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PRESENCE OF A-AMYLASES INRHIZOBACTERIAS ISOLATED FROM AMAZONIAN SOILS

***[1]Cassiane Minelli-Oliveira, [2]Mirna Sayuri Farias Miyamoto, [1]Spartaco Astolfi Filho, [2]Luiz Antonio de Oliveira and [1]José Odair Pereira**

[1]Universidade Federal do Amazonas. Av. General Rodrigo Octávio, 6200, Coroado I. CEP: 69077-000,
[2]Instituto Nacional de Pesquisas da Amazônia. Av. André Araújo, 2936, CEP: 69067-375,
Manaus, Amazonas, Brazil

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***Corresponding author:**
Cassiane Minelli-Oliveira

ABSTRACT

Several bacteria found in nature produce α-α-amylases. This research evaluated the presence of thermotolerant α-amylases in rhizobacteria isolated from legume nodules to use for the conversion of starch from amylaceous plants. Forty rhizobacteria were tested for the presence of these α-amylases, as well as for the thermotolerance characteristic. Nineteen bacteria produced an amylolytic halo and eleven were showed to be thermophilic. As they are not pathogenic to animals and plants and are thermotolerant, these rhizobacteria are potential and safe sources to produce α-amylases for use in the bioindustry. The α-amylases of the rhizobacteria INPA R001 (*Bradyrhizobium*sp) and INPA R020 (*Paenibacillus*sp) showed the highest rates of amylolysis.

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INTRODUCTION

Alpha-amylases (EC 3.2.1.1) can convert starch into by-products that can later be converted into alcohol in the fermentation process or else into other products of economic value. In nature, one of the main sources of α-amylase production is microorganisms, especially those present in soils. Programs to select new microbial sources for the production of enzymes are underway in all parts of the planet. Some examples are researches with nitrogenases, and hydrogenases present in rhizobacteria commonly referred to as rhizobia. These enzymes participate in the process of biological fixation of atmospheric nitrogen and, therefore, are of biotechnological interest in agronomic and forestry. However, we cannot neglect the potential of using these bacteria as sources of other enzymes of recognized industrial value, such as α-amylases, lipases, pectinases, phosphatases, nitrate reductase, citrase, tryptophanase, catalase, gelatinase, urease, caseinase, lysine decarboxylase and proteases (Oliveira *et al*., 2007; Chagas Jr *et al*., 2010; Kumari *et al*., 2010; Ahemad and Kibret, 2014). According to Li *et al*. (2013) and Gianfreda (2015), these enzymes play an important role in the rhizosphere environment of plants, as they contribute to altering the availability of nutrients by mineralizing organic compounds with the release of these elements in the soil, making them available to the plants. Enzymes and proteins can also be important for the nodulation process in legumes, according to studies by Robledo *et al*. (2012) and Salavati *et al*. (2012 a, b, c, 2013). Thus, the enzyme complex present in rhizobacteria can serve as a raw material for use in industries that deal with bioproducts and, because they are non-pathogenic to plants and animals, make the production process less costly, being safe and cheaper sources for these bioindustries. In view of this, the present study was carried out to assess the presence of α-amylases and characterize some properties of rhizobacterial isolates. In this research, activity at high temperatures was considered a desirable characteristic for bioindustries, which can use them at higher temperatures, free from most microbial contamination.

MATERIAL AND METHODS

Forty rhizobacteria isolates obtained from the Rhizobia Collection of the Laboratory of Ecology and Biotechnology of

Microorganisms of the Amazon/ INPA were tested for growth in a LB liquid medium (Sezonov*et al*., 2007) with 1% of starch, according to the methodology of Oliveira and Magalhães (1999).In addition, the presence of α-amylases was verified, using iodine vapor as an indicator of the presence of starch (Kopachena *et al*., 2008). A soft yellow zone around the colony in contrast to the blue medium indicated amylolytic activity of the bacteria (Buzzini and Martini, 2002). The bacteria that grew in a medium containing starch and that showed amylolytic activity were tested at a temperature of 55 °C for 48 hours. Thermotolerantsbacteria were tested by the iodine and turbidity tests in liquid medium containing starch, being evaluated on the day of the inoculum and, with 1, 2 and 3 days of incubation.

Table 1. Growth, amylase production and thermophilic sensitivity of rhizobacteria in starch medium

Rhizobacterias	Days[1]				Amylase[2]	Termophilic[3]
	3	6	9	12		
INPA R156	3.13	3.38	3.56	3.69	2	1
INPA R028	3.19	3.25	3.25	3.25	2	2
INPA R012	2.63	2.88	2.88	2.88	2	2
INPA R034	3.56	3.56	3.56	3.56	2	2
INPA R007	3.44	3.44	3.75	3.75	2	2
INPA R020	3.69	3.75	3.75	3.75	2	2
INPA R054	4	4	4	4	2	1
INPA R015	3.5	3.75	3.75	3.75	2	2
INPA R001	2.67	3.5	3.58	3.92	2	2
INPA R026	4	4	4	4	2	2
INPA R046	4	4	4	4	2	2
INPA R152	4	4	4	4	2	1
INPA R572	1.43	1.5	1.56	1.56	2	1
INPA R214	4	4	4	4	2	1
INPA R017	4	4	4	4	2	1
INPA R554	1	1	1	1	1	1
INPA R159	1	1.19	1.31	1.31	1	1
INPA R552	1	1	1	1	1	1
INPA R582	1.25	1.38	1.38	1.38	1	1
INPA R584	1	1.25	1.25	1.25	1	1
INPA R579	1.25	1.38	1.38	1.38	1	1
INPA R600	4	4	4	4	2	2
INPA R014	4	4	4	4	2	2
INPA R603	1.13	1.25	1.25	1.25	1	1
INPA R606	1	1	1	1	1	1
INPA R583	1.13	1.25	1.25	1.25	1	1
INPA R580	1.13	1.44	1.44	1.44	1	1
INPA R602	1	1.13	1.13	1.13	1	1
INPA R560	1.13	1.31	1.31	1.31	1	1
INPA R172	4	4	4	4	2	1
INPA R569	1	1.13	1.13	1.13	1	1
INPA R595	1	1.25	1.25	1.25	1	1
INPA R553	1	1	1	1	1	1
INPA R584	1.13	1.31	1.31	1.31	1	1
INPA R549	1	1.31	1.31	1.31	1	1
INPA R598	1	1.13	1.13	1.13	1	1
INPA R562	1.25	1.5	1.5	1.5	1	1
INPA R583	1.06	1.38	1.38	1.38	1	1
INPA R589	1	1	1	1	1	1
INPA R035	4	4	4	4	2	1

[(1)]Notes according to Oliveira and Magalhães (1999). [(2)] 1- No halo production 2- With halo production. (3) 1 - did not grow; 2 - grew.

Then, the halo formation test was carried out in the culture medium using iodine vapor to determine the amylolysis index (AI), defined as the ratio between the diameter of the colony and the diameter of the halo.

RESULTS AND DISCUSSION

Of the 40 rhizobacteria tested in the medium with starch as a carbon source, 17 showed values above 3.06, which according to Oliveira and Magalhães (1999), indicate high growths in the culture medium. The rhizobacteria INPA R012 showed a value between 2.06 and 3.0, being considered of medium growth, and 22 rhizobacteria showed values below 2.0 (Table 1), indicating low growth or absence of visual growth (value 1.0) according to Oliveira and Magalhães (1999). The best rhizobacteria from this test were INPA R054, INPA R026, INPA R046, INPA R152, INPA R214, INPA R017, INPA R600, INPA R014, INPA R172 and INPA R035, which had a value of 4.0 on the third day of growth. According to Oliveira and Magalhães (1999), microorganisms that present high values after three days of growth are the ones that are best adapted to the conditions of the medium environment, indicating, in the present case, that they are using starch more efficiently for their growth. The rhizobacteria INPA R156, INPA R028, INPA R034, INPA R007, INPA R020, INPA R015 and INPA R001 showed values between 3.06 to 3.90 up to 12 days of observation, which also shows them to be of excellent growth using the starch for their growth. These results reinforce those obtained by Oliveira *et al*. (2007), who also found amylolytic activity in rhizobia isolated from Amazonian soils, thus showing the potential that these bacteria present as suppliers of this enzyme of biotechnological interest. Because they are not pathogenic to plants and animals, these rhizobacteria can be a safe source for obtaining α-amylases for use in bioindustries. Regarding the production of α-amylase, it was observed that approximately 47.5% presented formation of a starch degradation halo using the iodine vapor method (Table 1). Table 1 also shows that all negative α-amylase rhizobacteria showed little (reached maximum growth values of 1.44) or no visual growth in the

Table 2. Iodine and turbidity tests in starch medium

Rhizobacterias	Iodinetest[a]				Turbiditytest[b]			
	Daysafterinoculum				Daysafterinoculum			
INPA R028	0	1	2	3	0	1	2	3
INPA R001	1	1	1	4	1	1	2	4
INPA R012	1	1	3	4	1	1	3	4
INPA R034	1	1	2	3	1	1	3	3
INPA R007	1	1	3	3	1	1	2	3
INPA R020	1	1	2	3	1	2	3	3
INPA R015	1	1	2	4	1	1	3	4
INPA R026	1	1	3	4	1	1	2	3
INPA R046	1	1	2	3	1	1	2	3
INPA R600	1	1	3	3	1	1	2	3
INPA R014	1	1	3	4	1	2	3	3
INPA R028	1	1	3	3	1	1	3	3

a - 1 dark blue; 2 - bluish purple; 3 - light purple; 4 - gray; 5 - yellow.
b - 1- cloudy; 2 - less cloudy; 3 - slightly cloudy; 4 - not cloudy

Table 3. Amylolysis Index of therhizobacterias

Rhizobacterias	Diameter		AI (dh/dc)
	Halo (dh)	Colony (dc)	
	---------- cm ----------		
INPA R028	1.3	1.0	1.3
INPA R001	1.4	0.5	2.8
INPA R012	1.0	0.7	1.4
INPA R034	0.8	0.6	1.3
INPA R007	0.8	0.5	1.6
INPA R020	1.2	0.5	2.4
INPA R015	0.9	0.6	1.5
INPA R026	0.8	0.5	1.6
INPA R046	1.0	0.8	1.2
INPA R600	1.5	1.0	1.5
INPA R014	1.1	0.8	1.4

medium (grade 1.0), confirming that their deficiencies in using starch as a source of carbon hindered their growth in this culture medium. According to López-López *et al*. (2015), thermostable enzymes, in general, have advantages for application in industry, since biotechnological processes conducted at high temperatures have a significantly reduced risk of contamination by mesophilic microorganisms, which are the majority in an industrial environment. In addition, they can increase the reaction speed due to a decrease in viscosity and an increase in the diffusion coefficient and substrate solubility (Andualema and Gessesse, 2012). Therefore, the interest in testing the growth of microorganisms at elevated temperatures. The results showed that 27.5% of the tested bacteria grew under a temperature of 55 °C, being considered thermophilic (Table 1). Considering the growth values in the medium and tolerance to high temperatures, it can be inferred that the most promising rhizobacteria as α-amylase suppliers are: INPA R028, INPA R012, INPA R034, INPA R007, INPA R020, INPA R015, INPA R001, INPA R026, INPA R046, INPA R600 and INPA R014. It was found that no bacteria had amylolytic activity that resulted in the appearance of yellow color during the experiment. However, after three days of observation, five showed a gray color and six a light purple color, which indicates a high activity of their enzymes (Table 2). By the sequence of colors and numbers (1-5) given, blue (1) represents absence of amylolysis and yellow (5), high enzymatic activity. As for turbidity, visually, a maximum activity value (note 4) was observed with three days of observation with three of the rhizobacteria (INPA R028, INPA R001, INPA R020), with the other eight showing note 3 (Table 2). Due to this methodology and observed numerical sequence (1-4), cloudy (1) represents absence of amylolysis and non-cloudy (4), high enzymatic activity, indicating that these bacteria showed high enzymatic activities.

The verification of the visual enzymatic activity of rhizobacteria considered thermophilic was identified by the amylolysis index (AI), determined through the relationship between the diameter of the halo and the diameter of the colony (Hankin and Anagnostakis, 1975). In this test it was found that the rhizobacteria INPA R001 showed the highest value, with an index of 2.8, followed by the rhizobacteria INPA R020 with 2.4, INPA R007 and INPA R026 with 1.6 and INPA R015, INPA R600 with 1.5 (Table 3). Comparing this experiment with the one carried out by Oliveira *et al*. (2007), who also used the Enzyme Index (EI) as a reference, where the ratio between the diameter of the hydrolysis halo and the diameter of the colony (expressed as enzyme index - IE) ≥ 2.0 indicates good production of extracellular enzymes, it was possible to verify that the tested rhizobacteria presented similar results and that INPA R001 and INPA R020 presented values greater than 2.0, showing the higher enzymatic activities among all them.

Conclusions

Of the 40 rhizobacteria isolates, 17 grew well in culture medium containing starch, 19 produced amylolytic halo and 11 were shown to be thermophilic. As they are not pathogenic to animals and plants and are thermotolerant, these rhizobacteria are potential and safe sources to produce α-amylases for use in the bioindustry. The α-amylases of the rhizobacteria INPA R001 (*Bradyrhizobium*sp) and INPA R020 (*Paenibacillus*sp) showed the highest rates of amylolysis.

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