ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 10, Issue, 01, pp. 33249-33251, January, 2020

RESEARCH ARTICLE OPEN ACCESS

EFFECT OF TEMPERATURE AND PH ON AMYLOLYTIC ACTIVITIES OF *BACILLUS ATROPHAEUS* AND *PAENIBACILLUS POLYMIXA* ISOLATED FROM AMAZONIAN SOILS

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ARTICLE INFO

Article History:
Received 17th October, 2019
Received in revised form
03rd November, 2019
Accepted 21st December, 2019
Published online 29th January, 2020

Key Words:

Microbial metabolism, Microbial amylases, Amazonian microrganisms, Thermotolerant amylases.

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ABSTRACT

The "Pro-Alcohol" program in Brazil is based on the use of sugarcane and, since the harvest is carried out once a year, there is a period of inactivity between harvests. As a result, several mills began to use in the off-season, starch producing species for alcohol production. In order to use these plant species, it is necessary the use of α-amylases. The search for thermotolerant amylases in bacteria isolated from Amazonian soils is an important strategy to reduce and even stop importing these enzymes. For this purpose, tests were conducted in solid and liquid media with two previously selected bacteria (*Paenibacillus polymyxa* ITAAM 10M and *Bacillus atrophaeus* ITAA 23M) using a 1% starch solution to detect starch degradation index. Then the enzymatic complexes of these bacteria were tested at different temperatures and pHs. Both bacteria enzymatic complexes were influenced by temperatures and pHs of the cultivation medium. The amylolysis indices of *Bacillus atrophaeus* ITAAM 23M and *Paenibacillus polymyxa* ITAAM 10M were respectively 1.5 and 1.2. The amylases of both bacteria differed in their chemical characteristics and were most active at 100 °C. The highest amylolytic activity demonstrated by amylases was 1.37 U.mL^{-1} and 1.18 U.mL^{-1} respectively for *P. polymyxa* and *B. atrophaeus*.

Citation: ***Cassiane Minelli-Oliveira, Mirna Sayuri Farias Miyamoto, Spartaco Astolfi Filho et al.*** **2020.** "Effect of temperature and ph on amylolytic activities of bacillus atrophaeus and paenibacillus polymixa isolated from amazonian soils", *International Journal of Development Research*, 10, (01), 33249-33251.

INTRODUCTION

Soil microorganisms, mainly fungi and bacteria, are the most common source of α-amylases for commercial purposes. Unlike plant and animal enzymes, microbial enzymes are not dependent on favorable weather conditions as they can be produced in fermentations where cultivation conditions are controlled and electronically monitored (Orlandelli *et al*., 2012). The currently marketed α-amylases are produced by *Bacilllus licheniformis* and *B. amyloliquefaciens* by fermentation processes and are mainly characterized by their high resistance to high temperatures. These enzymes are used in the textile, food, pharmaceutical, beverage, detergent, papermaking industries, and the production of ethanol (Curvelo-Santana *et al*., 2010). In the Brazilian Amazon, there are few studies on enzyme-producing microorganisms of economic importance (Oliveira *et al*., 2006; 2007; 2007b 2010).

Due to the industrial importance of α-amylases, studies have been carried out on enzyme complexes excreted by a strain of *Paenibacillus polymyxa* and one of *Bacillus atrophaeus*, previously tested for their ability to use starch as a carbon source at a temperature of 55 °C. The objective of this research was to obtain thermotolerant enzymes, of great interest for bioindustries, which can be used at high temperatures with less possibility of contamination by thermosensitive microorganisms.

MATERIALS AND METHODS

The strains ITAAM 10 M (*Paenibacillus polymyxa*) and ITAAM 23M (*Bacillus atrophaeus*) were previously isolated from different soils of the state of Amazonas and presented the highest growth rates using the methodology of Oliveira and Magalhães (1999) at a temperature of 55 °C in LB culture medium with 1% starch. Both bacteria were tested for hydrolysis halo formation in the culture medium using the

iodine vapor method to determine the amylolysis index (AI), defined as the ratio of colony diameter to halo diameter. According to the methodology, the iodine granules were placed in empty Petri dishes and, above them, the Petri dishes with the bacterial media grown in 1% starch LB medium, which were opened and turned upside down. Thus, it was possible to verify halo formation from iodine vapor (Hankin and Anagnostakis, 1975). After growing in 1% starch LB liquid medium, the cell number was counted with the aid of the Neubauer chamber, diluting the bacterial suspension with a starchless LB culture medium to a concentration equivalent to 10^6 cells.mL^{-1}. The inoculum was then centrifuged, the cells were discarded and the supernatant containing the amylase enzyme complex was used. Enzyme assays were performed by the Dextrinization method according to the methodology proposed by FUWA (1954), in which an enzyme unit (U) was defined as the amount of enzyme required to hydrolyze 0.1 g of starch. All temperature related tests were performed on a Thermomixer. The enzymatic complexes of the two bacteria were then tested at 20, 30, 40, 50, 60, 70, 80, 90 and 100 ° C for a period of 10 minutes to evaluate their effects on the amylase activities studied. The effects of acidity on the activities of these enzymes were also tested using sodium acetate buffer at pH 3.5, 4.5, 5.5 and 6.5.

RESULTS AND DISCUSSION

The qualitative enzymatic activity of the bacteria was determined by the amylolysis index. In this test (Table 1), it was found that *B. atrophaeus* ITAAM 23M had an index of 1.5 while that of *P. polymyxa* ITAAM 10M was 1.2. This test is widely used to evaluate the activity of amylases and serves as the initial comparison parameter of microorganisms producing these enzymes (Junqueira and Cervelatti (2013). Higher growths in the culture medium indicate higher starch degradation potentials, which can be observed with *P. polymyxa*.

Table 1. Amylolysis Index of both bacteria.

Bactérias	Diameters		AI (hd/cd)
	Halo (hd)	Colony (cd)	
	---------- cm --- ----------		
Paenibacillus polymyxa ITAAM 10M	2.9	2.4	1.2
Bacillus atrophaeus ITAAM 23M	1.8	1.2	1.5

The best temperature for *P. polymyxa* ITAAM 10M amylolytic complex activity was 100 °C (Figure 1), with 1.4 U.mL^{-1} followed by temperatures of 20 and 30 °C with 1.05 U.mL^{-1}. The activity exhibited a continuous decrease from 30 to 60 °C, followed by a gradual increase from 60 to 100 °C. It is possible that their starch reaction sites may be blocked almost entirely at 60 °C due to some special structural modification of the molecules, thus explaining this specific behavior. This assumption, however, requires further investigation. However, it is more likely that there is more than one α-amylase in the bacterial complex. Future experiments must be done to find out how many α-amylases are in the complex, as well as to isolate, purify and to characterize each one of them. However, the commercial use of α-amylase usually does not require purification of the enzymes, but in some industrial sectors, such as in pharmaceutical and clinical economic fields, they must be high purified (Souza and Magalhães, 2010). Enzymes purification also must be used in in studies of structure-function relationships and biochemical properties (Gupta *et al*., 2003).

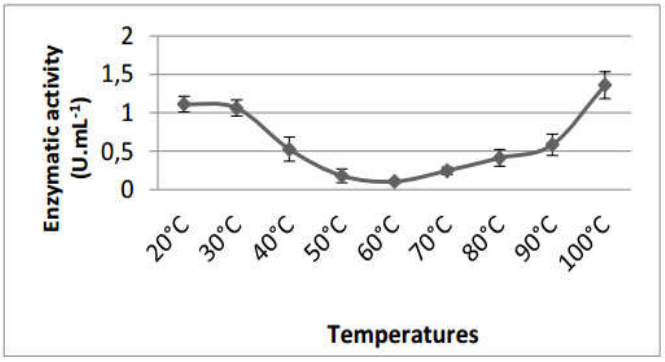

Figure 1. Amylolitic activity of *Bacillus atrophaeus* ITAAM 10M under differents temperatures and pH 6,5

B. atrophaeus ITAAM 23M enzyme (Figure 2) was also more effective at 100 °C, with 0.72 U.mL^{-1} followed by temperatures of 30 and 40 °C with 0.4 U.mL^{-1}, decreasing from 50 to 60 °C. From 60 to 90 °C there was almost zero activity, showing a difference compared to the amylases of the previous bacteria, suggesting they present different structures, not only in amino acid composition and distribution, but also in their spatial conformation. It is possible that in the bacterial extract there is more than one α-amylases, which would explain the activity decrease behavior with temperature increase from 20 to 90 °C, but increase of this activity when the temperature increases from 90 °C to 100 °C. According to Fujimoto *et al*. (1980), amylases have different structures, and *B. subtilis* α-amylase consists of a single polypeptide chain with 26% α-helix, 22% leaf β and dimensions of 35 AX 40 AX 70 A with three domains, characteristics that were observed in other α-amylases. After testing at different temperatures, tests at different pHs and three temperatures were conducted to evaluate the best pHs at each temperature.

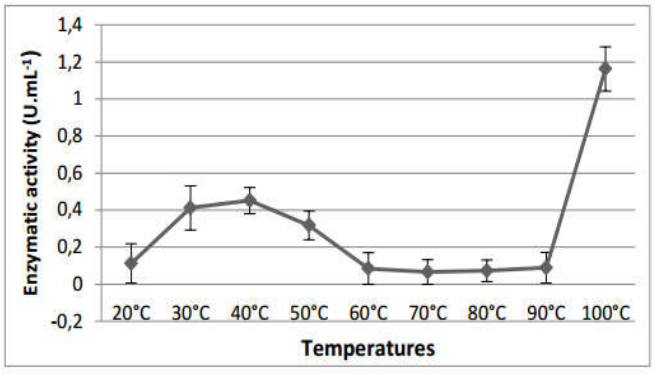

Figure 2. Amylolitic activity of *Bacillus atrophaeus* ITAAM 23M under differents temperatures and pH 6.5

The amylolytic activities of *P. polymyxa* ITAAM 10M (Figure 3) showed better performance at pH 4.5 and temperature of 100 °C, with 1.37 U.mL^{-1}. It was at this temperature that it also showed more activity than at other temperatures at pHs 3.5, 5.5 and 6.5. At 80 °C, amylolytic activity varied very little between pH 3.5 and 6.5. At 90 °C, the highest activity was at pH 3.5, with 0.9 U.mL^{-1}, dropping sharply to pH 4.5, decreasing less intensely to pH 6.5, when it had activity of 0, 1 U.mL^{-1}.

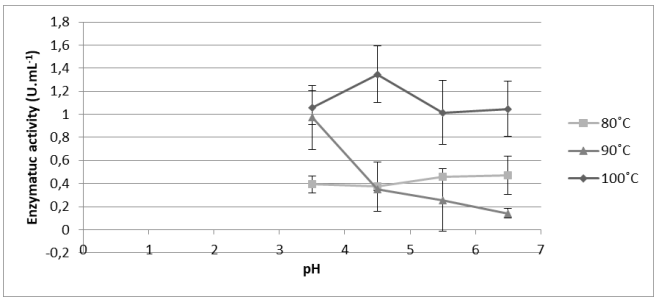

Figure 3. Amylolitic activity of *Paenibacillus polymyxa* ITAAM 10M under differents temperatures and pHs

The amylolytic activity of *B. atrophaeus* ITAAM 23M increased linearly as pH increased from 3.5 to 6.5 (Figure 4), except at 80 °C, when there was very little variation between pHs. This behavior was considerably different from that observed with ITAAM 10M, which means that the enzymes are quite different from each other.

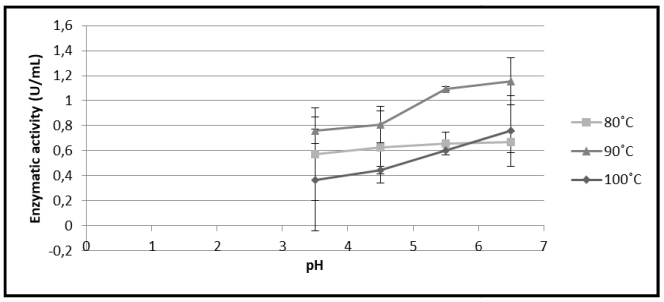

Figure 4. Amylolitic activity of *Bacillus atrophaeus* ITAAM 23M under differents temperatures and pHs

The highest amylolytic activities of ITAAM 23M occurred at 90 °C at all pHs analyzed. Comparing the behavior of these enzymes at temperatures of 80 °C and 100 °C, higher activity values were found at 80 °C at pH 3.5 and 4.5 and lower at 100 °C at pH 6.5. The structural effects of pH and temperature variations on these enzymes are similar to those found in globular proteins, as extreme pH changes may alter the enzyme structure due to charge repulsion (Hogget and Kellet, 1976). Minor changes in pH may result in dissociation of oligomeric enzymes. This is, for example, the case of the isoforms of PI and PII yeast hexokinase, which are dimeric at pHs less than 7.5 and monomeric when the pH is greater than 8.0 (Hogget and Kellet, 1976; Williams and Jones, 1976). In this case, the monomeric form is more active than the dimeric one, but there are cases in which dissociation of the enzyme results in its complete inactivation. On the other hand, changes in pH that do not fully affect the structure of an enzyme may reduce its activity just by affecting catalytic site residues (Thurikill *et al*., 2006).

Conclusions

- The amylolysis indices of *Bacillus atrophaeus* ITAAM 23M and *Paenibacillus polymyxa* ITAAM 10M were respectively 1.5 and 1.2.
- The α-amylases of both bacteria differed in their biological and characteristics.
- The α-amylases of these bacteria were most active at 100 °C.
- The highest amylolytic activity demonstrated by amylases was 1.37 $U.mL^{-1}$ and 1.18 $U.mL^{-1}$ respectively for *P. polymyxa* and *B. atrophaeus*.

Acknowledgments

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

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