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Articles

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Producción de amilasas por cepas de hongos anamorfos aislados de la hojarasca de *Quercus* sp

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Abstract: Anamorphic fungi is a group of microorganisms of great importance since they produce a wide variety of substances as part of their metabolism, as well as enzymes useful in the degradation of different substances. Some of these enzymes are amylases, which degrade starch to dextrin, maltose or free glucose. Therefore, having industrial application for the potential of 40 strains of anamorphic from *Quercus* sp, lead litter collected in the Municipal Regional Park Astillero Municipal de Tecpán, in Chimaltenango (N 14°46'48.81", O 91°0'27.42") and the Ecological Park Senderos de Alux, in San Lucas Sacatepéquez (N 14°36'43.41", O 90°38'15.92"). The production of amylases was evaluated through the index of enzymatic activity, which was evidenced by the detection of starch agar degradation halo on starch agar, as well as by the measurement of the amylolytic activity of the enzyme extracts. Of the strains evaluated, 37 (92.5%) produced amylases. The amylolytic activities of the anamorphic fungi native strains correspond with those reported for species of industrial use. The strain that showed the highest amylolytic activity (625 [13.09] UA/dl) was *Virgaria nigra* SL12517, which is similar to that reported for other fungi used in industrial processes. This shows the amylolytic potential of anamorphic fungi of *Querqus* sp leaf litter.

Keywords: enzymes, degradation, amylolytic capacity.

Resumen: *Los hongos anamorfos son un grupo de microorganismos de gran importancia ya que producen una amplia variedad de sustancias como parte de su metabolismo secundario, así como enzimas útiles en la degradación de diferentes sustratos. Algunas de estas enzimas son las amilasas, las cuales degradan el almidón a dextrina, maltosa o glucosa libre, por lo que tienen aplicación a nivel industrial en la fabricación de detergentes y textiles y en la producción de alimentos. En este estudio se evaluó el potencial de 40 cepas de hongo anamorfos para la producción de amilasas, las cuales fueron obtenidas a partir de hojarasca de Querqus sp del Astillero Municipal de Tecpán-Guatemala (N 14°46'48.81", O 91°0'27.42") y del Parque Ecológico Senderos de Alux, San Lucas Sacatepéquez (N 14°36'43.41", O 90°38'15.92"). La producción de amilasas se midió cualitativamente a través del índice de actividad enzimática, evidenciado por la detección de halos de degradación en agar almidón, y se cuantifico espectrofotométricamente con la medición de la actividad amilolítica de los extractos enzimáticos. De las cepas evaluadas 37 (92.5%) produjeron amilasas. Las*

actividades amilolíticas de las cepas nativas de hongos anamorfos coincidieron con las reportadas para especies de uso industrial. La cepa que presentó la mayor actividad amilolítica (625[13.09] UA/dl) fue Virgaria nigra SL12517, la cual es similar a la reportada en la literatura para otros hongos utilizados en procesos industriales. Los resultados de este estudio muestran un considerable potencial amilolítico en hongos anamorfos de la hojarasca de Querqus sp.
Palabras clave: *enzimas, degradación, capacidad amilolítica.*

Introduction

Amylases is a group of hydrolases that can break the α-glycosidic bond of starch. Amylases are also widely distributed in nature. They are found in plants, animal and microorganisms in which they have a nutritional function, since they allow the digestion of carbohydrates. This enzyme is divided in three important types: α-amylase (1,4-α-D-glucanglucanohydrolase), ß-amylase (1,4-α-D-glucano-maltohydrolase), and γ-amylase (1,4-α-D-glucanoglucohydrolase) (Ramachandran et al., 2004). Fungi mainly produce α-amylase, which randomly breaks the 1,4 α-D-glycosidic bond between adjacent glucose units in the linear amylose chain (Saleem & Ebrahim, 2013). Fungal α-amylase constitutes 25% of the worldwide enzyme market and is widely used in biotechnological processes, since it has the advantage of being profitable and it eases the modification and optimization of production processes (Karnwal & Nigam, 2013). This is the reason why α-amylase is useful in the food, beer, pancreatic enzymes, textiles and detergents industry (Gudynaite-Savitch & White, 2016).

Fungal amylases for industrial purpose are produced from fungi such as *Aspergillus*, *Penicillium* and *Rhizopus* isolated from substrates rich in starch (Pandey, Soccol & Mitchell, 2000). However, anamorphic fungi are also a group of interest, due to their capacity of degrading complex substrates such as leaf litter, which is composed mainly by cellulose, lignin and starch. Said capacity exists due to the potential that fungi have to produce enzymes such as cellulose, laccase and amylase (Gamboa & García, 2008; Rubbo & Kiesecker, 2004).

In spite of the biotechnological potential that the anamorphic fungi have for the production of enzymes, in Guatemala, there are not studies related to this, reason why the main objective of this research was to analyze the capacity of 40 strains of native saprotrophic anamorphic fungi for the production of α-amylase. The production capacity of this enzymes was measured qualitatively through the enzymatic activity rate, demonstrated by the detection of degradation halos in the starch agar. It was quantified by means of spectrophotometry, measuring the amylolytic activity of the enzymatic extracts obtained from wheat bran. It should be mentioned that the enzymes production at local level is important, mainly for the food, beer, textiles and detergents industry, since it will eliminate the importation costs from said enzymes. In addition to that, this study shows the capacity that the native anamorphic fungi have for the production of α-amylase.

Materials and Methods

Origin of the Strains:

We used 40 strains of native anamorphic fungi isolated from the leaf litter of Quercus sp from the Astillero Municipal de Tecpán-Guatemala, Chimaltenango (N 14° 46' 48.81", = 91° 0' 27.42") and from the Ecological Park Senderos de Alux, San Lucas Sacatepéquez, Sacatepéquez (N 14° 36' 43.41", O 90° 38' 15.92"), which are stored in the strain collection of saprotrophic and mycorrhizal fungi of the Microbiology Department of the Faculty of Chemistry and Pharmaceutical Sciences of the University of San Carlos of Guatemala. We made axenic cultures from the strains in potato dextrose agar (PDA), which were incubated for 15 days at 25 °C. After his, we took circles with 0.5 cm diameter from the mycelium of each one of the strains, and they were transferred in sterility conditions to starch agar at 1.0% for their adaptation and biomass production for the later assays (Lagunes et al., 2015).

Amylase Production in Starch Agar:

Fungal strains were inoculated once again in the starch agar medium and were incubated at 25 °C for 18 days. The amylase production was detected by the information of the hydrolysis halo formed around the colonies, by means of the addition of iodine in potassium iodide (0.026% I2 + 0.26% KI) as revealer. The enzymatic activity rate was estimated by means of the formula. We made five repetitions for each fungal strain (Pandey et al., 2000).

Amylase Production by Fermentation in Solid Phase and Enzymes Extraction:

From the fungal strains developed in Petri dishes with starch agar, we made a spore suspension or mycelium in Tween 80 at 0.01%. Each suspension was inoculated in 100 ml thread jars containing wheat bran (prepared with 10 g of wheat bran adding 5 ml of distilled water and sterilized for 15 minutes at 121 °C and 1 atm) and then incubated at 28 °C for seven days for the amylase production. The enzymes were extracted by adding 50 ml of an aqueous solution of NaCl at 1.0% to the jars with wheat bran. Then they were shaken in an orbital shaking incubator for 30 minutes. The obtained extracts were filtered and stored at 5 °C (Pandey et al., 2000).

Evaluation of the Amylolytic Activity of the Extracts:

The amylolytic activity of the extracts was evaluated with the addition of 0.5 ml of a soluble starch solution 0.2 M in sodium acetate 0.1 M and 10.0 µl of the aqueous extract, with later incubation at 37 °C for 30 minutes. After this time, we added an iodine solution in potassium iodide (0.006% I2 + 0.06% KI) in HCl 0.2 M, which reacted with the residual starch and was determined by means of the absorbance through spectrophotometry in a VWR V-1200 equipment at 640 nm (Queen, Rajalakshmi, & Komathi, 2017). The basal absorbance was measured after the preparation of the reagents. We used soluble starch as a positive control. The quantification was expressed in amylolytic units by deciliter (UA/dl) through the formula.

We made five repetitions for each strain. The amylolytic units determine the amount of enzymes contained in 100.0 ml of sample, which can hydrolyse 10.0 mg of starch in 30 minutes (Sarmiento, Vargas, Pedroza, Matiz, & Poutou, 2003).

Statistical Analysis:

The mean and the standard deviation were estimated for the activity rate and the enzymatic activity of each fungal strain. The normality of the data was evaluated with the Shapiro-Wilk test. To demonstrate if there was any difference in the activity rate and the enzymatic activity of the 40 strains, we carried out a variance analysis and the Multiple Comparison test of Duncan (.05 of significance). The results were processed in the program R® (Zaferanloo, Bhattacharjee, Ghorbani, Mahon, & Palombo, 2014).

Results

Forty strains of saprotrophic anamorphic fungi were isolated from the leaf litter of Quercus sp, and 37 produced amylases. Of these, *Virgarianigra* SL12517 showed the highest enzymatic activity (625.13 UA/dl), followed by *Aspergillus niger* SL14917 (621.63 UA/dl) and *Aspergillus* sp SL15317 (558.57 UA/dl). *Mariannaeaelegans* TP13819 showed the lowest activity (1.52 UA7dl), as well as the TP16919 strain (1.52 UA/dl). *Chloridium* sp SL10619, TP13019 and *Helicosporium* sp SL10819 did not show amylolytic activity. From the strains that produced amylases, 20 showed activity rate higher than 100, whereas the corresponding aqueous extracts showed enzymatic activity higher than 50 UA/dl (Table 1).

Table 1.
Activity rate and amylolytic activity of the strain extracts that show activity higher than 100 and enzymatic activity higher than 50 UA/dl.

Anamorphic fungi	**Activity rate**[1]	**Enzymatic activity**
		(UA/dl)[2]
Aspergillus sp SL15019	99.26 (1.84)	36.55 (4.00)[ab]
Beltrania querna SL10119	94.52 (23.42)	23.42 (1.61)[b]
Beltrania querna TP12819	98.88 (12.08)	32.18 (1.21)[ab]
Cladosporium sp TP16519	99.03 (26.04)	26.04 (6.58)[ab]
Mariannaea elegans TP13829	99.32 (1.49)	1.52 (4.35)[c]
Paecilomyces sp TP 17019	97.59 (12.66)	43.56 (1.59)[a]
Paecilomyces sp TP17219	96.00 (16.74)	15.53 (3.40)[b]
Penicillium sp SL15819	99.38 (5.10)	43.56 (5.56)[a]
Penicilium sp SL15919	98.77 (3.56)	40.06 (1.63)[a]
Penicillium sp SL16219	99.44 (6.17)	47.07 (1.87)[a]
Stachybotrina sp SL12019	98.33 (12.36)	5.02 (2.95)[c]
Stachybotrina sp TP14619	96.92 (11.67)	29.55 (1.84)[a]
Thozetella cubensis SL12219	97.59 (47.07)	47.07 (7.21)[a]
Thozetella nivea Sl12319	99.17 (5.41)	43.56 (4.58)[a]
Thozetella nivea TP14719	97.71 (9.75)	26.04 (8.75)[a]
TP16819 Strain	98.08 (10.77)	29.55 (8.12)[a]
TP16919 Strain	97.41 (8.40)	1.52 (1.71)[c]

1 The relation halo/colony was measured in millimeters at de 18th day of incubation, mean (standard deviation). 2 Amylolytic units in deciliter. a, b, c indicate a significant difference, based on the Multiple Comparison test of Duncan ($p < .05$), mean (standard deviation).

The *Aspergillus* sp SL15019 strains, *Beltrania querna* SL10119 and TP12819, *Cladosporium* sp TP 16519, *Mariannaea elegans* TP13919, *Paecilomyces* sp TP17019 and TP 17219, *Penicillium* sp SL15819, SL15919 and SL16219, *Stachybotryna* sp SL12019 and TP14619, Thozetella cubensis SL12219, *Thozetella nivea* SL12319 and TP14719, as well as the TP16819 and TP16919 strains show activity rate lower than 100, and their corresponding aqueous extracts show enzymatic activity lower than 50 UA/dl (Table 2).

Table 2.
Activity rate and amylolytic activity of the strain extracts that show activity lower than 100 and enzymatic activity lower than 50 UA/dl.

Anamorphic fungi	**Activity rate**[1]	**Enzymatic activity**
		(UA/dl)[2]
Aspergillus sp SL15019	99.26 (1.84)	36.55 (4.00)[ab]
Beltrania querna SL10119	94.52 (23.42)	23.42 (1.61)[b]
Beltrania querna TP12819	98.88 (12.08)	32.18 (1.21)[ab]
Cladosporium sp TP16519	99.03 (26.04)	26.04 (6.58)[ab]
Mariannaea elegans TP13829	99.32 (1.49)	1.52 (4.35)[c]
Paecilomyces sp TP 17019	97.59 (12.66)	43.56 (1.59)[a]
Paecilomyces sp TP17219	96.00 (16.74)	15.53 (3.40)[b]
Penicillium sp SL15819	99.38 (5.10)	43.56 (5.56)[a]
Penicilium sp SL15919	98.77 (3.56)	40.06 (1.63)[a]
Penicillium sp SL16219	99.44 (6.17)	47.07 (1.87)[a]
Stachybotrina sp SL12019	98.33 (12.36)	5.02 (2.95)[c]
Stachybotrina sp TP14619	96.92 (11.67)	29.55 (1.84)[a]
Thozetella cubensis SL12219	97.59 (47.07)	47.07 (7.21)[a]
Thozetella nivea Sl12319	99.17 (5.41)	43.56 (4.58)[a]
Thozetella nivea TP14719	97.71 (9.75)	26.04 (8.75)[a]
TP16819 Strain	98.08 (10.77)	29.55 (8.12)[a]
TP16919 Strain	97.41 (8.40)	1.52 (1.71)[c]

1 The relation halo/colony was measured in millimeters at de 18th day of incubation, mean (standard deviation). 2 Amylolytic units in deciliter. a, b, c indicate a significant difference, based on the Multiple Comparison test of Duncan ($p < .05$), mean (standard deviation).

Discussion

The amylolytic activity rate showed that the 92.5% of the strains of anamorphic fungi produced amylases. This strains were collected from the leaf litter of *Quercus* sp, which has glucans such as cellulose and starch as part of its composition and constitutes the main source of carbon for decomposer microorganisms in the first stages. Therefore we expected a high amylolytic potential from the studied anamorphic fungi.

Of the strains evaluated in this study, *V. nigra* SL12517 showed the highest amylolytic activity (625 UA/dl), which surpasses the ones found by Puri and Loveleen (2013) that produced α-amylases from wheat bran and obtained values between 272 UA/dl and 411 UA/dl. Likewise, *A. niger*, showed the second highest activity (621.63 UA/dl), which is comparable with the same species that Tester, Qi and Karkalas (2006) reported for the degradation of agroindustrial residues, where they demonstrated its effectiveness in raw starch decomposition under different pH and temperature conditions. *Aspergillus* has been widely used for α-amylase production due to its capacity to be used with different sources of carbon and tryptones, as well as its few nutritional requirements (Mathew, Vezhacharickal, Sajeshmar & Ashokan, 2016).

Spier, Woiciechowski, Vandenberge and Sccol (2006) and Villalba, Bula, Juan and Ávila (2008) found that *A. niger* has a high potential for amylase production in agroindustrial residues such as manioc starch, sugarcane bagasse and wheat bran. Regarding the other *Aspergillus* species, they show amylolytic activity between 183.60 and 558.57 UA/dl. It has been reported that this genus shows the capacity to degrade starch through the induction of substrates such as wheat or corn bran. It also shows a potential for its application in the food and textiles industry (Alva et al., 2007; ojsow, 2012; Mojsov et al., 2018).

Regarding the *Aspergillus* sp SL15217, *B. rhombica* SL10217, *Brachysporiella* sp SL10317, *Chaetopsinasplendida* SL10517, TP16819 and TP16919 strains, Paecilomyces sp TP17117 and *Penicillium* sp SL15417, SL16417, SL15717, SL16017, SL16117, SL16317, they show enzymatic activity between 100 and 400. This values are similar to those reported by Castro et al. (2011) about the capacity of the fungi genus *Penicillium*, *Rhizopus* and *Aspergillus* as amylase enzyme, cellulase and xylanase producers for industrial application. In addition, we observed a low enzymatic activity in *C. splendida* SL10517 and *Phyalocephalahumicola* SL11417.

On the other hand, Vanegas-Zamora, Méndez and Murillo (2018) used strains of *Aspergillus* sp, *Rhizopus oryazae* and *Penicillium* sp in subproducts of rice for α-amylase production and obtained results between 1000 and 4000 UA/dl. Mazumdar and Maumdar (2018), obtained activity up to 645 UA/dl from *Aspergillus oryzae* in banana peel. This amylolytic activity is higher than those obtained in this study because they optimized variables such as the fermentation period, incubation temperature, initial pH

of the medium and the concentration of the substrate.

It is important to mention that the potential of the native anamorphic fungi associated to the leaf litter of *Quercus* sp for the α-amylase production is promising for its industrial use by means of its large-scale production, using wheat bran and other agroindustrial residues. This can be possible since said fungi can be developed in substrates with few nutrients and they show activity comparable to the strains currently used in the α-amylase production.

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