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Vol. 15(28), pp. 1511-1519, 13 July, 2016
DOI: 10.5897/AJB2016.15360
Article Number: 877D12759445
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Characterization of α-amylase produced by the endophytic strain of *Penicillium digitatum* in solid state fermentation (SSF) and submerged fermentation (SMF)

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Received 25 March, 2016; Accepted 10 June, 2016

α-Amylases are enzymes responsible for breaking the α-1,4 bond in polysaccharides with three or more glucose units, occupying the second place in the most widely employed enzymes in industry in the world. The objective of this study was to compare the yields of α-amylase produced by the endophytic fungus, *Penicillium digitatum*, strain D1-FB, isolated from *Baccharis dracunculifolia* D.C. (Asteraceae), through the solid state fermentation (SSM) and submerged fermentation (SmF) processes, in addition to characterizing the produced enzyme. The two fermentations were conducted for 120 h, taking samples every 24 h to obtain the peaks of production. The enzymes were characterized according to their optimal pH and temperature for performance and stability regarding the incubation in the presence of ions, variations in pH and temperature. The maximum yield of the enzyme was observed with SSF, using rice bran as substrate after 72 h of fermentation, with 1,625 U/mL. The α-amylase had an optimal pH at 6.5 and optimal temperature at 37°C. All the ions resulted in a decrease in the activity of α-amylase in the concentration of 5 mM. The enzyme proved to be quite stable in a pH range of 6.0 to 7.5 and up to the temperature of 37°C, but it presented great drops in activity with temperatures above 45°C and in the presence of ions at the concentration of 5 mM.

Key words: *Penicillium digitatum*, α-amylase, starch, enzymes, endophytic.

INTRODUCTION

Starch is a polymer consisting of glucose molecules joined by α-1,4 and α-1,6 bonds. Two polysaccharides comprise the structure of starch: amylose and amylopectin (Figures 1A and B). The first (1A) is a linear molecule containing more than 6000 glucose units connected by glycosidic α-1,4 bonds, and the second (1B), very similar to glycogen (Myers et al., 2000), is highly branched, containing α-1,4 bonds between the of glucose monomers, and α-1,6 in the branching points at each 24-30 glucose residue (Brena et al., 1996).

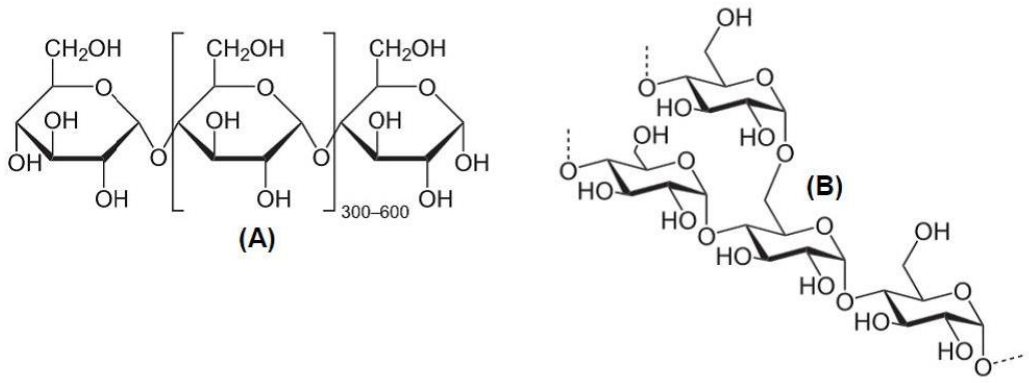

Figure 1. (A) Amylose Molecule. (B) Amylopectin Molecule. Corradini et al. (2005).

Amylases are responsible for the hydrolysis of the starch molecule and are widely distributed in nature. Starch is found mainly in seeds of cereals, such as corn, barley, wheat and rice, and in tubers or roots, such as potatoes and manioc, with the size and shape of the grains being specific for the different cereals (Moraes, 2004).

According to Gupta et al. (2003), amylases are divided into two groups, the endo-amylases and exo-amylases. Endo-amylases catalyze hydrolyses within the starch molecule in various points of the chain simultaneously. This action causes the formation of dextrins and small polymers composed of glucose units of various lengths, breaking the glycosidic α-1,4 bonds present on the inside of the amylose or amylopectin chains. α-Amylase is the most well-known endo-amylase. The exo-amylases known as amiloglicosidases or glicoamylases, hydrolyze glycosidic α-1,4 and α-1,6 bonds (Lorentz, 2005; Onofre et al., 2011, 2012).

Amylases are applied in the most varied industrial sectors that require the hydrolysis of starch, being mainly used in the food industry for the preparation of beers, jellies and to obtain free glucose for the most varied applications (Michelin, 2005).

In addition to the food industry, these enzymes can be used in the formulation of detergents and in the paper, pharmaceutical, fermentation and textiles industries (Oliveira et al., 2007). There are two processes for the production of microbial enzymes: solid state fermentation (FES) and submerged fermentation (SmF). SmF is traditionally used for the production of enzymes because it provides a better control of some important process parameters, such as pH and cell growth, in addition to facilitating the recovery of extracellular enzymes and the determination of biomass (Fernandes, 2006).

One of the main characteristics of solid-state fermentation is the use of substrates with low water activity, in which the conditions for growth are similar to the fungi's natural habitat. This facilitates their growth on the solid substrate and the production of large quantities of enzymes (Paris et al., 2008; Rocha, 2010).

The vast majority of microorganisms used in the solid-state fermentation are filamentous fungi (Silva, 2002). The reduced amount of water in the substrate greatly restricts the number of micro-organisms that are capable of adapting to this process, but fungi prove to be quite tolerant to this environment (Pandey et al., 2005; Fernandes, 2006). The objective of this study was to compare the production of α-amylase produced by the endophytic fungus, *Penicillium digitatum*, strain D1-FB, isolated from *Baccharis dracunculifolia* D.C. (Asteraceae), through the solid state fermentation (SSF) and submerged fermentation (SmF) processes, in addition to characterizing the produced enzyme.

MATERIALS AND METHODS

Micro-organism studied

For the realization of this work, the endophytic fungus *P. digitatum* strain D1-FB was used, isolated from *B. dracunculifolia* D.C. (Asteraceae) maintained in the mycology collection of the Microbiology Laboratory of the Regional Community University of Chapecó – UNOCHAPECÓ – Chapecó – Santa Catarina - Brazil.

Fermentation process for the production of α-amylases

Preparation of the inoculum

After growing the fungus on PDA for seven days, the cells were suspended in a phosphate 100 mM buffer, pH 7.0, and subjected to stirring in order to obtain a homogeneous solution. The cell concentration was determined by counting in a Neubauer chamber (Germano, 2000).

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Preparation of the fermentation media

Two different fermentation processes for the production of amylase were tested: SSF and SmF. The substrate of choice for SSF was rice bran. Each Erlenmeyer flask of 125 mL received 10 g of rice bran (Fa), with the production of the enzyme in pure bran (Fa) being compared with bran supplemented with 20% of manioc starch (Fm).

The moisture content of the media was guaranteed by a phosphate buffer 100 mM, pH 6.5, in the proportion of 65% for the medium containing pure bran, and of around 90% for the medium supplemented with manioc starch. In SmF, the medium was composed of 50 $g.L^{-1}$ of manioc starch, 0.2 $g.L^{-1}$ of $MgSO_4$ and 0.2 $g.L^{-1}$ of $ZnSO_4.7H_2O$, solubilized in a phosphate buffer 100 mM, pH 6.5. Two different sources of nitrogen were tested, one organic (urea) and the other inorganic ($NaNO_2$) at 2.5 $g.L^{-1}$.

The fermentation was conducted in Erlenmeyer flasks of 125 mL containing the medium at 20% of its maximum capacity. 10^8 spores were inoculated in all media. The flasks were incubated in a Shaker at 28°C and 150 rpm. Samples were taken every 24 h for four days for the evaluation of enzyme yields (Onofre et al., 2011; Onofre et al., 2012).

Recovery of the enzyme from the fermented media

For the recovery of the enzyme from the solid fermented medium, 5 mL of NaCl 1% solution was added for each gram of solid substrate and maintained under stirring for 1 h at 100 rpm. For the liquid medium, a solution of NaCl 1% was added until the volume reached 50 mL. After this process, the suspension was filtered to obtain the crude extract, and then centrifuged at 2000 rpm for 8 min, discarding the precipitate.

Enzymatic assay

The activity of α-amylase was determined by measuring the levels of reducing sugars in solution as a result of the action of the α-amylase on the starch. The activity was determined in samples in triplicate by quantifying the reducing sugars (glucose) with the Miller (1959) and Fernandes et al. (2007) method. A mixture containing 0.5 mL of enzymatic extract; 0.5 mL of a starch 0.5% solution in a Tris-HCl buffer 0.05M pH 8.5, and 0.2 mL of the same buffer was incubated at 90°C for 10 min. After this period, 1.0 mL of the Miller reagent (3, 5-dinitrosalicylic acid) was added to the reaction. The mixture was placed in boiling water for 10 min, then cooled in an ice bath for 5 min and 4.8 mL of distilled water was added. The developed color was measured with a SHIMADZU UV-mini 1240 spectrophotometer, using a wavelength of 540 nm. The same procedure was performed with the control, except that the miller reagent (3, 5-dinitrosalicylic acid) was added together with the enzyme to the starch 0.5% solution, and this mixture was placed in boiling water as described above. The content of reducing sugars was determined through a glucose curve. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute per mL from the soluble starch under the test conditions (Onofre et al., 2011; Onofre et al., 2012).

Characterization of the α-amylase

Optimal pH for enzymatic activity

For the assessment of the influence of pH on enzyme activity, the enzymatic assay was conducted according to item 2.3, while varying the addition of buffer solution: pH 5.0 - 6.0 (acetate buffer), 6.5 - 7.5 (phosphate buffer), 8.0 - 8.5 (Tris-HCl buffer) (Thys, 2004).

Optimal temperature for enzymatic activity

For the assessment of the influence of temperature on enzyme activity, the enzymatic assay was conducted according to item 2.3, while only varying the incubation temperature: 10, 20, 30, 37, 45 and 65°C (Silva, 2002).

Influence of ions on enzymatic activity

For the assessment of the influence of ions on enzyme activity the enzyme was incubated for 10 min at room temperature with the ions $CaCl_2$, $BaCl_2$, $HgCl_2$, $FeCl_3$ and $MgCl_2$ at concentrations of 1 and 5 mM, performing the enzymatic assay after this procedure, as described by Giongo (2006).

Thermal stability of α-amylase

The thermal stability of the amylase was tested by incubating the enzyme for 30 min at the temperatures of: 10, 20, 30, 37, 45 and 65°C, performing the enzymatic assay after this procedure as described by Rasiah and Rehm (2009).

Stability of α-amylase at different pH values

The stability of *α*-amylase at different pH values was tested by incubating the enzyme for 30 min in the following buffer solutions (100 mM): pH 5.0 - 6.0 (acetate buffer), 6.5 - 7.5 (phosphate buffer), 8.0 - 8.5 (Tris-HCl buffer), performing the enzymatic assay after this procedure as described by Thys (2004).

RESULTS AND DISCUSSION

Through the analysis of the fermentation processes, one can observe that the endophytic strain of *P. digitatum*, isolated from *B. dracunculifolia* D.C. (Asteraceae) had better results in the solid medium when compared with fermentation in the liquid medium, yielding 1625 U/mL through solid state fermentation in the medium containing only rice bran, while in submerged fermentation these values did not exceed 712 U/mL in the medium containing inorganic nitrogen (Liq/Ino). By comparing the data obtained in this study with those found by Spier (2005) working with *Penicillium* sp., one can see that he obtained similar results as those of this study, since he observed a production of 1690 U/mL of fungal amylases in SSF, with yields surpassing double the activity achieved in SmF. This same behavior was observed by Hu et al. (2013), who found that the fungus *Penicillium* sp. in a semi-solid medium had better results than in submerged fermentation and in a medium supplemented with an inorganic fraction ($NaNO_2$) of nitrogen.

In Figure 2, the enzyme yield data (in total units) in the different media over the course of 120 h at 24 h intervals is presented.

SSF has been described as an excellent option for the growth of the filamentous fungi. In general, fungi have a

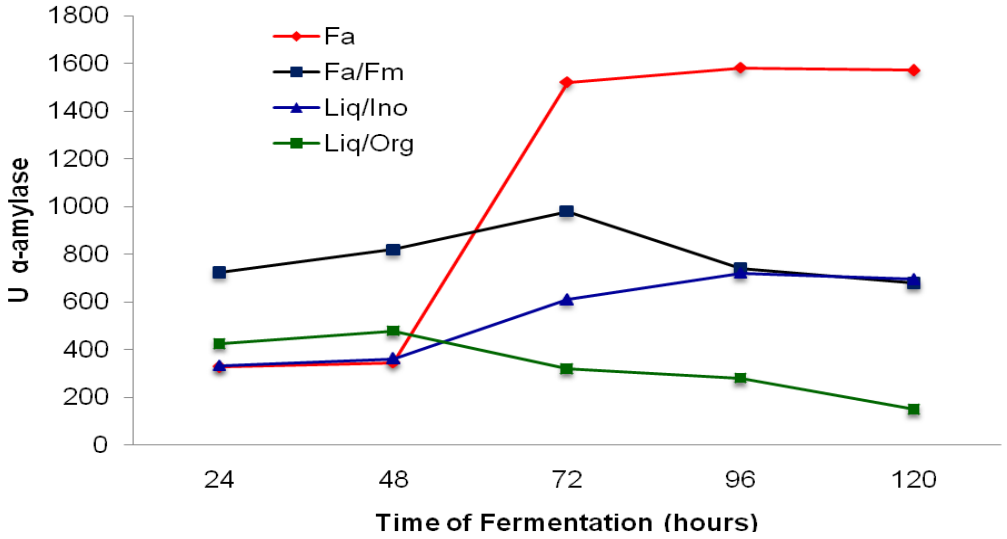

Figure 2. Yield of *α*-amylase as a function of time in solid media containing only rice bran (Fa) and supplemented with manioc starch (Fa/Fm), and in liquid medium with the organic nitrogen source urea (Liq/Org) and the inorganic source $NaNO_2$ (On/Innovation).

good ability to grow on solid substrates, which explains their good adaptation to SSF, and for this reason, they have been widely used in this process, mainly to obtain enzymes (Silva, 2002; Lei et al., 2014).

One of the main characteristics of SSF is the use of substrates with low water activity, in which the conditions for growth are similar to the natural habitat of fungi, which facilitates their growth on the solid substrate and the yield of large quantities of enzymes. The organic matter present in this material is used as a source of energy for growth, and the carbon is used for the synthesis of cellular biomass and of the products of the microbial metabolism (Mitchell and Lonsane, 1992; Hattori et al., 2013; Driss et al., 2014).

It should be noted, however, that the SSF technology should not be seen as a technique that replaces submerged fermentation. In fact, each technique has its own potential and particularities. However, there is a consensus about the need for ongoing research on the factors related to SSF in order to take advantage of the full potential of this technology (Pandey et al., 2001). According to Del Bianchi et al. (2001) and Pandey (2002), the control of certain variables is necessary to obtain products with constant and uniform characteristics. One can therefore state that the observation of these factors, and the correct handling in relation to each one of them, will certainty bring about better results in the solid state fermentation process. Environmental conditions, such as temperature, pH, water activity, oxygen level and the concentration of nutrients and products, significantly affect cell growth and product formation.

When the liquid fermentation media are compared, the fungus can be observed to have adapted better to the inorganic source of nitrogen, showing a maximum yield of 712 U/mL, while in the medium containing organic nitrogen the peak yield of total amylases was 438 U/mL. According to Gupta et al. (2003), organic nitrogen sources are preferred for the production of α-amylase by bacteria. On the other hand, various inorganic salts, such as ammonium sulfate, sodium nitrate and ammonium nitrate, have been reported in improved yields of α-amylase by fungi. Both the solid media had good enzyme yields: 1625 U/mL in the medium containing rice bran, and 932 U/mL in the medium containing rice bran supplemented with manioc starch.

One would expect that the medium supplemented by starch would have higher enzyme yields, since the presence of starch should induce the production of amylolytic enzymes, but this was not the behavior observed. This fact may have occurred due to the presence of starch, which gelatinized after heat treatment (sterilization), making the medium more compact. As such, this made aeration of the solid medium harder. Spier (2005) reported this same limitation with the use of starch in submerged cultivation, noting that enzyme activity decreased with the increase of the concentration of starch in the medium.

Characterization of the enzyme

Optimal pH for enzymatic activity

The *α*--amylase produced by SSF in the medium containing rice bran were used in the tests for characterization of the enzyme. Figures 3 and 4 show the activity of the enzyme as a function of pH and temperature, respectively. The enzyme showed optimal

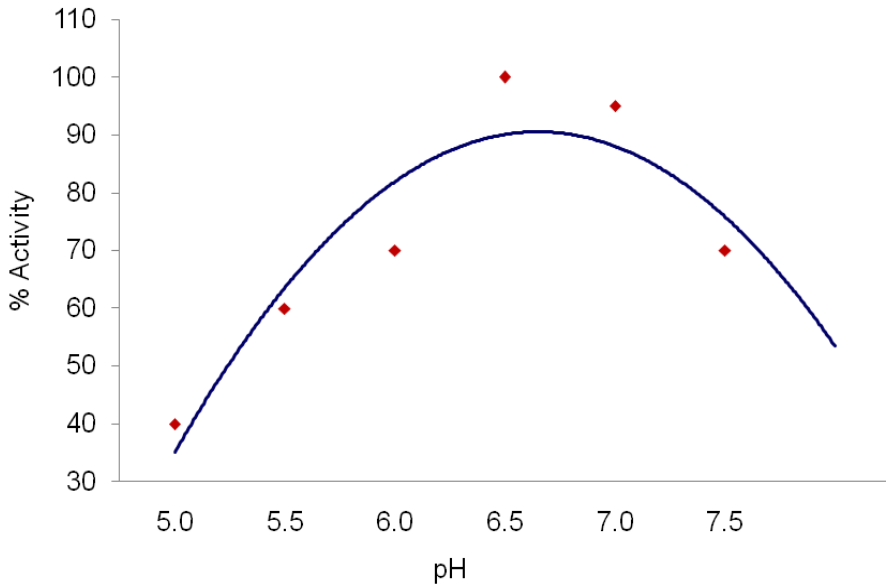

Figure 3. Curve representing the optimal pH for *α*-amylase activity.

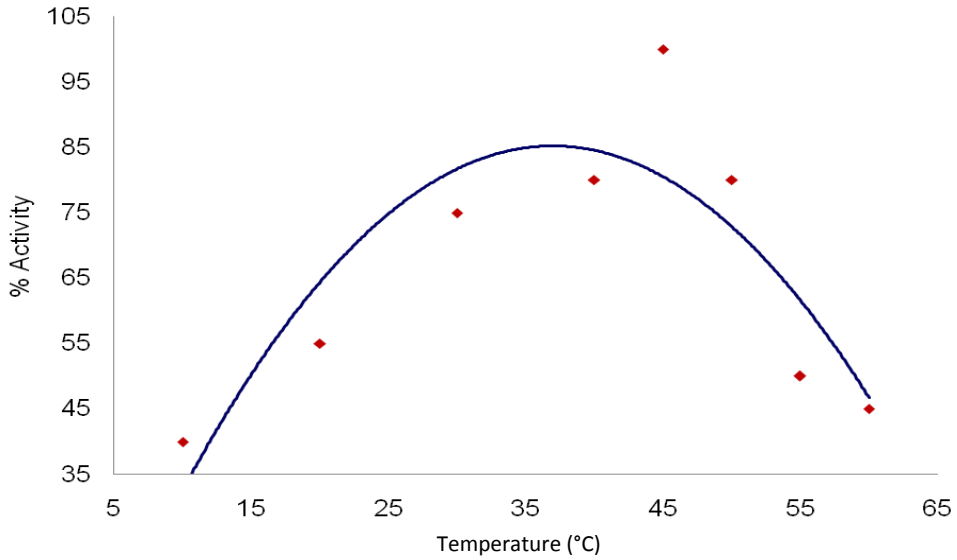

Figure 4. Curve representing the optimal temperature for *α*-amylase activity.

pH at 6.5, while maintaining 96% of its activity at pH 7.0, characterizing it as a neutral *α*-amylase. This data is consistent with Figueira et al. (2000) and Oliveira et al. (2010) who produced amylases with the fungi *Fusarium moniliforme* and *Aspergillus flavus,* obtaining optimal pH values close to 6.9, demonstrating the trend for the production of slightly neutral amylases by filamentous fungi. Spier (2005) on the other hand, obtained a higher yield of amylolytic enzymes with an initial pH equal to 4.0 when working with *Aspergillus niger*, which shows that each species of fungus may have a different behavior in specific pH.

According to Soccol (1992) and Onofre et al. (2011, 2012), the growth capacity of fungi is limited in extreme conditions of acidity and alkalinity. This characteristic is of extreme importance in fermentation processes since they show that under these conditions the vast majority of the bacteria responsible for the contamination of fermentation processes are inhibited. The optimal temperature was 37°C, with the enzyme being completely inactivated at the temperature of 65°C. Spier (2005) obtained similar results to those in this study for a fungal amylase,

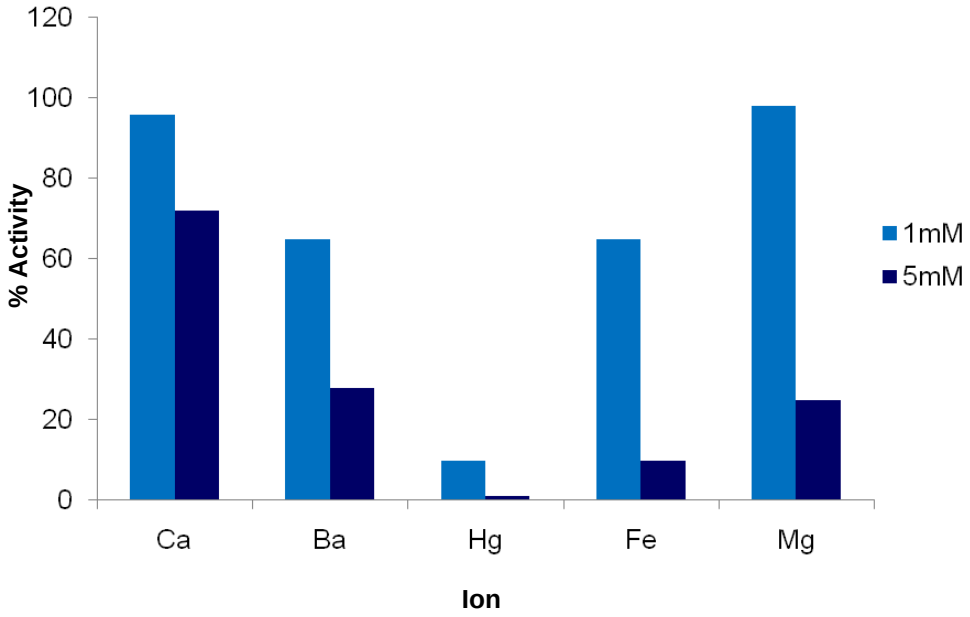

Figure 5. Influence of ions on enzyme activity at the concentrations of 1 and 5 mM.

reporting optimal pH at 6.0 and optimal temperature between 45-55°C. This behavior is also in agreement with Kundu et al. (1973) and Ueno et al. (1987) cited by Pandey et al. (2005), who reported that optimal yields of α-amylase were obtained at temperatures between 30 and 37°C.

Influence of Ions on enzymatic activity

Metal ions have a variety of functions in proteins. These ions may be directly involved in the catalytic process during the enzymatic reaction, or they may participate in electron transfer or redox reactions (Najafi et al., 2005). The stabilization of some enzymes can be induced, mainly, by such divalent ions as Ca^{2+}, Mn^{2+}, Zn^{2+} and Mg^{2+}. If used in low concentrations, these ions can stabilize the tertiary structure of the protein, promoting the formation of cross-links that provide a greater stability to it (Tomazic, 1991). Figure 5 shows the behavior of *α*-amylase regarding the incubation with some ions. All the ions tested at concentrations of 1 and 5 mM were associated with a drop in activity of the enzyme of at least 14%, with the ions Hg^{2+}, at both concentrations, and Fe^{3+}, at the concentration of 5 mM, leading to the complete loss of enzyme activity. The change in activity per ion is a very particular characteristic of each enzyme. Some enzymes may require divalent ions for their activation or as a cofactor, commonly Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+}, increasing their activity. However, some ions may generate a drastic drop in activity, such as Hg^{2+}, Fe^{3+}, and may lead to total inhibition (Giongo, 2006). It should be noted that high concentrations of ions can have an inhibitory effect. Some studies also show that the stability of α--amylase is compromised in the presence of small quantities of these ions (Yang and Liu, 2004; Bernhardsdotter et al., 2005; Hashim et al., 2005).

Thermal stability of α-amylase and at different pH values

pH is one of the most important factors that affect fermentation processes, since it can change the chemicals of the culture medium, ionize polar molecules, affect enzymatic reactions and the post-translation processes of enzymes. The pH of the culture medium can influence microbial growth, and induce the expression of genes that result in changes in phenotypes, such as changes in morphology, physiology or the expression of enzymes. The limitation of growth has been associated with a reduction in the production and/or activity of extracellular enzymes (Madigan et al., 2004). Figures 6 and 7 shows the stability of the enzyme regarding the incubation at different pH values and temperatures, respectively.

In the more extreme pH values tested, *α*-amylase underwent a reduction in enzyme activity of at least 24%, with it being completely inhibited at pH 8.5. In the 6.0 - 7.5 pH range, the enzyme proved to be quite stable, maintaining at least 95% of its activity, with this being the recommended pH range for the application of the enzyme.

Thermal resistance is considered one of the most important criteria for the industrial application of the enzyme, given that most processes require the use of

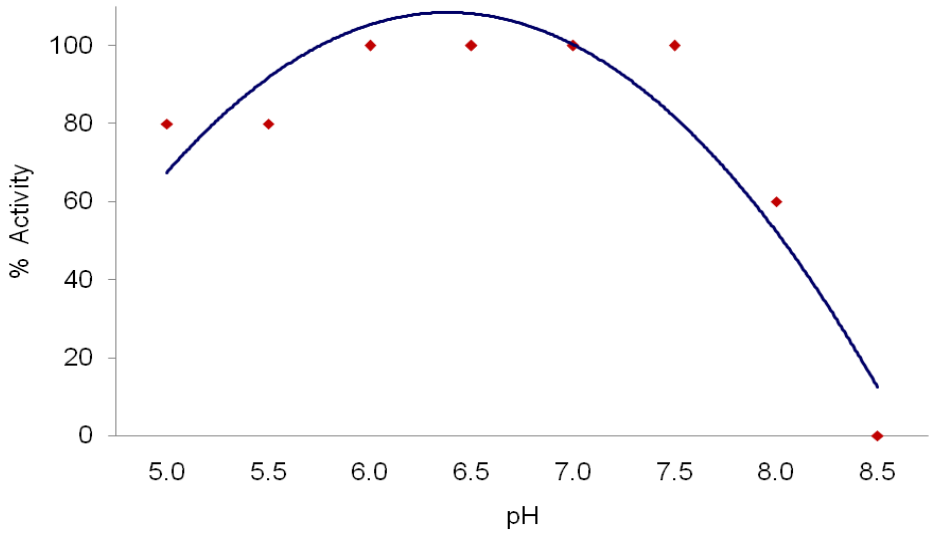

Figure 6. Stability of α-Amylase regarding incubation at different pH values.

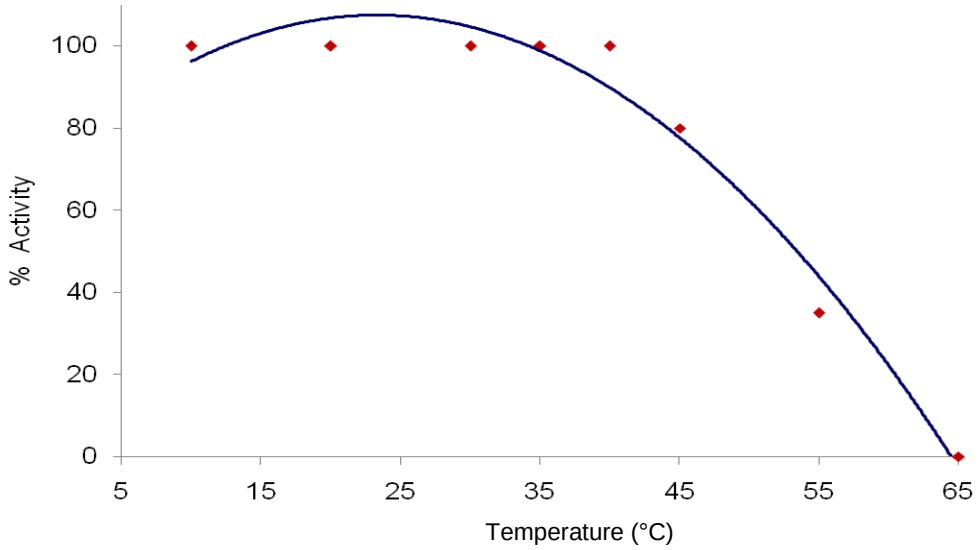

Figure 7. Stability of α-amylase regarding different incubation temperatures.

high temperatures (Ray and Nanda, 1996). The use of high temperatures for the activity of these enzymes, however, depends on the stability limit of the protein, since it will have a range within which its structure is maintained. Outside of this range, denaturation would occur, resulting in a loss of activity. An increase in the use of the α--amylase enzyme in biotechnology has occurred due to its extensive range of working conditions, including high temperatures, extreme pH, and the presence of surfactants and organic solvents (Tanaka and Hoshino, 2002).

When the enzyme was incubated at temperatures of 10 to 37°C, it preserved its full activity. When a temperature of 45°C was employed, the enzyme suffered a decline in residual activity, although this has been its optimal temperature of operation, indicating that the produced *α*-amylase should be applied at temperatures below 45°C to maintain its activity for a longer period of time.

Rasiah and Rehm (2009) obtained amylase which proved quite stable at 85°C. Figueira et al. (2000) produced amylases with the fungi *Fusarium moniliforme* and *A. flavus,* obtaining thermal stability close to 20°C. Fungal amylases are more sensitive to the elevation of temperature, tending to suffer a drastic drop in activity at temperatures above 50°C (Spier, 2005). This behavior was observed and is represented in Figure 6.

Understanding the thermo-stability of enzymes will promote their addition to products that need to go through some form of heat treatment during their processing, while preventing inactivation or with the intentional inactivation of the enzyme when the expected result of its catalytic activity has been obtained.

Conclusion

With the results obtained, it is possible to conclude that the production of α-amylase through SSF was more efficient than the production of the enzyme through SmF, obtaining more than twice the activity found in SmF with SSF, with values of 1625 and 712 U/mL for SSF and SmF, respectively. At 120 h of solid state fermentation using rice bran as substrate, 1625 U/mL was obtained, demonstrating the excellent α-amylase secretion ability of the D1-FB strain of the endophytic fungus, *P. digitatum*, isolated from *B. dracunculifolia* D.C. (Asteraceae). The enzyme characterization tests revealed an optimal pH at 6.5 and an optimal temperature at 45°C. The α-amylase produced was stable in the neutral pH range, but it showed great drops in activity with temperatures above 45°C and in the presence of ions at a concentration of 5 mM. These results allow for the conclusion that the produced α-amylase may be a new enzyme alternative for application in industries that require the saccharification of starch.

Conflict of interests

The authors have not declared any conflicts of interests.

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