylases. Cleavages in α (1-6) bond of starch are unlikely to have occurred. The pH and the temperature used are outside the range of the activity of the debranching enzymes and limit dextrinases, and in addition, the concentration of these enzymes is small due to a significant deactivation that occurs during the kilning of the malt. Thus is possible to infer that the main sugars yielded in the hydrolysis were glucose, maltose and linear and branched dextrins [20, 21].

The Figure 6 shows the content of reducing sugars generated as a function of the hydrolysis time for the different extract concentrations used. The concentration of 0.5 mL of extract showed a greater amount of reducing sugars at the end of the reaction. However, at concentrations of 1.0 and 1.5 mL, sugar production was much faster and decreased over time. According to the Michaelis-Menten equation for the rate of an enzymatic reaction, the higher the concentration of substrate and enzyme, the higher the reaction rate, and therefore more product will be formed. However, the curves in Figure 6 show a behavior contrary to the Michaelis-Menten kinetics. According to the initial rates (Table 3), the tests carried out with the highest amounts of enzyme extract (1.0 and 0.5 mL) promoted a faster product formation. Although, after 20 minutes of reaction there is a significant drop in the concentration of reducing sugars. In the test performed with 0.5 ml of malt extract, there was a less significant decrease in the concentration of sugars.

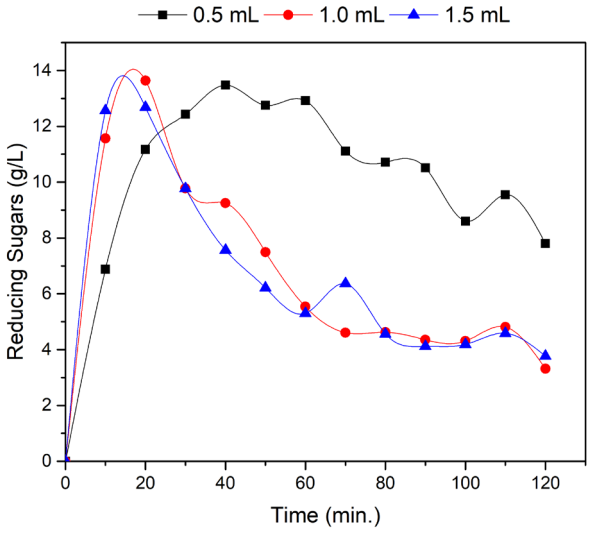

Fig. 6. Reducing sugars produced as function of the hydrolysis time for each malt extract concentration.

Table 3. Initial rates for the malt extract concentrations.

Malt extract concentration (mL)	Initial rate ($g.L^{-1}.s^{-1}$)
0.5	0.0115
1.0	0.0193
1.5	0.0209

It is suggested that this decrease in the concentration of sugars occurred due to a transglycosylation reaction between the substrate and the sugars generated. Retaining glycosyl hydrolases, as α-amylase, have the ability to catalyze hydrolysis and transglycosylation reactions. The reaction catalyzed by these enzymes consists of a two-step mechanism for both hydrolysis and transglycosylation. First, the active site containing a carboxyl and carboxylate group involves the substrate (glycosidic bond of starch). The oxygen in the carboxylate attacks the anomeric carbon at the same time as the oxygen in the glycosidic bond deprotons the carboxyl and the glycosidic bond is broken. In the second stage of the mechanism, it is defined whether hydrolysis or transglycosylation will occur. If water attacks carbon linked to the enzyme, hydrolysis occurs. However, when sugars such as glucose and maltose are present in the reaction medium, the hydroxyls of these sugars are able to attack the intermediate as well and an oligosaccharide is obtained [17, 22, 23]. Both mechanisms are shown in Figure 7.

The literature reports that both retaining hydrolases, as α-

amylase, and inverting hydrolases, such β-amylase are able to catalyze hydrolysis and transglycosylation reactions. Mótyán [22] and collaborators evaluated the transglycosylation activity catalyzed by barley α-amylase with oligosaccharides. The authors found that both natural α-amylase and those with a mutant active site showed transferase activity. Vester-Christensen [24] and coworkers reported transglycosylation catalyzed by limit dextrinase in reactions with oligosaccharides. Fazekas and collaborators [25] observed a phenomenon similar to that shown in Figure 6. In hydrolysis reactions of oligosaccharides containing chromophor groups, the authors observed that the concentration of products with lower degrees of polymerization decreased after the beginning of hydrolysis while the concentration of some oligosaccharides increased, which indicates that transglycosylation occurred. Mangas-Sanchéz and coworkers [26] evaluated the catalytic power of transglycosylation of different glucosidases on maltose and cellobiose. The results showed that in the beginning there is a decrease in the concentration of maltose and an increase in the concentration of glucose and panose. These results show that there was a competition between hydrolysis and transglycosylation. The increase in glucose concentration indicates the occurrence of hydrolysis. The formation of panose, an oligosaccharide, shows that there has been transglycosylation.

Literature reports of transferase activity of different amylases make plausible the hypothesis that the decrease in the concentration of reducing sugars during the reaction (Figure 6) occurred due to a transglycosylation reaction between the products generated and the substrate. The decrease in the content of reducing sugars it is more pronounced for the tests with 1.0 and 1.5 mL of extract, because the rapid formation of sugars allows them to attack the substrate bound to the enzyme, instead of water, obtaining an oligosaccharide. For the test performed with 0.5 mL of extract, the initial rate shows that the formation of sugars is slower, and therefore there is a predominance in the hydrolysis reaction over transglycosylation. However, it is possible to infer that the reactions of hydrolysis and transglycosylation compete with each other during the 120 minutes of the test, since the three curves in Figure 6 show throughout the reaction consumption and product formation.

Therefore, it is suggested that hydrolysis performed with 0.5 mL of enzyme extract was more effective in the generation of sugars due to hydrolysis being predominant over transglycosylation. Due to the greater efficiency in the production of sugars, the hydrolysis condition with 0.5 mL of enzymatic extract was subjected to alcoholic fermentation and the results obtained are shown in Table 4.

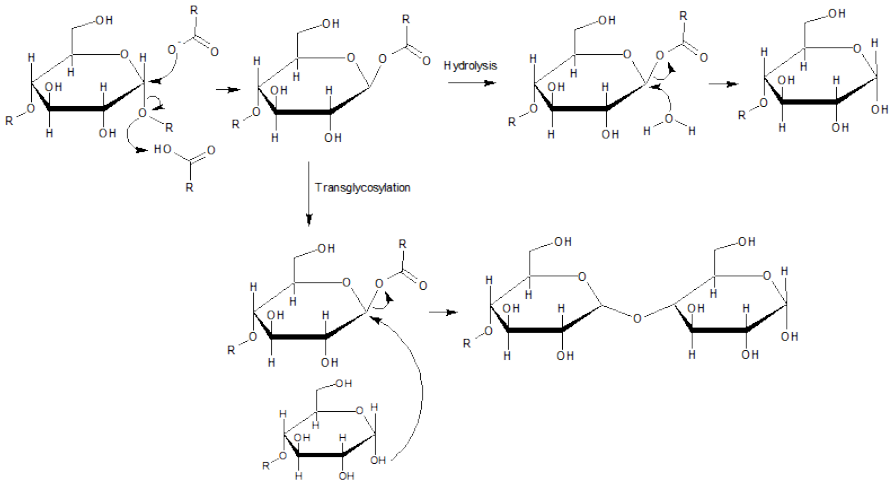

Fig. 7. Hydrolysis and transglycosylation mechanism catalyzed by a retaining enzyme.

The high fermentation efficiency found shows that approximately 88% of the sugars generated were transformed into ethanol. Efficiencies close to 100% are difficult to achieve, since part of the sugars can be consumed in the aerobic respiration, and, in addition, these molecules have other functions within the yeast metabolism, and thus are consumed in different metabolic routes in the cell [14, 27].

Table 4. Ethanol content and alcoholic fermentation efficiency

Malt extract (mL)	Reducing Sugars (g/L)	Ethanol Content (g/L)	Y_{real} (g/g)	Efficiency (%)
0.5	8.56 ± 2.01	7.74 ± 3.19	0.452	88.6%

The fermentation efficiency is close to that reported by Santana [14] who used different types of malt for the hydrolysis of cassava starch. Despite the high efficiency, the ethanol content found is lower than the values found in other studies [6, 14]. This is due to the low content of reducing sugars in the wort. The inhibition of amylolytic enzymes by the hydrolysis products did not allow for a higher conversion efficiency of starch to sugars, which results in low ethanol production.

3. Material and Methods

In this work, Pilsen Barley Malt, acquired from the Cooperativa Agrária Agroindustrial located in the city of Guarapuava in the state of Paraná, was used as source of amylases. The cassava starch was purchased from local stores.

3.1 Malt milling

The Barley Malt was previously milled in order to increase the surface area and enzymes exposure for the hydrolysis reactions. The milling was carried out with a roller grinder suitable for malts.

3.2 Preparation of barley malt extract

The amylases were extracted according to the methodology proposed by Farias [13] with modifications using 15 g of milled malt in contact with 15 mL of 0.1 M TRIS-HCl buffer solution. The mixture was left to stand for 1 hour and then was centrifuged at 3500 for 10 minutes. The supernatant was subjected to simple filtration, frozen and then stored until use in hydrolysis reactions.

3.3 Scanning electron microscopy (SEM)

The cassava starch morphology was evaluated with a Scanning Electron Microscope Tescan Vega 3 with SE detector and tungsten filament at 20 kV and working distance from 10 to 15 mm.

3.4 Enzymatic hydrolysis of manioc starch

The hydrolysis of cassava starch was carried out using suspensions of starch in water with a concentration of 10% (w/V). The suspension was buffered to pH 5.3 with a Sorensen buffer to promote the optimal conditions for the amylases activities [21]. Then, the suspension was heated to 70 °C to promote gelatinization of cassava starch. The system was cooled to 65 °C, temperature where there is greater activity of β-amylase and α-amylase [21], and then barley malt was added, starting the hydrolysis, which lasted 2 hours. Two sources of amylase were used in this work: barley malt and the extract of this grain. Three concentrations of each amylase source were tested in the hydrolysis reaction, and they are shown in Table 1 and Table 2.

3.5 Kinetic evaluation

To elucidate the results obtained, a kinetic evaluation was performed for the reactions made with the enzymatic extract of malt. For this, the hydrolysis was carried out in the same way as described in section 3.3. During the reaction, 1.0 mL aliquots were removed every 20 minutes and 3 drops of 0.5 mol/L solution of NaOH were added to stop the reaction and then the reducing sugars was quantified. Initial rates were measured using the same method. However, 1 mL aliquots were collected every 60 s for 600 s totaling 10 points for determining the initial rates.

3.6 Reducing sugars quantification

The reducing sugars produced after the hydrolysis were quantified by the Miller method with DNS reagent. [19] An aliquot of 1 mL of the hydrolysate was added to 1 mL of the DNS reagent, stirred and heated in a water bath at approximately 100 °C. The quantification was made using a glucose calibration curve, which the concentration varied from 0.1 to 0.5 g/L, and the absorbance was measured at the wavelength of 540 nm.

3.7 Alcoholic fermentation

The apparatus used in this step was sterilized with 70% ethanol (w/w) and dried at 100 °C to complete elimination of unwanted microorganisms. The volume of work was expanded in the proportion of 1: 5, therefore suspensions of 500 mL were used. The fermentation was carried out using the yeast *Saccharomyces cerevisiae* from the commercial yeast Nottingham of the Lallemand® brand at a concentration of 5 g/L. The yeasts were previously activated in 20 mL of water in the temperature range of 35 - 40 °C for 20 minutes. The hydrolysis was carried out according to the same methodology previously described in section 3.3 using 10% (m/V) of cassava starch and 0.5 mL of malt extract. Ammonium sulfate, magnesium sulfate heptahydrated and dibasic potassium phosphate at concentrations of 5; 0.2 and 0.1 g / L respectively was added to the obtained wort. The same was transferred to a fermenter one where the alcoholic fermentation was carried out for 7 days at 20 °C.

The ethanol content was determined from the NBR 13920 standard as described by Cantos-Lopes et al. [10] The fermentation efficiency was calculated using the real yield and the theoretical yield of the reaction. The calculation was done using the two equations below:

$$Y_{theoretical} = \frac{2.46}{180} \qquad (1)$$

$$\varepsilon = \frac{Y_{real}}{Y_{theoretical}} 100 \qquad (2)$$

Being Y_{real} the ratio between the mass of ethanol obtained from the fermentation by the amount of reducing sugars produced at the hydrolysis, and ε the alcoholic fermentation efficiency.

4. Conclusions

Both milled barley malt and malt extract were able to promote the enzymatic hydrolysis of cassava starch. Nevertheless, the lowest concentration of malt extract yielded a higher value of reducing sugars. A probable reason for the better performance of the enzyme extract in the hydrolysis reaction is the more homogeneous distribution of amylases and the greater contact of these enzymes with the substrate. It is possible that transglycosylation reactions competed with hydrolysis, which resulted in a low yield in reducing sugars. The alcoholic fermentation generated a low ethanol content; however, its efficiency was high, which shows a punctuality for the production of bioethanol from this source of sugars.

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Author Contributions

Conceptualization, Felipe Staciaki da Luz; Everson do Prado Banczek; Methodology, Felipe Staciaki da Luz; Renata Nascimento Caetano; Adrielle Ferreira Bueno Investigation, Felipe Staciaki da Luz; Everson do Prado Banczek; Resources, Everson do Prado Banczek; Paulo Rogério Pinto Rodrigues; Data curation, Felipe Staciaki da Luz; Adrielle Ferreira Bueno; Renata Nascimento Caetano; Everson do Prado Banczek; Writing—original draft preparation, Felipe Staciaki da Luz; Writing—review and editing, Felipe Staciaki da Luz; Everson do Prado Banczek; Paulo Rogério Pinto Rodrigues; Supervision, Everson do Prado Banczek; Project administration, Everson do Prado Banczek; All authors have read and agreed to the published version of the manuscript.

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