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PRODUCTION AND CHARACTERIZATION OF GLUCOAMYLASE FROM FUNGUS *ASPERGILLUS AWAMORI* EXPRESSED IN YEAST *SACCHAROMYCES CEREVISIAE* USING DIFFERENT CARBON SOURCES

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ABSTRACT

Glucoamylase is widely used in the food industry to produce high glucose syrup, and also in fermentation processes for production beer and ethanol. In this work the productivity of the glucoamylase of *Aspergillus awamori* expressed by the yeast *Saccharomyces cerevisiae*, produced in submerged fermentation using different starches, was evaluated and characterized physico-chemically. The enzyme presented high specific activity, 13.8 U/mgprotein or 2.9 U/mgbiomass, after 48 h of fermentation using soluble starch as substrate. Glucoamylase presented optimum activity at temperature of 55ºC, and, in the substratum absence, the thermostability was for 1h at 50ºC. The optimum pH of activity was pH 3.5 - 4.0 and the pH stability between 5.0 and 7.0. The half life at 65ºC was at 30.2 min, and the thermal energy of denaturation was 234.3 KJ mol^{-1}. The hydrolysis of different substrate showed the enzyme's preference for the substrate with a larger polymerization degree. The gelatinized corn starch was the substratum most susceptible to the enzymatic action.

Key words: Glucoamylase, *Aspergillus awamori*, *Saccharomyces cerevisiae*, Corn starch, Potato starch, Cassava starch.

INTRODUCTION

Glucoamylase (α-1,4-glucan glucohydrolase, amyloglucosidase, EC 3.2.1.3) is of great importance to the fermentation and food industries for saccharification of starch and other related oligosaccharides. Glucoamylase (GA) consecutively hydrolyzes α-1,4 glycosidic bonds from the non-reducing ends of starch, resulting in the production of glucose. To a lesser extent, it also has the ability to hydrolyze α-1,6 linkages, also resulting in glucose as the end-product (24). The enzyme is produced by a variety of microorganisms, and *Aspergillus niger*, *Aspergillus awamori* and *Rhizopus oryzae* have been considered the most important for industrial application (5).

The principal industrial use of GA is in the production of glucose, which in turn serves as a feedstock for biological fermentations in the production of ethanol or in the production of high fructose syrups (33,34). It also improves barley mash for beer production (36). GA is a key enzyme too in the production of sake and soy sauce. Specially, in sake brewing the enzyme is considered to be most important because the rate of fermentation is dependent on the activity of the GA (18).

Works using a recombinant gene expression reported the isolation and characterization of the GA cDNA and expression in *S. cerevisiae*. Innis *et al*. (13), successfully constructed a plasmid (pAC1) in which a GA gene (pGAC9) was inserted. This plasmid was used to transform *Saccharomyces cerevisiae* that expressed a free GA in the culture medium. In the study, the autors discuss the plasmid construction, the expression of GA and its structural characteristics, which showed similarity to its native form in *Aspergillus*.

Saccharomyces cerevisiae is an attractive host for the production of heterologous proteins. It possesses a well defined

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secretory pathway and performs post-translational modification such as glycosylation, making it a suitable host for the production of eukaryotic proteins (19). Moreover, it's the best studied organism, and the most frequently employed yeast in industrial processes.

This recombinant GA has been used in many works of molecular biology (6,8,20,21) mainly in regard to improving stability (8,20,22) or selectivity (6), but there are very few studies about the production of this enzyme in liquid fermentation employing different varieties of starches as a source of carbon.

Corn starch is the principal raw material used to obtain glucose syrup in the USA and Europe. Therefore, almost all scientific papers about GA have been done using this substrate to evaluate the GA's action. Recent fermentation studies have shown that nutrient source and type of fermentation can affect the enzyme's properties (15,27). GA secretion is greatly dependent on the type of starch (30).

The aim of this study was to produce a wild type GA from *Aspergillus awamori* expressed by *Saccharomyces cerevisiae* from four different sources of carbon and to characterize the physical chemical parameters of this enzyme.

MATERIALS AND METHODS

Microorganism

S. cerevisiae strain C468 (α *leu*2-3 *leu* 2-112 *his*3-11 *his* 3-15 *mal*$^-$) containing plasmid YEpPM18, a yeast episomal expression vector containing the wild-type GA cDNA from *A. awamori* created by Cole *et al*. (4), who was in the Cetus corporation, which donated this strain to the Ford's group, which donated this system strain to our research group. The culture was maintained in 10% glycerol solution at - 80ºC.

Culture Medium

Was used: 2% starch the different sources: soluble starch (Synth, SP, Brazil); cassava starch; corn starch and potato starch (Cargill, Brazil). The starches were gelatinized at 100ºC for 3 min and, afterwards, the following items were added in each starch solution: 0.5% ammonium sulphate (Merck, Darmstadt, Germany), 2% glucose (Synth), 0.17% "Yeast nitrogen base without aminoacids" (Difco, Detroit, MI, USA), L-histidin 20 mg/L (Sigma, St. Louis, MO, USA), thus constituting four diverse culture medium regarding the source of starch.

Substrates for enzymatic activity

Starch from corn, potato and cassava were obtained from Department of Food Engineer and Technology - IBILCE/UNESP. Oligosaccharides maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose and also synthetic substrates – ρ-nitrophenyl-α-D-glucopyranoside and ρ-nitrophenyl-β-D-glucopyranoside were obtained from Sigma, and sucrose from Merck.

Growth conditions

The strain was inoculated in Petri dish containing medium SD + His (2% glucose, 2% soluble starch, 0.5% ammonium sulphate, 0.17% "Yeast nitrogen base without aminoacids", L-histidin 20 mg/L and 2% agar) and incubated at 30ºC for 5 days.

Enzyme production

One colony was transferred to a 125 mL Erlenmeyer flask containing 20 mL of each culture medium with different starches. The flasks were incubated in a rotary shaker at 180 rpm for 16-20 h at 30ºC. One mL of this suspension was used as inoculum and transferred to others 125 mL Erlenmeyer flask containing 20 mL of the different media, and these were incubated according to the conditions described below, for 7 days. Flasks with the fermented material were taken every 24 h. The biomass was separated by centrifugation at 10000 g for 10 min at 5ºC, and the supernatant was used to evaluate GA activity.

Assay of GA activity

Glucose liberated from starch by GA was estimated by peroxidase/glucose-oxidase enzymatic method described by Bergmeyer and Bernt (2) with modification. The reaction mixture was made up of 0.4 mL of 0.5% soluble starch (Merck) solution in 0.2 M sodium acetate buffer, pH 4.5 and 0.1 mL diluted crude enzyme. After enzymatic reaction at 50ºC for 10 min, samples were cooled in an ice water bath. From this reaction mixture, an aliquot of 0.2 mL was taken and mixed with 0.3 mL of 0.2 M phosphate buffer, pH 7.0. One mL of the glucose oxidase reagent was added and the mixture was incubated at 37ºC for 1 h, and the absorbance was measured at 500 nm. One unit of GA was defined as the amount of enzyme required to release 1 μmol of glucose/min under the assay conditions and activity was expressed as U/mL. The activity was also expressed as $U/mg_{biomass}$, and $U/mg_{proteins}$.

Microbial biomass quantification

The cellular mass was suspended and homogenized with 20 mL of distilled water, following centrifugation at 10000 g for 10 min at 5ºC, and the supernatant was put aside during three cycles. Finally the biomass was suspended with 20 mL of distilled water and part of this suspension (1 mL) was transferred to eppendorfs tube of known weight in triplicate. These were centrifuged at 10000 g for 10 min and the supernatant was put aside. Cellular mass was dried at 60ºC until constant weight.

Quantitative protein determination

Protein content of enzyme solution was measured following the method of Hartree-Lowry (9), using bovine serum albumin (BSA) as standard.

Quantitative reducing sugar determination

Reducing sugar was measured following the method of Somogy (35), using glucose as standard.

Optimum pH and temperature

The optimum pH was determined by measuring activity at 55ºC for 10 min using various buffers. The following 0.1 M buffer systems were used: citrate-NaOH (pH2.5); sodium acetate (pH 3.0-5.5); citrate-phosphate (pH 6.0-7.0); Tris-HCl (pH 7.5-8.5) and glicine-NaOH (pH 9.0-10.5). The optimum temperature was assayed by measuring activity at pH 4.0 (0.1 M acetate) over different temperatures ranging from 25 to 95ºC.

pH stability and thermostability

For pH stability the crude enzyme was dispersed (1:1) in 0.1M buffer solutions pH 3.0-5.0 (sodium acetate), pH 5.5-7.0 (citrate-phosphate), pH 7.5-8.5 (Tris-HCl) and pH 9.0-10.5 (glicine-NaOH) and incubated at 25ºC for 24 h. An aliquot was used to determine the remaining activity at the optimum pH (4.0) and temperature (55ºC). To test the heat stability of the enzyme, the GA solution in buffer acetate pH 4.0 was incubated at various temperatures ranging from 10 to 90ºC for 60 min. The reaction was stopped in ice-cold water and the remaining activity was measured at pH (4.0) and temperature (55ºC).

Evaluation of enzyme action on different substrates

Hydrolytic action of GA was evaluated on corn, potato and cassava starches and on oligosaccharides with two to seven glucose units: maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. Activity was also assayed on synthetic substrates ρ-nitrophenyl-α-D-glucopyranoside and ρ-nitrophenyl-β-D-glucopyranoside and on 0.5% sucrose. Enzyme activity was determined at optimum activity conditions.

Irreversible Thermoinactivation kinetics

Kinetics was carried out according to Rashid and Siddiqui (32), with modifications. Enzymatic extracts were incubated in 0.2 M acetate buffer, pH 4.0 at 65, 70 and 75ºC. Seven samples were withdrawn periodically from each temperature, cooled in an ice water bath and enzyme activity was determined.

Analyses of thermodynamic parameters of enzymatic denaturation

Thermodynamic parameters of denaturation, free energy, enthalpy and entropy were determined according to Rashid and Siddiqui (32).

RESULTS AND DISCUSSION

The best substrate for GA production was the potato starch, where GA production was more significant at the end of fermentation, with enzymatic activity of 8.3 U/mL (Fig. 1A). The soluble starch was also a good substrate for the enzyme production with 7.0 U/mL, followed by cassava starch and corn starch with 4.9 and 2.7 U/mL, respectively (Fig. 1A). These results are very expressive when compared to others in the literature. For example, the hybrid strain CL-9 of *Saccharomyces diastaticus* produced GA extracellular with 1.06 U/mL of activity in a medium containing glucose and starch (30), *S. cerevisiae* expressing a GA from *A. awamori* that exhibited 0.09 U/mL of activity (7) and, a recombinant *S. cerevisiae* strains *Stell7* produced GA with activity of 1.5 U/mL (16). Regarding biomass production, the peak was observed at 72 h of cultivation with 3.0 mg/mL (Fig. 1A). The hybrid strain DI-20 of *Saccharomyces diastaticus* produced 4.5 mg/mL cellular mass after 72 h of growth (30). Biomass decrease was accompanied by an increase of GA secretion, which was also observed by Peres *et al*. (30).

Reducing sugar concentration and pH value of the culture medium are illustrated in Fig. 1B. After 24 h of fermentation, pH was highly reduced, decreasing to a limit of 2.4. Reducing sugar concentration was high in the first 24 h, however after this period it was drastically reduced. The comparison between variations of pH and reducing sugar concentration shows that while sugar and nutrients were consumed by the yeast, an acidification of the medium occurred. This intense reduction of pH is possibly due to the intake of ammonium salts, or to the release of metabolites produced by the microorganism, with a possible release of organic acids and ethanol. Regarding protein concentration (Fig. 1B) it can be seen that it is high in the beginning of cultivation probably due to the presence of amino acid histidin in the culture medium. The protein secretion was close to the peak of GA production at the end of fermentation.

The enzyme exhibited specific activity of 2.9 U/$mg_{biomass}$ in medium of soluble starch (Table 1). In other studies, results were inferior, such as for the hybrid strain CL-9 of *S. diastaticus* that exhibited specific activity of 0.68 U/$mg_{biomass}$ in medium containing starch, whereas strain *Saccharomyces* DI-20 (17) exhibited 2.5 U/$mg_{biomass}$. The recombinant *S. cerevisiae* strains *Stell7* produced specific enzyme activity 0,055 U/$mg_{biomass}$ (16). Regarding specific activity in U/mg of protein, the results were also very significant with 13.8 U/$mg_{protein}$ in the medium contain soluble starch and 14.5 U/$mg_{protein}$ in the medium of cassava (Table 1). The high specific activity found is important, because it probably shows that the greater number of the enzymes secreted was GA.

Physico chemical characterization of GA from *A. awamori* expressed in *S. cerevisiae*

GA exhibited optimum activity at pH values between 3.5 and 4.0 (Fig. 2). It has been reported that fungal GAs act better in acid pH (28). It can be pointed out that the enzyme produced by *Aspergillus* sp N-2 exhibited optimum pH in the range of 3.5 - 4.5 (38). Optimum pH for GA from *Aspergillus niger* was 4.8 (14). Commercial preparations of GAs from *A. niger* exhibit

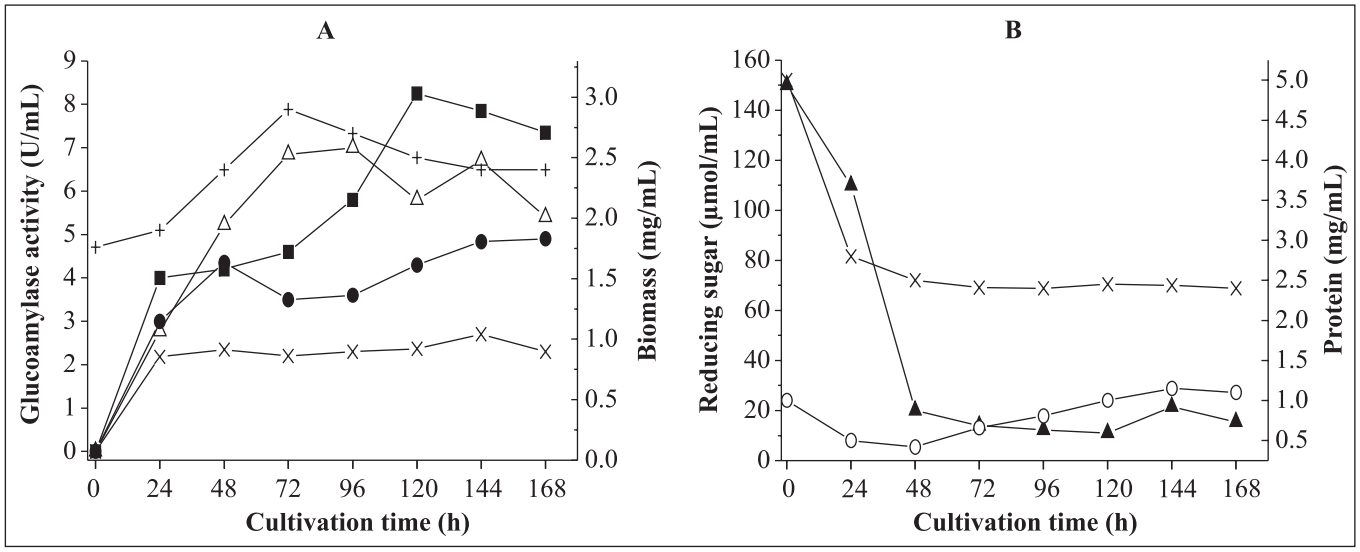

Figure 1. GA production by yeast *S. cerevisiae* in medium with different starches. **(A)** Soluble starch –Δ; Cassava starch –●; Potato starch –■; Corn starch –x, and biomass in soluble starch –+ (For cellular mass determination, its was dried at 60ºC until constant weight); **(B)** pH variation in the cultivation medium –x, protein concentration –○ and reducing sugar –▲ (values were determined using soluble starch as substrate).

Table 1. Specific activity of GA from *Aspergillus awamori* expressed in *Saccharomyces cerevisiae* in medium with different starches.

Parameters	Recombinant GA			
	soluble starch	cassava starch	potato starch	corn starch
Specific activity (U/mg $_{protein}$)	13.8	14.5	14.2	7.8
Specific activity (U/mg $_{biomass}$)	2.9	_	_	_

_ values not determinate.

optimum pH in the range of 3.5 - 5.0 (29). To conclude, the value for optimum pH found for the enzyme studied in this work is in the range of other reported works available in literature. Regarding stability, the enzyme kept 80% of its activity between pH 5.0 and 7.0. When in alkaline pH, its activity was reduced (Fig. 2). These results are in agreement to GAs from other works, since fungal enzymes are generally stable in a range from acid to neutral pH. GA from *Paecilomyces variotii* was stable in pH between 3.0 to 7.5 (25) and native GA from *Aspergillus niger* was stable in pH between 2.0 to 7.0 (14).

Optimum temperature of fungal GAs is generally in the range of 50 to 60ºC (28). Optimum temperature of recombinant GA of this work was 55ºC (Fig. 3). Above this temperature, activity decreased and at 60ºC it lost approximately 20% of activity and at 65ºC it kept approximately 50% of residual activity. Anto *et al*. (1) described a GA from *Aspergillus* sp HA-2 that exhibited optimum temperature at 55ºC. GA from *Aspergillus oryzae* cultivated in submerged fermentation exhibited optimum temperature of activity at 65ºC, and the enzyme produced in solid state fermentation at 56ºC (10). GA exhibited inferior tolerance towards temperature increase when compared to the value obtained for optimum temperature, being stable up to 50ºC in the absence of substrate for 1h (Fig. 3) and above this temperature stability decreased gradually.

Thermodynamic parameters of the enzyme

Half life of the enzyme at 65ºC was 30.2 min, and the activation energy (ΔG) was of 102.5 KJ mol^{-1}, lower than the one found by Rajoka *et al*. (31) and Li *et al*. (20). Entropy value (ΔS) was of 381.53 J mol^{-1} k^{-1}, very close to the value found in the study of Flory *et al*. (7). Thermoinactivation process of GA is caused by the incorrect conformation of the protein (26), where thermal inactivation was caused by the rupture of non covalent bonds followed by its concomitant unfolding (Table 2).

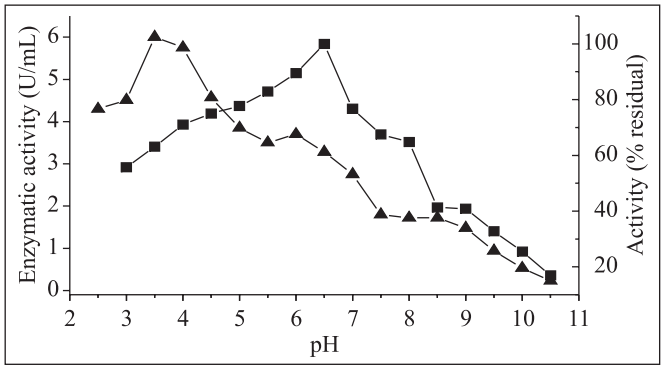

Figure 2. Effect of pH on enzyme. Optimum pH (▲) and stability (■). Buffer were: citrate-NaOH pH 2.5; sodium-acetate pH 3.0 to 5.0; citrate-phosphate pH 5.5 to 7.0; Tris-HCl pH 7.5 to 8.5 and glicine-NaOH pH 9.0 to 10.5. Activity was measured with 0.5% soluble starch as substrate.

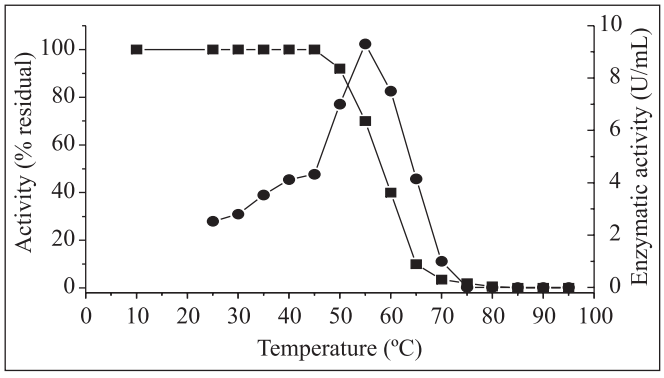

Figure 3. Effect of temperature on enzyme. Optimum temperature (●) and stability (■). Activity was measured with 0.5% soluble starch as substrate.

Evaluation of glucoamylase action on different substrates

Hydrolysis of different starch sources showed that that gelatinized corn starch was more susceptible to enzymatic action exhibiting hydrolytic activity of 9.5 U/mL. As expected, raw starch exhibited lower enzymatic activity of approximately 2.7 U/mL (Fig. 4), since granule susceptibility to enzymatic action is increased when starch is in its gelatinized form (3). The enzyme did not hydrolyze synthetic substrates *ρ*-nitrophenyl α-D-glucopyranoside and *ρ*-nitrophenyl β-D-glucopyranoside and also sucrose. Besides, the enzyme exhibited preference for substrates with higher levels of polymerization, being maltoheptaose the one that underwent the highest hydrolysis rate. These results confirm conclusively that this is a true GA enzyme and no a maltase or α-amylase.

These data are typical of GA since enzymatic activity increased with substrates molecular weight. Hydrolysis rate of α-1.4 bonds increased with molecular weight, being more efficient in larger sequences than maltopentaose and about five times smaller in maltose (23). GA is an enzyme that is able to hydrolyze 100% of starch into glucose (11).

According to Thanarathan *et al.* (37) the difference in the action of amilolytic enzymes on different types of starches is related to differences in molecular composition, specially in amylose and amilopectin content, and on the size of their chains. Other factors may interfere in the action mechanism of amylases on starch granule. The association between granules components makes it more or less susceptible to enzyme attach. Another factor of great importance regarding enzyme activity on starch granules is their porosity (12).

Table 2. Kinetic and thermodynamic properties of GA from *Aspergillus awamori* expressed in *Saccharomyces cerevisiae.*

Parameters	Recombinant GA
Half life (min) 65ºC	30.2
K_d (min^{-1})	0,0003
E_{ad} (KJ mol^{-1})	234.3
ΔG (KJ mol^{-1}) 65ºC	102.5
ΔH (KJ mol^{-1}) 65ºC	231.48
ΔS (J $mol^{-1}K^{-1}$) 65ºC	381.53

Free energy of activation for irreversible inactivation of GA (ΔG) = -RT ln (K_d h/K_B T); enthalpy of GA (ΔH) = E_a – RT; entropy of GA (ΔS) = ΔH – ΔG/T, and denaturation energy (E_{ad}) = -R (slope) were determined according to Rashid and Siddiqui (32). Activity was measured with 0.5% soluble starch as substrate.

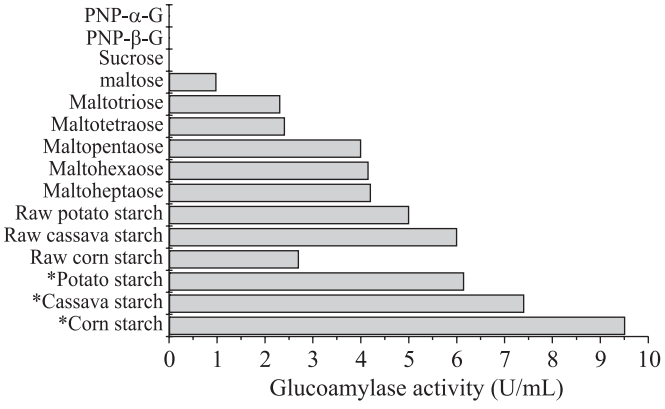

Figure 4. Enzymatic activity of GA on different substrates at 55ºC and pH 4.0 for 10 min;
*Starches were gelatinized until 70ºC with agitation. Each value represents the mean of three experiments. PNP-α-G (*ρ*-nitrophenyl-α-D-glucopyranoside) and, PNP-β-G (*ρ*-nitrophenyl-β-D-glucopyranoside).

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RESUMO

Produção e caracterização da glucoamilase do fungo *Aspergillus awamori* expressa em levedura *Saccharomyces cerevisiae* usando diferentes fontes de carbono

A glucoamilase é amplamente utilizada na indústria de alimentos no processamento do amido para a produção de xarope com alto teor de glicose e também muito empregada nos processos de fermentação para produção de cerveja e etanol. Neste trabalho a glucoamilase de *Aspergillus awamori* expressa em *Saccharomyces cerevisiae* produzida sob fermentação líquida foi avaliada quanto à produtividade em diferentes amidos e caracterizada físico-quimicamente. A enzima apresentou alta atividade específica de 13,8 U/$mg_{proteína}$ e de 2,9 U/$mg_{biomassa}$ ao final de 48 h de fermentação em meio contendo amido solúvel. A glucoamilase apresentou temperatura ótima de atividade a 55ºC, e temperatura de desnaturação térmica na ausência de substrato por 1h a 50ºC. O pH ótimo de atividade foi na faixa de 3,5 - 4,0 e a estabilidade ao pH entre os valores 5,0 e 7,0. A meia vida a 65ºC foi 30,2 min., e a energia de desnaturação foi de 234.3 KJ mol^{-1}. A hidrólise em diferentes substratos mostrou a preferência da enzima pelos substratos com maior grau de polimerização, sendo o amido de milho gelatinizado o substrato preferencial à ação enzimática.

Palavras-chave: Glucoamilase, *Aspergillus awamori*, *Saccharomyces cerevisiae*, Amido de milho, Amido de batata, Amido de mandioca.

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