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Full Length Research Paper

Amylolytic enzymes produced by the fungus *Colletotrichum gloeosporioides* in rice semi-solid fermentation

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***Amylase*s are enzymes that hydrolyze starch, releasing several products including dextrines and small polymers of glucose units. This work investigated the capacity of the endophytic fungus *Colletotrichum gloeosporioides* of producing the amylolytic enzymes α-*amylase* and *glucoamylase* through fermentation in semi-solid medium of residues of the processing of rice, without supplementation. The α-*amylase* activity was determined according to the methodology of Pandey et al. (2005) and that of *glucoamylase* was determined through the release of reducing sugars, dosed by the DNS method described by Soccol (1992). The results show that the fungus *C. gloeosporioides* showed the highest peaks of enzymatic production at 96 h, where 563.32 U/g of α-*amylase* and 345.30 U/g of *glucoamylase* were obtained at 28°C and pH 5.49. With these data it is concluded that the fungus *C. gloeosporioides* has great capacity of producing amylolytic enzymes (α-*amylase* and *glucoamylase*) through fermentation in rice-based solid state without supplementation.**

Key words: Starch, biotechnology, enzymes, processes, endophytic, rice.

INTRODUCTION

The *amylase*s, which act on starch and polysaccharides to hydrolyze the glycoside bonds α-1,4 and α-1,6, can be divided into α-*amylase*s, which disrupt the inner bonds of the substrate (*endoamylases*); α-*amylase*s, which hydrolyze units from the non-reducing ends of the substrate (*exoamylases*) and *glucoamylases* (*amyloglucosidases*), which release glucose units from the non-reducing end of starch molecules (Gupta et al., 2003; Norouzian et al., 2006).

*Amylase*s have a great importance in biotechnology with a wide spectrum of applications, such as in textile industry, paper and cellulose, leather, detergents, beer, liquor, bread, and children cereals, *liquification* and conversion of starch to sugar, animal chow, fermentation industry (vitamins, amino acids, antibiotics), chemical and pharmaceutical industries (Pandey et al., 1999; Butzen and Haefele, 2008).

Currently, the screening and identification of filamentous fungi capable of secreting extracellular enzymes with biotechnological potential are activities of great importance. The confirmation of the potential for enzyme secretion by a species and the analysis of the conditions of production lead to a possible improvement of the environmental conditions favoring the maximal exploration of this capacity. Therefore, the search for new chemical compounds with biological activity highlights the economical and social importance of this research and its applications in the many areas of interest.

The microbial capacity of hydrolyzing different organic compounds, either natural or synthetic, as well as inorganic, to extract nutritional and energy sources, made possible the employment of these biological agents,

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through sanitary engineering, as a solution to the problems generated by the wastes released on the environment. The outstanding capacity of degradation of microorganisms is the consequence of the evolution of the enzymatic systems of prokaryote and eukaryote cells, which have co-existed with a wide variety of natural substances of diverse origins (Vasconcelos et al., 2003). This diversity of potential substrates for microbial growth resulted, then, in the appearance of enzymes capable of transforming organic molecules of rather distinct structures. This response of the metabolism of certain microorganisms undoubtedly confers some additional advantages to the microbial cells, such as exploration of new ecologic niches and energy sources. In addition, despite the importance given to the *amylase*s, few are the information concerning these enzymes from endophytic fungi, especially their function, molecular and kinetic properties (Spier, 2005).

Despite that traditionally enzymes are produced through submerse fermentation because of the easier control of the process, the fermentation in semi-solid medium has some advantages over the former (Santos et al., 2006), for instance: the culture medium is simpler and less expensive, often made up of non-refined agricultural residues, like the residues from the processing of rice, which are frequently disposed of by the industries; less production of residues by the fermentation; less use of water, preventing the contamination through bacteria that generally demand larger amounts of liquid in the medium; less energy input; easy aeration with greater diffusion of gases; possibility of production of spores and mushrooms, which cannot be obtained through submerse fermentation (Hölker et al., 2004; Kwiatkowski et al., 2006).

Fungi from the genus *Colletotrichum* are ascomycetes found in every environment, especially in association with plants, either as pathogens, symbionts or endophitic. Tavares (2004) described that the conidia of the fungus *Colletotrichum gloesporioides* are salmon-colored, straight, cylindrical, with obtuse apex and truncated base.

Assis et al. (2010) report that *C. gloeosporioides* has a large potential for producing the α-*amylase* and *glucoamylase* enzymes. The *amylase*s are enzymes that hydrolyze starch releasing several products, including dextrines and small polymers of glucose units.

The use of residues of rice processing is desirable because they can represent up to 22% of the grain weight. The inadequate destination of these residues can cause large passive environmental issues for the industries. One of the destinations of these residues can be the feeding of domestic animals, when associated to other nutrients. In this way they become a low-cost substrate for fungal growth and the obtention of enzymes of biotechnological interest.

The present work evaluated the capacity of the endophytic fungus *C. gloeosporioides* of producing the amylolytic enzymes α-*amylase* and *glucoamylase* through fermentation in semi-solid medium composed of residues of the processing of rice.

MATERIALS AND METHODS

Microorganism under study

For this investigation it was used the endophytic fungus *C. gloesporioides*, lineage D4-FB, isolated from *Baccharis dracunculifolia* D.C. (Asteraceae) between 2008 and 2009 and kept in the fungus library of the Laboratory of Microbiology of the Paranaense University– UNIPAR – Unit Campus of Francisco Beltrão – Paraná – Brazil.

Fermentation process

In the production of the enzymes α-*amylase* and *amyloglucosidase*, it was used as the solid fermentation in 250 ml Erlenmeyer flasks. The following culture media was employed for the enzymatic production: 100 ml distilled water for each 50 g of rice residue. The pH was adjusted to 6.8 and this medium was sterilized at 121°C for 15 min. The medium was inoculated with the spore suspension at a ratio of 10^7 spores per gram of rice. After being homogenized and mixed in the Erlenmeyer, the medium was incubated at 28°C for 144 h (Pandey et al., 2005).

Analysis of the fermented substrate

Each 24 h, samples of five grams were mixed with 50 ml of distilled water. This suspension remained under agitation during 30 min. Next, it was filtered to remove the solid debris, yielding a clear extract used for pH determination. The extract was centrifuged at 3000 rpm for 15 min and the supernatant was used to determine the enzymatic activity.

pH

The pH was measured on a suspension obtained after homogenization of five grams of fermented material in 50 ml of distilled water, which was agitated continually for 30 min.

Dosage of the amylolytic activity of the α-*amylase*

The activity of the α-*amylase* was determined measuring the concentration of starch through iodine dosage. One unit of α-□*amylase* is defined as the amount of enzyme capable of hydrolyzing 10 mg of starch in 30 min under the conditions described by Soccol (1992). The calculations for the determination of the α-□*amylase* activity were carried out according to the methodology described by Pandey et al. (2005).

Glucoamylase

The *glucoamylase* activity was determined through the release of reducing sugars, dosed through the DNS method (Miller, 1959) cited by Costa (1996), described by Soccol (1992) and Pandey et al. (2005). The sugars were expressed as glucose equivalents. One unit of *glucoamylase* was defined as the amount of enzyme releasing 1 µmol of reducing sugar (expressed as glucose) per minute under the conditions of the assay (Alazard and Raimbault, 1981), cited by Soccol (1992). The calculations for the

Table 1. Units of enzyme produced during the periods from 24 to 144 h of fermentation, at 28°C.

Time (h)	*α-Amylase* (U/g)*	*Glucoamylase (*U/g)	pH
24	145.95±28.95D#	28.80±10.23E	6.29
48	392.90±23.98B	78.75±18.43D	6.57
72	510.67±35.76A	342.90±15.87A	6.05
96	563.32±45.54A	345.30±14.98A	5.49
120	498.54±43.65B	278.98±36.45B	5.35
144	328.23±28.56C	178.65±30.23C	5.30

*Means followed by capital letters on the column do not differ at the level of 5% by Tukey´s test. #Mean of the activities obtained from triplicates. Mean from the enzymatic activities ± standard deviation.

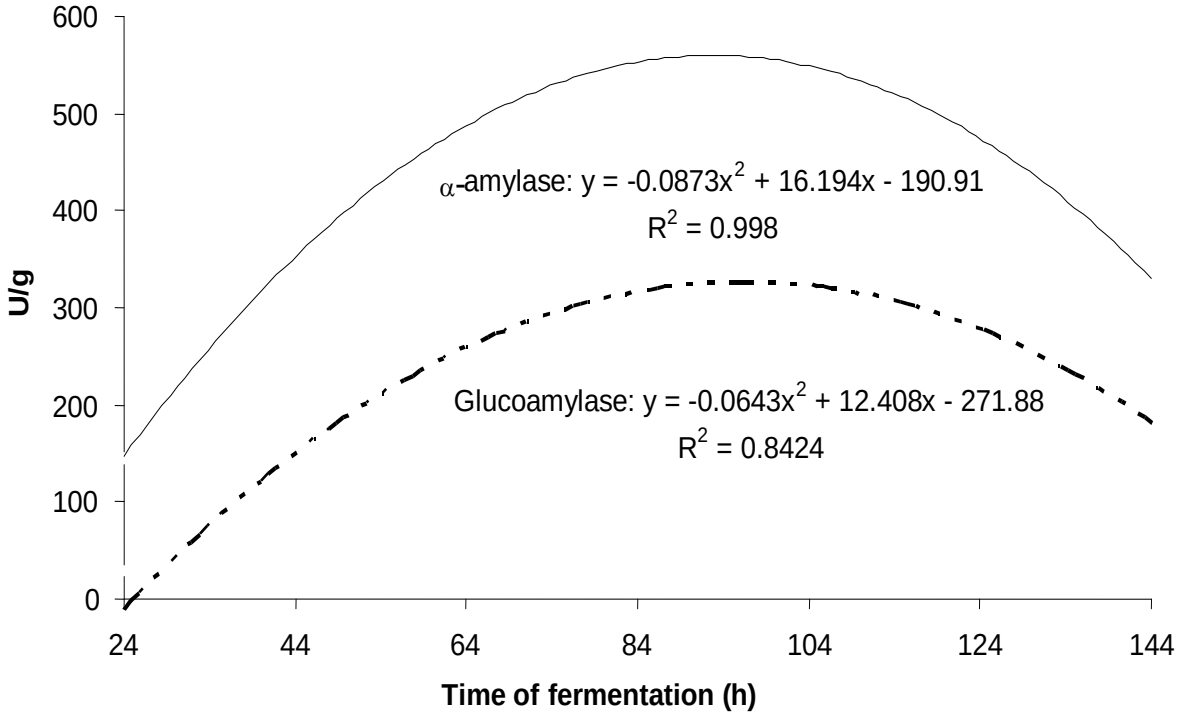

Figure 1. Kinetic of the production of the enzymes α-*amylase and amyloglucosidase* at 28°C, initial pH 6.8, rice-based substrate without supplementation relative to time (hours) and the production of enzymes U/g of substrate.

determination of the α-*amylase* activity were carried out according to the methodology described by Pandey et al. (2005).

Statistical analysis

The statistical analysis was carried out with the Statistical software, version 5.0. The analyses of variance were carried out according to the rules of the ANOVA. The significant differences between the means were determined through Tukey´s test at the level of 5%. All the activities were made in triplicate.

RESULTS AND DISCUSSION

The determination of the enzymatic activity produced by *C. gloeosporioides* was expressed in units of activity (UI – International Units) as the amount of enzyme catalyzing the transformation of 1 µmol of product per minute for each gram of fermented substrate. These data are presented in Table 1 and in Figure 1.

With the data obtained, it is observed that the fungus *C. gloeosporioides* inoculated in rice-based culture medium caused hydrolysis of the substrate in response to the action of the enzymes produced. The high level of starch makes rice an excellent source of carbon, in addition of being an excellent source of starch, indispensable for the synthesis of *amylase*s. Fellows (1994) states the need for the presence of a source of starch to induce the production of *amylase*s by filamentous fungi.

The analysis of the data in Table 1 and Figure 1 reveals that the best time for the production of the two

enzymes was from 72 to 96 h, with peak production of 510.67±35.76 and 342.90±15.87 U/g of fermented substrate, respectively for α-amylase and *glucoamylase*. At 96 h the values were higher but statistically equal to those at 72 h of fermentation at the level of 5% by Tukey´s test: 563.32±45.54 and 345.30±14.98 U/g of fermented substrate for α-amylase and *glucoamylase*, respectively. After this time, the values became lower at 120 and 144 h, this behavior being observed in the expression of both enzymes. At 24 and 48 h, it was observed that the values for both α-*amylase* and *glucoamylase* were increasing, indication a strong gene expression for both enzymes.

As for *glucoamylase*, it is noticed that the initial values at 24 h are statistically lower by Tukey´s test than those obtained at 48 h, because these values were 28.80±10.23 and 78.75±18.43 U/g of fermented substrate for 24 and 48 h, respectively. The results obtained allowed the construction of a curve representing the kinetics of the behavior of the two enzymes during the whole fermentative process. The following variables were considered: rice-based substrate, temperature of 28°C and initial pH 6.8, and the equations representing this behavior were y = -0.0873x2 + 16.194x – 190.91 with R^2 = 0.998 for α-*amylase* and y = -0.0643x2 + 12.408x – 271.88 with R^2 = 0.8424 for *glucoamylase*.

For the mathematical model observed, there is the ratio of the relative expression of the error in the process, which is given by R^2 and was very close to 1.00 in the quantification of α-amylase, indicating a small variation in the increasing expression of the gene for this enzyme during the period of observation. This points to a system with stable control of the variables and efficient process of enzyme quantification (Pfaffl, 2001).

The ratio between the values of R^2 of the gene expressing the production of the enzymes under study normalizes the expression of this gene. If the reference gene has a stable behavior, R^2 will be 1.00 and the value of R^2 can be used for the general analysis of the behavior of the whole process (Pfaffl, 2001). The results obtained reveal that rice was an excellent substrate for the production of amylolytic enzymes, because it induced the fungus *C. gloeosporioides* to express the genes for the two enzymes assayed.

Norouzian et al. (2006) reported that some fungal lineages produce enzymes capable of hydrolyzing starch and releasing glucose. Other authors also state the need of the presence of a source of starch for the induction of *amylase* production by fungi, yeast and bacteria (Fellows, 1994; Pandey et al., 1999; Gupta et al., 2003). The microorganisms do not directly uptake complex molecules such as starch, a polysaccharide. In the absence of another usable component in the medium, the microorganism synthesizes those specific enzymes that degrade the complex substrate to simpler molecules – in this instance *amylase*s – so that these can convert the starch from the rice into usable sugars, thus guaranteeing the growth and development of the microorganism (Gupta et al., 2003).

According to Pandey et al. (1999), some factors can interfere in the gene expression of *amylase*s, such as the accumulation of substrate, which can interfere in the microbial respiration as well as in the aeration of the medium. Spier et al. (2004) reported the importance of empty spaces among particles to facilitate the transfer of gases and heat, of the size of the particles and of the assembly of the fermentation medium on solid substrates, leading to a greater production of *amylase*s through fermentation with fungal cultures. When studying several substrates with or without supplementation for the production of extracellular *amylase*s by *Aspergillus oryzae* through solid fermentation, (Galvez, 2005) obtained *amylase* activity of 255 U/g of starch-based substrate, under the same conditions of this work, after 72 h of fermentation, indicating values inferior to those reported here with the fungus *C. gloeosporioides,* which presented values of 510.67 U/g of substrate, demonstrating that it is an excellent amylolytic fungus.

Another important variable to be assessed is temperature, because it interferes with the optimal conditions for the activity of an enzyme (Lehninger, 2006). In this work temperature was initially set at 28°C. According to Pandey et al. (2005), excellent yields of α-*amylase* were reached with temperatures between 28 and 37°C. This same author obtained the best productions of α-amylase by *Aspergillus niger* at temperatures of 28 to 30°C, coinciding with the conditions of the fermentation process used in this work. The monitoring of the pH is also an important variable concerning enzyme activity, because according to Soccol (1992) and Soccol et al. (2005), the fungus has a limited capacity for growth in extreme conditions of acidity or alkalinity. This characteristic is of extreme importance for the fermentative processes, because they show that under these conditions most of the bacteria responsible for the contamination of the fermentative processes are inhibited.

In this work, initial pH was 6.8. This pH was changed during the process from 6.8 to 6.29 in the first 24 h, reaching 5.49 at 96 h of fermentation and keeping close to 5.30 until the end of the process. It is observed that the best productions of both enzymes were observed at 72 and 96 h – pH 6.05 and 5.49, respectively – indicating that the fungus *C. gloeosporioides* expresses its genes for these amylolytic enzymes in more acidic environments. These results are in accordance with the data obtained by Costa (1996), who studied the influence of pH in the production of *amyloglucosidase* by *A. niger* by solid fermentation in rice bran, and verified an optimal pH of 5.6 for the production of this enzyme. In comparison, the fungus *C. gloeosporioides* has preference for pH 5.5 for the production of α-amylase and *glucoamylase.*

In face of the results obtained, it is verified the capacity

of *C. gloeosporioides* of producing the amylolytic enzymes α-*amylase* and *glucoamylase,* using residues of the processing of rice without supplementation as substrate, under the conditions of this work for solid-state fermentation.

Conclusion

After this investigation, it could be concluded that the fungus *C. gloeosporioides* demonstrated capacity for the production of *amylase*s (α-*amylase* and *glucoamylase)* through rice-based solid-state fermentation without supplementation. The highest activity of α-*amylase* and *glucoamylase* was at 72 and 96 h, with 510.67±35.76 and 563.32±45.54 U/g of substrate for α-*amylase* and 342.90±15.87 and 345.30±14.98 for *glucoamylase*, and was reached at pH of enzymatic incubation of 6.05 and 5.49 and incubation temperature of 28°C.

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