VOLUME-8, ISSUE-3, MARCH-2019 • PRINT ISSN No 2277 - 8160

Original Research Paper

Physiology

IN SEMI-SOLID STATE FERMENTATION α-AMYLASE AND AMYLOGLUCOSIDASE PRODUCTION WITH THE FUNGUS *CYLINDROCLADIUM SP.*

Siddhartha Sankar Dash

Govt approved Permanent Part Time Teacher, Dept. of Physiology, Midnapore College, Midnapore -721101, West Bengal *Corresponding Author

ABSTRACT

We know that Amylases have a wide range of applications and very important. Now a days semi-solid state fermentation is increasingly used for the production of enzymes, since it requires simple technology and costs are very low. In this regard, it is worth emphasizing the importance of rice as a substrate, due to its readily availability and low cost. This study evaluated the ability of the fungus *Cylindrocladium* sp.*how* to produce α-amylase and amyloglucosidase. The studies were carried out with the fungus strain *Cylindrocladium* sp. with a rice-based substrate. The material was kept in an oven at 28 °C for 96 hours and it is measured every 24 hours. The DNS reagent method was adopted for the quantification of amyloglucosidase enzymes, . The α-amylase activity was determined by measuring the concentration of starch using iodine dosage. Analysis of the data obtained in this study showed that the fungus *Cylindrocladium* sp. produces á-amylase and amyloglucosidase enzymes; peak production for α-amylase was at 72 hours with 245.02 U/g, and at 92 hours for amyloglucosidase with 73.58 U/g. Through the interpretation of these results it was possible to conclude that the fungus *Cylindrocladium* sp. produces relevant values of the enzymes α-amylase and amyloglucosidase.

KEYWORDS : Starch, Bioprocess, Biotechnology, Enzymes.

1. Introduction

The use of fungi in bioprocesses has grown in importance due to the production of several enzymes with different physico-chemical characteristics and excellent potential for industrial application. According to Bon *et al.*[1] Brazil's foreign market for enzymes in 2005 was valued at 147.2 million dollars, accounting for just 3.7% of the international market. Imports accounted for 126.6 million dollars (86%) and exports for 20.6 million dollars. Thus, it is important to study and find new species of microorganisms that produce enzymes with biotechnological potential.

The use of fungi in bioprocesses has grown in importance due to the production of several enzymes with different physico-chemical characteristics and excellent potential for industrial application during recent decades. Some of these characteristics include the possibility for large-scale synthesis, as well as the ease with which they are excreted to the external environment[2].

Even though it is still very little known, the microbial biodiversity is responsible for producing hundreds of industrial materials such as vaccines, enzymes, antibiotics, besides being a source of food, representing tens of billions of dollars worldwide[3,4,5].

Amylases are some of the most important industrial enzymes[3] and currently are of great importance in biotechnology; they were first produced at the beginning of the 20th century due to industrial interest in the production of glucose from starchy materials[4]. Among the amylolytic enzymes, the most important is á-amylase because it plays an essential role in the conversion of starch to products with low molecular weight, which can be used by other enzymes of the same group[5].

It is being used as additives in detergents, they can be used in the saccharification of starch and in the food, fermentation, paper and textile industries. With the advent of new biotechnological frontiers, the spectrum of amylase application has expanded to many other areas, including clinical, pharmaceutical, medical and chemical-analytical fields[6].

Amylolytic enzymes are used in the baking industry to give products a larger volume, better color and smooth texture. In the production of glucose and fructose, enzymes are used to hydrolyze the starch molecules. In the paper industry, amylases are used to protect the paper against mechanical damage and improve the finish. Amylases have also been used in foods for infants and added to cereals to lower their viscosity and are used by breweries to produce clear beer[7,8].

For the degradation of the starch molecule amylases are responsible and are widely found in nature. Although amylases can be derived from various sources, including plants, animals and microorganisms, there is a great industrial demand for microbial enzymes[9]. Nowadays, large quantities of microbial amylases are commercially available and hydrolysis of starch has been used inprocessing industries[10, 11].

According to Pandey *et al.*[12], although industrial enzymes of microbial origin are mainly produced by submerged fermentation (SmF), solid state fermentation (SSF) is an important method and is already used in some countries.

The SSF process has great potential for enzyme production, and is of interest because raw fermented solid can be used as a substrate[13]. In addition, the procedures are of particular economic interest for countries with abundant agroindustrial waste and biomass, as this waste can be used as a low cost raw material[14]. One possible substrate, which is easily available and cheap in Brazil, is rice and, in particular, the residues from refining rice.

Several species of *Cylindrocladium* sp. have been detected in Brazil, most of them pathogenic to forest species and agronomic crops of major importance[15]. However, they have also been found in plants, and may be pathogenic, saprophytic, endophytic and epiphytic[16].

The aim of this study was to evaluate the ability of producing α-amylase and amyloglucosidase amylolitic enzymes using solid state fermentation with a rice substrate. In view of the high applicability of amylolytic enzymes and the limited number of studies that use the fungus *Cylindrocladium* sp. for their production.

2. Material and Methods

2.1. Studied Microorganism

This study used the endophytic fungus *Cylindrocladium* sp, strain D8-FB, isolated from *Baccharis dracunculifolia*D.C. (Asteraceae), from 2014 to 2016 and kept in the mycology collection of the Microbiology Laboratory in Midnapore Medical College, west Bengal

2.2. Fermentation Process

For the production of the α-amylase and amyloglucosidase enzymes, solid fermentation was used in 250 mL Erlenmeyer flasks. The medium for the production of the enzymes consisted of 100 mL of distilled water to 50 grams of rice. The pH was adjusted to 6.8 and the medium was autoclaved at 121 ℃ for 15 minutes. This medium

was inoculated with spore suspension at a rate of 10^7 spores per gram of rice. After being homogenized and mixed in the Erlenmeyer flask, it was incubated at 28 °C for 96 hours.

2.3. Analysis of the Fermented Substrate

Five grams of the sample were collected for Every 24 hours, and mixed with 50 mL of distilled water. This suspension was under constant agitation for 30 minutes. It was then filtered to remove solids to yield a clear extract, used for determining the pH. The extract was centrifuged at 3,000 revolutions for 15 minutes and the supernatant was used to determine enzyme activity.

2.4. pH

The pH was measured on a suspension obtained after homogenization of 5 grams of ferment in 50 ml of distilled water, which was constantly agitated for 30 minutes.

2.5. Dosage of α-amylase Amylase Activity

An α-amylase unit is defined as the amount of enzyme capable of hydrolyzing 10 mg of starch in 30 minutes under the conditions described by Soccol[17]. The α-amylase activity was determined by measuring the concentration of starch per dosage of iodine. The calculations for the determination of α-amylase activity were carried out following the methodology described by Pandey *et al.*[12].

2.6. Amyloglucosidase

One unit of amyloglucosidase was defined as the amount of enzyme released by 1 ìmol of reducing sugar (expressed as glucose) per minute under the test conditions. The calculations for the determination of á-amylase activity were carried out following the methodology described by Pandey *et al.[*12].

Amyloglucosidase activity was determined from the release of reducing sugars, measured by the DNS method of the Miller[18] according to Costa[19], described by Soccol,[17] and by Pandey *et al.[*12]. Sugars were expressed as glucose equivalents.

2.7. Statistical Analysis

The statistical analysis was carried out using the program *Statistica,* version 5.0. Analyses of variance were in line with Anova standards. Significant differences between the means were determined by the Tukey's test at a 5% significance level. All activities were carried out in triplicate.

3. Results and Discussion

The following values were found for the production of enzymes after interpreting the results,. These results are presented in Table 1. Table 1 presents the following values for production of á-amylase: 139.87±23.28; 260.03±24.39; 240.30±34.26; 245.02±22.33 and 107.27±23.29 U/g at times of 12, 24, 48, 72 and 96 hours respectively. The production peak for α-amylase occurred at 24 hours, and was statistically similar to the production at 48 and 72 hours. However, there is a decline in enzyme production at 96 hours of fermentation. For amyloglucosidase, the values were: 2.34±1.04; 3.52±1.05; 9.62±3.12; 73.58±8.95 and 12.45±3.32 at 12, 24, 48, 72 and 96 hours, respectively. The yield was statistically similar at 12 and 24 hours, and the peak was at 96 hours (73.58 U/g at a pH of 5.49).

Table 1. Units of enzyme produced during the periods 12-96 hours of fermentation at 28 ℃

Time (hours)	α-amylase (UI/g)*	Amiloglicosidase (UI/g)	pH
12	139.87±23.28B	2.34±1.04C	6.32
24	260.03±24.39A	3.52±1.05C	6.29
48	240.30±34.26A	9.62±3.12B	6.57
72	245.02±22.33A	12.45±3.32B	6.05
96	107.27±23.29B	73.58±8.95A	5.49

* Means followed by the same capital letters in the same column do not differ, at a 5% level using the Tukey test.
± Mean of activities obtained from dosages carried out in triplicate. Mean of enzymatic activities ± standard deviation

Figure 1 shows the kinetic behavior of the fungus Cylindrocladium sp. inoculated onto a semi-solid rice-based medium. Analysis of the kinetic behavior of the two enzymes reveals that they had a polygonal behavior. For á-amylase this behavior was y = -0.0763x2 + 7.6191x + 80.457 and for amyloglucosidase it was: y = 0.0185x2 – 1.2611x + 19.292.

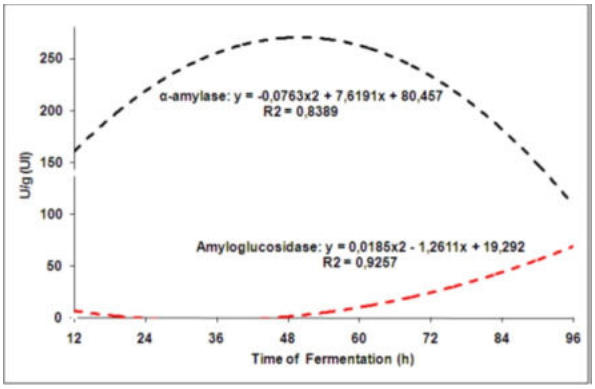

Figure 1. Kinetics of production of the enzymes α-amylase and amyloglucosidase under the conditions of 28 °C, initial pH of 6.8, and a rice-based substrate No studies in the literature were found that use the fungus *Cylindrocladium* sp. in fermentation processes to evaluate its potential in enzyme production; therefore, the results can only be discussed by comparing them with studies of other species of fungi.

This study's results can be compared with those found by Spier[8] for *Rhizopus* sp. and *Aspergillus* sp. using the same fermentation process and a substrate of cassava starch mixed with cane sugar bagasse,as a substrate.Spier obtained yields of 20.38 ± 3.21 and 530.55 ± 11.85 U/g of substrate for á-amylase and for amyloglucosidase the yields were 43.09 ± 9.23 to 770.66 ± 17.22 U/g, respectively for each fungus. The results in this study are lower than those found by Spier[8]. This difference may be due to factors such as temperature, since the culture conditions adopted by Spier[8] were 30 ℃ which is higher than the temperature used in this study (28 ℃). Temperature is an important physical property that can interfere in enzymatic production. Most studies show that the production of amylases by fungi take place within the temperature range of 25-37 ℃ [19].

The study by Paris *et al.*[13] evaluated the behavior of the fungus *Aspergillus casiellus* for the production of amylase (using a substrate of conventional soybean meal, fermentation period of 144 hours and temperature of 28 ℃). They found that this fungus was able to produce 13.73 U/g of amylase, lower than the values obtained in our study. This difference may be related to the substrate used by Paris *et al.*[13], because soybeans contain low levels of starch. According to some authors, it is important that there is a source of starch to induce the production of amylases by fungi[10].

A study carried out by Figueira *et al.[*20] evaluated the amylase activity of *Aspergillus flavus* and *Fusarium moniliforme* in fermentation based on corn broth, at 25 ℃ for 15 days. They had better results for *Aspergillus flavus,* which produced 1,373.21 U/mg, than those obtained in this study; but worse results for *Fusarium moniliforme,* which produced 90.0 U/mg. This difference may be related to the type of substrate and the species of the fungus used in the process.

According Pandey *et al.*[11,12], amylolytic enzymes are also produced by filamentous fungi, are preferred species of *Aspergillus* and *Rhizopus*. Pandey *et al.*[11-14], discloses that species such as *Aspergillus niger, A. oryzae, A. awamori, Fusarum oxysporum, Humicola insolens, Mucor pusillus, Trichoderma viride* are species-producing á-amylase and *Aspergillus niger, A. fumigatus, A. saitri, A. terreus, A. foetidus* and *Rhizopus foetidus*, *A. delemer*species of fungi are used for the production of amyloglucosidase, and Soccol *et al.*[4]

emphasize the amyloglucosidase (glucoamylase), in most cases, is mainly produced by fungi *Aspergillus* sp., *Rhizopus* sp. and *Endomyces* sp.

The results of this study and comparison with the abovementioned authors are evidence that optimizing the fermentation media by adding a source of nutrients to the substrate could lead to better production of fungal amylase.

Finally, this work aims at contributing to the understanding of the interactions endophyte /plant and new perspectives on the biotechnological potential of endophytic microorganisms plants, virtually unexplored in this field, however, as seen, with great potential.

4. Conclusions

After interpreting the results it was concluded that at all the observation periods, the fungus *Cylindrocladium* sp. produced α-amylase and amyloglucosidase. The peak production of α-amylase was at 72 hours with 245.02 ± 22.33 unit for each gram of fermented substrate, while amyloglucosidase peaked at 96 hours with 73.58 ± 8.95 units per gram of substrate.

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