



Obtaining and evaluation of enzymatic extract from *Aspergillus spp.* by saccharification of sour cassava starch liqued

Obtención y evaluación de un extracto enzimático de *Aspergillus spp.* para la sacarificación de almidón de yuca licuado

Recolha e avaliação de um extracto de enzima de *Aspergillus spp.* para a sacarificação da mandioca amido liquefeito

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Abstract

The saccharification enzymes are more expensive than liquefaction enzymes for that reason are sought strategies that allow the supply of these enzymes at low cost. The objectives of this study were to evolve saccharolytic enzymatic extracts from native strains, to select an extract and to determine the best variables for the production of glucose syrups from liquefied bitter cassava starch. Thirteen mushrooms isolated from sour cassava (*Manihot* sp.) were evaluated for their saccharolytic activity, hydrolysis of maltose, glucose production and biomass formation in submerged fermentation. *Aspergillus* A1 was selected because it had the highest volumetric activity (0.09UI.L⁻¹). During solid-state fermentation, the presence of proteins was corroborated by electrophoresis SDS-PAGE. Through various experiments, the best experimental condition were pH 4.0, agitation 0r.p.m and temperature 55.0°C; the effect of cofactors Cu²⁺, K⁺, Mg²⁺ and Na⁺ was evaluated, Mg²⁺ increases the activity in 1.32UImg⁻¹ (32.4%). The thermal stability at 55.0°C is 120 minutes. Finally, the saccharolytic capacity of the enzymatic extract was confirmed using liquefied cassava starch.

Keywords: Glucoamylase, *Aspergillus*, Saccharification, Syrup Glucose.

Resumen

Comercialmente las enzimas de sacarificación son más costosas que las de licuefacción, por tal razón se buscan estrategias que permitan el suministro de estas enzimas a bajo costo. Los objetivos de este estudio fueron evaluar extractos enzimáticos sacarolíticos a partir de cepas nativas, seleccionar un extracto y determinar las mejores variables para la producción de jarabes de glucosa. Para ello, en trece hongos filamentosos aislados de yuca amarga *Manihot* sp. fueron evaluadas la actividad sacarolítica, la hidrólisis de maltosa, la producción de glucosa y la formación de biomasa bajo condiciones de fermentación sumergida. El aislamiento identificado como *Aspergillus* A1 fue seleccionado por presentar la más alta actividad volumétrica (0,09UI.L⁻¹). Durante la fermentación en estado sólido se corroboró la presencia de proteínas mediante el método de electroforesis SDS-PAGE y se evidenció una banda de mayor intensidad con peso molecular entre 60 y 70kDa. Para el extracto enzimático de *Aspergillus* A1 se determinó que las mejores condiciones experimentales de sacarificación, con el uso de maltosa como sustrato, fueron pH 4,0, temperatura 55,0°C, y sin agitación. Igualmente en la evaluación del efecto de los cofactores Cu²⁺, K⁺, Mg²⁺ y Na⁺ en concentraciones de 1 mM, se observó que todos incrementan la

actividad enzimática especialmente el Mg^{2+} , el cual la aumenta en $1,32\text{ UI.mg}^{-1}$ (32,4%). La estabilidad térmica de la proteína a $55,0^{\circ}\text{C}$ fue de 120 minutos. La capacidad sacarolítica del extracto enzimático fue confirmada usando como sustrato almidón de yuca amarga licuado.

Palabras clave: Glucoamilasa, *Aspergillus*, Sacarificación, jarabes de glucosa.

Resumo

Comercialmente as enzimas de sacarificação são mais custosas do que as de liquefação, por esta razão procuram-se estratégias que permitam fornecer este tipo de enzimas a baixo custo. Os objetivos deste estudo foram avaliar extratos enzimáticos sacarolíticos de cepas nativas, selecionar um extrato e determinar as melhores variáveis para a produção de xaropes de glicose. Para isto, de treze fungos filamentosos isolados de mandioca amarga *Manihot* sp., foram avaliadas a atividade sacarolítica, a hidrólise de maltose, a produção de glicose e a formação de biomassa em condições de fermentação submersa. O isolamento *Aspergillus* A1 foi selecionado por apresentar a maior atividade volumétrica ($0,091\text{ UI.L}^{-1}$). Durante a fermentação em superfície a presença de proteínas foi confirmada pelo método de eletroforese de SDS-PAGE junto com uma banda de maior intensidade com peso molecular entre os 60 e 70kDa. Para os preparados enzimáticos de *Aspergillus* A1 determinou-se que as melhores condições experimentais de sacarificação usando maltose como substrato foram pH 4,0, temperatura de $55,0^{\circ}\text{C}$, sem agitação. Igualmente na avaliação do efeito dos cofatores Cu^{2+} , K^{+} , Mg^{2+} e Na^{+} em concentrações de 1 mM, observou-se que todos incrementam a atividade enzimática principalmente o Mg^{2+} em $1,32\text{ UI.mg}^{-1}$ (32,4%) em relação ao controle. A estabilidade térmica da proteína de $55,0^{\circ}\text{C}$ é de 120 minutos. A capacidade sacarolítica do extracto enzimático foi confirmada usando substrato hidrolisado de mandioca amarga.

Palavras-chave: Glucoamilase, *Aspergillus*, Sacarificação, xarope de glicosa.

Introduction

World cassava production has increased in the last years, reaching 270.2 million of tons by 2014 [1]. Starch from sour cassava is a cheap source to produce ethanol. Starch pretreatment process, involve the breaking of amylose and amylopectin by enzymes, being these the costliest step in the ethanol production from starch, around 47% of total value, mainly by the enzymes price [2].

One example is the use of saccharolytic enzymes for the hydrolysis of starch in obtaining fermentable syrups for bio-ethanol production, which reduces time and generates fewer residues compared with traditional acid hydrolysis [3]. Enzymatic extracts are commercially available for liquefaction and saccharification of corn and sorghum starch, high-cost raw materials that compromise food security. However, for cassava starch, which is not suitable for human consumption, no commercial enzymes for hydrolysis exist in the market. In addition, sour cassavas abound greatly in Latin America.

Glucose syrups can be obtained via chemical or enzymatic hydrolysis of starch [4]. In chemical hydrolysis, the use of acids increases the cost of production, unwanted products are formed, and wastes can pollute the environment. In contrast,

enzymatic hydrolysis is a cheaper alternative that produces less pollution. However, due to the complexity of the structure of starch, it is necessary to incorporate heat treatment and the use of different enzymes to ensure the highest conversion of this polymer to glucose [5].

The enzymatic hydrolysis of starch is carried out in two steps: liquefaction and saccharification. In the first step, α -amylase (EC 3.2.1.1) is used to make cuts at random into the internal structure of starch due to endo-enzyme activity, generating many products such as pullulan, dextrin and glucose. The pullulan and dextrin are not viable to be used in the alcoholic fermentation process because they are not absorbed by fermenting microorganisms. Saccharification can occur by two enzymes: pullulanase (EC 3.2.1.41), which only breaks α -1,6 bonds and produces mostly dextrins, disaccharides such as maltose and a very small amount of glucose; and glucoamylase (EC 3.2.1.3 and EC 3.2.1.20), which hydrolyzes α -1,4 glycosidic terminal bonds of dextrins from starch liquefaction, forming mainly glucose. The pullulanases are produced mostly by bacteria, and glucoamylases are produced by fungi [6].

Enzymatic saccharification is expensive and thermally unstable. The enzymatic process is

performed at high temperatures because this improves solubility, decreases viscosity of the starch, reduces microbial contamination, decreases the process time and reduces production costs [7]. The bio-fuel industry seeks to replace the enzymes used in the industrial processes of starch hydrolysis with enzymes having higher thermostability [8]. These enzymes can be obtained from wild type microorganisms because in some cases, native enzymes exhibit better catalytic activity than commercial enzymes [9].

For production of enzymes with saccharolytic activity from filamentous fungi, the genera *Aspergillus* and *Rizhopus* have been the most studied using solid state fermentation (SSF) [10]. Native fungal enzymes also have been shown to viably be used for glucose obtaining on an industrial scale.

The aim of this project was the isolation, identification and selection of native filamentous fungi with saccharolytic activity, and to obtain an enzymatic extract from the optimal isolation to be used in glucose syrup production from maltose and cassava starch.

Materials and methods

For primary isolation of fungi, sour cassava (*Manihot sp*) was collected with evident fungal colonization from two farms in rural Barrancabermeja, Santander, Colombia. Ten grams of sour cassava was chopped, homogenized and diluted in 90ml of sterile saline solution. Each suspension from cassava homogenized dilutions were taken to obtain fungal colonies by surface culture. Sub-cultures were made to obtain axenic strains from the isolations at 25°C [11]. The culture media used contained maltose (5g.L⁻¹) as the only carbon source, rose bengal to restrict microbial growth and chloramphenicol (0.1g.L⁻¹) as a broad-spectrum antibiotic.

Genus of Isolated fungi were identified by macro- and microscopic characteristics using taxonomic keys. The culture media for identification was potato dextrose agar (PDA).

Submerged fermentation (SF) was performed in flasks of 250mL in sterile conditions, each flask contained 50mL of basal media in deionized water with the following per L: maltose (5), KH₂PO₄ (5), MgSO₄.7H₂O (5), peptone (2) and yeast extract (1). SF was made to determine maltose consumption and production of glucose, protein and biomass. Each flask was adjusted to a final concentration of 10⁵ propagules mL⁻¹. Each batch of SSF isolations

had twenty-five flasks, the fermentation was held with agitation at 150rpm and incubate at 25°C for 72 hours. Every eight hours, one flask and two replicates were taken to obtain the enzymatic extracts, the biomass was separated from the broth using filtration through a Büchner funnel with Whatman No. 1 filter paper, and the enzymatic extract was obtained by vacuum.

A first screening of the enzymatic extract was performed by evaluating maltose hydrolysis and glucose production using 3,5-dinitrosalicylic acid (DNS) [12] and glucose oxidase (Biosystems®) [13], respectively. Volumetric productivity was calculated based on glucose production. The maltose concentration was calculated via subtraction of the concentration of reducing sugars and glucose. The production of glucose, protein by Bradford method [14], and biomass by dry weight and the hydrolysis of maltose were determined in a second screening. The isolation with the best results was named *Aspergillus* A1. The enzymatic volumetric activity (UI.L⁻¹) of the enzymatic extract from this isolation was quantified (Figure 1). The enzymatic volumetric activity (UI.L⁻¹) was defined as micromoles of glucose produced per ml in one minute at 50°C and 200rpm.

To increase the protein concentration from the enzymatic extract, SSF of *Aspergillus* A1 was performed in a column bioreactor with a lignocellulosic support sterilized at 121°C for 15min. The column bioreactor was filled with 8cm of support and 150ml of broth. The crude enzymatic extract was obtained by pressing the lignocellulosic polymer, followed by centrifugation at 10000 × g for 15 minutes and finally filtering (Millipore, cellulose 0.45μm).

Electrophoresis SDS-PAGE was performed on enzymatic extracts obtained by SSF of *Aspergillus* A1 to determine the molecular weight of the proteins present in the extract. A Fermentas PageRuler of 10 to 200kDa was used as a molecular weight standard. Samples were run at 120 volts in 12% polyacrylamide gels using Coomassie blue as the dye.

Analysis of variance was performed to establish the highest values of enzymatic activity of the enzymatic extract from *Aspergillus* A1 at a pH 3-9, a temperature of 30-90°C and an agitation of 0-600rpm. The results of this analysis were used to determine the best experimental conditions for maltose hydrolysis. The independent variables were pH 4.0; 4.5 and 5.0; 16mmol.L⁻¹ buffer sodium acetate and 0,1molL⁻¹ citric acid; temperatures of 55, 60 and 65°C; and agitation at 0, 200 and 400rpm. The experiments were performed in tubes

containing 1mL of enzymatic extract and 1mL of maltose at a concentration of 5mgmL^{-1} . The response variable was the glucose concentration versus time. Each assay was performed in triplicate, and the results were analyzed using Statgraphics software.

The effect of 1mmolL^{-1} aliquots of cofactors Cu^{2+} , K^+ , Mg^{2+} and Na^+ on enzymatic extract activity was determined per the methodology of Bhatti *et al.* [15]. This was performed based on the optimized

conditions found in the experimental design. The thermal stability of the enzymatic extract was also evaluated at 55°C through glucose quantification every 15 minutes for 3 hours.

Finally, the saccharolytic activity of the selected enzymatic extract was evaluated using the optimized parameters for the best cofactor on fermentable syrups obtained from liquefied cassava starch. Liquefied cassava starch was obtained according to the methodology described by Ruiz *et al.* [16].

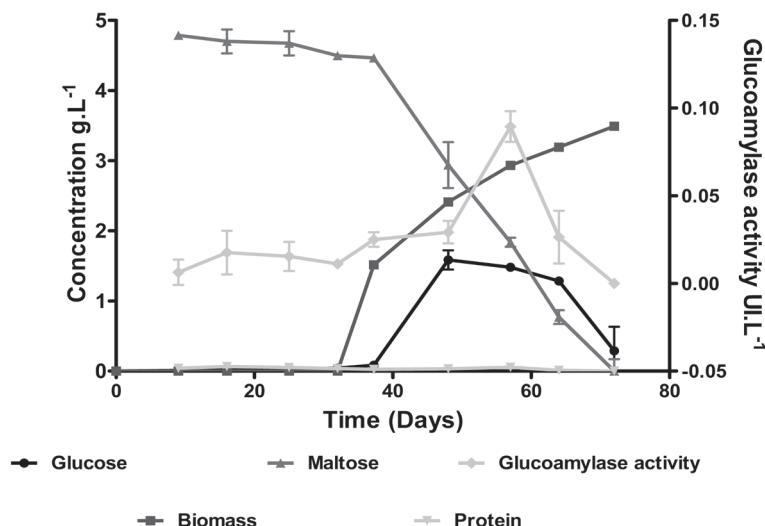


Figure 1. Growth curve of *Aspergillus* A1 submerged fermentation.

Results and Discussion

From samples of sour cassava, it was possible to obtain 13 fungal isolations belonging to the genera *Fusarium*, *Penicillium*, *Geotrichum*, *Scopularipsis* and *Aspergillus*, and all the isolations showed saccharolytic activity. The two isolations with the best volumetric productivity were *Aspergillus* A1 and *Aspergillus* A12 with 0.07 and 0.05gL^{-1} , respectively. The isolation that showed the best production of glucose from maltose was *Aspergillus* A1 (1.3gL^{-1}) between 50 and 60 hours of fermentation. This agrees with the highest volumetric enzymatic activity from extract obtained at the same time, despite a low protein concentration, as shown in Figure 1.

Based on the volumetric activity, glucose production from maltose hydrolysis was confirmed in all fungal isolations, with the fungi of genera *Aspergillus* having the best productivity. The previous results confirm that the *Aspergillus* genus had the highest glucose syrup production by submerged fermentation [17,18]. Authors such as Riaz *et al.* [19] and O'Brien *et al.* [20], have suggested several

species of *Aspergillus*, including *A. awamori*, *A. foetidus*, *A. niger*, *A. oryzae*, *A. terreus*, for glucose syrups obtaining from polysaccharides.

The enzymatic extracts obtained from submerged fermentation of *Aspergillus* A1 did not reach a higher concentration of protein (0.05gL^{-1}). This contrasted with SSF, where it was possible to obtain concentrations greater than 0.05gL^{-1} .

The increase in protein concentration in the enzymatic extract was possible with the change from submerged fermentation to solid fermentation with a lignocellulose support. With this variation, the protein concentration reached 0.06gL^{-1} . This is consistent with the work of Rodríguez *et al.* [21], and Singhania *et al.* [22], who concluded that use of SSF for obtaining enzymatic extracts is better than the concentrations achieved by submerged fermentation. The electrophoresis SDS-PAGE results of enzymatic extracts obtained from SSF of *Aspergillus* A1 showed 11 bands with different molecular weights. The band with a molecular weight between 60 and 70kDa had the highest intensity, as shown in Figure 2.

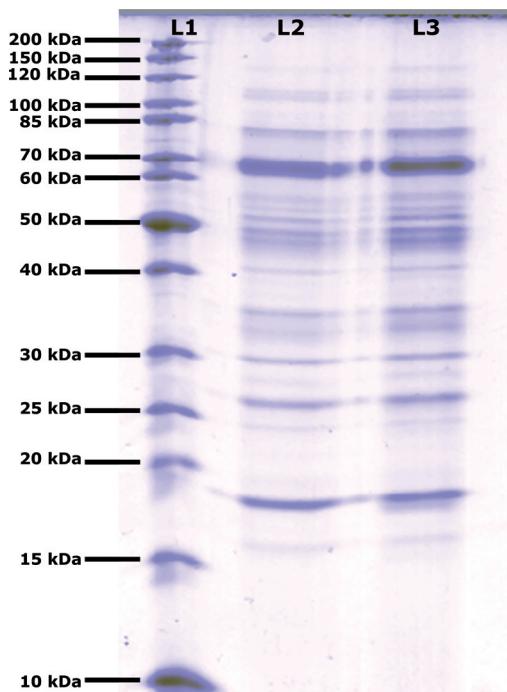


Figure 2. Polyacrylamide gel electrophoresis of enzymatic extract from *Aspergillus* A1 obtained by SSF. Line 1 Molecular weight marker; Lines 2 and 3 enzymatic extract from *Aspergillus* A1 from two different bioreactors. Coomassie brilliant blue G-250 staining.

The bands observed in gels from SDS-PAGE obtained from different SSF of enzymatic extracts from *Aspergillus* A1 were not different from each other, guaranteeing the reproducibility of SSF, as shown in Figure 2. The band with the highest intensity occurred between 60 and 70 kDa. These results corroborate those of Norouzian *et al.*,⁸ who established the molecular weights of fungal glucoamylases to be within a range of 48 to 90 kDa. da Silva *et al.* [23] and Bagheri *et al.* [24] reported the molecular weight of glucoamylase from *Aspergillus niveus* to be 76 kDa.

Analysis of variance showed that the best saccharolytic activity of the extract at pH values between 4 and 5, agitation between 200 and 600 rpm and a temperature of 60°C. By using these values, it was possible to define the variables for the experimental design matrix.

As can be seen in Figure 3, the best enzymatic extract activity from *Aspergillus* A1 with respect to pH occurs at 20 minutes, (4.4 UI·mg⁻¹) at a pH between 4 and 5 and at 60°C. Under these conditions, the activity reached 3.5 μmol·L⁻¹. These results are comparable to those of Bagheri *et al.* [24], who reported that the highest activity of glucoamylase occurs at a pH between 4.5 and 6.5 and at temperatures between 50 and 70°C.

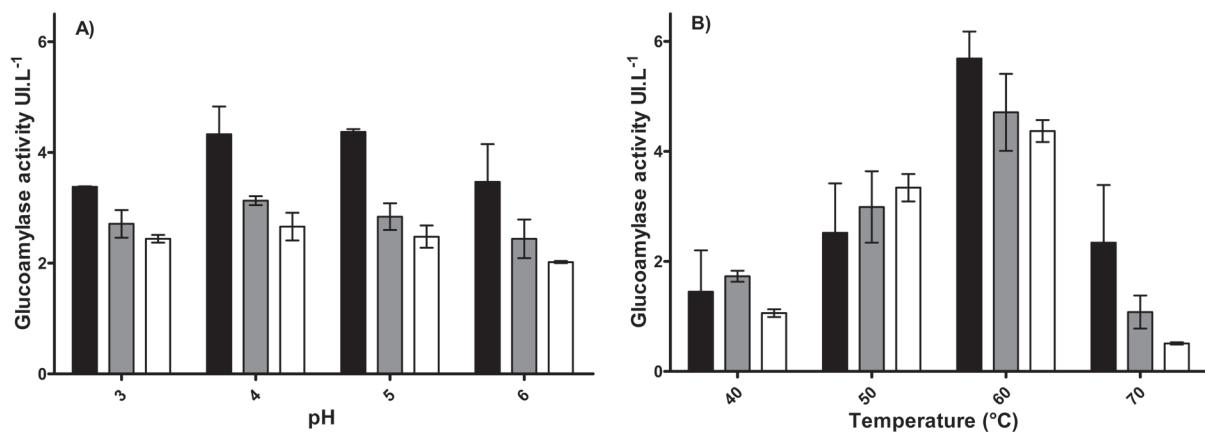


Figure 3. Glucoamylase activity under the effect of different values of pH 3 – 6 (A) and temperature 40–70°C (B), using maltose to concentration 5 mg·mL⁻¹ like a substrate. (black bars) glucoamylase activity at 20 min; (grey bars) glucoamylase activity at 40 min and (white bars) glucoamylase activity at 60 min.

The highest values of enzymatic activity and glucose obtained from maltose were at pH 4 (4.4UI.mg⁻¹ and 6.8 μ mol.mL⁻¹, respectively) and a temperature of 60°C (5.6UI.mg⁻¹ and 3.5 μ mol.mL⁻¹, respectively), as shown in Figure 3.

From these experimental design results, seven effects were found with a P value lower than 0.05 and confidence level of 95%. The results indicate that there was little or no interaction between the

temperature and agitation. This is contrary to what is normally observed regarding interactions of pH/temperature and pH/agitation, with the latter typically being the most significant.

The polynomial obtained for glucoamylase activity (Equation 1) showed an R-squared value of 89.1% and an adjusted R-squared value of 87.7%, which could explain the variability in enzymatic activity with a standard error of 0.4% and an absolute error of 0.3%.

$$\begin{aligned} \text{Glucoamylase activity} = & 103.29 - 52.81.pH + 0.79.Temperature - 0.016.rpm + 5.04pH^2 + \\ & 0.074.pH.Temperature + 0.002.pH.rpm - 0.009.Temperature^2 + 0.00000F5.Temperature.rpm + \\ & 0.000003.rpm^2 \end{aligned} \quad (1)$$

From the mathematical model obtained to predict enzymatic activity, pH and pH2 were the most influential factors. From the response surface and surface contour (Figure 4) it can be concluded that the best saccharolytic activity obtained by

the enzymatic extract was at an agitation of 0rpm. Enzymatic activity was increased when the agitation increased to 4rpm at an average temperature of 55°C [25].

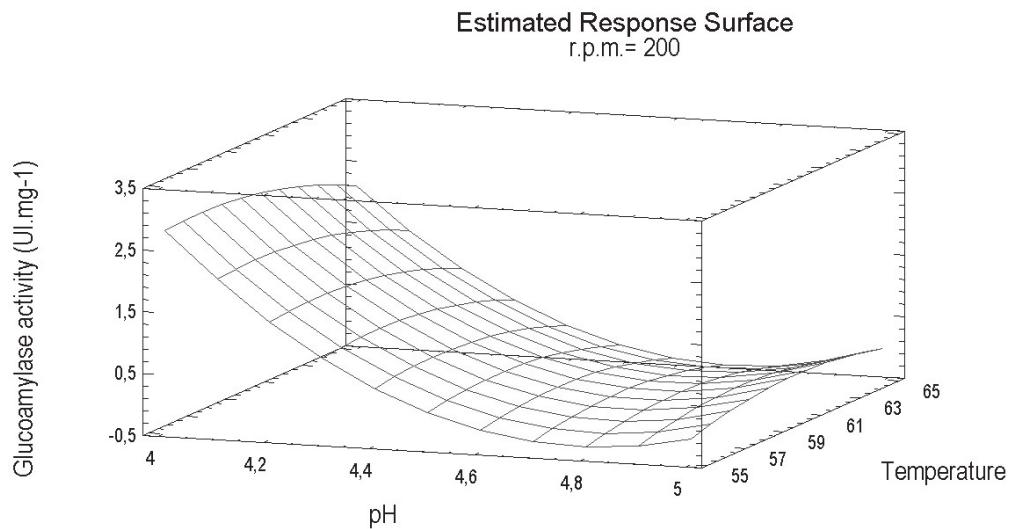


Figure 4. Response surface estimated of the combined effects of pH and temperature of enzymatic extract from *Aspergillus A1*, without agitation, 5mgmL⁻¹ of maltose concentration at 15 minutes of reaction.

Enzymatic cofactor evaluation showed that all cofactors evaluated had a positive influence on enzymatic activity. The Mg²⁺ ion was found to increase enzymatic activity by 32%, resulting in a value of 4.15UI.mg⁻¹.

Figure 5 shows that the use of metallic ions such as enzymatic cofactors increases enzymatic

activity compared to the control. Mg²⁺ was the most influential factor, resulting in a 32.4% increase in enzymatic activity. This result agrees with that reported by Bhatti *et al* [15] and Benassi *et al* [26], who found a 176% increase in enzymatic activity using manganese ions in glucoamylase from *Fusarium phoenicis*.

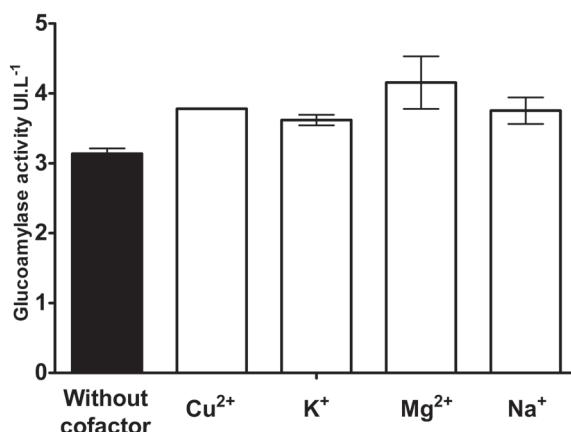


Figure 5. Effects of various metal ions at 1mmol concentration on glucoamylase activity, (black bar) control.

The thermal stability assay showed three periods of variation. The first 30 minutes of enzymatic activity peaked at $5.6\text{UI}.\text{mg}^{-1}$, followed by a decrease by 22%, and then the activity remained stable until 120 minutes. The enzymatic activity finally decreased to an average of 56% at the completion of the study.

The half-life determined for the enzymatic extract from *Aspergillus* A1 was 60 minutes of incubation at 60°C , which was a similar time to that obtained by Sutthirak *et al* [27], who determined that the greatest decrease in enzyme activity occurred after two hours (Figure 6).

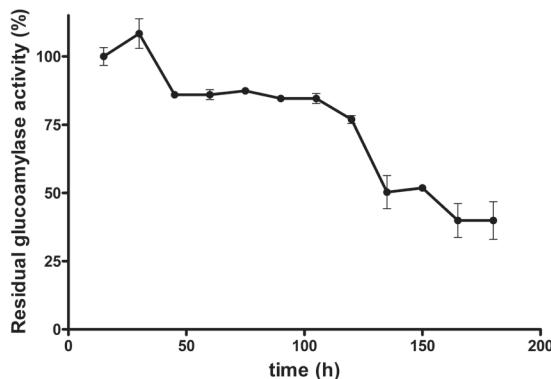


Figure 6. Thermal stability of enzymatic extract from *Aspergillus* A1, $5\text{mg}.\text{ml}^{-1}$ maltose concentration.

In saccharification assays of liquefied cassava starch, it has been found that saccharolytic activity has a glucose/enzymatic activity ratio of 0.9 for enzymatic extracts from *Aspergillus* A1. This is lower than that obtained by Ruiz *et al* [16], who

found a value of 3.7 for purified commercial glucoamylase Spirizyme ® Fuel.

From 13 fungal isolations identified from decomposed cassava, the *Aspergillus* genera had the highest saccharolytic activity. From this group, it was possible to obtain an enzymatic extract with glucoamylase enzyme activity.

The SSF make it possible to obtain a higher protein concentration in enzymatic extracts from *Aspergillus* A1 for quantification and observation of the proteins by Bradford assay and electrophoresis, respectively.

The best parameters for maltose hydrolysis by enzymatic extracts from *Aspergillus* A1 were a pH of 4.0; a temperature of 55°C without agitation and $1\text{mmol}.\text{L}^{-1}$ of Mg^{2+} cofactor.

It was confirmed that the enzymatic extract from native *Aspergillus* sp. could undergo saccharification of liquefied cassava starch.

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