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Research Paper

Thermal-Stability of Enzyme Activity and its Application in The Hydrolysis of Starchy Residue From Mandioca Processing

Science

KEYWORDS : Cassava, *Mandioca*, Starch residue, amylase, *Trichoderma reesei.*

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ABSTRACT

Natural occurring fungi have been investigated as producers of amylase degrading starchy residue from mandioca (Cassava) flour processing. A high degrading starch fungi strain was isolated and identified as Trichoderma reesei CFIBA001, and was capable to hydrolyze starchy residue completely (20 g l-1) in three days of incubation at 30oC and 160rpm. The amylase present in the crude extract showed high activity with thermal-stability over 3h at 60oC with pH 5.5. This is an important result for a fungus strain with the potentiality to use in the exploitation of the mandioca´s residue discarded from mandioca flour manufactory to produce sugars that could be used in the bioetanol producing process.

INTRODUCTION

Mandioca's starchy root produce more raw starch per unit of land than any other staple crop, and grows almost exclusively in arid and semiarid tropics (Nassar et al., 2002). Brazil is the second most important producer of *mandioca* in the world and has been developing technologies to use its starch, which is composed of unbranched amylase (20±5%) and branched amylopectin (80±5%) both of which can be hydrolyzed either with acids or enzymatically to release glucose and maltooligosacchrides (González et al., 2008). In this way, the absence of low cost technologies that promote the digestion and starchy residue saccharification is a limiting obstacle in the use of recalcitrant biomasses. For this reason, crude enzymatic technology is suggested, initially to hydrolyze the residues and after that to promote the alcoholic fermentation with commercial yeast.

Amylase belongs to the family of glycoside hydrolyze enzymes that break down starch by acting on α-1,4-glycosidic bonds. The α-amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) is an important enzyme that cleave at random locations on the starch chain, ultimately yielding maltotriose and maltose, glucose and "limit dextrin" from amylose and amylopectin, and different α-amylase sources had been investigated to produce robust protein and high activity for liquefying cassava starch (Michelin et al., 2008, Liu and Xu, 2008). Liquefaction of starch by enzymatic method has many advantages over other methods as it is safer for both the environment and consumers, and the reaction can be, more specifically controlled under the mild and moderate conditions thus consequently may result in efficient process and fewer by-products.

Industrial production of starch hydrolysis sugars and oligosaccharides such as glucose syrup or substrate for bioethanol processing, basically, involves liquefaction and saccharification processes of starch. Liquefaction and saccharification require the starch granules to be extensively gelatinized at high temperature (above 70ºC). Typically, enzyme hydrolysis of granular starch yields low degree of conversion to fermentable sugars. Although combination of α-amylase and glucoamylase enhances the release of fermentable sugar (glucose) continuously from granular starch. If the degree of conversion of native starch could be further increased, it would be very useful in the industrial process of fermentable sugars for bioethanol and other bioactive molecule productions (Shariffa et al., 2009).

The present study aims to describe the isolation and identification of a fungus producing amylase isolated from cassava root for using in the hydrolysis of starch, and reports the interesting thermo-stable a-amylase activity capable to degrade row starch residue from *mandioca* in a single step.

METHODS

Microorganisms

The filamentous fungi were isolated from *mandioca* roots (*Manihot sculenta*) brought from Belém-Pará- Brazil. Samples of smashed piece from cassava was analysed for microbial composition. Serial dilution of the samples in 9 g l^{-1} sodium chloride solution were used for microbial colonies isolation using PYS-agar (5 g l^{-1} Peptone, 5 g l^{-1} yeast extract, 10 g l^{-1} *mandioca* starch) supplemented with 50 mg l^{-1} chloraphenicol according to (Oguntoyinbo, 2008). Aliquots (0.2 ml) of appropriate dilution were plated in triplicate and incubated for 72h at 30º C. Fungi colonies were isolated and transferred to PYS. The isolated fungi colonies were maintained at 30º C, on PYS plate medium. For long-term storage fungi strains were maintained at 5% glycerol solution at 4ºC. Slices of 3 mm^2 from 5 day-old cultures were inoculated onto PYS plate or into 0.5 ml of cryotube glycerol solution. Grown cultures were maintained at 4ºC.

Screening test for starch degradation on plate agar

The PYS- agar medium contained 1.0% (w/v) mandioca starch, 0.5% (w/v) of peptone (Oxoid), 0.5% (w/v) of yeast extract (Oxoid) and 2.0% (w/v) of agar (Oxoid) was used. A piece of mould pure culture was transferred onto the centre of a plate of PYS-agar medium and incubated at 30ºC for 72 h. Visualization of starch degradation was done by flooding with a 1:3 (lugol solution-Merk: distilled water). Clearing of the typically blue coloration of the starch with iodine indicated starch degradation (Dung at al., 2006; Chen et al., 2005).

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Protein assay
The protein content was measured at 595nm at 30 °C (Bradford, 1972). Bovine Serum Albumin was used as standard.

Preparation of crude extract enzyme
Inoculum of the fungus was transferred into 100 ml Conical flask containing 250 ml or 25 ml of the liquid medium containing 5% peptone (w/v), 5% yeast extract (w/v), 20% (w/v) *mandioca* starch as only carbon source, without pH adjustment. The culture was incubated at 30°C and 160 rpm for 4 days in the darkness. The culture was centrifuged at 10,000g for 10 min at 4°C and the supernatant was dialyzed overnight at 4°C. The crude extract centrifuged and filtrated was dialyzed overnight against distilled water. A 12.000 KDa pore membrane was used. The dialyzed crud extract was used as the enzyme source for the assays (the same procedure was repeated for other fungi when necessary).

Effect of Temperature on the activity and stability of the enzyme crud extract
The stability of the enzyme activity was determined as follows: dialyzed enzyme crude extract was mixed with the 50 mM McIlvaine buffer (pH 5.5). The effect of temperature on α-amylase activity was determined over the range of 30–80°C incubated for 10 min or at 60°C incubated up to 4 hours and at 30 min time intervals, tubes were withdrawn and boiled in a bath for 5 min and placed in an ice-water bath. The remaining amylase activity was determined as previously described.

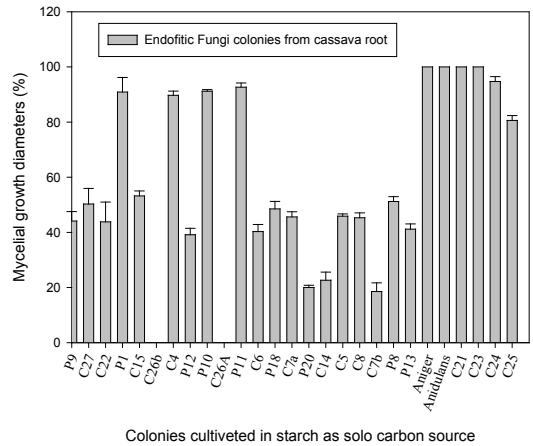
Raw starch hydrolysis
A reaction mixture in a total volume of 500 ml containing 10g of raw starch granules and medium 1 inoculated with C25, maintained in a bottle of 1L, was incubated at 30 °C and 160 rpm for 4 days. 2 ml of aliquots Samples were taken and centrifuged at 3,000 rpm for 3 min at 4°C (to assure not precipitate the gelatinized starch), and the reducing sugar or starch in the supernatant, without interfering particles, was quantified as described previously.

RESULTS
Isolation, identification and screening
Isolation of the fungi from *mandioca* roots was proceeded as follows: Colony counts made on the dilution plates of the bark and pulp from the root suggested (30) different colonies fungi microflora (16 from bark and 9 from pulp) under the conditions of the experiment. The colonies were isolated and cultivated as describe previously. All colonies were submitted to α-amylase assay activity and simultaneously, preserved at glycerol solution 5% at 4°C, as described previously. A total of 23 out of 25 fungi strains (92%) possessed growth development in starch as only carbon source (Figure 1), and good over-producers of amylase activity were selected for further study. *Aspergillus niger and A. nidulans* were use as patterns of growth during seven days incubation. The mycelial growth diameter reached 8.5 cm corresponding to 100%.

The mycelia growth diameter and amylase activity (halo formation with lugol) were assayed at the 7th and 3rd days of incubation, respectively, and colonies P1, P8, P11 and C25 were selected because presented the highest halos of activity. Although growth developments had not reached more than 51.18% for P8.

During growth on agar plate containing starch as substrate, the strains formed a large digesting halo around their colonies after staining with lugol solution showing that theses fungi strains secreted starch-digesting enzymes. The highly halo producing fungi were selected from screening on agar plate.

Figure 1. Development of mycelia fungi growth diameter on agar plate with starch as only carbon source.



Selection of high α-amylase activities strains in liquid medium
Individual colony from selected P1, P8, P11, C15, C24 and C25 was submitted to liquid medium cultivated in conic flask of 100ml containing 25ml of SPY (Starch 20g l^{-1}, Peptone 5g l^{-1}, Yeast extract 5g l^{-1}). Aliquot of 1ml was harvested from the medium and submitted to residual starch analysis after 4 days of incubation. Only P1, P11 and C25 presented 98%, 96% and 100% of starch degrading, and were selected on these conditions, respectively. C24, C15 and C8 showed poorly starch degrading, and a blank was used as control.

The fermented media of P1, P11 and C25 were individually submitted to dialysis and α-amylase activity was assayed as describe previously using 200 μL of dialyzed medium. All three samples were filtrated, centrifuged at 5000 rpm by 10 min, and dialyzed overnight at 4°C. Strain C25 presented the highest activity**.** The protein contents for all strains were of P1=42.5 mg/mL, P11=51.1 mg/mL and C25=27 mg/mL. The amount of starch hydrolysis reached: P1=0.49 g/L, P11=1.18 g/L and C25=1.12 g/L.

The fungal colony C25 was selected and cultivated in 1 liter bottle with 500 ml liquid medium 1 or 2. During the course of starch degradation, C25 cultivated in medium-1 produced a few proteins in the culture (SDS-PAGE, not shown), and little concentration amount when compared with medium 2. This result confirmed that medium-1 was more selective for this kind of amylase activity, and suggested the possibility to use it as adequate for this work, Figure 2. This fungus was identified as *Trichoderma reesei* MIBA001.

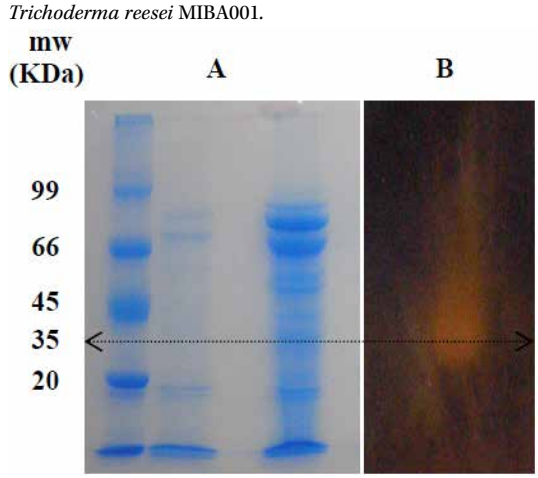


Figure 2. SDS-PAGE and Zymogram of α-amylase present in Crud extract. Figure 2 - A shows the patterns of known mo-

lecular weight (Dalton): 99.000 (Phosphorylase b from rabbit muscle), 66.000 (Albumim from bovine serum), 45.000 (Ovalbumim from chicken egg white), 20.100 (Trypsin inhibitor from soybean). The crud extract was concentrate 40x and applied on gel with 10 and 20 µl, respectively.

Evaluation of α-amylase activity was made at the 4th day of incubation, C25 in medium 1 presented 2% of remaining starch. It was observed that media 2 at the 4th day presented more amount of protein then media1. Although medium 2 lost activity by the time (data not shown) while C25 medium 1 activity was stable. Although C25 showed a better activity for both media-1 and 2, however media-1 presented lower amount of protein, which is provable to have the high specific activity and less interfering proteins. For this reason, medium-1 was selected to cultivate the strain C25 for further study in this work.

Effect of pH on the enzyme activity and stability

The enzyme was optimally active at pH 5.5, and more than 90% of the activity was displayed in the pH range of 4.5–5.5. The enzyme showed the highest activity at 55ºC, Table 5.

Effect of temperature on the enzyme activity and stability

The optimum temperature for the enzyme activity was between 55–65ºC for starch degradation, and decreased sharply above this temperature.

Time stability of the enzyme activity at 60ºC reaction

The enzyme showed optimum degrading starch activity stability at 60ºC of reaction, and maintained 40% of the original activity after 3h of incubation at the same temperature, Figure 3. From these results, the enzyme seemed to have considerable thermostability, which can be favorable in industrial operations.

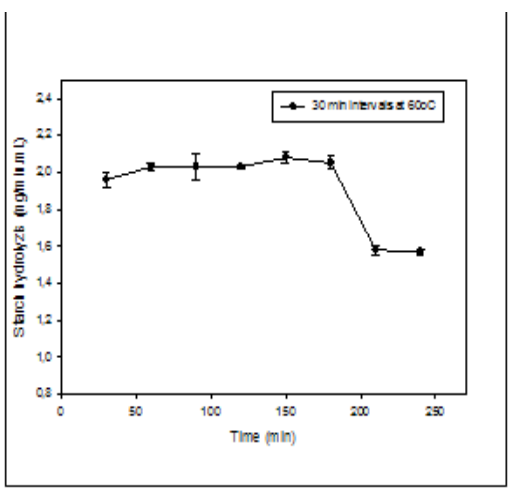


Figure 3. Thermostability of a-amylase activity assayed at pH 5.5 and 60ºC

CASE STUDY

In the present study, amylase produce fungus was evaluated in the hydrolysis of row starch in one-step. High degrading starch is found and the producer was identified as *T. reesei*.

CONCLUSIONS

The present study has yielded a raw starch digesting a-amylase produced by fungi *Trichoderma reesei MIBA001* isolated from pulp cassava. Compared with other α-amylases reported previously, the enzyme in this research performed some differences in properties, which are valuable for industrial starch liquefaction. From the application point of view, this microorganism should be an important producer of this enzyme to be applied as a potential candidate to bring about a complete hydrolysis of high concentration raw starchy *mandioca´s* residue. It was concluded that both organic and inorganic nutrients presented in the media were required for *T. reesei* to produce α-amylase with a level of selectivity and potential to be use in a starchy mandioca residue hydrolysis process.

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